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DYNAMICS OF MOLECULES AND MEMBRANES

DURING THE EARLY STAGES OF FOCAL ADHESION FORMATION

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Sensation and transduction of forces by endothelial cells are part of their inherent role as permeability barriers and regulators of blood flow in arteries, capillaries, and veins. Because of this proximity to blood flow, they are continuously exposed to mechanical forces that exhibit a wide range of spatial and temporal scales and directions. Among the key sensing mechanisms of the cell are focal adhesions (FA), and the plasma membrane. Dynamic and elusive in nature, membrane rafts have not been associated with being mechanosensitive, nor of being mechanically coupled to other mechanosensors. However, there is mounting evidence that focal adhesion formation and stability is sensitive to membrane composition. In order to test the hypothesis that membrane rafts participate in mechanosensation we developed a technique where single endothelial cells were deformed via FAs induced at the tip of a fibronectin (FN)-functionalized nanoelectrode probe. The dynamics of nascent FA assembly was assessed using time-lapsed fluorescent images of transfected RFP talin. We further hypothesized that membrane rafts, by virtue of their attachment to the cytoskeleton and high mobility in the plasma membrane, were mechanically coupled to FAs. To determine the kinetics and sequential order of raft and talin mechanosensitivity, time lapsed confocal fluorescent images were taken of cells during mechanical manipulation of a single induced FA by the nanoelectrode probe. Remote mechanosensing by rafts, sequential kinetics, and long term reversible accumulation was observed. These results demonstrate that rafts are directly mechanosensitive, mechanically coupled to focal adhesions, and that raft mobility may enable the earliest events related to FA mechanosensing. To better
understand adhesion and the role of membrane composition, an optical trap was used to manipulate a FN functionalized bead near a cell in order to monitor dynamic adhesion events and particle tracking of DIC images was used to assess surface fluctuations and adhesion. Cholesterol depletion, which disrupts membrane rafts, reduced adhesion time, suggesting a role of the membrane in adhesion. Additionally, an increase in effective diffusion of functionalized beads upon early contact points to the importance of membrane fluctuations in adhesion. Together, these findings provide us with insight to a new mechanosensor, the membrane raft, as well as a more holistic understanding of adhesion that involves adhesion proteins, their dynamics, and the role of the plasma membrane in their transport and accessibility.
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I am grateful and humbled to herewith present the culmination of my work.
Chapter 1

INTRODUCTION

1.1 Endothelial cell structure

Endothelial cells line the insides of arteries, veins, capillaries and lymphatic’s as a monolayer. These cells have important roles in physiological homeostasis, in the permeability of blood vessels, and in the mediation of a variety of physiological and mechanical stimuli. Interaction between endothelial cells and adjacent hemodynamic forces, such as shear stress, involves several endothelial structures including: i) a surface glycocalyx layer composed of glycosaminoglycan’s, glycolipids, and glycoproteins responsible for modulating EC-leukocyte adhesiveness and permeability to large molecules, ii) a plasma membrane composed of a lipid bilayer and integral and peripheral membrane proteins, that acts as a physical barrier and maintains physiological homeostasis of the cell, iii) a 3-component cytoskeleton (actin filaments, microtubules, intermediate filaments), which plays an important role in maintaining the shape of endothelial cells, iv) focal adhesion complexes responsible for cell signaling and adhesion to the basement membrane, and v) cell (tight and gap) junctions responsible for cell-cell adhesion and communication. (Figure 1-1)
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1.2 Mechanotransduction

Of the various cells in our body which are subjected to mechanical forces, endothelial cells (ECs) have a feature that despite being in a variety of vascular locations, they have the ability to respond to local changes in blood flow. In addition, endothelial cells can respond to acute and sustained changes of hemodynamic shear stresses which act at the apical cell surface. ECs regulate normal vascular function by: i) modulating vascular tone in response to acute changes in blood flow\(^3\)\(^{10}\) and, ii) remodeling vascular wall structure in response to chronic hemodynamic changes\(^\text{11,13}.\)

Mechanotransduction has been measured from seconds to hours of exposure to flow. Rapid responses include potassium channel activation, intracellular calcium release, G-protein activation, and stimulation of protein kinases\(^3\)\(^{37,42,17,22,23}.\) Other responses include MAP kinase signaling, altered gene expression, changes in the cytoskeleton, focal adhesion translocations, as well as glycocalyx, and cell junction responses\(^\text{19,20,13,46}.\)

A typical example of mechanotransduction occurs when forces that act on the apical surface are transmitted by the cytoskeleton to other locations where signaling can occur. Sites where integral membrane proteins and the cytoskeleton are connected and where this force would be transduced include: focal adhesions, intercellular junctions, caveolae, and the nuclear membrane\(^\text{13}.\) There are numerous other players in mechanotransduction including membrane ion channels, cilia, tight and gap junctional
complexes, nuclear tethered nesprins, all sharing the capability to sense, transduce, and adapt to force. Furthermore, studies have shown that EC function is not only affected by force but also by temporal and spatial gradients in shear stress\textsuperscript{8,25,47}. Frequency responses vary from a range of changes in membrane fluidity, to gene transcription modulation, to focal adhesion translocation\textsuperscript{25,33}. Approaches to studying the response of cells to mechanical forces include: i) the use of a group of cells which are subjected to some deformation or physical stress and then assayed as a group, and ii) approaches which assay cellular responses of a single cell\textsuperscript{25}. Some approaches measure cell generated traction forces, while others consider the forces between the extracellular matrix and the endothelial cell\textsuperscript{16}. To begin to decipher these events in mechanotransduction by identifying, characterizing, and understanding how mechanical forces contribute to the cell will depend on the development of new techniques to measure and manipulate forces in cells and tissues.

1.3 Endothelial plasma membrane

The cellular plasma membrane is made up of a 5-nm thick lipid core that houses various carbohydrates and proteins that are in either the outer leaflet, the inner leaflet, or are transmembrane proteins (Figure 1-2). The core is made up of two phospholipid monolayers which are stabilized by hydrophobic and van der Waals interactions\textsuperscript{35}. The multiple combinations of lipid hydrophilic headgroups and hydrophobic acyl chains, permit the inclusion of various molecules like glycerophospholipids, sphingolipids,
glycolipids and sterols\textsuperscript{15}. In addition, complex lipids are not distributed randomly in the membrane but form lipid domains, where sphingolipids and cholesterol are segregated in dipalmitoyl phosphatidyl choline-rich membrane areas. These domains are involved in the trafficking and sorting of specific proteins, as well as in signal transduction processes\textsuperscript{6}. Therefore, it has been observed that there is a link between the composition of lipid membrane domains and their biological and functional properties. However, the molecular basis and implications of these links to cellular function are not yet fully understood.

Figure 1-2: \textbf{The plasma membrane.} It is composed of a wide variety of phospholipids containing transmembrane proteins, peripheral membrane proteins, lipid domains, and GPI-anchored proteins sitting in lipid rafts (dark gray headgroups). (nicerweb.net)
1.4 Membrane Rafts and GM1 Gangliosides

Among the inhabitants of the membrane are "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes"\textsuperscript{38}. These domains are called membrane rafts. Small rafts are able to form larger stabilized platforms by means of protein-protein and protein-lipid interactions\textsuperscript{28, 48, 4, 21}.

Membrane rafts may also function as general signaling compartments, allowing the clustering of proteins such as integrins\textsuperscript{41}, which regulate cytoskeletal organization. The composition of these domains, besides being rich in sterol- and sphingolipids, is also associated with the inclusion of gangliosides. Gangliosides are complex glycolipids that have a strong amphiphilic character because of their large saccharidic headgroup and the double-tailed hydrophobic moiety. They are particularly abundant in the plasma membrane, where they are inserted into the external leaflet (Figure 1-3), with the hydrophobic ceramide moiety and with the oligosaccharide chain protruding into the extracellular space. The lipid moiety of gangliosides, shared with all sphingolipids, is called ceramide. The more common name of this is sphingosine\textsuperscript{29,43}, which is connected to a fatty acid by an amide linkage.
Figure 1-3: **Lipid Raft.**  (A) Depiction of a lipid raft with proteins (multimedia.harvard.edu)  (B) Gangliosides insert in the outer leaflet of the membrane.  (C) Top: Ganglioside oligosacharide chains (Sonnino, S. et al. 2008) Bottom: GM1 Ganglioside Structure (lipidlibrary.aocs.org/Lipid)
The geometry of the monomer inserted into the membrane is mostly determined by the very large surface area occupied by the oligosaccharide chain. There are different oligosaccharide chains associated with various gangliosides. The oligosaccharides depicted lack the membrane-anchoring ceramide, which would be attached to the terminal Glc in the GM1 ganglioside. The GM1, which is the most abundant and commonly used ganglioside to identify membrane rafts, has been widely used to fluorescently label rafts. This is possible by taking advantage of the strong affinity between GM1 and cholera toxin subunit B. The GM1 ligand, which we will also use in these studies to label membrane rafts, contains five carbohydrate residues and its shape resembles the letter “Y.” GM1 retains essentially the same conformation in all binding sites with Cholera Toxin subunit B.

1.5 Membrane modification through cholesterol depletion

Cholesterol depletion in cells can be achieved by treatment with cyclodextrins which are cyclic oligosaccharides consisting of α-(1–4)-linked D-glycopyranose units and typically exist as hexamers (αCDs), heptamers (βCDs) or octomers (γCDs). These compounds are potent carriers of hydrophobic compounds due to the fact that they contain a hydrophobic cavity which may encapsulate various hydrophobic molecules (Figure 1-4). Among cyclodextrins, β-cyclodextrins have the highest affinity for inclusion of cholesterol and Methyl-β-cyclodextrins (MβCD) and 2-hydroxyl-β-CD (2OHpβCD) are the most common vehicles to deplete cells of cholesterol. The extent to which cholesterol depletion takes place is a function of the βCD derivative used, its
concentration, incubation time, temperature and cell type. Another important factor to consider is that if cells are treated for short times, the majority of depletion will occur at membrane raft sites, whereas if cells are treated for long times, cholesterol will be depleted in all domains. For long exposures, 80–90% of total cellular cholesterol can be removed\textsuperscript{48}, providing a useful tool for modification of membrane composition.

\begin{figure}
\centering
\includegraphics[width=0.6\textwidth]{figure1.png}
\caption{The mechanism of methyl beta-cyclodextrin (MβCD) in cholesterol depletion and enrichment. A simplified scheme of the effects of MβCD on lipid membranes. Cylinders represent MβCD, dark-headgroup phospholipids sphingomyelin, open circle-headgroup lipids represent “other” phospholipids. (A) MβCD approaches the membranes and captures cholesterol from both raft and nonraft regions. The mechanism of capture likely involves a membrane fluctuation-induced partial protrusion of cholesterol molecules into the aqueous phase (adapted from Zidovetzki, R 2007).}
\end{figure}
1.6 Focal Adhesions

1.6.1 Focal Adhesion Structure and Formation

Inhabiting mostly the basal side of the cell, we find focal adhesion sites. Much like our feet, and just as complex, they serve to allow the cell to contact and adhere its body to the surface it rests on. Focal adhesion sites have many other roles besides being one of the main surface sensing organelles. They are endowed with an extraordinary degree of molecular complexity. They are a collection of more than 90 proteins which can be classified into structural proteins (non-enzymatic proteins), and regulating proteins (enzymatic proteins)\textsuperscript{39}.

They can be defined as an integrin-mediated cell–substrate adhesion structure that anchors the ends of actin filaments (stress fibers) and mediates strong attachments to substrates\textsuperscript{19,32}. They also function as an integrin-signaling platform\textsuperscript{45}. Focal complexes

Figure 1-5: Focal Adhesion Site - essential components. (Adapted from Puklin-Faucher, E. and Sheetz, M.P. 2009)
undergo a series of remodeling steps during the process of maturing into a focal adhesion\textsuperscript{7}. Once matured, structural proteins are recruited (Figure 1-5). These include integrins, extra cellular matrix (ECM) proteins such as fibronectin, vitronectin, laminin, and intra-cellular proteins such as talin, vinculin, paxillin, and zyxin.

\textbf{1.6.2 Integrin Activation}

Integrin activation, involves a conformational reorganization of the integrin–integrin dimer such that its affinity to the matrix ligand is radically increased. It is essential for the initiation of focal adhesions\textsuperscript{44}, and is associated with integrin recognition by talin\textsuperscript{18,12}. Two groups of proteins, the talins and the kindlins, each of which bind to cytoplasmic domains of integrins and connect them with the actin cytoskeleton, are vital for integrin activation\textsuperscript{19,14}. As it can be seen in Figure 1-6, the activation of the beta chain of the integrin undergoes a conformation change by 20\textdegree\textsuperscript{1} which is also associated with the binding of talin to this chain. Hence, talin can be used as a marker for focal adhesions sites since it will bind to integrins when they are active.

Therefore, assays for focal adhesion formation and remodeling are often carried out by fluorescently labeling talin, since it is at the focal adhesion sites where there is accumulation of activated integrins\textsuperscript{1,2,3,9,10}. 
Integrin transmembrane domains can induce integrin clustering by the respective homooligomerization of the α and β transmembrane domains\textsuperscript{31}. Integrin clustering requires the formation of the ternary complex consisting of activated integrins, immobilized ligands, talin, and PI(4,5)P\textsubscript{2}. (Figure 1-7)

Figure 1-6: **Conformational reorganization in integrin activation.** Activation of integrins is associated with integrin recognition by talin. (Adapted from www.jin-lab.org and Anthis, M et. al, 2009)
1.6.3 Focal Adhesion Complex

The molecular nature of the earliest integrin adhesion complexes is not clear, but they comprise at least two molecules of talin that connect two integrin– integrin dimers with actin. Such hypothetical adhesion nanocomplexes resemble the talin-dependent, 2 pN 'slip bonds' that are formed between fibronectin and the cytoskeleton. Integrin receptors, respond to biochemical and physical characteristics of the microenvironment which result in local alterations in cytoskeletal dynamics and the generation of mechanical force. Focal complexes, or nascent matrix adhesions, are transient structures.
that either disappear or develop into fully grown, mature focal adhesions. The molecular nature of this transition is still unknown, but involves differences in protein composition, phosphorylation and dynamics.

Recruitment of additional components promotes the clustering of elementary nanocomplexes and reinforcement of the integrin–cytoskeleton bonds. Binding of vinculin to talin triggers the clustering of activated integrins through the vinculin tail. Vinculin head regulates integrin dynamics and clustering and the tail regulates the link to actin. (Figure 1-8) Once vinculin becomes activated, the conformational changes leading to a switch from low- to high-affinity binding of vinculin to talin stabilizes an active conformation of integrins in FAs, resulting in reduced FA turnover and growth$^{2,34}$.

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**Figure 1-8:** **Recruitment of additional components** that promote the clustering of nanocomplexes as well as reinforcement of integrin-cytoskeleton bonds (adapted from Milewic, DM et al 2006)
1.7 Multi-point Scanning Confocal Fluorescence Microscopy

The most common conventional confocal microscopes use a single laser beam to scan a specimen, however this method is limited by its low speed scanning capability. For high speed, real time, confocal image acquisition, a multi-point scanning setup is a better choice. Using the traditional Nipkow disks to raster scan the specimen with the light illuminating through the holes; numerous small points of light scan the specimen when the disk is spinning. However, the disks have a limitation of a very low (1-2 %) light efficiency and fixed pinholes.

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**Figure 1-9: Principles of microlens-enhanced multi-point scanning.** A Multi-point scanning system has 70 % light efficiency vs. a single beam scanning system with 1-2% light efficiency. (Adapted from yokogawa.com)
This limitation has been overcome by using a disk containing microlens arrays in combination with the Nipkow disk, thereby dramatically improving the light efficiency to approximately 70%. The combination of multi-point scanning and microlens-enhanced technologies permits real-time confocal imaging of live cells. Comparable to the spinning disc confocal technology is the 2-D array multibeam laser confocal scanner VT-Infinity3 system from Visitech. This system uses nanoscale technology to adjust the pinhole size of 2500 pinholes with software-selectable 10- to 64-μm-diameter holes. This permits different objectives to be used and allows for variance in the degree of confocality. This technology uses an acousto-optic tunable filter (AOTF) laser control. The AOTF module allows for computer control of laser line selection, individual line intensity and high speed shuttering by operating in microseconds.

Figure 1-10: Light path of the multi-point confocal VT Infinity 3 scanner. (www.visitech.co.uk)
The microscope has matched micro-lens and pinhole arrays to achieve optimum performance and a single, double-sided galvanometer mirror to scan the sample, de-scan the fluorescent light returned from the sample, and re-scan that fluorescent light into the camera to create multi-dimensional, confocal images in real-time. The basic optical path consists of a stationary micro-lens array illuminated with an expanded laser beam. A galvanometer mirror scans the array over the sample and de-scans the returning fluorescence light. This light is separated from the illuminating beam by a dichroic mirror, and passes through a stationary pinhole array to create the confocal image. This image is re-scanned in perfect synchronization by the reverse side of the galvanometer mirror onto an ultra-sensitive EMCCD camera. The galvanometer scanner is synchronized to the selected camera capture parameters, both exposure time and frame capture rate. Fast scanning performance derives from the fact that the galvanometer needs to scan only in one direction.
1.8 Significance and approach

How can we determine how sensitive the cell is and why does it matter? Many studies have focused on the response of cells to applied forces, and there is no doubt that the cell responds in many forms: mechanically, dynamically, biochemically, and its response is both spatially and temporally dependent. However to gain a deeper understanding of the ongoing interplay between molecular mechanisms of sensation and stimuli, studies at the single cell level must be carried out. In the work presented in this thesis, our goal was to uncover the spatial and temporal nature of the cellular response to adhesion at the cellular and molecular level and to understand how adhesion is mediated by membrane rafts and the cellular membrane.

1.9 Main results of this study

We have introduced a new method for induction and manipulation of a nascent focal adhesion on a single endothelial cell using a functionalized nanoelectrode probe. In doing this we have observed that binding of the probe onto the cell surface was dependent on the nanoelectrode size, fibronectin concentration, and binding reaction time between probe and cell, which was accomplished through integrin-fibronectin linkages. By coupling this with high speed confocal microscopy, we have shown that it is now possible to assess the dynamics of assembly of the individual constituents of focal adhesions from the moment of probe contact onward.
In addition, our results provide evidence that membrane rafts exhibit both passive and active response to deformation in a spatially and directionally dependent manner, and that there exist two subpopulations of rafts. Focal adhesion deformation led to long range deformation of immobile rafts followed by active recoil of a subpopulation of displaced rafts. Another important finding was that initial adhesion between the FN-probe and the cell induced rapid accumulation of GM1 at the probe site, whereas talin accumulated subsequently. Lastly, long term deformation of FAs led to continual GM1 accumulation at the probe site that was reversible upon removal of the deformation. Together these findings have uncovered the membrane raft as a new mechanosensor.

The roles of the membrane in early dynamics of sensation are also presented in this work. Using as readout the position of a functionalized bead manipulated with an optical trap, dynamic characteristic profiles of binding were obtained. The observed decrease in binding time upon cholesterol depletion suggests that the membrane is involved in focal adhesion formation and stability. Furthermore, during initial bead cell contact, an increase in mean squared displacement of functionalized bead positions was evident, indicating that there is an increase in effective diffusion for functionalized beads upon initial contact. We hypothesize that this increase in diffusion is caused by membrane surface fluctuations.

Together, the results of this work provide insight into the truly dynamic nature of the cell, especially during initial contact with its environment.
1.10 REFERENCES


Chapter 2

FOCAL ADHESION INDUCTION AT THE TIP OF A FUNCTIONALIZED NANOELECTRODE

Foreword

The following chapter is taken from the manuscript: “FOCAL ADHESION INDUCTION AT THE TIP OF A FUNCTIONALIZED NANOELECTRODE”, Daniela E Fuentes, Chilman Bae, and Peter J. Butler, CAMB-S-11-00076, 2011. Published for the special issue honoring the 2011 National Medal of Science winner Dr. Shu Chien.

2.1 SYNOPSIS

Cells dynamically interact with their physical micro-environment through the assembly of nascent focal contacts and focal adhesions. The dynamics and mechanics of these contact points are controlled by transmembrane integrins and an array of intracellular adaptor proteins. In order to study the mechanics and dynamics of focal adhesion assembly, we have developed a technique for the timed induction of a nascent focal adhesion. Bovine aortic endothelial cells were approached at the apical surface by a nanoelectrode whose position was controlled with a resolution of 10s of nanometers using changes in electrode current to monitor distance from the cell surface. Since this probe was functionalized with fibronectin, a focal contact formed at the contact location.
Nascent focal adhesion assembly was confirmed using time-lapse confocal fluorescent images of red fluorescent protein (RFP) – tagged talin, an adapter protein that binds to activated integrins. Binding to the cell was verified by noting a lack of change of electrode current upon retraction of the electrode. This study demonstrates that functionalized nanoelectrodes can enable precisely-timed induction and 3-D mechanical manipulation of focal adhesions and the assay of the detailed molecular kinetics of their assembly.

2.2 INTRODUCTION

There exist many force spectroscopy techniques that can be used to characterize and manipulate single cells. These include atomic force microscopy (AFM)\(^6,21,48\), optical tweezers\(^11,31,50,58\), magnetic tweezers\(^45,56,12,27\), biomembrane force probes\(^22\), and micro-needle manipulation\(^16\). Each of these methods is well suited to a particular force magnitude and positional accuracy and, collectively, they have contributed immensely to our understanding of how cells sense force and how they exert forces on their surroundings. Such data has led to a detailed delineation of the mechanisms cells use to organize their environment, an organization which leads to physiological phenomena including bone remodeling, blood vessel caliber control, and embryonic development\(^44\) and pathophysiological phenomena such as cancer, atherosclerosis, hypertension, and arthritis\(^19\). Although force spectroscopy has become ubiquitous because of its potential to uncover details of mechanobiology, there remain some needed functionalities. First, it would be advantageous to define the precise timing of the transition between non-contact
and contact of the force probe with the cell so that molecular assembly kinetics resulting from initial contact could be measured. Second, the probe site should be on the order of the typical size of a focal adhesion, which is a micron or less. Third, the system should be able to apply prescribed deformations to cells at a prescribed location in order to test the hypothesis that mechanically-induced changes in cells are spatially heterogeneous. The technique to accomplish these design goals must be compatible with multiple microscopy imaging techniques and be able to be implemented in an environmentally controlled cellular sample chamber, in order to maintain cells under physiological conditions. One imaging modality that can delineate cell surface topography at the nanometer level is scanning ion conductance microscopy (SICM)\textsuperscript{28}. In this method an electrode is brought close to a sample, and scanned laterally. During this scanning, the electrode current is read continuously and the pipette position is controlled in a feedback manner to maintain constant electrode distance from the sample. Motions necessary to maintain ion current are converted to sample topography. The lateral spatial resolution of this method is limited primarily by the size of the pipette tip, which can be as small as 10 nm, and the axial resolution (distance from cell) can be as small as 10s of nm subject only to the ability to measure small current flow through the electrode. Thus, SCIM provides a means to have nanometer-scale topographical information in a non-contact mode\textsuperscript{34}. Use of a non-contact mode has a particular advantage since cellular topography can be imaged over many hours without damaging the cells and without inducing changes in the cell that might be related to contact and force application\textsuperscript{25}. 
To date there are no reports on the use of SICM with functionalized electrodes. Such an innovation could assist in assessing the role of adhesion events in a number of contexts. In addition, taking advantage of SICM’s ability to detect distance from current means that contact timing and location can be quantified and prescribed. This method would enable experiments that seek to test the role of contact and deformation on the kinetics of reactions on the surface and inside cells. In this study we have developed such a tool. By functionalizing a SICM nanoprobe with fibronectin and mounting the system on a high speed confocal microscope we show that we can induce nascent focal adhesion formation on the apical surface of a cell with precise timing and position. Furthermore, by transfecting the cell with fluorescent focal adhesion proteins, we show that the dynamics of protein recruitment can be assessed from the moment of contact onward. Such information will allow us in future studies, to determine the precise kinetic parameters related to molecular transport to focal adhesions. Furthermore, we show that prescribed deformations can be applied to the cells. Such deformations should yield insight into long-range coupling of cellular constituents as well as provide the capability to assess the role of deformation in the kinetics of focal adhesion assembly.
2.3 MATERIALS AND METHODS

2.3.1 Experimental Setup

A schematic representation of the experimental setup is shown in the Figure 2-1 (top). Bovine aortic endothelial cells (BAECs) were cultured and plated on a temperature controlled chamber (Bioptechs, Butler, PA, USA) and placed on a piezoelectric stage with resolution of 0.2 nm and 100 µm range of motion (NanoView & NanoDrive, Mad City Labs, Madison, WI, USA), which was, in turn, mounted on a joystick-controlled my stage with 20 nm resolution and 25 mm range of motion (Mad City Labs Inc., Madison, WI, USA). Both stages were controlled with dedicated Lab VIEW-based software programs. The piezoelectric stage was also controlled by Voxcell imaging software (Visitech International, Sunderland, UK).
An inverted Olympus IX71 microscope with a 100 W halogen light provided bright field illumination for phase contrast which was used for initial positioning of the probe. Imaging was done with an oil-immersion objective (Plan APON60X/1.45NA, TIRFM-2, WD0.15 mm). A multibeam laser confocal scanner (VT-Infinity3; Visitech International, Sunderland, UK) was used to visualize the real-time changes of fluorescence in cells. A high-performance electron multiplying cooled charge-coupled device (EMCCD) digital imaging camera (Sensicam-EM; Cooke Corporation, Romulus, MI, USA) performed image capture. The nanoelectrode probe was mounted on a computer-controlled micromanipulator (MP-285; Sutter Instruments, Novato, CA, USA). A patch clamp amplifier (Model 2400, A-M Systems, Inc., Carlsborg, WA, USA) with a 20 MΩ probe were used to monitor current changes through the nanoelectrode and to apply computer-controlled external voltages to it. A LabVIEW-based software program
that received data from a data acquisition board (NI PCI 6229, National Instruments, Austin, TX, USA) was used to analyze amplifier signals. Combined, this system enabled feedback positional control of the nanoelectrode through micro-manipulation, piezoelectric stage positioning, ion conductance monitoring, and data acquisition.

2.3.2 Probe Development

The probe was prepared from fire-polished, thin wall borosilicate glass capillaries (O.D. 1.0 mm, I.D. 0.78 mm, Sutter instrument, Novato, CA, USA) with an internal electrode filament (Figure 2-1 middle). The capillary was pulled with a PUL-1 micropipette puller (World Precision Instruments, Florida, USA) so that the pipette had a shank as short as possible in order for it to resist deflection during nano-manipulation, while maintaining a small tip size (Figure 2-1 middle). Finite element analysis of tip deformation showed that the lateral spring constant was about 100 nN/nm (data not shown). Taper length was found to correlate inversely with probe diameter, meaning the longer the taper length, the smaller the probe diameter (data not shown). The probes were fabricated such that tip inner radii were approximately 250 nm for a “small” (Figure 2-1 middle), 500 nm for a “medium”, and 1 µm for a “large” sized probe, as verified by electron microscopy. The electrode resistance was <50 MΩ.
2.3.2 Probe set-up

In order to minimize interference with the optical light path of the microscope, the capillary was bent by approximately 45° using the heated filament of the PUL-1 puller thereby decreasing obstruction of phase contrast microscopy. The tip was filled by capillary action along the filament of the glass pipette.

The back of the probe was first immersed into CO₂-independent media and allowed to passively back fill for approximately 1 min. The remaining tapered region was back filled using a 34 gauge MicroFil (World precision instruments, Inc. Sarasota, FL, USA). Two electrode holders were tested, the QSW-A10P (Warner Instruments, Hamden, CT, USA) which uses a Ag wire, and the KCl bridge electrode holder (model ALA PPH-KCL-BNC, ALA Scientific Instruments, Inc.,Westbury, NY, USA). The latter was used for the functionalized probe experiments as it had a more stable DC current (less drift) and less noise (data not shown).
2.3.3 Fibronectin Functionalization of the Nanoelectrode

Fibronectin (FN) was diluted with DPBS under sterile conditions to concentrations of 0, 0.01, 0.05, 0.1, and 0.5 mg/ml. All functionalization steps, including incubation, were carried out inside a cell culture hood under sterile conditions. Borosilicate glass nanoelectrode probes were cleaned in \( \text{H}_2\text{O/ EtOH 80\% (v/v)} \) solution and rinsed twice in autoclaved ddH\(_2\)O. The tips were filled with CO\(_2\)-independent media, and micropipettes were placed in holders. Tips were then submerged in FN solution and the setup was then placed inside a 37\(^\circ\)C incubator for 4 h. When necessary, visualization of the functionalized nanoelectrode was achieved by fluorescently labeling FN using the AlexaFluor 568 Protein Labeling Kit (Molecular Probes A10238) (Figure 2-1 middle).

In order to find the optimal concentration for binding of the nanoelectrode probe, probes functionalized using FN concentrations of 0, 0.01, 0.05, 0.1, or 0.5 mg/ml were brought to the cell, allowed to bind, and the ion current was monitored during the retraction phase, \( (t_3 \text{ in Figure 2-1, bottom}) \). Specifically, the probe was halted when the initial current dropped by 2\% (e.g., from 2.02 to 1.98 nA) while a 1 mV \( V_m \) was applied. This % current drop ensured cell contact was made with minimal indentation and falls between the <2\% drop in current used in non-contact mode SICM and the drop of 20\% used in patch clamp indentation experiments. After contact, binding was assessed by measuring the current during retraction of the probe after 10 min of reaction time.
2.3.4 Cell Culture and Transfections

All *in vitro* experiments were performed on bovine aortic endothelial cells (BAECs) (VEC technologies, Rensselaer, NY). BAECs were sub-cultured between passages 3–10 in T-25 flasks with MCDB-131 complete medium (VEC technologies, Rensselaer, NY) while maintained at 37 °C in a gas mixture of 95% air and 5% CO2 with 90% humidity. The cells were then seeded onto chambered coverglasses and placed in the incubator for 2 h, after which new MCDB-131 media was added. BAECs were incubated overnight. Cellular transfection of red fluorescent protein (RFP)-talin fusion sequences were performed the day before imaging using BacMam technology (Invitrogen) at a ratio of approximately 10 particles of BacMam per cell.

2.3.5 Assessing Probe-Induced Focal Adhesion Assembly

In order to measure the time course of molecular scale activation in newly formed focal adhesions, the FN-functionalized probe was allowed to come into contact with BAECs transfected with RFP-talin. Probes were positioned over the cell to be contacted, the cell was raised to meet the tip of the probe using the piezoelectric stage, while monitoring the current between the tip and the cell as the approach phase \( (t_1) \) progressed (Figure 2-1, bottom). After contact, as recognized by the electronic signature of the approach phase, the reaction phase \( (t_2) \) began and the FN-functionalized tip was allowed to bind to the cell for intervals of 0, 1, 5, 10, and 15 min. The third phase was retraction
(t₃), where the cell was moved away from the probe using the piezoelectric stage, while monitoring the current between the probe and the cell. To assess talin accumulation, time-lapsed confocal images of RFP fluorescence were collected during the adhesion phase

2.3.6 Data Analysis and Statistics

Images were processed using ImageJ software. Average pixel values in regions of interest of time-lapse images remote from the probe contact point were determined in order to characterize degradation of RFP fluorescence due to photo bleaching. These average values were added back to values of RFP fluorescence evaluated at the probe site. The resulting values were then normalized to initial intensity and then plotted with respect to time. Where indicated, compiled data is represented as mean ± standard deviation. Statistical significance was evaluated using a Student’s t-test at the p<0.05 level.
2.4 RESULTS

2.4.1 Defining Probe-cell Contact

A 2% decrease (e.g., 0.04 nA) from the initial current (e.g., 2.02 nA) was used to define the timing of probe-cell contact. This 2% decrease was 4 times greater than the system noise, calculated at 0.01 nA (RMS). The uncertainty in position of contact was approximately 40 nm (RMS/slope). The decrease in current began when the probe was within 500 nm of cell surface (Figure 2-2). Once contact was achieved, the position of the probe and the cell was maintained. In control experiments using a non-functionalized electrode, retraction of the probe resulted in an increase in current that mirrored the decrease of current during the approach phase (Figure 2-2), whereas current was maintained after retraction of functionalized probes. These results demonstrate that current transients were good indicators of adhesion.
Figure 2-2: **Approach of probe to the cell.** This figure depicts the changes in current accompanying the last few nanometers of approach and retraction. During the approach phase, the piezoelectric stage moved the cell towards the probe and the current of the nanoelectrode was continuously monitored. The current displayed a marked decrease once the probe was near the cell surface, and achieved probe-cell contact as defined by a 2% total percentage drop from the initial current value. During the retraction phase, the piezo-electric stage moved the cell away from the probe and the current increased when the probe was retracted from the cell surface. For a cell that was bound to the probe, current did not increase during retraction.
2.4.2 Nascent Focal Adhesion Formation

The FN-functionalized nanoelectrode probe was allowed to come into contact with the cell using the electronic signature shown in Figure 2-2. After probe-cell contact, the reaction phase took place, allowing for the induction of a nascent focal adhesion at the site of probe-cell contact. The time of contact was defined as $t = 0$. Initially, there was no evidence of a focal adhesion at the probe tip (Figure 2-3 A). However, over a total reaction time of 15 min, the time lapsed images (Figure 2-3 C) of red fluorescence indicated the accumulation of talin resulting from the formation of a nascent focal adhesion at the site of probe-cell contact. Accumulation of talin is a marker for activated integrins, which bind to FN during focal adhesion formation (illustrated in Figs. 3D–3F). Thus, in addition to the electronic signature during approach, talin accumulation provides unambiguous verification of probe-cell contact and binding.
Figure 2-3: **Induction of focal adhesions upon probe contact:** Confocal images at the apical surface verify nascent focal adhesion formation (A and C). At the time the probe made contact with the cell (t = 0) (A) no RFP-talin was present on the apical surface of the cell at the probe-cell contact site (circle). After a reaction phase of t = 15 min (B) during which the fibronectin-functionalized probe remained in contact with the cell, presence of an induced apical nascent focal adhesion was observed via accumulation of RFP-talin at the site of probe cell contact (C, circle). This induction likely followed outside-in signaling via integrins induced by the fibronectin functionalized probe (D, E, F). (SFK-Src Family Kinase, FAK-Focal Adhesion Kinase)
2.4.3 Remote Remodeling of Focal Adhesions

Upon contact, the time-lapsed images of RFP-talin revealed not only the induction of an apical focal adhesion at the probe site (circle) as seen in Figure 2-3 (C) and in Figure 2-4 (t = 80 s), but also the remote remodeling of basal focal adhesion sites. A z-stack of confocal images was used to distinguish between apical vs. basal focal adhesion sites. Selected time points allowed for the concurrent visualization of initial focal adhesion sites pseudo-colored in blue at time t = 0 s (contact time), and real-time location of focal adhesion sites pseudocolored in red during the reaction time of 15 min. The overlay of initial locations (blue) and subsequent locations (red) can be seen in each of the panels of Figure 2-4. By t = 20 s remodeling of basal focal adhesion sites was evident. Partial overlap is denoted by the purple region at t = 20 s and t = 40 s. At t = 60 s complete translocation of basal focal adhesion sites occurred, as all sites had new locations. Notably, at time = 80 s, RFP-talin accumulation at the probe tip was observed, suggesting that a nascent apical focal adhesion was forming at the site of probe-cell contact (circle). At subsequent time points (t = 3 min to t = 15 min) continued remodeling of remote basal focal adhesion sites occurred in response to probe-cell contact and binding. Non-contacted control cells did not exhibit appreciable basal remodeling over the 15 min observation time (data not shown).
Figure 2-4: **Real-time response of basal focal adhesions to apical probe-cell contact during reaction phase.** Selected time-lapse images of RFP-talin display concurrent initial location of focal adhesion sites (pseudo-colored in blue at t = 0 or contact time) overlaid with real time location of sites (pseudo-colored in red). At time = 0 s, no accumulation of RFP-talin at the probe site (circle) was evident. Remodeling of remote basal focal adhesion sites was observed in response to contact as early as the first time point of 20 s and throughout the reaction time of 15 min. At time = 80 s a nascent apical focal adhesion site was observed at the site of probe-cell contact. Subsequent time points (t = 3 min to t = 15 min) displayed continued remodeling and translocation of remote basal focal adhesion sites.
2.4.4 Kinetics of Talin Accumulation

Using an image frame rate of 2 s\(^{-1}\), the kinetics of RFP-talin accumulation was assayed during the first 90 s of formation of the nascent focal adhesion. Average fluorescence intensity was measured for each time point, corrected for photo bleaching and normalized to initial values. The accumulation profile for talin as seen in Figure 2-5 displays delayed accumulation followed by a rapid increase starting at 65 s and reaching a plateau at 82 s. The average fluorescent intensity increased by approximately 50% from its initial value. These results demonstrate that the ability to monitor the kinetics of formation of the nascent focal adhesion site subsequent to a precisely defined initial contact time point.
Figure 2-5: **Time course of talin accumulation at probe site.** Time course of transfected RFP-talin was measured using average fluorescence intensity at the probe site. Following probe contact with the cell, there was little to no accumulation for about 65 s. Subsequently, talin accumulated rapidly reaching a 50% increase which plateaued at 82 s.
2.4.5 Further Verification of Probe-Cell Binding

The slope of the retraction electronic signature, images from phase microscopy, and confocal fluorescence verified that current failed to increase upon retraction of a bound probe whereas current increased upon retraction when the probe was not bound (Figure 2-6 A). Even at a distance of 5 µm from the initial point of contact, the electronic signature was able to distinguish between a bound and an unbound cell. Concurrently, brightfield images were able to show that if the probe was displaced further, the cell remained bound to the probe (Figures 2-6 B – 2-6 C). Fluorescence images of RFP-talin showed that when the probe was moved laterally, the focal adhesion moved with it (Figs. 2-6 D – 2-6 E). These results suggest that electrode current can be used to monitor the extent of binding even during deformation of the cell by the probe.
Figure 2-6: **Verification of probe-cell binding at nascent focal adhesion**: Three simultaneous metrics were used to verify that the probe remained bound to the cell through the induced nascent focal adhesion. (A) Current increases upon probe retraction from cells were substantially smaller for a bound cell (bottom curve) vs. an unbound cell (top curve). Final current value was subtracted from raw current values so that each curve would start at 0 nA. (B, C) Phase contrast images demonstrate that the cell remained bound to the probe. (D, E) Fluorescence microscopy images reveal that the focal adhesion remained bound to the probe as the probe was moved during lateral displacement.
2.4.6 Optimization of Probe-Cell Binding

Lastly, a few key parameters were optimized. For SICM imaging and probe positioning, the scanning image resolution and sensitivity depends on the tip diameter and shape. Since the potential to bind to the cell also depends on the probe size, we determined the effect of the probe tip size on the ion current pattern of phase $t_3$. We categorized the probe tip sizes as “small,” corresponding to a tip radius $<250$ nm and probe electrical resistance $>20$ M$\Omega$, and “medium,” corresponding to tip radii between 250 and 500 nm with a probe electrical resistance of 10–20 M$\Omega$. Experiments were conducted with different reaction times (1, 5, and 10 min) and with different FN concentrations (0.01, 0.05, and 0.1 mg/ml). First, increasing FN concentration decreased the slope of the current vs. retraction distance curve (Figure 2-7a). Thus the slope can be used to assess degree of adhesion. Second, as shown in Figure 2-7b, the success rate increased as the FN concentration increased. With a 10 min reaction phase, a medium probe size, and 1 mV $V_m$, the success rates were 42.8, 50, and 75% at FN concentrations of 0.01, 0.05, and 0.1 mg/ml, respectively. Third, successful probe-cell binding was observed to be dependent on probe size (Figure 2-7c). With 0.01 mg/ml of FN, 10 min of reaction time, and 1 mV $V_m$, the success rates for binding were 12.5% and 70% for using small and medium sized probes, respectively. Finally, we determined the effect of reaction time on the success rate of binding. As shown in Figure 2-7d, the success rate increased as the FN reaction time increased. With 0.05 mg/ml FN concentration, medium probe size, and 1 mV $V_m$, the success rates were 0, 33.3, and 50% for reaction times of 0, 5, and 10 min, respectively.
Figure 2-7: **Parameters affecting probe-cell binding**: (a) Slope of current vs. retraction distance after binding as a function of fibronectin concentration. Small slope indicates tighter binding between pipette and cell surface. (b) Success rate of binding increases as a function of fibronectin concentration (c) Binding success rate increases as probe size increases, (d) Increased reaction time increases success rate of binding.
2.5 DISCUSSION

The main findings of this study are that ion conductance through a nanoelectrode can be used to prescribe the time and location of induction of a focal adhesion, induced focal adhesions can be mechanically manipulated and current transients through the electrode provide a readout of the degree and integrity of adhesion. These findings were supported by assays for focal adhesion formation and remodeling indicated by fluorescently labeled talin, which accumulates at focal adhesion sites subsequent to activation and dimerization of integrins. This technique was designed to enable detailed delineation of the identity and kinetics of the numerous proteins that participate in focal adhesion formation and reinforcement upon mechanical manipulation.

Focal contacts undergo a series of remodeling steps during the process of maturing into focal adhesions. Once matured, structural proteins are recruited including integrins, extracellular matrix proteins such as fibronectin, vitronectin, laminin, and intracellular proteins such as talin and vinculin. Integrin activation is essential for the initiation of focal adhesions in that conformational reorganization of the integrin dimer increases its affinity to the matrix ligand. This activation is associated with integrin recognition by talin and results in binding of talin to the cytoplasmic domains of integrins and connection with the actin cytoskeleton. Focal adhesions can be induced by outside-in signaling, they function as both adhesion and signal transduction organelles, and they serve to inform the cell about the chemical makeup and mechanics of its surroundings.
Focal adhesions form due to accumulation of focal adhesion proteins and it is believed that focal adhesion proteins, themselves, are sensitive to applied forces. For example, Bell,\(^5\) proposed that the rate constant governing receptor-ligand binding was dependent on mechanical force. Numerous other studies have elucidated tethering bond kinetics, slip bonds, catch bonds, and unbinding kinetics\(^{13,33,36,37}\) of focal adhesion proteins.\(^{4,36,52}\) Less well understood is the dependence on force of the kinetics of accumulation of focal adhesion proteins, especially during the initial stages of focal adhesion formation. Although previous studies have provided insight into the development of focal adhesions from nascent focal contacts,\(^{4,36,52}\) there are no published reports of the kinetics of focal contact formation in response to adhesion, likely due to the difficulty in defining the initial contact time. In this study we introduce a technique in which induction of focal contact formation is precisely timed and provide preliminary data on the real-time kinetics of talin accumulation in response to initial probe cell contact, in a spatially localized manner. We also show that the same electrode can be used to apply a prescribed deformation to the newly formed focal adhesion.

This study is based on SICM and provides a means for outside-in induction of a single nascent apical focal adhesion in a single bovine aortic endothelial cell using nanoelectrode probe-cell contact. SICM was invented by Hansma et al.,\(^{28}\) and has been further developed to image and analyze surface topography of live cells. SICM is a non-optical imaging method that uses an electrolyte-filled nanopipette as a scanning probe to image cell surface structures with resolution at the nanometer level. SICM records the
ionic current through the probe as the probe is scanned over the surface of a sample immersed in an electrolyte solution. The current drop used in SICM to define probe cell contact is typically 2% or less of the initial current. SICM employs a feedback loop to maintain a constant distance between the pipette and the surface, so that the displacement of the scanning pipette normal to the sample during a scan represents the topography of the surface.

While SICM emphasizes the non-contact nature of probe positioning we have functionalized our nanopipette and used the positioning sensitivity (on the order of 40 nm) to minimally contact the cell surface with molecular specificity. In addition, by using the electronic signature of the nanopipette electrode, we can assess the integrity of the attachment of the cell surface with the pipette, in real time, and monitor detachment.

A previous study by Riveline et al.\textsuperscript{49} also used nanopipettes functionalized with fibronectin for cell manipulation. In that study, remote focal adhesions moved toward the pipette in response to large mechanical perturbation of the cell surface. This observation is in contrast with focal adhesion movement away from the probe observed in our experiments. It is possible that the large deformations used in the Riveline caused focal adhesion translocation in the direction of the force, which in the large deformation case, is toward the pipette.
In the current study, focal adhesions translocated without applied force. Therefore, it is possible that the new adhesion point induced by the pipette led to cytoskeletal remodeling and subsequent changes in force distribution in the cell. Although it would be difficult to predict the directions of forces in this instance, these changes in force distribution could have led to basal focal adhesion remodeling. Consistent with this interpretation, in related studies by Mathur et al., remote sensing of basal focal adhesions in response to small forces applied by AFM showed that that the cell responds globally to the localized applied force. Our results, therefore, are consistent with other’s observations that focal adhesions respond to applied external force. However, our method explores the additional phenomenon of the response of the cell to minimal force application. It uses SICM technology to provide a new tool for timed induction of focal adhesions with nm-scale deformation ranging from near zero nm to micron scale. Such studies will be useful in elucidating the early events of focal adhesion formation and reinforcement in response to force.

SICM is a technique complementary to AFM for assessing topography of cells, and to optical and magnetic tweezers for force application. For example, the AFM, a member of the scanning probe family of microscopes, manipulates a cantilevered probe (tip radius ranging from a few nanometers to a few micrometers) for tension or compression loading. A slightly larger version of the AFM technique facilitates microscale indentation of an entire cell. Conventional AFM can detect forces in the range of 10–100 pN and has a displacement range of 0.5–100 nm. One disadvantage of
AFM is that it is difficult to study biological processes and structures non-intrusively since by definition the cantilever must be deflected in order to define contact.\textsuperscript{42} In our implementation of SICM, current through the electrode provides the distance measurement and contact is initiated only at prescribed times and locations.

Optical tweezers, can measure forces on a $\mu$m scale bead of up to 100 pN\textsuperscript{41,48} with sub-millisecond temporal resolution. When the laser beam is focused on a dielectric particle, the particle experiences a three-dimensional restoring force directed toward the center of the focused beam.\textsuperscript{2,48} Even though optical tweezers have high versatility and precision, focused laser light can induce cell damage caused by local heating.\textsuperscript{42,46,53} More importantly, trap stiffness depends on the gradient of the optical field, which can be refracted by contaminating substances and adherent cells. Magnetic tweezers, on the other hand, use a magnetic field gradient to exert and measure pico- to nanonewton forces on magnetic particles.\textsuperscript{26,42} The core advantage of magnetic tweezers is that it permits parallel single-bead measurement because a magnetic field can exert force everywhere, which would be difficult to achieve with other force spectroscopy techniques.\textsuperscript{18} Some limitations are that the bandwidth and sensitivity are limited by video-based detection and high-current electromagnets can generate undesirable Joule heating.\textsuperscript{14,42} Studies such as optical and magnetic tweezers which use particles as probes\textsuperscript{30} must also contend with internalization of particles due to phagocytosis, the relatively non-uniform distribution of beads, and large (3–6 $\mu$m$^2$) and ambiguous contact
area between the bead and cell membrane.\cite{ref43} In contrast, the nanoelectrode probe used in this study cannot be phagocytosed or pulled due to its shape and stiffness.

### 2.6 SUMMARY

In summary, we introduce a new method for induction and manipulation of a focal adhesion on a single endothelial cell using a functionalized nanoelectrode probe. We observed that binding of the probe onto the cell surface was dependent on the nanoelectrode size, fibronectin concentration, and binding reaction time between probe and cell, which was accomplished through integrin-fibronectin linkages. Furthermore, by coupling the technique with high speed confocal microscopy, we show that it is possible to assess the dynamics of assembly of the individual constituents of focal adhesions from the moment of probe contact onward. This new capability should allow for the development of new kinetic models of focal adhesion assembly in the presence and absence of force, thus opening up new areas of research in the integration of engineering principles of molecular transport and biological sensing of the cellular microenvironment.
2.7 REFERENCES


Chapter 3

COORDINATED MECHANOSENSITIVITY OF MEMBRANE RAFTS AND FOCAL ADHESIONS

Foreword

The following chapter is taken from the cellular and biomolecular engineering special issue showcasing Outstanding Papers of the 2011 Biomedical Engineering Society. The manuscript is entitled: “Coordinated mechanosensitivity of membrane rafts and focal adhesions” Daniela Fuentes and Peter J. Butler, Cellular and Biomolecular Engineering, March 2012.

3.1 SYNOPSIS

Endothelial cells sense mechanical forces of blood flow through mechanisms that involve focal adhesions (FAs). The mechanosensitive pathways that originate from FA-associated integrin activation may involve membrane rafts, small cholesterol- and sphigolipid-rich domains that are either immobilized, by virtue of their attachment to the cytoskeleton, or highly mobile in the plane of the plasma membrane. In this study, we fluorescently labeled non-mobile and mobile populations of GM1, a ganglioside associated with lipid rafts, and transfected cells with the red fluorescent protein- (RFP-) talin, an indicator of integrin activation at FAs, in order to determine the kinetics and sequential order of raft and talin mechanosensitivity. Cells were imaged under confocal microscopy during mechanical manipulation of a FA induced by a
fibronectin (FN)-functionalized nanoelectrode with feedback control of position. First, FA deformation led to long range deformation of immobile rafts followed by active recoil of a subpopulation of displaced rafts. Second, initial adhesion between the FN-probe and the cell induced rapid accumulation of GM1 at the probe site with a time constant of 1.7 seconds. Talin accumulated approximately 20 seconds later with a time constant of 0.6 seconds. Third, a 1µm deformation of the focal adhesion lead to immediate (0.3 sec) increase in GM1 fluorescence and a later (6 sec) increase in talin. Fourth, long term deformation of FAs led to continual GM1 accumulation at the probe site that was reversed upon removal of the deformation. These results demonstrate that rafts are directly mechanosensitive and that raft mobility may enable the earliest events related to FA mechanosensing and reinforcement upon force application.

3.2 INTRODUCTION

The cellular plasma membrane is a heterogeneous mixture of lipids that dynamically coalesces into domains according to lipid preferences for liquid-ordered and liquid-disordered phases. Membrane rafts are an important type of liquid-ordered domain that are highly transient, tens of nanometers in size, and enriched in cholesterol and sphingolipids. Rafts dynamically combine and disperse to facilitate protein sequestration and protein-protein and protein-lipid interactions. These domains, besides being rich in cholesterol, are associated with the ganglioside GM1, a
glycosphingolipid that, when tagged with fluorescence, can be used as a marker of lipid rafts\textsuperscript{32,52,57}. Membrane rafts may also play a role in cell mechanics\textsuperscript{44,56} as they can modulate the clustering of integrins\textsuperscript{9} which regulate cytoskeletal organization, membrane trafficking\textsuperscript{24,62} and connections to the extracellular matrix\textsuperscript{29,55} via focal adhesions. The association of cholesterol-rich domains and integrins and involvement of Rac and Rho\textsuperscript{14,16,17,63} with membrane rafts suggests that rafts play a role in focal adhesion assembly and reinforcement, although this hypothesis has not yet been tested directly. Raft lipids may regulate protein localization and function through enhanced affinity between lipids and specific amino acid sequences in a protein’s extracellular\textsuperscript{73}, transmembrane\textsuperscript{61}, or intracellular domains\textsuperscript{13}. Such spatially and temporally regulated lipid-protein interaction that facilitates protein -raft association may permit the cell to regulate polarized sorting and signal transduction processes.

Rafts associate with integrins and may facilitate mechanosensation through integrin ligation and clustering, which are physiologically and clinically relevant to vascular tone regulation and shear-induced gene expression leading to atherogenesis. For example, Jalali \textit{et al.} showed that shear stress caused an increase in new ligand binding of $\beta_1$ integrins in and around focal adhesions (FAs) of endothelial cells (ECs) plated on fibronectin (FN) and an increase in ligand binding of $\beta_3$ integrins in ECs plated on vitronectin\textsuperscript{39}. In \textit{ex vivo} arteriolar preparations, activation of the vitronectin receptor, $\alpha_v\beta_3$-integrin, and FN receptor, $\alpha_5\beta_1$-integrin, induced coronary arteriolar dilation by stimulating endothelial production of cyclooxygenase-derived prostaglandins\textsuperscript{33} which
dilate blood vessels\textsuperscript{6,22}. Thus integrin-matrix interactions at FAs are required to initiate the signaling pathway leading to shear stress-induced vasodilation and blood pressure regulation. Integrins are associated with rafts and nucleate actin polymerization [reviewed in\textsuperscript{44}] by concentrating phophatidylinositol 4,5 biphosphate (PIP\textsubscript{2})\textsuperscript{43}. FAs are also cholesterol rich microdomains, and $\beta_1$ integrins are required for raft formation\textsuperscript{63,17} and signaling through Rac-1\textsuperscript{17}. Wang and colleagues found that Src-activation colocalized with Lyn, a raft marker\textsuperscript{48} supporting an emerging picture of rafts as dynamic nanodomains that cluster the necessary critical mass of receptors\textsuperscript{68} for downstream signaling through important mechanotransduction pathways, such as mitogen activated protein kinases (MAPK)\textsuperscript{60}, with time scales of formation of 20ms and length scales of tens of nanometers\textsuperscript{19}. Finally, EC membrane microdomains are themselves known to be differentially sensitive to fluid shear stress\textsuperscript{67} making rafts a central focus of mechanosensation leading to vasoregulation and atherogenesis.

Despite the convergence of research on focal adhesion mechanosensing and lipid raft function, to date no studies have provided direct evidence for mechanical coupling between membrane rafts and focal adhesions nor has the dynamic kinetic response of rafts to mechanical perturbation of focal adhesions been elucidated. This study was undertaken to directly measure mechanical coupling between induced focal adhesions and stable lipid rafts and to measure the kinetics of mobile raft coalescence at focal adhesions upon FA formation and mechanical perturbation (Figure 3-1).
The study was enabled by a newly developed technique based on scanning ion conductance microscopy in which a FN-functionalized nanoelectrode was used to induce a focal adhesion and the precise timing of adhesion was determined through analysis of current through the electrode\textsuperscript{23}. Subsequently, adhesion formation was followed by

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**Figure 3-1: The membrane raft and focal adhesion connection:** Membrane rafts, rich in sphingolipids and cholesterol are identified by ganglioside marker GM1. Transmembrane proteins in some membrane rafts render a raft anchored via linkage to the cytoskeleton. In contrast, mobile rafts have no direct or indirect connection to the cytoskeleton. Focal adhesion sites, areas of clustered activated integrins linked to the cytoskeleton via talin, are known mechanotransducers. The connection between membrane rafts and focal adhesions may involve both the cytoskeleton and the membrane.
nanomechanical manipulation of the induced focal adhesion. Fluorescently labeled GM1 was used to assay raft dynamics and red fluorescent protein (RFP) - talin was used to assay focal adhesion formation and integrity, since talin binds to activated integrins through enhanced affinity between talin and integrin–integrin dimers$^{1,2,4,7,8}$.

3.3 MATERIALS AND METHODS

3.3.1 Cell Culture and fluorescence labeling of rafts and talin

All in vitro experiments were performed on bovine aortic endothelial cells (BAECs) (VEC technologies, Rensselaer, NY) sub-cultured between passages 3-10 with MCDB-131 complete medium (VEC technologies, Rensselaer, NY) at 37°C in a gas mixture of 95% air and 5% CO$_2$ with 90% humidity. Red fluorescent protein (RFP)–talin fusion plasmids (Invitrogen) were transfected into cells using BacMam technology (Invitrogen). The cells were then seeded onto chambered coverglasses or temperature-controlled chambers (Bioptechs, Butler, PA, USA) and placed in the incubator for 2 hours, after which new MCDB-131 media was added. BAECs were incubated overnight prior to experiments. Membrane rafts were labeled by conjugating GM1 with Alexa Fluor 488-tagged recombinant cholera toxin-subunit B (CT-B) (Invitrogen) with the cells at 4 °C for 15 minutes, rinsed three times with DPBS, and followed by CT-B crosslinking
with anti-cholera toxin subunit B antibody (anti–CT-B) (Invitrogen) at 4 °C for 10 minutes. Cells were slowly brought back to room temperature prior to experiments.

### 3.3.2 Experimental Setup

A schematic representation of the experimental setup is shown in Figure 3-2 and has been previously described. Briefly, chambers containing cultured BAECs were placed on a piezoelectric stage (NanoView & NanoDrive, Mad City Labs, Madison, WI, USA). An inverted Olympus IX71 microscope with a 100W halogen light provided brightfield illumination for phase contrast. Fluorescence imaging was done with an oil-immersion objective (PlanAPO 60X/1.45 NA). Cellular fluorescence was assayed using a laser scanning confocal scanner system (VT-Infinity3, Visitech International, Sunderland, UK) coupled to an EMCCD digital imaging camera (Sensicam-EM; Cooke Corporation, Romulus, MI, USA).
The nanoelectrode probe was functionalized with fibronectin as previously described\textsuperscript{24} and was mounted on a computer-controlled micromanipulator (MP-285; Sutter Instruments, Novato, CA, USA). A patch clamp amplifier (Model 2400, A-M Systems, Inc., Carlsborg, WA, USA) connected to a 20MΩ probe detected current changes through the nanoelectrode. Together with a multifunction data acquisition board (NI PCI 6229, National Instruments, Austin, TX, USA) and LabVIEW software, the system coordinated nanoelectrode positional feedback control of the micro-manipulator and piezoelectric stage position using electrode current as the feedback process variable.

### 3.3.3 Induction and manipulation of focal adhesions using functionalized electrodes

Functionalized nanoelectrode probes were brought near the cell using the piezoelectric stage, while monitoring the current between the tip and the cell (Figure 3-2B). After contact, the FN-coated probe was allowed to bind to the cell for 15 minutes at a
location approximately halfway between the nucleus and the outer edge in order to later capture the membrane raft response of the majority of the apical plane. FA deformation (Figure 3-2 C) was accomplished by displacing the adhered probe parallel to the apical cell membrane by 1 µm. The probe was displaced in the positive x direction which corresponded to a focal adhesion translocation away from the nucleus approximately perpendicular to either the major or minor axis of the cell. In order to measure the time course of molecular-scale activation in newly formed FAs, the FN-functionalized probe was allowed to come into contact with BAECs transfected with RFP-talin. Where indicated, compiled data is represented as mean ± standard deviation with n≥3 (different cells from different cultures). Statistical significance was evaluated using a Student’s t-test at the p<0.05 level.

3.3.4 Tracking of Membrane Rafts and Kinetic assays of GM1 and talin accumulation

Real time locations of membrane rafts were individually mapped using ImageJ tracking functions. In the case of passive displacement and active recoil, individual raft locations were manually tracked one at a time and frame-by-frame using the "Manual Tracking" plugin. For control cells the "Spot Tracker 2D" plugin was used with a 3 pixel square size for centering. For GM1 and talin kinetics, regions of interest from time-lapsed images were quantified and the fluorescence reduction values were added back to
values assessed near the electrode adhesion point. The resulting values were then normalized by initial intensity and plotted with respect to time.
3.4 RESULTS

3.4.1 Rafts passively displace in response to FA deformation

Individual apical raft trajectories were tracked during focal adhesion deformation and while deformation was maintained (Figure 3-3 A). Displacements from multiple cells were normalized by assigning the origin to the probe site and plotting raft locations relative to the probe site. Raft locations and displacements were then plotted on a common figure (Figure 3-3 A) and analyzed together (Figure 3-3 B). First, rafts simultaneously displaced in response to focal adhesion displacement. This simultaneous response occurred not only in rafts located near the probe-cell contact point but also in remote rafts. Figure 3-3 B depicts responses of individual rafts as a function of their distance from the probe versus their passive displacement magnitude. This passive response could be divided into two subpopulations, rafts that were located between the probe and nucleus and rafts located on the opposite side of the nucleus. Rafts that were on the same side of the nucleus as the probe displaced with magnitudes which decreased as the distance between the membrane raft and the location of the probe increased (green group (hexagons)). Displacements of rafts that were located on the opposite side of the nucleus (red group (pentagons)) were nearly identical to displacements of rafts from control cells in which no focal adhesion displacement was induced (blue group (diamonds)).
Figure 3-3: **Passive membrane raft response.** (A) Rafts from multiple cells were tracked and overlaid to create a composite image of raft trajectories (n=4 cells), in response to a 1 µm displacement of a focal adhesion. The probe (circle) was displaced in the positive x direction which corresponds to a focal adhesion translocation away from the nucleus approximately perpendicular to the main axis of the cell. The probe was initially located approximately halfway between the nucleus and the outer edge of the cell. Individual trajectories initiated at the point furthest from the probe location (circle) and terminate closer to the probe. (B) Distance to probe vs. displacement magnitude of individual rafts. Green group (hexagons) corresponds to rafts that are located on the same side as the probe (relative to the nucleus); the red group (pentagons) corresponds to rafts located behind the nucleus (relative to the probe); control rafts are in blue (diamonds).
3.4.2 Rafts recoil after FA deformation and passive raft displacement

Subsequent to focal adhesion displacement the probe was maintained in its position and cells were imaged over time using confocal microscopy in order to capture the response of apical membrane rafts. Within the first minute after deformation a small subpopulation of rafts exhibited displacement in the opposite direction of the displacement applied by the probe, indicating raft recoil (Figure 3-4 A). The trajectories are plotted from the raft location closer to the probe location and terminate where the raft finished its motion. The subsequent remodeling is indicated by the overlapped tracked displacements resulting in a clustered trajectory endpoint. The magnitude of the recoil was greater for rafts that were closer to the probe, and smaller for more remote locations (Figure 3-4B). 21% percent of all rafts exhibited recoil. However, considering that the location of all active rafts that recoiled coincided spatially with the probe side of the cell (relative to the nucleus) out of the population of rafts located on the probe side, 38% exhibited active recoil.
Figure 3-4: **Active response: Recoil.** (A) After deformation the probe was stationary and membrane rafts moved in the direction opposite to deformation, indicative of active recoil. Individual trajectories initiated closer to the probe location and terminate with subsequent continued remodeling as indicated by the overlapped tracked displacements resulting in a clustered trajectory endpoint. (B) Distance to probe vs. displacement of individual active rafts is plotted with the green group corresponding to rafts that are located on the same side as the probe (relative to the nucleus), and control rafts in blue.
3.4.3 Directions of passive response and active recoil coincided with FA deformation direction

We defined the displacement of the probe as the positive x-direction, for which 0 degrees was assigned as a reference direction, meaning that any deformation that is aligned with the direction of probe displacement would be aligned to the 0 degree mark. The raft displacement angles relative to the horizontal (0º) were calculated and used to determine directionality as functions of magnitude of displacement and distance from the probe. Figure 3-5 contains these directionality plots and illustrates the angle of individual raft displacements with respect to the horizontal in degrees, the magnitude of displacement (as indicated by concentric circles), and distance from the probe (as mapped out by color gradients in each area). Local passive response of rafts was closely aligned with the direction of the applied FA displacement as demonstrated by the small angles ranging from 0 up to 30º, include the entire range of displacements from 0 to 1 µm, and are populated by local rafts up to 12.5 µm away (Figure 3-5 A). In addition, the local passive raft response aligned predominantly with the direction of focal adhesion displacement.
Figure 3-5: **Directional dependence of passive and active membrane raft response.**

(A) Passive response aligned with the direction of FA displacement (aligned with 0°). (B) Active response was in the opposite direction of displacement ranging from angles between 90° - 270°, and covering a range of displacements up to 0.8 µm. (C) Control rafts do not exhibit a particular directionality as their displacements cover the full 360° range, and their magnitudes of displacement are less than 0.2 µm.
In contrast, rafts recoiled in the opposite direction of the focal adhesion displacement, as can be seen by the majority of responses exhibiting angles greater than 90° and less than 270° (Figure 3-5B). The magnitudes of recoil range from 0.1 to 0.8 µm and occurred over a distance of 1.6 to 12.7 µm from the probe with an average ratio of passive displacement to active recoil of 1.06 ± 0.05. Both the active and the passive responses were different than rafts in control cells (Figure 3-5 C) where the magnitudes of displacements were less than 200 nm. Rafts in control cells (probe-cell contact without deformation) did not exhibit directionality of movement, and their angles relative to the horizontal were spread throughout the full 360 º range. Thus, membrane rafts sensed focal adhesion translocation by passively displacing towards the probe, and actively recoiled in a direction away from the probe. In addition, the magnitude of passive and active responses was greater the closer the rafts were to the site of FA deformation.

3.4.4 Rafts and talin rapidly and sequentially accumulate at new focal adhesions

The FN-functionalized electrode probe contacted the apical cell surface with the t=0 time point defined by a 2% drop of current through the electrode\(^{18}\) (final portion of current drop is shown in Figure 3-2 B). After contact, a rapid increase in GM1 accumulation occurred around the probe site followed by accumulation of talin (Figure 3-6 A). Within the first 5 seconds after contact, GM1 fluorescence rapidly increased and reached a plateau at 10 seconds (Figure 3-6 A, green group) that was 14±4.6% greater
than control (Figure 3-6 B, left). The control group assayed the GM1 intensities on the apical membrane in absence of probe cell contact. All intensities were normalized relative to their initial intensity. The characteristic time constant of GM1 increase was 1.7 sec, as calculated using an exponential recovery fitting model (not shown). In addition, rapid accumulation of GM1 coincided with the probe location and the surrounding area as illustrated in Figure 3-6 C. Kinetics of transfected RFP-talin accumulation after probe-cell contact was also assayed. Initially, talin did not increase near the probe (Figure 3-6 A). After 20 seconds, however, talin accumulated rapidly with a characteristic time constant of 0.57 ± 0.13 seconds and reached a plateau at 30 seconds with an increase of 2.26 ± 1.14 fold (Figure 3-6 B).
Figure 3-6: Kinetic response of membrane rafts and talin upon contact. (A) GM1 fluorescence accumulated with a time constant of 1.68 seconds, reached a plateau at 10 seconds. This increase was followed by accumulation of talin at 20 sec that reached a plateau at 30 seconds. (B) On average, GM1 accumulation increased 14±4.6% whereas talin increased by 2.26±1.14% (n=3). (C) (Top) GM1 accumulation around the probe (circle) is represented by 3-D intensity maps (Bottom).
3.4.5 Rafts and talin rapidly and sequentially accumulate after focal adhesion deformation

Upon 1µm apical focal adhesions deformation, GM1 accumulated by 15±3.8% within 5 seconds around the probe site followed by accumulation of talin (Figure 3-7 B). Increase in accumulation was measured relative to the control samples which assayed accumulation prior to deformation. The characteristic time constant of GM1 response upon deformation was 0.3 sec, as calculated by fitting an exponential recovery model to data in Figure 3-7 A (not shown). After the displacement neither the probe nor the cell were moved, and fluorescence was measured continually during the first minute. After 6 seconds, talin begins rapid accumulation reaching a 11 ± 3.1% increase with a characteristic time constant of 0.16 ± 0.04 seconds. Thus, GM1 accumulation occurred within milliseconds after focal adhesion deformation followed later by talin accumulation.
Figure 3-7: **Kinetic response of membrane rafts and talin upon deformation.** (A) Upon FA-deformation, GM1 accumulated with a characteristic time constant of 0.3 seconds and reached a plateau at 3 seconds followed by accumulation of talin at 7 sec reaching a plateau at 10 seconds. (B) GM1 accumulation increased 15± 3.8% whereas talin increased by 11± 3.1% (n=3).
3.4.6 Deformation induces long term accumulation of membrane rafts that is reversible

Over tens of minutes after deformation, GM1 accumulation occurred first around the probe site (t = 163, 357, and 1419 seconds) and continued to increase radially away from the probe (t = 2288 s; Figure 3-8A). The region of interest (indicated in the t=2288 time point in Figure 3-8 A) was analyzed for fluorescence intensity from t = 0 to t = 2288s. GM1 continuously and monotonically increased after deformation in and around the probe site (Figure 3-8 B and insets). On average, GM1 increased by 7.9 ± 1.6% from the 10 to 15 minute time points (Figure 3-8 C) relative to control raft intensities prior to deformation. After maintaining FA deformation for about 30 minutes, the nanoelectrode probe was brought back to its initial position. Removal of deformation was accompanied by a decrease of GM1 fluorescence (Figure 3-9 A) that was radially dependent (insets), and leveled off after 10 minutes. Average decrease in accumulation upon reversal was 41.4± 7.4% (Figure 3-9 B) relative to control GM1 intensity prior to reversal.
A

$\begin{align*}
\text{t = 0 s} & \quad \text{t = 67 s} & \quad \text{t = 163 s} \\
\text{t = 357 s} & \quad \text{t = 1419 s} & \quad \text{t = 2288 s}
\end{align*}$

B

![Graph showing the average fluorescence intensity over time]

C

![Bar chart comparing control and deformation]

- **CONTROL (10 min)**
- **DEFORMATION (10-15 min)**
Figure 3-8: **Deformation induces long term accumulation of membrane rafts.** (A) Selected frames of time-lapsed images of GM1 display six time points starting at $t = 0$ with initial and final locations of the probe (circles). GM1 accumulation continued to increase first around the probe site ($t = 163, 357, and 1419$ seconds) and continued to grow radially outward (away from the probe) and inward (inside the probe). (B) Long term kinetic response of GM1 accumulation exhibited a continuous increase; insets represent 3-D displaying of intensity and radial geometry of accumulation. (C) An average increase of $7.9±1.6\%$ (n=3) in intensity from the initial value was observed at time points ranging from 10 - 15 minutes after deformation.
Figure 3-9: **GM1 fluorescence decreases upon reversal of deformation.** (A) Reversal of GM1 accumulation was continuous and leveled off after 10 minutes. The radial decrease is depicted by the insets showing 3-D representation of GM1 fluorescence intensity around the probe site. (B) Average decrease in accumulation upon reversal was 41.4±7.4% (n=3).
3.5 DISCUSSION

The main findings of this study are that non-mobile rafts displace passively in response to focal adhesion deformation with magnitudes that decrease with increasing distance from the focal adhesion. Displaced rafts actively recoil back to their original positions, particularly for those rafts that exhibited initial larger displacements. For the region of the cell located behind the nucleus (relative to the probe location), the nucleus appears to prevent raft displacements. The mobile subset of rafts (also labeled by cholera toxin) rapidly accumulates after fibronectin has made contact with the cell, increases in accumulation upon focal adhesion deformation, continues to increase over 30 minutes of sustained deformation, and disperses when deformation is released. With respect to short time kinetics, rafts respond immediately to adhesion and deformation, while talin accumulates on the order of tens of seconds later. Thus mobile rafts participate in focal adhesion formation and reinforcement at very early times and prior to talin accumulation.

3.5.1 Mechanical Coupling from Focal Adhesions to Local and Remote Rafts

The observation that focal adhesion mechanical manipulation induces directional passive deformation of lipid rafts suggests that there is mechanical coupling between focal adhesions and rafts. Potential sources of this mechanical coupling include the plasma membrane spectrin (fodrin) submembranous cytoskeleton, and the internal
cytoskeleton composed of actin, microtubules, and intermediate filaments. It has been previously reported that lipid modifications such as glycosylphosphatidylinositol (GPI) anchors, palmitoylation, or myristoylation can target proteins to lipid rafts. \(^{42,64}\) In turn, GPI-anchored proteins, as well as integrins, are associated with the cytoskeleton. \(^{28,66,68}\) In addition, cytoskeletal components are linked to membrane rafts \(^{3,21,28,31,49,51,58,65,66}\) and membrane rafts may be necessary for the coupling of the membrane to the cytoskeleton \(^{44}\) as manipulation of membrane cholesterol content alters cytoskeleton-raft association. It is also known that when association of ezrin (which interacts with actin filaments via actin binding sites) with lipid rafts was decreased, the ability of lipid rafts to coalescence into larger signaling platforms was enhanced \(^{28,31}\) suggesting that rafts were freed from their cytoskeletal constraints. These indications that rafts are anchored are consistent with a hypothesis that rafts can mechanosense locally and remotely through their connection to the cytoskeleton. The fact that raft displacement decreased with increasing distance from the displaced focal adhesion suggests that this displacement was passive, much like the passive deformation of remote points to a point displacement in a large elastic sheet (results from finite element simulation, not shown). Additionally, the lack of displacement of rafts on the side of the nucleus opposite the displaced focal adhesion is consistent with passive deformation that would be expected if the nucleus was considerably stiffer than the rest of the cytoplasm. \(^{20}\) In summary, a subpopulation of anchored rafts responds locally and remotely with directional and spatial dependence to deformation of a focal adhesion. This mechanosensation is similar to other modes of
cytoskeleton-linked mechanosensation which occurs at long distances from the point of applied force\textsuperscript{35-37,41,71,72}.

A subset of membrane rafts recoiled within minutes after initial passive deformation. This recoil was in the direction away from the probe and was greatest for those rafts that experienced the greatest initial deformation. Furthermore, Figure 4 (A) illustrates raft recoil in a representative cell with a general orientation along the major axis of the cell and in a direction opposite to probe displacement. This behavior was evident regardless of whether the probe was pulled in a direction along the major or minor axis of the cell. Recoil of rafts was also observed to be parallel to the direction of displacement but in the opposite direction. One possible explanation for this phenomenon is that initial deformation worked against the action of a molecular motor clutch, such as an isoform of myosin, that was bound to the raft and cytoskeleton, and altered the directionality of the complex, resulting in motor activity being oriented in the direction opposite to the passive deformation\textsuperscript{10,12,18,26,34,45,54}. Previous studies have shown that resistance of the cell to pulling of the apical membrane using an optical trap may be due to the involvement of Src family kinases (SFK) in that SFK activation is vital for the fibronectin rigidity sensing process\textsuperscript{40,70}. However, we cannot discount the possibility that the recoil was passive and resulted from the detachment of a spring-like mechanism. Interestingly however, active cellular recoil has also been previously reported to occur within seconds in response to applied stress\textsuperscript{11} by a myosin-dependent mechanism,
suggesting that mechanisms of active recoil in response to mechanical stress exist in cells.

3.5.2 Dynamic Kinetic Response: Mobility of Membrane Rafts

Adhesion led to immediate recruitment of mobile membrane rafts. This coalescence occurred in the absence of force application and preceded the recruitment of talin, suggesting that recruitment of rafts occurs prior to the activation of integrins. Upon adhesion, membrane rafts are known to be involved in the aggregation of integrins, and upon integrin-mediated detachment, rafts internalize or dissolve upon integrin-related processes leading to the internalization and dissociation of lipid rafts. Upon detachment, the cell also triggers the release of phosphorylated caveolin from focal adhesions, which in turn permits its association with caveolae in order to induce the endocytosis of lipid rafts. Other studies have also observed that when cells detach, integrin-mediated processes lead to increased integrin avidity. We propose, therefore, that rafts participate in the reinforcement of focal adhesions after initial integrin ligation by coalescing additional integrins leading to increased integrin avidity. This coalescence of integrins allows the additional integrins to undergo activation and the cells to firmly adhere. In a previous study, we found that contact time needed to be sustained for at least 5 minutes suggesting that firm adhesion depended on the kinetics of integrin accumulation, which may be a diffusion-limited process. The observation that rafts accumulate before talin supports this hypothesis.
However, it remains to be determined whether integrin activation leads to raft coalescence or whether raft coalescence occurs first. However, raft coalescence increased with applied force suggesting that the membrane, with the integrated participation of lipid and integrin components, is an early mechanosensor.

Indications for the coalescence of rafts also come both from studies in model membranes as well as live cell studies. As the diffusion of membrane rafts are inhibited by initial integrin ligation by fibronectin, the membrane becomes more inhomogeneous leading to diffusion of lipids and GPI-anchored proteins down concentration gradients, which in turn focuses membrane constituents leading to enhanced bioactivity. Essentially, the dissociation and association of rafts in response to contact, deformation, and reversal of deformation, indicate that rafts are responsive to force within seconds and over tens of minutes. This kinetic spatial and temporal dependence of membrane raft mechanosensation demonstrates that the plasma membrane is involved in the formation and reinforcement of focal adhesions in response to force.

We further report that rafts increased in concentration in and around the focal adhesion upon deformation. This increase occurred with a time constant of 0.3 seconds and continued over tens of minutes as deformation was held constant. In addition, rafts dispersed after the deformation was released but adhesion was maintained. These results suggest that rafts participate in focal adhesion reinforcement upon force application. While raft association with focal adhesions has been documented, there do not appear to
be any reports that rafts participate in focal adhesion reinforcement upon force application. Currently the mechanism of this reinforcement remains unclear. While it is possible that the commonly implicated players in focal adhesion reinforcement, Rho, Rac, and actin, may play a role, these intracellular proteins generally are not thought to appear until minutes after force application\textsuperscript{29}. The time constant of 0.3 seconds for initial raft recruitment after force suggests that this phenomenon is one of the earliest reported and is comparable to the time constants for force-dependent ion channel activation\textsuperscript{50}. We propose that raft mobility and coalescence is a necessary component of focal adhesion reinforcement upon force application and provides an additional mechanism of force sensing by cells.
3.6 SUMMARY

In summary, we provide evidence that membrane rafts participate in focal adhesion development at sites of extracellular matrix–integrin attachment and that rafts respond to mechanical deformation of focal adhesions. Relatively non-mobile raft response was both passive and active and occurred in a spatially and directionally dependent manner. Highly mobile subpopulations of membrane rafts reversibly accumulated at the probe site in response to adhesion and deformation. Furthermore, GM1 accumulated significantly earlier than talin both upon contact and upon deformation. In conclusion, we have demonstrated direct mechanosensation of membrane rafts to mechanical perturbation of focal adhesions thus opening up a new avenue of investigation of mechanotransduction that focuses on force induced raft mobility and raft-dependent signaling.
3.7 REFERENCES


4.1 INTRODUCTION

Cell adhesion plays a role in regulating a wide variety of physiological processes ranging from tissue function to signaling across cell membranes \(^{28,33,54}\). The ability of cells to adhere to their surroundings through adhesion complexes involve a complex level of structural and molecular diversity \(^{32,54}\). Focal adhesion complexes \(^{21,47,53}\) have been shown to be induced not only on contact with ECM proteins \(^{15,18,19}\) but also upon application of force \(^{2,3,21,37,39,46}\). Among factors that influence adhesion are the duration and size of contact area, distribution of substrate, as well as membrane composition \(^{11,16,16,23,28,49,55}\).

The mechanism of adhesion and mechanosensation has many key players in addition to focal adhesions, one of which is the highly heterogeneous and dynamic plasma membrane. The composition of the plasma membrane inherently causes conformational changes in proteins but it’s also capable of sorting proteins through lipid domains such as rafts \(^{38}\). Membrane treatments have been shown to cause disruption not only of membrane proteins, but also changes in cytoskeletal organization \(^{17}\) cellular permeability, morphological changes, and local viscous domains \(^{14,20,35,36,41,42,50,57}\). Of interest to this study, previous work has shown that modification of cellular cholesterol
content changes the size of focal adhesion sites and adhesion area \(^{40}\). In addition, the existence and role of membrane fluctuations \(^{13,27,34,45,58}\) may have an adverse effect on adhesion. As such, for example, Brownian motion of nucleated cell envelopes has been shown to hinder adhesion \(^{58}\).

Being able to assess the real-time response of the cell as adhesion bonds are formed is a key piece to a better understanding of cellular adhesion. Our goal for this study was not to assess the formation of focal adhesions, which we have previously shown \(^{18,19}\), but rather to understand the dynamic response of the cell to the event of binding. In this study we seek to better understand the adhesion event and the role of membrane composition in adhesion. To achieve this, we used an optical trap to manipulate a functionalized bead and allowed it to make contact with the cell. The position of the bead was tracked before, during, and after making contact with the cell surface, thereby allowing us to observe the dynamic adhesion as the motion of the bead changed from being in a trapped state to an adhered state. We first characterized the distinct readouts of bead trajectories for a free bead, a trapped bead, and an adhered bead. Subsequently, the characteristic profiles of adhesion were found, suggesting that arrest of the bead occurs rapidly but in stages, as shown by the temporary initial binding followed by later final adhesion.

Furthermore, we sought to shed light on the role of membrane composition on adhesion upon binding by depleting the membrane of cholesterol as assaying for
adhesion. This treatment resulted in a decrease in binding time but an increase in the rate of adhesion, meaning that although the bead bound sooner in time, it took longer to change to an adhered state. Interestingly, we also observed that an increase in the standard deviation of functionalized bead positions was evident during initial bead cell contact. Finally, by assessing the adhesion event through the mean squared displacement of the bead, the results indicate that there is an increase in effective diffusion for functionalized beads upon contact, which may be a result of membrane fluctuations.
4.2 MATERIALS AND METHODS

4.2.1 Experimental Setup

A schematic representation of the experimental setup is shown in Figure 2-1. Bovine aortic endothelial cells (BAECs) were cultured on a temperature controlled chamber (Bioptechs, Butler, PA, USA) which was placed on a piezoelectric stage with resolution of 0.2 nm and 100 µm range of motion in x, y, and z directions (NanoView & NanoDrive, Mad City Labs, Madison, WI, USA). The stage position was controlled with a dedicated LabVIEW-based software program or by the Voxcell imaging software (Visitech International, Sunderland, UK). An inverted Olympus IX71 microscope with a 100 W halogen light provided brightfield illumination. Cells were imaged under DIC microscopy using a 60X PlanApo oil immersion objective, NA= 1.45 and a water-immersion condenser (0.90NA). Time-lapse images of the bead and cells were acquired using a high-resolution 12 bit CCD camera (Sensicam QE; Cooke Corp, MI) at 24.58 frames per second with exposure times of 15 ms with an image resolution of 123 nm/pixel. Fluorescence imaging of Alexa Fluor 568 labeled fibronectin functionalized beads was done with an oil-immersion objective (PlanAPO N 60X/1.45 NA, TIRFM-2, WD 0.15 mm) using a 2-D array multibeam laser confocal scanner VT-Infinity3 system (Visitech International, Sunderland, UK). A high-performance electron multiplying cooled charge-coupled device (EMCCD) digital imaging camera (Sensicam-EM; Cooke Corporation, Romulus, MI, USA) performed image capture.
Figure 4-1: **Experimental Setup.**
The optical trap (or laser tweezers) had as source an 850 nm 50 mW fiber coupled laser diode model iFLEX-1000 (Qioptiq, formerly Pointe Source, Rochester, NY). The laser path followed the fiber optic cable and exiting through a collimated diode at ~ 1 mm diameter, the laser was then reflected by two x-y steering mirrors, entered a compact beam expander consisting of two lenses which expanded and collimated the beam from 1 mm to ~8 mm in diameter, and then passed through a fine tuner and a shutter. The fine tuner was used to manually adjust the length of the beam expander, thereby adjusting the collimation, resulting in the ability to fine tune the focal point location of the trap. After exiting the shutter, the laser was reflected off a dichroic mirror (reflects wavelengths < 730 nm) and then entered the back aperture of the 60X 1.45 NA oil immersion objective, resulting in a highly focused trap.
4.2.2 Protein Labeling

Visualization of the fibronectin on beads was achieved by fluorescently labeling biotinylated fibronectin. Protein labeling was carried out with the Alexa Fluor 568 Protein Labeling Kit (Molecular Probes A10238). Fibronectin protein buffer exchange of Tris with DPBS was carried out using a desalting column (Thermo Fisher Scientific, Rockford, IL). All steps, except centrifugation, were carried out under sterile conditions inside a cell culture hood. The column was equilibrated with DPBS, and fibronectin was loaded in the center of the resin bed followed by centrifugation at 1,000 g for 2 minutes.

Alexa Fluor 568 labeling of fibronectin was carried out according to the manufacturer's protocol. Briefly, a 1M bicarbonate solution was prepared with ddH₂O. Fibronectin was diluted with DPBS to 2 mg/ml to which 50 μl of the bicarbonate solution was added. This solution was added to a vial of reactive dye and stirred for 1 h at room temperature. Protein purification was carried out using column exchange chromatography according to the manufacturer's protocol.
4.2.3 Fibronectin functionalization of polystyrene beads

Stable binding of fibronectin to polystyrene beads was achieved through use of the avidin biotin complex. Avidin coated yellow fluorescent polystyrene beads (SPHERO fluorescent particles, Spherotech Lake Forest, IL) of original concentration of 0.1% w/v were diluted by 8:1000 in 0.22 µm filtered water. Sterile fibronectin from bovine plasma (Sigma Aldrich, St. Louis, MO) was used at a concentration of 1 mg/mL in 0.5 M NaCl, 0.05 M Tris, pH 7.5. Biotinylated fibronectin was diluted with DPBS under sterile conditions to a concentration of 0.1 mg/ml.

All functionalization steps, including incubation, were carried out inside a cell culture hood under sterile conditions. Avidin coated beads were washed by pipetting up and down in sterile filtered ddH2O. After spinning down to remove the ddH2O, the beads were incubated with a 0.1 mg/mL labeled biotinylated fibronectin solution for one hour at 37 °C with gentle rocking. The bead solution was briefly spun down and the functionalized beads were resuspended in sterile DPBS or MCDB-131 cell culture media. The overall functionalization ensemble consists of, in order of attachment, polystyrene bead, avidin, biotin, fibronectin-Alexa Fluor 568 (Figure 4-2 A). Bead functionalization was validated using confocal fluorescence microscopy and DIC (Figure 4-2).
Figure 4-2: **Functionalization of 2 µm polystyrene beads and verification of protein labeling.** (A) Bead functionalization was achieved using avidin coated beads and Alexa Fluor 568-labeled biotinylated fibronectin. (B) DIC image of functionalized bead. (C) Fluorescence image of bead with Alexa Fluor 568-labeled fibronectin.
4.2.4 Cell Culture

All in vitro experiments were performed on bovine aortic endothelial cells (BAECs) (VEC technologies, Rensselaer, NY). BAECs were sub-cultured between passages 3-10 in T-25 flasks with MCDB-131 complete medium (VEC technologies, Rensselaer, NY) while maintained at 37°C in a gas mixture of 95% air and 5% CO₂ with 90% humidity. For imaging, the media was changed to HEPES buffered, phenol red-free DMEM media.

4.2.5 Cholesterol Depletion of Cell Membrane

All in vitro experiments were performed on bovine aortic endothelial cells (BAECs) (VEC technologies, Rensselaer, NY). BAECs were sub-cultured between passages 3-10 in T-25 flasks with MCDB-131 complete medium (VEC technologies, Rensselaer, NY) and maintained at 37°C in a gas mixture of 95% air and 5% CO₂ with 90% humidity. Endothelial cells were treated with methyl-β-cyclodextrin (MβCD) following established protocols. Briefly after rinsing with DMEM without serum, the cells were incubated for 1 hour in sterile 20 mM methyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO) in DMEM without serum and washed three times with serum-free DMEM. For imaging, the media was changed to HEPES buffered, phenol red-free DMEM media.
4.2.6 Functionalized bead-cell adhesion assays

Functionalized beads were positioned over the cell to be contacted, the bead was lowered to meet the apical surface of the cell, while monitoring the DIC image until the apical membrane in the region of interest came into focus (Figure 4-3). Time lapse images were collected before, during, and after contact. Imaging was done at 11.47 fps for images taken with a 15 ms exposure and 45 ms delay. Since the system had 8 GB memory limit, the delay was added to decrease the frame rate and increase the length of time images could be continually acquired. Before contact, a series of images were taken while the bead was held above the surface of the cell as depicted in Figure 4-3 (A). This initial time above the cell was equivalent to ~ 20 seconds (0.33 minutes). The bead was then lowered onto the surface of the cell during continuous imaging. Initial contact was assessed by noting that the apical membrane, as observed through the DIC image, came into focus as the bead was lowered. Care was taken to ensure that touching the membrane did not dislocate the bead from its axial position in the trap. Imaging was continual until the camera ran out of memory, spanning a total of 12,116 frames. Image files were then immediately exported and time lapse imaging continued in this periodic manner for a total time of >50 minutes resulting in a data set of > 36,000 points. Due to this memory limitation, the data sets for adhesion contain a gap corresponding to the time image files were being exported to disk.
Initial verification of bead cell binding was done by either turning the laser off and observing whether the bead remained adhered to the cell, or by attempting to lift the bead up and away from the cell (data not shown). If adhesion occurred, the bead remained on the surface of the cell. Further verification of binding was done post data collection through particle tracking.

Control experiments for functionalized binding were carried out using non-functionalized beads and followed the same procedure of imaging before, during, and after contact with the same image capture parameters. The non-functionalized beads were allowed to contact the surface of the cell for the same periods of time as functionalized beads, however upon verification of binding, beads did not exhibit adhesion as evidence by the fact that the bead freely diffused away from the cell when the trap was turned off (data not shown).

Higher image capture frequency was done to assay the standard deviation of bead position upon initial bead cell contact. Imaging was carried out before, during, and after contact at 24.58 fps with a 15 ms exposure with no delay. Before contact, a series of 500 images (~20 seconds) was captured. This initial time period was followed by trap-assisted bead binding (for 1000 frames), followed by continuous imaging for 3-5 minutes. Sample sets of n > 12 were collected per treatment of cell or bead condition.
Figure 4-3: **Adhesion assays using the optical trap.** (A) A trapped bead was brought above the surface of the cell. (B) 500 time lapsed images (equivalent to 20 seconds) were acquired during which the bead remained trapped above the surface of the cell. (C) The bead was brought down by the trap until it was allowed to make contact with the apical surface of the cell (D) Imaging was continuous during approach, contact, and subsequent periods during which the fibronectin functionalized bead formed stable adhesion to the endothelial cell.
4.2.7 Particle tracking and data processing

Two-dimensional particle tracking of beads in images was employed in order to measure the time course of adhesion. 2D trajectories of functionalized beads were tracked for beads that were free in solution, optically trapped, adhered to glass, or in contact with the apical surface of an endothelial cell. Bead trajectories were measured using an intensity weighted, centroid-based particle tracking algorithm custom-written in LabVIEW 7.0 (National Instruments, Austin, TX)\textsuperscript{12}. The program uses cross correlation-based tracking and the resulting tracked trajectories have a precision of 1-2 nm. A second LabVIEW program was used to calculate the centroid values of bead positions. Displacements of beads over time were then converted to time-averaged mean squared displacements (MSD), $\langle \Delta r^2(\tau) \rangle$ for various lag times $\tau$,

$$\langle \Delta r^2(\tau) \rangle = \left[ \langle (x(t + \tau) - x(t))^2 \rangle + \langle (y(t + \tau) - y(t))^2 \rangle \right]$$

(4-1)

Individual MSD vs. $\tau$ (Tau) plots were plotted for each case: a trapped bead, a bead contacting glass, and functionalized beads contacting cell that were untreated or treated with methyl-$\beta$-cyclodextrin to deplete cholesterol. Slopes for each lag time of MSD vs. $\tau$ plots were calculated for each individual repetition ($n>12$) per each of the five cases using a custom made LabVIEW program. Ensemble averaged slopes of MSD vs. $\tau$ of all cases were also calculated using LabVIEW and used to plot the change in slope of the MSD over time.
In addition, initial data processing of bead position centroid values was carried out in order to address thermal and mechanical drift inherent of the experimental setup. Drift and fluctuations in focus required periodic adjustment, causing peaks in the raw bead positions. Therefore, to eliminate this noise bead positions were smoothed using a moving average of 3 points. The running standard deviation of this data deviation was then calculated with a window of 10 points. This running standard of deviation was plotted vs. time and used as the parameter which monitored changes in bead state as a result of changing bead conditions.
4.3 RESULTS

4.3.1 Spatio-temporal characteristic profiles of bead positions exhibit distinct variances

Individual functionalized bead trajectories were tracked for three bead conditions: free in solution, optically trapped, and adhered to glass (Figure 4-4 A). Free bead trajectories had a standard deviation with a mean of $105 \pm 38$ nm, whereas a trapped bead had a mean of $27 \pm 8$ nm. In contrast an adhered bead had a standard deviation with a mean of $1 \pm 1$ nm. We can observe that not only does the mean of the standard deviation decrease as a bead changes state from free to trapped to adhered (Figure 4-4 B), but also the second order standard deviation (the standard deviation of the standard deviation as shown by the error bars of Figure 4-4 (B) decreases. These results suggest that the standard deviation of bead positions detectable with our experimental setup are sufficient to identify adhesion events as a bead transitions from a trapped state to an adhered bead.
Figure 4-4: **Spatio-temporal characteristic profiles of bead positions.** (A) Three spatial states of a fibronectin functionalized bead were tracked over time: a free bead in solution, an optically trapped bead, and an adhered bead onto glass. The standard deviations of the averaged bead position for all three cases vs. time have distinguishable profiles which characterize each state. (B) The mean of the standard deviation of bead positions was found to be significantly different, with values of 105 \( \pm \) 38 nm, 27 \( \pm \) 8 nm, and 0.9 \( \pm \) 0.7 nm for the free, trapped, and adhered bead respectively.
4.3.2 Adhesion profiles of initial and final binding

In order to form an adhesion, a biotinilated Alexa Fluor 568 labeled fibronectin functionalized bead was trapped, brought near the cell, and allowed to make contact with the apical surface. Time lapsed images were acquired, before, during, and after contact, in order to ascertain two dimensional bead trajectories over time. Binding, as assessed through the decrease in the standard deviation of bead positions, was observed to occur for the fibronectin coated beads for $t > 25$ minutes after contact. As can be seen in Figure 4-5, a bead in contact with a single endothelial cell has a starting profile similar to a trapped bead. During the first 13 minutes, the standard deviation of the bead in contact with the cell was $29 \pm 9$ nm, whereas when the bead is adhered to the cell (for times greater than 25 minutes) the standard deviation of the bead position is $3 \pm 1$ nm. Statistical significance was evaluated using a one-way ANOVA at the $p < 0.05$ level and the means were found to be significantly different. These profiles show the starting and ending points of adhesion of a fibronectin functionalized bead to a single endothelial cell.
Figure 4-5: **Adhesion profiles of initial and final binding.** (A) Standard deviation of the position of a functionalized bead in contact with the cell was initially observed to have a profile akin to a trapped bead, with a slightly higher mean of $29 \pm 9$ nm. This plateau was typically observed to span $t > 15$ min. In contrast, for $t > 25$ minutes, adhered bead profiles on the surface of the cell were observed to have a standard deviation mean of $3 \pm 1$ nm. Statistical significance was evaluated using a one-way ANOVA at the $p < 0.05$ level and the means were found to be significantly different. These initial and final binding profiles show the starting and ending points of adhesion of a fibronectin functionalized bead to a single endothelial cell. (Inset) Composite DIC and confocal fluorescence image of an Alexa Fluor 568 labeled fibronectin functionalized bead adhered to an endothelial cell.
4.3.3 Dynamic adhesion profiles

In order to follow the dynamics of adhesion, time lapsed images of the functionalized bead were acquired before, during, and after contact with the cell, spanning a total period of 50 minutes. The maximum number of images attainable at any one consecutive interval with our region of interest was 12,116, which with the settings in our system allowed for a total data collection time of 17.6 minutes. The gaps in data collection represent the time intervals during which this data was being exported to hard disk.

For the representative case of the fibronectin functionalized bead shown in Figure 4-6, after a plateau in the standard deviation of the bead position after contact, we observed an initial binding event at $t = 29.5$ minutes as shown by the rapid decrease in the standard deviation. Following this initial adhesion event, an increase in the standard deviation indicates that the initial binding event was temporary. Final adhesion was observed at $t > 43$ minutes as indicated by the stable decrease in the standard deviation.
Figure 4-6: **Dynamic adhesion profile of a fibronectin functionalized bead binding onto an endothelial cell.** Time lapsed images of a functionalized bead were taken before, during, and after contact and the standard deviation of the averaged bead positions was plotted vs. time. Initially a plateau in the standard deviation was observed during the first 20 minutes. Adhesion as assessed through a rapid decrease in the standard deviation was primarily observed at $t = 29.5$ min, and followed by a temporary increase, final adhesion was observed at $t > 43$ min. Note: Data points were collected in sets of 12,116 images, gaps in the data set represent intervals where image data was being exported.
Figure 4-7: **Three dimensional spatio-temporal representation of a functionalized bead binding to an endothelial cell.** Standard deviation of x and y bead positions plotted vs. time for a fibronectin (FN) functionalized bead binding to a cell. Standard deviation in both x and y positions decrease over time as the bead forms a stable adhesion to the cell surface.
4.3.4 Cholesterol depletion of membrane reduces adhesion time

Cholesterol depleted endothelial cell membranes were obtained by treating cells with methyl beta-cyclodextrin (MβCD) which stimulates cholesterol efflux. As previously mentioned, in order to follow the dynamics of adhesion, time lapsed images of the functionalized bead were acquired before, during, and after contact with the cell during a total period of 50 minutes. The standard of deviation of bead positions vs. time for the characteristic adhesion of a cholesterol depleted cell is plotted in Figure 4-8. Adhesion of the fibronectin functionalized bead onto the cholesterol depleted membrane was observed to initially occur at ~ t = 8 min and the standard deviation of bead positions continued to decrease steadily, eventually at t > 40 min a stable adhesion was formed. Note that as before, gaps in the data set correspond to periods of time when the time lapsed images were being exported.
Figure 4-8: **Effect of cholesterol depletion of the endothelial cell membrane changes adhesion profile.** Cells were treated with methyl beta-cyclodextrin (MβCD) to stimulate cholesterol efflux, thereby resulting in cholesterol depleted cell membranes. Adhesion of a fibronectin functionalized bead was observed to initially occur at $\sim t = 8$ min and the standard deviation of bead positions continued to decrease steadily. When $t > 40$ min a stable adhesion was formed.
4.3.5 Characteristic time of adhesion is dependent on membrane composition

A sigmoidal fit was carried out in order to find the characteristic time of adhesion of the fibronectin functionalized bead to the endothelial cell surface. For the case of the functionalized bead binding to an untreated endothelial cell, the fit converged after 50 iterations from which a half maximal effective concentration (EC50) was calculated. This value refers to the level at which a response was observed halfway between the baseline and maximum and occurred after an exposure time of 29.5 min (Figure 4-9). Similarly, a sigmoidal fit was also carried out to characterize the time constant for adhesion of a functionalized bead onto a cholesterol depleted endothelial cell treated with methyl beta-cyclodextrin (MβCD) (Figure 4-10). The characteristic time (EC50) for the MBCD-treated cell was 6.9 min. These results suggest that depleting cholesterol in cells reduces the time it takes for fibronectin-mediated adhesion.
Figure 4-9: The characteristic time of adhesion of a fibronectin functionalized bead to an endothelial cell. A sigmoidal fit was carried out to characterize the time constant for adhesion of a functionalized bead onto an endothelial cell. The half maximal effective concentration (EC50) indicates the time at which standard deviation was halfway between the baseline and maximum. In this example, EC50 was 29.5 min.
Figure 4-10: The characteristic time of adhesion of a fibronectin functionalized bead to a cholesterol depleted endothelial cell. A sigmoidal fit was carried out to characterize the time constant for adhesion of a functionalized bead onto an endothelial cell treated with methyl beta-cyclodextrin (MβCD). The half maximal effective concentration (EC50) corresponded to 6.9 min.
4.3.6 Standard deviation of fibronectin functionalized beads increases upon initial bead cell contact

An increase in the standard deviation of bead positions was observed upon initial bead cell contact. This increase was relative to the initial trajectory of a trapped bead above the cell prior to contact. Positioning of beads was carried out by placing the bead above the cell (but not making contact) and holding the bead in place for 500 frames (corresponding to \( t = 0 - 0.33 \) minutes). Therefore, each experiment has an initial trapped bead trajectory characteristic of the trapped bead above the cell surface. This initial period can be seen in Figure 4-11 A, where we can observe the three dimensional plot of both x and y bead positions over time as the bead is held above the cell surface. After this initial period the bead was brought down to contact the cell and the first 20 seconds of contact corresponded to frames 1000 - 1500. This places the initial 20 seconds of contact time at \( t = 0.67 - 1 \) min. Figure 4-11 B is a plot of precisely this initial period of bead cell contact. (Note: both panels (A) and (B) of Figure 4-11 represent the same experimental bead and cell but during different conditions of contact). In panel (B) we can observe that the range of bead positions is greater than in (A), in other words the variance of the bead positions after initial contact with the cell was observed to increase.
Figure 4-11: **Increased standard deviation in functionalized bead positions occurs upon bead cell contact.** (A) A trapped functionalized bead is held above the cell for 0.33 minutes and its x and y trajectories are plotted vs. time. (B) The bead is allowed to make initial contact with the cell at \( t > 0.67 \) minutes, and the initial period of bead cell contact is plotted for a period of 0.33 minutes. The range and variance of bead positions during this initial contact period was observed to be higher than the motion of the trapped bead above the cell.
Variance of bead positions increases upon bead cell contact. Five scenarios were compared and the variance of beads upon initial cell contact was found to be higher than control beads. First, the variance for a control trapped bead without contact had a mean of $1481 \pm 313$ nm, which was similar to the variance observed for a bead in contact with the glass whose variance had a mean of $1496 \pm 276$ nm. In contrast a bead in contact with the cell had an increased variance with a mean of $2343 \pm 482$. For fibronectin functionalized beads in contact with the cell for untreated and MβCD treated cells the variance was observed to be $2522 \pm 394$ nm and $2715 \pm 341$ nm respectively. For each scenario $n>10$. 
4.3.7 Mean square displacements of fibronectin functionalized beads increases upon bead cell contact

Mean squared displacements were calculated for each bead trajectory with a maximum tau value of 10 sec. The mean squared displacement was then plotted vs. Tau for individual lag times for a trapped bead as shown in Figure 4-13 A-K. For each different lag time (A-K), note that the slopes of the mean squared displacement vs. Tau plots are approximately linear. This linearity is characteristic of the brownian motion observed in a Newtonian fluid, in which the trapped bead was immersed. We can also observe that although linear, the slopes of each lag time change in magnitude.

Similar plots were generated for each individual bead state (trapped bead, bead contacting glass, bead contacting cell, FN bead contacting MβCD treated and untreated cell). A representative example of the mean squared displacement vs. Tau plots for a functionalized bead in contact with the cell is shown in Figure 4-14. As before, note that the slopes change for different lag times (A-K), however during the point of initial contact, which corresponds to 4-14 B we observed a marked increase in the slope.
Figure 4-13: **Trapped Bead Mean Square Displacements vs. Tau plotted for individual lag times.** Linear profiles were observed for the individual lag times where the Mean Squared Displacement was plotted vs. Tau, indicating the brownian motion of the trapped bead. Note also that for each lag time, a different MSD slope can be observed.
4.3.8 Ensemble average slopes of mean square displacements indicate fibronectin functionalized beads have a higher diffusion upon contact

Figure 4-14: Mean Square Displacements vs. Tau plotted for individual lag times of a fibronectin functionalized bead in contact with a MβCD treated cell. Linear and nonlinear(E,F) profiles were observed for the individual lag times where the Mean Squared Displacement was plotted vs. Tau. Notably there was in large increase in the slope of (B) which corresponds to the lag time when the bead was first in contact with the cell.

The individual slopes for each lag time for individual bead conditions were averaged and plotted vs. time. The resulting plot as seen in Figure 4-15, enables us to observe that for the second lag time, which corresponds to initial bead cell contact, there is a marked increase in slope for the fibronectin functionalized beads. This increase in slope of the MSD vs. time plot indicates that there is an associated increase in diffusion for both the fibronectin bead in contact with the cell and with a MβCD cell.
Figure 4-15: **Average slopes of Mean Squared Displacements of bead trajectories vs. time.** MSD vs. Tau plots of individual cells were averaged for each of five cases: a fibronectin functionalized bead in contact with a MβCD treated cell which had the highest increase in slope for the second lag time. A fibronectin bead in contact with the cell, also exhibiting a high slope for this early lag time. A bead in contact with the glass, a bead contacting the cell, and a trapped bead, all with varying but not significantly high slopes. The increase in the average slopes indicates that there is an increased effective diffusion for the fibronectin beads in contact with the cell irrespective of membrane treatment.
4.4 DISCUSSION

We have shown that cellular adhesion to extracellular matrix proteins is dynamic, dependent on membrane composition, and that initial adhesion events point to an increased effective diffusion. We have been able to track adhesion events with our system by being able to distinguish the states of the functionalized bead, and we have obtained characteristic profiles of three conditions of a functionalized bead: a free bead in solution, an optically trapped bead, and an adhered bead onto glass (Figure 4-4 A). With these profiles established, we were then able to follow the event of adhesion between a fibronectin functionalized bead and an endothelial cell, and observed the dynamic binding events which lead to a stable adhesion. In particular, we observed that initial adhesion for the example shown in Figure 4-4 C (which occurred at 29.5 minutes) was rapid but temporary, potentially indicating that the adhesion was not stable enough to overcome the brownian motion of the bead. The effect of brownian motion impeding adhesion has been previously seen in other systems. In our case, stable adhesion did not occur until after 43 minutes. To shed light on these results, we must consider the events occurring when the fibronectin bead is binding to the endothelial cell. First, it has been well established that fibronectin coated probes will lead to focal adhesion formation, second the formation of focal adhesions formation is a process that involves maturation of the focal adhesion complex from nascent focal complexes to stable focal adhesions. This process of adhesion is what this study seeks to better
understand. Although in the study we have not directly measured the presence of focal adhesions as a result of the fibronectin coated bead contacting the cell, we may infer that this process of bead adhesion involves formation of focal adhesions as has been previously seen when fibronectin coated beads⁵ or probes¹⁸,¹⁹ contact the cell surface. Initially the temporary adhesion may not be stable enough to overcome the motion of the bead, implying that either the focal adhesions being formed may involve weaker nascent focal complexes which are known to dissipate³²,53 and or that the more mature focal adhesions being formed are not strong enough to hold on to the bead and arrest its motion. It is also interesting to note, that the event of adhesion is rapid, once it occurs. This observation is consistent with our previous studies¹⁸,¹⁹ of talin kinetics which used a fibronectin functionalized nanoelectrode probe which contacted the cell, and we observed a rapid increase in talin accumulation, thus verifying focal adhesion formation. Both the present and previous studies provide an insight into the kinetics of adhesion and its dynamic nature. In this study we also observed that although the initial adhesion event was temporary, over time the cell is able to steadily bind the bead after 43 minutes as observed in the examples in Figure 4-2 A and Figure 4-3. This timing is consistent with previous studies which have placed the time of final adhesion of a bead at approximately 50-60 minutes. This timing is not in conflict with our previous studies¹⁸,¹⁹ which place the initial time of talin accumulation on the order of seconds, because in the present study we are not assessing the initial point of molecular kinetic accumulation, but rather the overall process and final adhesion of a bead with brownian motion. Thus, our data agrees with preceding studies²¹ that suggest that the initial adhesive complexes formed by
fibronectin receptors such as integrins translate into focal complex formation. We have observed that there is a transition between a temporary weaker adhesion and a more permanent one. This correlates with previous observations that the strength of focal complexes is less than that of focal adhesions. Principally, this study elucidates the dynamic nature of cellular adhesion.

We have also shown that the role of membrane composition, in particular cholesterol depletion, changes the characteristic adhesion profile and shortens the binding time for endothelial cells. As we observed in Figure 4-8 and 4-10, the half maximal effective concentration for the MβCD cholesterol depleted cell occurred at 6.9 minutes, though the rate of adhesion was not as rapid as for the untreated cell. This result is surprising since cholesterol is a principal component of membrane rafts. Membrane rafts are domains which dynamically combine and disperse to facilitate protein sequestration and protein-protein and protein-lipid interactions and modulate the clustering of integrins which regulate connections to the extracellular matrix via focal adhesions. It might therefore be expected that the disruption of rafts by depletion of cholesterol may inhibit the clustering of integrins thereby inhibiting focal adhesion formation and adhesion. However, the present study shows that there may be other mechanisms at play that may overcome this inhibition and reduce binding time despite cholesterol depletion. By depleting the membrane of cholesterol, membrane stiffness increases which then could decrease membrane undulations or increase the bending rigidity. Elucidating the role of the membrane in mechanosensation has shown
the evident relationship between membrane stress and membrane biophysical properties. Furthermore, cholesterol depletion has been shown to affect the interaction between the cytoskeleton and the membrane\textsuperscript{40} which thus increases traction stresses of the acto-myosin complex. The larger traction forces result in larger focal adhesions\textsuperscript{40} which may explain why we observe a shorter time for adhesion for cholesterol depleted cells.

The increase in variance upon initial bead-cell contact is another interesting finding, in that one would expect that binding events or interaction with the glycocalyx may dampen the brownian motion of the bead. A variance increase upon contact with the cell would have to account for a component with large enough fluctuations to affect the beads motion. Membrane fluctuations have been measured and observed in various cell types \textsuperscript{4,26,29,34,45,56}, and the oscillation amplitude ranges from 350 nm for human erythrocytes, to 20 nm for fibroblasts \textsuperscript{34}. Among various cellular activities which can cause height fluctuations are actin polymerization, actin-myosin-based contractions, the rearrangement of the structure from intermediate filaments under the plasma membrane, assembly or disassembly of large protein complexes, endocytotic and exocytotic activities, and intracellular transport \textsuperscript{30,34,44,56}.

Membrane fluctuations have also been shown to be ATP-dependent and correlated with the network structure of the underlying cytoskeleton\textsuperscript{43}, and can be related to the dynamic remodeling of the spectrin network. The junctional complexes of the spectrin network that join six spectrin polymers by a short, actin segment and protein-4.1
undergo ATP-induced remodeling taking the form of local associations and dissociations of spectrin filaments within the network or between the cytoskeleton and the lipid membrane\textsuperscript{27}. This remodeling of the cytoskeletal attachment would therefore result in a local release of the cytoskeleton-induced membrane tension thus resulting in local bilayer deformation on the scale of 83 nm\textsuperscript{27,43}. All of these previous studies provide evidence that the source, magnitude, and dynamic nature of membrane fluctuations may be the source of our observations. Although our system is not able to directly measure membrane fluctuations, this phenomena provides a possible mechanism for our observation that there is an initial increase in variance of the bead upon contact with the membrane.

We have also observed that the initial increase in displacement, as measured by the calculated mean squared displacement of bead positions, is temporary and as adhesion progresses the motion of the bead is quiesced. The mean squared displacement is related to diffusion, and for three dimensions is defined by the following equation:

$$\langle \bar{x}^2 \rangle = -\frac{6kT}{\gamma} \left( \frac{m}{\gamma} \left( \exp \left( \frac{-\gamma t}{m} \right) - 1 \right) - t \right)$$

where $\gamma$ is the friction coefficient, $\kappa$ is Boltzmann’s constant, $T$ is the temperature in Kelvin, and $t$ is time. For long times, or when

$$\left( \frac{m}{\gamma} \left( \exp \left( \frac{-\gamma t}{m} \right) - 1 \right) \right) \ll t$$
equation 4-2 can be simplified to

\[ \langle \bar{x}^2 \rangle = \frac{6kT}{\gamma} t \] (4-4)

recalling that the diffusion coefficient \( D \) is given by

\[ D = \frac{kT}{\gamma} \] (4-5)

equation 4-4 can be rewritten as

\[ \langle \bar{x}^2 \rangle = 6Dt \] (4-6)

and for two dimensions, the mean squared displacement is given by

\[ \langle \bar{x}^2 \rangle = 4Dt \] (4-7)

We can therefore observe that the relationship between the mean squared displacement with time (at long times) is proportional to the diffusion coefficient and is linear in time. In addition, when plotting the mean squared displacement vs. time, the slope is proportional to the diffusion coefficient.
Our results indicate that a significant change in the slope of the mean squared displacement occurs at the lag time when the functionalized bead contacts the cell as seen in the example plotted in Figure 4-14 B. This is in contrast to mean squared displacement plots of a trapped bead (as shown in Figure 4-13 B) where we observe no sudden changes in the magnitude of the slope. As previously discussed, we took an ensemble average of the slopes at each lag time for each of the five scenarios: a trapped bead, a bead contacting glass, and functionalized beads contacting the cell that were untreated or treated with methyl-β-cyclodextrin to deplete cholesterol (Figure 4-15). The results concur that there is an increase in the slopes of the mean square displacement when the functionalized bead makes contact with the cell, irrespective of cholesterol depletion. This increase in slope in turn may be linked to an increased diffusion of the functionalized bead when in initial contact with the membrane, resulting in a temporary eightfold increase of diffusion coefficient from approximately 100 nm$^2$/s to 800 nm$^2$/s. The source for this increase may be due to membrane fluctuations which as previously discussed play a dynamic role as these fluctuations may allow the cell to probe its environment both chemically and mechanically$^{45}$. 
4.5 SUMMARY

In summary, we sought to better understand focal adhesion formation. In order to do this we used as readout the position of a functionalized bead manipulated with an optical trap. We characterized trajectories of a free bead, a trapped bead, and an adhered bead. The characteristic profiles of binding were found for adhesion of a fibronectin functionalized bead to the cell. Binding appears to occur rapidly and in stages, as shown by the temporary initial binding and final adhesion at later time periods. We also wanted to shed light on the role of the membrane composition in binding, for which we used a treatment that depleted the cell of cholesterol. This treatment resulted in a decrease in binding time but an increase in the rate of adhesion suggesting that endothelial cellular adhesion to an extracellular matrix protein like fibronectin is cholesterol dependent. Interestingly, we also observed that an increase in the standard deviation of functionalized bead positions was evident during initial bead cell contact. The use of the mean squared displacement in elucidating the role of diffusion in the adhesion events indicates that there is an increase in effective diffusion for functionalized beads. These findings suggest that adhesion may be influenced by membrane fluctuations.
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Chapter 5

CONCLUSION AND FUTURE DIRECTIONS

5.1 CONCLUSION

The ability of cells to sense and respond to their environment is crucial to all life. Human endothelial cells, which line all of our blood vessels, have this ability to sense force and it is crucial to our vascular health. As such, the response of endothelial cells is linked to atherosclerosis and heart disease\textsuperscript{7,8}. To gain a better understanding of the ability of cells to sense and transduce their mechanical environment, it is imperative to study the cellular and molecular events in real time at the single cell level. New techniques and instrumentation are constantly allowing us to delve deeper into the dynamic response of single cells.

The work presented in this thesis is aimed at gaining a better understanding of the sensitivity of endothelial cells to their mechanical environment at the molecular and single cell level. We have introduced a new method for induction and manipulation of a nascent focal adhesion on a single endothelial cell using a functionalized nanoelectrode probe. By coupling this with high speed confocal microscopy, we have shown that it is now possible to assess the dynamics of assembly of the individual constituents of focal
adhesions from the moment of probe contact onward. This new capability should facilitate the development of new kinetic models of focal adhesion assembly in the presence and absence of force, thus opening up new areas of research in the integration of engineering principles of molecular transport and cell mechanics and biological sensing of the cellular microenvironment.

We have explored new avenues of mechanosensation that hypothesized mechanical coupling between membrane rafts and focal adhesions. The present work provides the first evidence that membrane rafts are mechanosensitive, thus uncovering a novel elusive mechanosensor. Furthermore we have shown that membrane rafts exhibit both passive and active response to deformation in a spatially and directionally dependent manner, and that there exist two subpopulations of rafts. Dynamics of the response of membrane rafts to deformation has provided us with evidence that rafts may be among the earliest responders to force and deformation. Having demonstrated direct mechanosensation of membrane rafts to mechanical perturbation of single FAs will open new areas of investigation of mechanotransduction that focuses on force-induced raft mobility and raft-dependent signaling.

The early dynamics of sensation have also been elucidated in this work as we sought to better understand the event of adhesion. Using as readout the bead position of a functionalized bead manipulated with an optical trap, dynamic characteristic profiles of binding were obtained. The observed decreases in binding time upon cholesterol depletion suggests that the membrane is involved in focal adhesion formation and
stability. In addition, we have also shown that during initial bead cell contact, an increase in mean squared displacement of functionalized bead positions was evident, indicating that for functionalized beads there is an increase in effective diffusion upon initial contact. These findings suggest that the cellular event of adhesion is highly dynamic and clearly dependent on membrane composition, thus opening the doors to new questions about what the role of the membrane is during the early stages of adhesion, and what role membrane fluctuations play in adhesion.

All together, the overarching contribution of this work has been to uncover that the cellular response to adhesion via focal adhesions is truly dynamic in nature in that the early events of adhesion reveal sequential dynamics of membrane rafts and focal adhesion proteins, that the membrane composition mediates adhesion formation, and that membrane fluctuations may govern adhesion of endothelial cells to their extracellular matrix.
5.2 FUTURE DIRECTIONS

The fact that the variance of the bead increased when it came in contact with the cell was not expected. However, this might be explained by membrane fluctuations. Membrane fluctuations could provide a mechanism of rapid protein sorting as certain proteins prefer certain membrane curvatures. Membrane fluctuations are controlled by the cell, suggesting that they are important for cellular health. The trap strength was typically on the order of 10 pN/µm and these fluctuations were on the order of 0.04 µm yielding a force by the membrane of approximately 0.4 pN. This very small force may not be sufficient to activate proteins, but it may be enough to regulate an adhesion event.

This suggests that a fruitful direction might be to assess the role of membrane fluctuations on adhesion. Such an investigation should involve the role of mechanical stress in lipid sorting and recruitment or dissipation of proteins associated with focal adhesion complexes such as talin, paxillin, actin, microtubules, zyxin, membrane rafts, and spectrin\(^1\)\(^4\)\(^,\)\(^,\)\(^,\)\(^6\)\(^,\)\(^,\)\(^10\)\(^,\)\(^,\)\(^12\)\(^,\)\(^,\)\(^14\). In addition, the interplay of these components needs to be addressed in order to have a more complete understanding of the dynamics of adhesion and mechanosensation. Measurement of the dynamic response of each of these components (using fluorescence confocal microscopy) to mechanical stress will require protein labels with high stable fluorescence lifetime so that one can obtain high frame rates and improved capture of early dynamic events upon stress or force application. Fluorophores need to withstand bleaching for longer term studies.
Moreover, discerning the role of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ in response to stimuli, as well as adhesion formation, would be another important component to consider. Previously, $\alpha_5\beta_1$ has been associated with influencing adhesion strength, while $\alpha_v\beta_3$ was associated with reinforcement in response to an applied force. Future studies may address the role of each integrin by blocking each integrin and repeating the adhesion assays carried out in this thesis. Inhibition of integrin $\alpha_5\beta_1$ may be carried out with inhibitory antibody against integrin $\alpha_5\beta_1$ (Thermo Fisher Scientific, Waltham, MA), whereas inhibition of $\alpha_v\beta_3$ may be obtained with the cyclic GPenRGDSPCA (GPen) peptide. In addition, functionalization with other ligands such as vitronectin which is known to bind to $\alpha_v\beta_3$, collagen which does not have specificity to either, and laminin which has integrin receptors dissimilar to fibronectin, would serve to decouple the effects of each integrin on the dynamics of adhesion.

Moreover, in order to allow membrane fluctuations to have a greater impact on bead position, experiments could be carried out by changing the current operating parameters in order to maximize bead displacement. The strength of the trap could be adjusted by changing the alignment of the laser in order to weaken the stabilization of the bead position by the trap, thereby allowing the potential effect of membrane fluctuations to have a higher impact on the bead variance. In conjunction, the size of the bead could be decreased in order to decrease the mass, thereby potentially increasing the variance of the bead in response to an equivalent membrane fluctuation. Changing ligands, as
previously mentioned, will also serve to decouple the potential increase in variance due to binding and unbinding events.

While fluctuations of the cell membrane could arise from sequential coupling and decoupling of the membrane from the spectrin cytoskeleton, the thermally driven motion of the thin membrane may be insufficient to significantly displace the much more massive 4.4 picogram bead. On the other hand, active pulsations of the cell surface could affect bead position as well as overall motor driven motions of the cell. Future directions could include modeling of the mechanics of adhesion between the bead and cell to address the role of bead/membrane/cell thermal motion and inertia on adhesion.

Key improvements in the setup should address significant drift and noise issues. These would include the ability to autofocus the system as coverslip drift was a significant problem that was overcome, to date, manually. Temperature stabilization of the objective and microscope is also needed in order to avoid thermal drift of the sample. These issues could be addressed with fiduciary markers on the surface of the sample in order to subtract out the noise as has been previously demonstrated. In addition, changing membrane composition with enrichment of cholesterol or depletion of it, as well as using cholesterol lowering drugs to further explore the effect on adhesion complexes would shed light on the role of membrane composition and whether it enhances or impedes adhesion via focal adhesion sites. Together, these new directions may lead us to continue the journey toward a better understanding of cellular adhesion and mechanosensation at the single cell level.
5.3 REFERENCES


# VITA

## DANIELA ELIZABETH FUENTES

### Education

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### Awards

- Best podium presentation-30th Annual Scientific BMES-SPRBM Conference 2012
- Outstanding Paper - Cellular and Molecular Bioengineering - Annual BMES Conference 2011
- Mary C. W. Fellowship 1999 - 2000
- Bunton Waller Fellowship 2006 - 2010

### Conferences / Presentations / Publications


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**Fuentes, D.E., Bae, C. and Butler, P.J.** "Nanoscale mechanical manipulation of the endothelial cell surface reveals mechanical coupling of membrane rafts." Biomedical Engineering Society Annual Meeting (BMES), Austin,TX. October 2010. (Oral Presentation).

### Experience

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