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Mechanotransduction of Matrix Stiffness Regulates Cell Adhesion Strength: An Analysis Using Biomaterial Surfaces with Tunable Mechanical and Chemical Properties

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Mechanotransduction of Matrix Stiffness Regulates Cell Adhesion Strength: An Analysis Using Biomaterial Surfaces with Tunable Mechanical and Chemical Properties

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering
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Keywords: Poly (dimethylsiloxane), Fibroblast, Focal adhesion, Wettability

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DEDICATION

This work is dedicated to my late father,

Lovely mom, Great husband,

And to my kids; the joy of my life,

Love you ALL.
ACKNOWLEDGMENTS

(Praise be to Allah through whose mercy all good things are accomplished)

It is still hard to believe that I have reached the end of my journey in USF studenthood, the goal that I left my family and country for, the dream that my late father wished to come true, the mission which was so tough and impossible in the beginning. It would not have been imaginable to do this work without the support from kind people surrounding me.

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Cells have the ability to sense the rigidity of the extracellular matrix which directly affects the control of cellular functions in development, wound healing and malignant transformation. Polydimethylsiloxane elastomers are useful model biomaterials for mechanotransduction studies because they possess several advantages including ease of fabrication, tunable elasticity and modifiable surface chemistry. In this work, we are investigating the influence of matrix stiffness on adhesion strength and the mechanosensory structures that regulate these processes. In addition, the effect of surface modifications to this elastic substrate system on other physical properties such as local stiffness and topography will be analyzed. Based on previous research, we hypothesized that cell adhesion dependent processes will be regulated by matrix stiffness, but that surface chemistry influences on protein adsorption could provide overriding regulatory signals. The results of this research will provide insight into the interconnected processes of mechanosensing and cell adhesion strengthening, and reveal criteria for designing instructive biomaterials with specific mechanical and chemical properties.
CHAPTER 1: INTRODUCTION

1.1 Research Significance

Fully understanding cell responses to the properties of biomaterials is an essential step in the development of new materials for medical applications. For instance, cells have the ability to sense the rigidity of the extracellular matrix which directly affects the control of cellular functions. Using biomaterials to mimic extracellular matrices (ECM), numerous studies have demonstrated that cell functions can be regulated by the manipulation of substrate parameters, such as substrate rigidity, chemistry, and topography. Changing biomaterial chemistry affects cell activity and cell growth phenomena via regulating protein adsorption and/ or conformation. On other hand; it has been agreed that controlling substrate topography allowed improving tissue repair and cellular engineering research, and basically this is a nanoscale change that affect cell-cell interaction. Recent research focused on the effect of substrate mechanical properties on cell function found that biomaterial rigidity controls cell adhesion and consequently controls cell morphology, differentiation, migration, and proliferation.

Cell adhesion to the extracellular matrix (ECM) is counted as crucial step in which this step could boost a cascade of multi cellular events that direct cell fate. Cell survival and function such as cell proliferation, differentiation, and migration are dominated by Integrin-mediated cell adhesion to the ECM. And even though substantial contributions have been made in identifying the practical components engaged in cell adhesion, there is still a gap in the understanding of the biomaterial factors that could regulate cell mechanotransduction (the processes when cells sense
the mechanical rigidity of a biomaterial and convert this sensing to biochemical signals that produce specific cellular responses).

To the best knowledge of the author, the relationship between mechanotransduction of matrix stiffness and cell adhesion strength has not been studied. In this work, tunable biomaterial with controllable stiffness and surface chemistry has been coated with specific ECM. Quantitative assays for adhesion strength and a morphological characteristic has been conducted to investigate this relationship.

This work is focused on using a model biomaterial system to investigate the relationship between mechanotransduction of matrix stiffness and the events that regulate cell adhesion to extracellular matrices. Both bulk mechanical properties and the surface properties of the biomaterial and its influences on protein adsorption and conformation were key factors in this study to understand structure-function relationships between adhesive interfaces and cell behavior.

1.2 Objective

The main objective of this doctoral dissertation research was to characterize a model biomaterial system and use it to investigate mechanotransduction events that regulate cell adhesion. The biomaterial properties, including surface chemistry, topography, and rigidity, were analyzed and their influences on protein adsorption, conformation, and cell spreading were quantified. The long term goal of this work was to understand structure-function relationships between adhesive interfaces and cell behavior so that cell instructive materials can be designed.

For this dissertation work, it was hypothesized that cell adhesion, an essential cell function required for survival and determination of cell fate, depends strongly on matrix
mechanical properties. The overall objective was accomplished by testing this central hypothesis through the following specific aims.

1.2.1 Aim 1

Identify the relationship between the mechanical properties of the biomaterial and the extent of polymer network crosslinking. To achieve this aim, several elastomer base to crosslinker weight ratios were prepared to produce a wide range of different biomaterial stiffness. Multiple mechanical testing methods that measured at a range of length scales were then applied to measure the elastic modulus of this biomaterial as a function of crosslinker percent. Afterwards, a model was fit to get a clear relationship between the elastic modulus of this biomaterial and the percentage of its crosslinker.

1.2.2 Aim 2

Modify the surface of the biomaterial to keep surface chemistry constant while varying the bulk mechanical stiffness. Thereby we aimed to independently analyze the effects of mechanical and chemical properties on cell adhesion. Chemical, mechanical, and topographic surface characterization methods were applied to confirm the achievement of this aim.

1.2.3 Aim 3

Examine the effect of matrix stiffness on protein adsorption and adhesion receptor binding. The adhesive protein fibronectin was adsorbed to all normal and modified biomaterial surfaces and then the effect of biomaterial properties on protein adsorption and conformation was analyzed. Since the conformation of fibronectin is known to regulate which specific adhesion receptors bind to it through its cell binding domain, an established assay that mimics receptor binding to the cell binding domain of fibronectin was employed. In addition, the
function of integrin receptor binding and actin-myosin contraction in regulating matrix stiffness-regulated adhesion was quantified.

1.2.4 Aim 4

Analyze the effect of substrate properties on cell adhesion and cell morphology using a stiffness-tunable silicone substrate. Mouse embryonic fibroblast cells were seeded on fibronectin-coated normal and modified biomaterial surfaces of different stiffness. Cell adhesion strength was quantified with a spinning disk device and cell spreading and morphology were analyzed with fluorescent microscopy.

1.3 Dissertation Summary

Chapter 1 and 2 of this dissertation provide the fundamental framework for conducting this research work as it represents a comprehensive investigation of extra- and intracellular elements that control mechanotransduction and cell adhesion. The significance and objectives of this research are clearly described in chapter 1, while a comprehensive review of literature that covers relevant topics including protein-biomaterial interaction, cell adhesion, and more is discussed in chapter 2.

Chapter 3 explains the change in silicone elastomer stiffness by changing base to elastomer weight ratio. A comparison between different mechanical testing methods and scales is made in this chapter where as a wide range of base to elastomer ratios were tested. After that a sigmoid model fit was created for each test to simplify the relation between silicone elastomer stiffness and the crosslinker percentage.

Chapter 4 describes how a specific surface modification was applied to the silicone surface to control its chemical surface properties, and after that normal and modified surfaces were characterized with a variety of techniques. The contact angle was measured for each
surface stiffness, atomic force microscopy (AFM) was used to identify changes in surface stiffness after surface modification, and last, the surface morphology was examined by measuring surface roughness of all surfaces.

Chapter 5 focuses on protein adsorption and conformation on the top of surfaces of different stiffness. A Quartz Crystal Microbalance with dissipation was used to observe the change in fibronectin adsorption on different PDMS stiffness of normal and modified surfaces. For protein conformation testing an antibody assay that mimics cell binding to fibronectin was used.

Chapter 6 focuses on the measurement of cell adhesion strength using a spinning disk device. This device applies a range of well characterized forces that are sufficient to detach strongly adhered cells on the matrix. The cell adhesion strength was measured as a function of time to fully cover the factors that affect cell adhesion. Cell morphology was also quantified after adhering cells reached steady state adhesion strength and morphology on top of the PDMS surface. A fluorescent microscope was used to measure cell spreading area and to compare this area as a function of PDMS stiffness and modification. Furthermore, cell contractility was inhibited to understand the role of mechanotransduction in adhesion strength. Focal adhesion assembly was also studied for normal cell seeding and with cell contractility inhibition.

Finally, in Chapter 7 a brief summary and conclusion of overall results of this dissertation work are presented.
CHAPTER 2: BACKGROUND

2.1 Matrix Adhesion Protein

The ex vivo assembly of specialized cells into tissues and organs is the ultimate goal of tissue engineering. To achieve the construction of complex tissues, the adhesion and communication of cells (the smallest basic unit of each living system) with each other and with their surroundings must be regulated\(^1\). The adhesion of cells with their surrounding protein scaffold, which is known as the extracellular matrix (ECM) is very important to provide a specific organization of these cells in the tissue. Moreover, cell adhesion is central to numerous basic cell functions, such as cell proliferation, migration, and differentiation\(^2\), figure2.1.

In addition to cell-ECM adhesion, some cells may be linked with each other at junctions between their plasma membranes. These junctions connect tissue cells and present a pericellular pathway that allows the diffusion of certain molecules at certain conditions across the cells, and provide channels of intercellular passage to exchange small ions and molecules between cells\(^1\). Cell-cell adhesion and cell-ECM adhesion are similar in some features. For example, cytoskeleton linkage is jointed in both of the adhesions, depends on the same signaling molecules, and uses the same protein families to control cell functions\(^2\).

Proteins, which consist of covalently connected amino acids are biological polymers that play an essential role in mediating a cell’s activities. The adsorption of proteins onto biomaterial surfaces is regulated by the properties of the material surface. In fact proteins are not penetrated through biomaterial, but rather accumulated and precipitated on the surface by electrostatic and
hydrophobic interactions, followed by secondary events including molecular spreading by conformational alterations\(^3,4\). Cell-material interactions are governed by these proteins and their structural conformation. In addition to the effect of protein primary structure on protein adsorption, studies verified that protein adsorption can be regulated as a function of biomaterial surface properties such as surface hydrophobicity, potential, topography, and composition\(^5-8\).

Thermodynamically, proteins are typically more favorable to adsorb on hydrophobic surfaces, in which the non-polar groups are more available\(^9-11\). Whereas for hydrophilic surfaces, in general proteins are less favorable to adsorb due to the presence of water molecules on these surfaces that are highly organized and tightly bound to the surface\(^12\). Therefore portions are subjected to conformational change to be adsorbed and overcome the energy obstacle. This change results in increasing the protein substrate contact by changing the tertiary structure of the protein. In general, a conformational change results in a more stable state rather than the initial dissolved stage and it can be highly affected by surface wettability. Furthermore, protein activity will be changed because of the conformational change in which the binding functionality sequence; i.e. cell binding domains such as the tripeptide RGD might be more exposed to the cells or hidden from them which will directly affect cell adhesion (figure 2.2)\(^10,13,14\).

### 2.2 Cell-Matrix Interaction

Cells rely on an intermediate adhesive layer to interact with any artificial substrate. This layer typically consists of adsorbed protein matrix such as adhesion proteins and growth factors. Previous study proved the functionality of this protein network in cell fate including the role of ECM in secretion and storage of growth factors proteins, support of cell adhesion, and transduction of cell signaling\(^15\). For instance, a variety of proteins exist in the basement membrane, the ECM underlying epithelial linings, including vitronectin, fibrinogen, fibronectin,
collagen I, collagen IV, and more\textsuperscript{16}. Moreover, basement membrane of endothelial cell comprises some ECM proteins as collagen IV, fibronectin, and laminin\textsuperscript{17}. Integrins are a type of transmembrane receptor protein with mechanosensory properties which identify and bind specifically to specific motifs with a certain sequence of amino acids such as arginine-glycine-aspartic acid (RGD) tripeptide found in several matrix proteins like laminin, fibronectin and vitronectin\textsuperscript{18}.

Cell adhesion strengthening theory was demonstrated as a process of two steps starting with initial integrin-ligand binding and followed by quick establishment\textsuperscript{19}. Previous studies reported that the initial step in cell adhesion strengthening involves three periods; first: the initial attachment and spreading, whereas the binding of integrin-ligand started and associated with an increase in cell-substrate contact area. Second, recruitment and increase in number of integrin-ligand bonds. And last, the interactions with cell cytoskeletal components, which include intracellular proteins that support the distribution of adhesive force in sites known as focal adhesion assemblies\textsuperscript{20-23}. These studies demonstrated the important functions of individual cell adhesion and spreading components. Gallant et al. (2005) examined cell adhesion strength and delivered a simple cell adhesion strengthening mechanism by using a spinning disk device\textsuperscript{24}. The spinning disk depends on applying a laminar force on cells attached to a biomaterial surface in order to compute their adhesion strength\textsuperscript{25}. Garcia and Gallant (2003) used a hydrodynamic shear assay in their study and found that cell adhesion started in the beginning with initial integrin binding and then followed by fast strengthening as a result of focal adhesion assembly creation\textsuperscript{26}. Gallant et al. (2005) stated that the area of cell adhesion and the adhesive time changes as a nonlinear relationship with the adhesion strength, where a controlled adhesive area and specific integrin binding were examined in their work, in addition to studying focal adhesion assembly.
sites\textsuperscript{24}. In 2007, Gallant and Garcia provided a simple mathematical model supporting their explanation of integrin distribution and clustering to create focal adhesion assembly\textsuperscript{27}. More recently, Elineni and Gallant (2011, 2014) showed that cell shape and the distribution of focal adhesion sites at peripheral locations enhance adhesion strength\textsuperscript{28,29}. Together these studies provide a picture of how cell interactions with the ECM and changes in their morphology regulate the strength of their adhesion.

2.3 Cell Adhesion Strength Measurements

Several quantitative assays for cell adhesion have been developed, and these can be classified according to the detachment force that is applied. These include centrifugation, hydrodynamic shear, and micromanipulation, and each has its specific advantages and limitations\textsuperscript{30}.

Centrifugation measurement applies a normal force that depends on difference in density between the cell and the medium. In this method, cells are seeded in a multiwell plate and then subjected to a centrifugation force, making sure that the top surface of the plate is facing out. The attached cells will detach as a result of applying normal force. The remaining cells are then counted by fluorescent imaging or radioactive labeling\textsuperscript{31}. This method is usually repeated more than one time with varied rotation speed because only a single force can be applied in each experiment. This assay is preferred when a comparison between different conditions of biomaterial treatment is required, but it is limited to small force range and used mostly to measure weak cell adhesion. This method is mostly used to measure cell adhesion strength during the initial period of adhesion\textsuperscript{19}.

Hydrodynamic shear assays are type of testing that includes the use of specific fluidic shear stresses on biomaterial surface that has been seeded with known cell density. In general,
there are many techniques that apply hydrodynamic shear on the attached cells, but typically three main kinds are used including radial flow chamber, the parallel plate flow chamber, and spinning disk device.

Radial Flow Chamber assay is one of this technique where the flow of hydrodynamic force is a radial flow created by the flow of fluid from chamber center to the outward wall over the attached cells. In this case, the maximum shear stress exists in the chamber center and decreases towards the chamber walls. This stress decreases nonlinearly with increasing circular distance from chamber center to the outward wall\textsuperscript{32}. Studies of mammalian cells adhesion were made using this configuration of radial flow chamber to investigate the effect of changing fibronectin protein concentration on mammalian cells adhesion\textsuperscript{33,34}.

Parallel Plate Flow Chamber is a method where a rubber gasket or PDMS channel is used to seal between two glass plates. A fluid with given flow speed is applied and a constant shear stress is gained along the length of the channel. While this shear stress is varied across the width relative to the size of the channel, this speed variation can be limited by using specific plate size. This method is characterized by the ability of live observation of cells detachment process by mounting the plates on a microscope\textsuperscript{35}. Different studies were made using this method including the characterization of fibroblasts cells of human skin while adhering on glass, and testing cell adhesion on cartilage and on dentin\textsuperscript{36-38}.

Spinning disk device assay is carried out by immersing adhered cells in a specific spinning buffer and rotating the disk at a prescribed speed. The result is a range of hydrodynamic shear stress on the attached cells proportional to the radial position. This stress is maximum on the outward of the attached sample and is minimum in the center of this sample. Cell adhesion strength is defined as the shear stress that is required to detach 50\% of the seeded cells. Due to
the ability to produce large detachment forces and apply a range of force in a single experiment, this method has been used in numerous studies to extensively investigate cell adhesion strength\textsuperscript{24,26,39-42}.

\textit{Micromanipulation} which is in contrast to studying cell population in hydrodynamic shear or centrifugation assays, in this method typically only one single cell is involved in the study. Two different techniques are usually used in the measurement; these are cytodetachment and micropipette aspiration. Atomic Force Microscope or other similar instruments are used in cytodetachment technique to measure cell adhesion force by recording the probe elastic deformation. On the other hand, cell adhesion force is measured by pressure aspiration in micropipette aspiration technique. There are also some other techniques that have been used to detect single cell mechanics such as, microplates and magnetic tweezers\textsuperscript{43-45}; however, each of these methods requires specialized equipment.

\textbf{2.4 Cell Adhesion on Biomaterial Surface in Vitro}

In vivo, most cell types require adhesion to the ECM to survive and function. Similarly, these cells need to attach and spread on matrix-coated biomaterial surfaces to survive and grow in vitro \textsuperscript{46}. This adhesion is modulated by integrin receptor binding to the adsorbed proteins; thus significant research has been conducted on how biomaterial surface properties such as surface wettability, stiffness, roughness, chemistry, and charge regulate protein adsorption and cell adhesion\textsuperscript{47,48}.

Proteins that are already available in the serum are adsorbed on the biomaterial surface before cells approach this surface. The amount and the conformation of adsorbed protein also play a significant role in cell adhesion and could control whether or not cells are able to attach. Cells use their integrins that are available in cell membrane to connect with ECM protein like
vitronectin and fibronectin. Despite years of research on protein-surface interactions, no universal models of adsorption have emerged. Further complicating matters, different cell types often have different responses to similar substrates.

2.4.1 Effect of Surface Wettability on Cell Adhesion

Proteins have a folded structure in solution to reduce their entropy that resulted from the interaction of water molecules with the hydrophobic side of protein chain. In general, the core of the folded protein consists of the hydrophobic non polar amino acid part of protein, while the external side is consisted of a polar acidic and basic part of the protein structure. The large quantity of the nonpolar groups of protein structure make proteins more favorable to be adsorbed on the surface of hydrophobic biomaterial. On hydrophilic surfaces, water molecules are bounded tightly on the surface resulting in a large energy barrier that prevents protein adsorption on the surface. As a result, the amount of proteins adsorbed on hydrophobic surfaces is typically higher when compared with that adsorbed in hydrophilic surfaces. Protein conformation changes occur to overcome the energy barrier that is connected with protein adsorption on hydrophilic surfaces. This conformation change results in an increased system entropy as the secondary structure of protein is changed and the contact area between this protein and biomaterial surface is increased. Surface wettability affects the final structure of adsorbed protein that undergoes a change in its structure during the adsorption process until reaching a stable state.

2.4.2 Effect of Surface Stiffness on Cell Adhesion

Previous studies reported that biomaterial stiffness has a significant role on cell traction force generation, migration and spreading. Typically, tunable polymeric biomaterials are used to investigate the effect of surface stiffness on cell response. These materials are
characterized by their ability of changing the bulk stiffness by changing the amount of crosslinking used such as polydimethylsiloxane and polyacrylamide gels. It has been demonstrated that cells adhere tightly and spread widely on stiffer biomaterial surface compared with soft surfaces\textsuperscript{56,58-60}. For soft biomaterial with elastic modulus in the range from 16 to 30 KPa, it is hard for cells to adhere and consequently could not spread well\textsuperscript{59,61}. In fact, cells usually start spreading when they attach to a substrate with similar elastic modulus. Therefore, a biomaterial with mechanical stiffness higher than the elastic modulus of cells is considered as an ideal biomaterial for cell adhesion and spreading\textsuperscript{59,61}.

2.4.3 Effect of Surface Roughness on Cell Adhesion

Surface topography can influence cell behavior as well. Cells are surrounded with a wide range of roughnesses in their natural environment; therefore a high surface roughness biomaterial could be related to better cell adhesion\textsuperscript{62-64}. This can be partially explained as an increase in surface area is provided with rougher surfaces than smooth surfaces, and this rough surface delivers more space for proteins to be adsorbed. At the nanoscale surface wettability may also vary with surface roughness\textsuperscript{65}. At larger length scales, cell orientation can be affected by topographical features. This phenomena is known as contact guidance. Studies have reported that cells choose the same orientation of surface groves\textsuperscript{66, 67}. In general, the effect of surface roughness on cell activities depends on cell type, as some kinds of cells prefer to adhere and spread on smooth surface rather than rough surfaces depending on their natural physiological environment\textsuperscript{68}.

2.5 Material of Interest

Polydimethylsiloxane (PDMS) is a synthetic silicone polymer, which has a wide range of uses in biomaterials research for several reasons including its biocompatibility and lack of
toxicity. It also has adequate mechanical stiffness for biological applications. The mechanical properties can be controlled by changing the weight percentage of the cross linker, curing time, and the curing temperature\textsuperscript{69-71}. PDMS is inexpensive, can be produced at low temperature polymerization, and it is easily fabricated into different shapes and sizes. PDMS is also considered as a flexible polymer with a SiO (CH\textsubscript{3})\textsubscript{2} repeating siloxane units (figure 2.3 and 2.4). All of these characteristics make PDMS biomaterial advantageous for many biomedical applications\textsuperscript{72,73}.

For instance, microfluidic devices have drawn great attention to PDMS because of its easy fabrication of small volumes for biological applications. However, PDMS displays some drawbacks including those small hydrophobic molecules tend to adsorb onto PDMS surfaces making it difficult to direct integrate electrodes on its surface\textsuperscript{74}.

The ability to tune the mechanical properties of PDMS provides an avenue to investigate the effect of mechanical stiffness on cell behavior\textsuperscript{75}. The influence of matrix stiffness has been established as a key modulator of cellular functions including stem cell differentiation, tissue regeneration, and gene expression\textsuperscript{76,77}.

Thus, model material systems that have manipulated mechanical and chemical properties are desired for mechanotransduction studies. There are many factors that can affect PDMS properties such as mixing time of base and crosslinker, curing time and temperature, base to crosslinking ratio, surfaces roughness, and surface modification\textsuperscript{78-80}. Significant understanding of how many parameters could change the PDMS properties will results in a deeper understanding of mechanobiology and advance the development of biomedical applications such as microfluidic and lab on a chip devices\textsuperscript{74,81,82}. 
2.5.1 PDMS Surface Modification

UV-radiation and gaseous plasmas, especially oxygen plasma are examples of the common high energy sources that have been used to increase PDMS hydrophilicity and promote biocompatibility\textsuperscript{83-85}. In this modification technique, bonds within the PDMS backbone are broken via the energetic photons, ions, or electrons that originate from the plasma. Any volatile organic species on the top surface of PDMS including carbon-containing fragments will leave, while any stable radicals and low-molecular weight polymer chains will stay on the PDMS’s surface. As a result, a recombination of silicon and oxygen radicals will happen through Si-O-Si bonding, building oxygen enriched silica-like layer on the top of PDMS surface that has different surface properties than the bulk PDMS\textsuperscript{86-88}. The surface wettability of silica-like layer is higher than normal PDMS surface, resulting in lower hydrophobicity and enhanced cell adhesion\textsuperscript{89}. Unless another treatment is subjected to the silica-like layer, this layer will not be permanent and the change in surface hydrophobicity will recover in several minutes after the plasma treatment. This recovery is usually a result of low molecular weight diffusion to the surface\textsuperscript{87,90,91}.

The change in surface composition of PDMS polymer by increasing oxygen content after plasma treatment has been examined with many different techniques such as: X-ray photoelectron spectroscopy (XPS), neutron refractometry, static secondary ion mass spectrometry (SSIMS), and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)\textsuperscript{86,92-94}.

Figure 2.3 shows the change in PDMS structure after plasma treatment, where three different layers are created. The third layer which is the bulk layer, stays the same and no change happened in this zone as it was not affected by plasma. The middle layer consists of low molecular weight fragments that resulted from the break of polymer backbone when carbon-
containing fragments left the surface. Lastly, the silica-like layer is located on the top of the PDMS surface. Minutes after plasma treatment, the low molecular weight fragments will diffuse to the surface leading to PDMS surface recovery.

![Diagram showing the effects of cell adhesion on other cell functions](image)

**Figure 2.1** Cell adhesion affects other cell functions
Figure 2.2 The effect of surface wettability on protein conformation and cell attachment
Figure 2.3 Chemical structure of PDMS

Figure 2.4 PDMS crosslinking
Figure 2.5 PDMS layer structure after plasma treatment
CHAPTER 3: CONTROLLING BULK ELASTIC MODULUS OF SILICONE ELASTOMER

3.1 Introduction

Polydimethylsiloxane (PDMS) is a synthetic silicone polymer, which is widely used in biomaterials research for several reasons, including biocompatibility and lack of toxicity. PDMS also has adequate mechanical stiffness for biological applications. PDMS mechanical properties can be controlled by changing the weight percentage of the crosslinker, curing time or temperature$^{69,95,96}$. PDMS is inexpensive and can be easily fabricated into different shapes and sizes, which makes it attractive for many biomedical applications$^{97,98}$.

Characterizing mechanical properties of cured PDMS is an essential step for using it in medical applications, since biomaterials mechanical properties have a notable effect on cells and tissues response$^{99,100}$. However, measuring the elastic modulus of PDMS is challenging for several reasons. First, many experimental factors can affect the measured data, such as the loading rate and the sample geometry. Second, during the tensile test PDMS undergoes large deformation under quite low load before its measured stiffness increases. Third, PDMS can be formed with stiffness gradients$^{101}$, or have its surface spatially modified$^{102}$, rendering it non-uniform and unsuitable for tensile testing. Furthermore, PDMS is a high surface energy material,

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thus the adhesion force between the PDMS surface and the tip during a compression test can affect the measurement.

This study focuses on measuring the elastic modulus of PDMS over a wide range of physiologically relevant stiffness, using compression testing at the macro and microscopic length scales. All the samples were prepared using the same procedure, and two different methods at different scales were used to confirm the change of PDMS elastic modulus as a function of the crosslinking percentage. The results were compared with the tensile tests, and the sigmoid stiffness dependence on crosslinking was obtained, providing a simple way to predict elastic modulus over a wide range of crosslinking percentage.

3.2 Theoretical and Background

3.2.1 The Macroscale Compression Model

There are multiple chemical and physical factors, which can affect the mechanical properties of PDMS, or limit the ability to measure these properties\textsuperscript{103-105}. Chemically all the samples should be prepared in the same way. The strain rate and the sample dimensions have an obvious effect on the measured elastic modulus with the macroscale test, which has been detected in previous work using the same compression device as in this study\textsuperscript{103}. In order to avoid the influence of the sample shape and the loading rate on the resulting data, an elastic half-space model has been used in this paper. This model was first offered by Lambe and Whitman in 1969, and consists of applying a constant compression load on top of a planar sample\textsuperscript{106}. In this model, a uniform load is applied over a small area of the soft sample. Sample thickness and the diameter of the loaded area should not be higher than the \(\frac{1}{4}\) of the diameter of the total tested surface\textsuperscript{107}. Normal force and displacement were recorded and used to solve the following equation for the elastic modulus\textsuperscript{108}:

\[ E = \frac{P}{4h} \left( \frac{d}{d+h} \right)^3 \]

\[ h \ll d \]

\[ E \approx \frac{P}{4h} \left( \frac{1}{d} \right)^3 \]

\[ E = \frac{P}{4h} \left( \frac{1}{d} \right)^3 \]
\[ E = \frac{2(1-\nu^2)qa}{w} \]

Here, \( E \) is the elastic modulus in MPa, \( \nu \) is the Poisson’s ratio (0.49 for PDMS) \(^{107}\), \( w \) is the recorded displacement, \( q \) is the applied load density, i.e. stress, and \( a \) is the radius of the circular contact area under load.

For comparison, PDMS strips were subjected to a macroscopic tensile test. Hooke’s law was used to calculate the elastic modulus:

\[ E = \frac{qL}{\Delta L} \]

Here, \( L \) is the initial sample length and \( \Delta L \) is the change of the sample length as a result of the applied stress, \( q \).

**3.2.2 The Nano-JKR Model**

For the microscale compression testing using an indenter, the measured stiffness is highly sensitive to the contact area between the probe and the sample. The surface energy of this soft material results in the adhesion force, which causes pull-in and pull-off events, obscuring the point of initial contact and thus affecting the contact area estimation. These factors present major challenges for the microscale characterization of soft materials, including PDMS \(^{109,110}\).

Ebenstein described a Johnson-Kendall-Roberts (nano-JKR) method using the spherical tip to overcome these limitations \(^{111}\). This model requires collecting the full loading and unloading force-displacement curve (Figure 3.1(a)). The spherical tip should be placed well above the sample surface, so that the full tip-surface interaction during the initial approach and loading is captured. The data collection should continue during the unloading to capture the tip pull-off event, until no force is sensed by the indenter. This model is known as the nano-JKR force curve and only requires two data points from the unloading portion of the curve to calculate
the reduced modulus, \( P_0 \) at \( \delta_0 \) and \( Padh \) at \( \delta_{adh} \) (Figure 3.1(a)). The first point is when the unloading force equals zero (\( P_0 \) and \( \delta_0 \)), whereas \( P \) is the applied force and \( \delta \) is the displacement. The second point is recorded when the unloading curve reaches the minimum force, which represents the adhesion force (\( Padh \) and \( \delta_{adh} \))\textsuperscript{112,113}. By knowing these two points, the following equation allows to calculate the reduced modulus, \( Er^{111} \):

\[
E_r = \frac{-0.95P_{adh}(\delta_0 - \delta_{adh})^{3/2}}{\sqrt{R}}
\]

here, \( R \) is the spherical tip radius, and the minus sign accounts for the measured negative pull-off force. Using the reduced modulus, \( Er \), and the Poisson’s ratio, \( \nu \), the elastic modulus, \( E \), can be calculated as:

\[
E = (1 - \nu^2)E_r
\]

The Nano-JKR force curve model is applicable only for materials with low elastic modulus and high surface energy, and a large diameter spherical probe tip is required (\( R \geq 30 \mu m \))\textsuperscript{112}. The special spherical tip with the 80 \( \mu m \) diameter was made for these measurements, shown in Figure 3.1(b).

3.3 Material and Methods

3.3.1 Sample Preparation

PDMS was purchased from Dow Corning Corporation as a kit of two components (Sylgard 184, Dow Corning Corporation, Midland, MI USA), prepolymer base and crosslinker. The components were mixed and cured to form the elastomer network. Five different PDMS base to elastomer weight ratios were tested in this experiment, 10:1, 11.5:1, 16.5:1, 20:1, 30:1, 40:1, and 50:1. These PDMS samples were prepared by well mixing of the polymer base with the crosslinker, and using different weight ratios of the curing agent to get different polymer
stiffness. It was manually mixed for 15 minutes for the higher cross linker amounts and 30 minutes for the lower cross linker amounts. All PDMS mixtures were degassed using a vacuum pump, and then poured over clean polystyrene Petri dishes. All the samples were about 1-2 mm thick. They were cured at 65 °C for 20-24 hours. Parallel samples from the same three preparations were used for all three methods. In some, but not all cases, identical samples were tested with both compression methods.

3.3.2 Macroscale Compression Test

In this study a custom-built load-displacement measuring device was used to run a compression test and measure the elastic modulus\textsuperscript{114}. PDMS samples were peeled from the Petri dishes and then subjected to a constant load of 20 g, and after 15 sec from loading the maximum displacements were recorded. By knowing the Poisson’s ratio of the PDMS samples (0.49), equation (1) was used to calculate the elastic modulus for each sample. Three different samples were tested for each stiffness and 5 different positions of each sample were loaded, then the average resulting value was calculated as the elastic modulus.

3.3.3 Macroscale Tensile Test

Pelham and Wang’s procedure was followed for tensile testing\textsuperscript{115}. The thickness of the PDMS samples that were subjected to tensile load was about 2 mm, while the cross section area of these samples ranged from $6.8 \times 10^{-6}$ m$^2$ to $1.6 \times 10^{-5}$ m$^2$. During tensile testing the force between 1 N and 9 N for the stiffer strips and between 0.5 N and 4 N for the softest samples was used with the maximum strain of 1.3. About 30 seconds after loading, a minimum of 5 displacements were recorded for PDMS samples stretched by the applied force, and the elastic modulus was estimated from the linear slope of the stress versus strain plot. Figure 3.2 represents schematic diagrams of macroscale compression and tensile test setups.
3.3.4 Microscale Nano-JKR Force Curve Test

Cured PDMS polymer was peeled and cut into 1x1 cm$^2$ square sample with a knife, and then placed on the nanoindenter stage. Hysitron Triboindenter (Hysitron, USA) was used for the nano-JKR experiments equipped with the custom-built spherical tip. To make the spherical tip, 80±2 µm borosilicate glass microsphere (Corpuscular, Cold Spring, NY) was glued onto the end of 1 cm long tungsten wire with 550 µm radius. The radii of the glass microsphere and the tungsten wire were measured using the Nikon Eclipse Ti-U microscope. The optical image of the tip is shown in Figure 3.1(b). The setup of this test is showed in figure 3.1(c).

According to a previous study, slow loading rate (≤100 nm/s) is preferred to provide accurate adhesion force measurements.$^{109}$ In this study all samples were loaded under constant rate of 60 nm/s, and the loading started from about 2 µm above the sample surface. Force-displacement curves were recorded for five different stiffness PDMS samples, and 3 samples were tested for each stiffness (crosslinking percentage).

3.4 Results

Measuring the elastic modulus of the PDMS samples of different stiffness is challenging because of its tendency for large deformation. The PDMS samples with the crosslinking ratio of 30:1 or higher were increasingly tacky. Thus, the maximum ratio tested was 50:1 base to crosslinker weight ratio. The minimum base to crosslinking ratio tested was 10:1. In general, most PDMS for biological applications is made with the weight ratios no higher than 10:1.$^{69}$ When the 5:1 and 2.5:1 ratios were tested at the macroscale, there were no noticeable differences between those samples and the 10:1 PDMS (data not shown). In fact, the elastic modulus of the higher base to crosslinking weight ratio was slightly less than the elastic modulus of the 10:1 PDMS, and close to the elastic modulus of 20:1 PDMS. This behavior has been reported
previously, presumably due to voids or inhomogeneities caused by the excess crosslinker\textsuperscript{116}. All measured elastic moduli were plotted as a function of the crosslinking percentage, which is the inverse of the base to the crosslinker weight ratio.

\subsection*{3.4.1 Macroscale Testing Results}

Consistent with the PDMS material stiffness increase by adding more crosslinker, there was a 10 to 85 fold increase in the elastic modulus when the crosslinking ratio increased from 1.96\% (50:1 PDMS) to 9\% (10:1 PDMS). Figures 3.3 and 3.4 present the data range of the compression test and tensile test results, respectively. The corresponding average values are listed in Tables 3.1 and 3.2 for compression and tensile tests respectively. 1.75±0.08 MPa was the maximum elastic modulus measured for the 10:1 PDMS, while the lower 0.17±0.009 MPa value of the elastic modulus was measured for the highest tested crosslinking ratio of 50:1. Tensile testing showed a similar trend, however, the elastic modulus increased over 85 fold from 0.018±0.0011 MPa to 1.545±0.122 MPa with the higher crosslinker percentage. This difference between the measured elastic properties in tension vs. compression can be attributed to the less compliant samples behaving more like a liquid rather than a solid in tension. While it is possible to measure water stiffness in hydrostatic compression, a tensile test on water would be challenging with larger contribution of the surface tension forces. Ashby argued that a true solid would have an elastic modulus above 1 GPa, thus the samples tested here do not qualify as the “true” solids, according to this approach\textsuperscript{117}.

\subsection*{3.4.2 Microscale Testing Results}

As expected, changing the testing scale and procedure, changed the resulting elastic modulus values. However, the change of PDMS stiffness still has a similar trend with the increasing crosslinking percentage (Figure 3.5 and Table 3.3). In general, the measured elastic
modulus values using the Nano-JKR force curve method are less than those measured with the macroscale tests, in which the elastic modulus of the 10:1 PDMS samples is $1.24 \pm 0.08$ MPa and for the 50:1 PDMS it is $0.1 \pm 0.02$ MPa.

### 3.5 Modulus Dependence on the Crosslinker Percentage

Wang et al. stated that the PDMS elastic modulus, $E$, in MPa can be estimated from the base to crosslinker weight ratio, $n$, as\textsuperscript{114}:

$$E = \frac{20}{n}$$

However, this approach can be used for PDMS with the narrow base to crosslinker weight ratio range, not higher than 10:1. The 10:1 is the optimal base to crosslinker weight ratio, and adding more crosslinker does not necessarily make the PDMS network stiffer, as would be predicted by equation (5).

In the macroscale test, it was obvious that the PDMS elastic modulus only slightly changed by increasing the crosslinking percentage more than 5%, which can be counted as the plateau region. In addition, below 2% crosslinking, the elastic modulus seems to be slightly affected by decreasing the crosslinking percentage. On the other hand, reducing the crosslinking percentage from 5% to 2.5% resulted in the elastic modulus decrease of about 9 fold. It can be inferred that the elastic modulus changed as a sigmoid function with respect to the crosslinking percentage. This non-linear behavior is explained by polymer gelation and network formation theory; specifically the distance between crosslinks and the extent of network formation\textsuperscript{118}. As crosslinks are introduced the polymer solution transitions from a liquid to a gel. Further increases lead to an interconnected network that behaves as an elastic solid with high failure strain (e.g. elastomers or rubbers). This increasing elastic behavior plateaus as the available crosslinking...
sites on the base polymer are saturated. For this reason, the Boltzmann equation was used to fit the sigmoid curve to the data in Figure 3.3:

\[
E = E_0 + \frac{E_1}{1 + \exp\left(\frac{X - X_0}{b}\right)}
\]

here, \(E\) is the PDMS elastic modulus in MPa at the crosslinking percentage \(X\); \(E_0\) is the minimum value of the elastic modulus, \(E_1\) is the maximum minus the minimum value of the elastic modulus (total elastic modulus range); \(X_0\) is the crosslinking percentage halfway between the highest and lowest value of the elastic modulus, and \(b\) is a constant related to the slope of the center portion of the curve.

For the macroscopic compression test data: \(E_1 = 1.68\) MPa, \(E_0 = 0.042\) MPa, \(X_0 = 3.49\), and \(b = 0.62\). The \(R^2\) value for the equation (6) fit equals 0.97. For the macroscopic tensile test data: \(E_1 = 1.51\) MPa, \(E_0 = 9.8 \times 10^{-10}\) MPa, \(X_0 = 5.8\), and \(b = 1.23\). The \(R^2\) value for the equation (6) fit equals 0.98.

The same sigmoid trend of the PDMS stiffness was observed with the microscale nano-JKR testing. However, slightly lower values of the elastic modulus were measured. Equation 6 can be also used for the microscale test to calculate the elastic modulus of PDMS samples at any stiffness, but with different values of the fitting parameter of equation (6). The Sigma Plot software version 11.2 was used to fit the data and calculate the corresponding parameters: \(E_1 = 1.16\) MPa, \(E_0 = 0.11\) MPa, \(X_0 = 4.66\), and \(b = 0.788\). The \(R^2\) value for the equation (6) fit is 0.96 (Figure 3.5).

**3.6 Discussion**

It is very important to identify the changes that would happen in the elastic modulus because of changing the base to crosslinker weight ratio, and measure it at different stiffness,
taking into account all the parameters that could affect the results. Generally, tensile testing is the gold standard to measure the elastic modulus, but it is not applicable for all materials, especially for characterization of biomaterials with gradient properties and/or materials with surface modification. For this reason, it was essential to find another testing method that can measure the elastic modulus of soft and tacky materials with high spatial resolution, and provide reliable data.

Using the Lambe and Whitman’s model and the nano-JKR force curve method is useful to avoid the effects of material shape and thickness. The distance between the tested surface and the probe has a great effect on the measured values, and this effect was totally avoided by applying the Lambe and Whitman’s model and the nano-JKR force curve method. The adhesion force between the PDMS samples and the indenter tip was considered only in the nano-JKR testing, while in the macroscale compression testing it was not, which resulted in higher elastic modulus values when using macroscale compression testing.

Even though the mechanical properties of PDMS polymer have been previously investigated, none of these studies focused on the change of the elastic modulus over a wider range of the crosslinking weight ratios\textsuperscript{119-121}. Most of the studies tested 2 or maximum 3 different stiffnesses, which is not enough to fully understand the effect of the crosslinking percentage on the elastic modulus of PDMS. In this study, working with a wider range of crosslinking ratios was beneficial to reveal the sigmoid trend of the PDMS elastic properties, and to predict the elastic modulus over a larger range of the crosslinking percentage using different test methods.

3.7 Conclusions

Two important points can be concluded from this study. First, PDMS material is stiffer under the macroscale compression test than the tensile and microscale tests. This may be a result of the adhesion force between the PDMS sample and the compressing tip, which was not taken
into account during the compression test. Thus, the macroscale compression test can be considered a good method to estimate the elastic modulus, but it is not sensitive enough to be used for the softer materials with the elastic modulus of less than 1 MPa. Second, these data show that the microscale nanoindentation results are close in magnitude and trend to the tensile test results for the stiffer samples, meaning that the nanoindentation nano-JKR test can be applied to measure the elastic modulus of PDMS material instead of the tensile test to avoid its difficulties. Nanoindentation also provides an easy way to measure the elastic modulus of PDMS samples with mechanical gradients, which cannot be achieved with regular tensile testing.
Figure 3.1 (a) Load-displacement indentation and pull-off curves for the 20:1 PDMS obtained with the spherical 80 µm diameter tip; (b) Optical image of the custom-made 80 µm diameter spherical tip; (c) test setup
Figure 3.2 Schematic diagrams of macroscale (a) compression and (b) tensile test setups.
Figure 3.3 The macroscale PDMS compression test elastic modulus results and the corresponding sigmoid cure fit.

<table>
<thead>
<tr>
<th>Base to crosslinker ratio</th>
<th>10:1</th>
<th>11.5:1</th>
<th>16.5:1</th>
<th>20:1</th>
<th>30:1</th>
<th>40:1</th>
<th>50:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crosslinking, wt%</td>
<td>9.00</td>
<td>8.00</td>
<td>6.00</td>
<td>4.76</td>
<td>3.22</td>
<td>2.43</td>
<td>1.96</td>
</tr>
<tr>
<td>$E$, MPa</td>
<td>1.75</td>
<td>1.74</td>
<td>1.72</td>
<td>1.70</td>
<td>0.83</td>
<td>0.33</td>
<td>0.17</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.08</td>
<td>.009</td>
<td>.011</td>
<td>0.05</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Figure 3.4 The macroscale PDMS tensile test elastic modulus results and the corresponding sigmoid cure fit

Table 3.2 PDMS macroscale tensile test elastic modulus results.

<table>
<thead>
<tr>
<th>Base to crosslinker ratio</th>
<th>10:1</th>
<th>11.5:1</th>
<th>16.5:1</th>
<th>20:1</th>
<th>30:1</th>
<th>40:1</th>
<th>50:1</th>
</tr>
</thead>
<tbody>
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<td>Crosslinking, wt%</td>
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<td>8.00</td>
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<td>4.76</td>
<td>3.22</td>
<td>2.43</td>
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<tr>
<td>$E$, MPa</td>
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<td>0.07</td>
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</tr>
</tbody>
</table>
Figure 3.5 The microscale PDMS nano-JKR test elastic modulus results and the corresponding sigmoid cure fit.

Table 3.3 PDMS microscale nano-JKR test elastic modulus results.

<table>
<thead>
<tr>
<th>Base to crosslinker ratio</th>
<th>10:1</th>
<th>11.5:1</th>
<th>16.5:1</th>
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<th>30:1</th>
<th>40:1</th>
<th>50:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crosslinking, wt%</td>
<td>9.00</td>
<td>8.00</td>
<td>6.00</td>
<td>4.76</td>
<td>3.22</td>
<td>2.43</td>
<td>1.96</td>
</tr>
<tr>
<td>$E$, MPa</td>
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<td>1.21</td>
<td>1.135</td>
<td>0.83</td>
<td>0.28</td>
<td>0.17</td>
<td>0.10</td>
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<tr>
<td>Standard deviation</td>
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<td>0.19</td>
<td>0.10</td>
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<td>0.03</td>
<td>0.02</td>
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</tbody>
</table>
CHAPTER 4: MATERIAL CHARACTERIZATION AND SURFACE MODIFICATION

4.1 Introduction

Numerous studies have demonstrated that cell functions can be regulated by the manipulation of substrate parameters that mimic their native matrices, such as substrate rigidity, chemistry, and topography. For example, changing the chemical composition of a biomaterial affects the growth rate of adhered cells because the protein adsorption is changed\122. It has been agreed that controlling nanoscale substrate topography improved tissue repair by affecting cell-cell interactions\123. Recent research focused on proving the effect of substrate mechanical properties on cell function, in which biomaterial rigidity controls cell adhesion and consequently controls cell morphology, differentiation, migration, and proliferation\69,78,124,125.\n
Since this work was dedicated to fully study the factors affecting cell adhesion to surfaces coated biomaterial matrix, surface wettability, rigidity, and roughness of the biomaterial were characterized in this chapter as a function of crosslinker ratio for normal and modified PDMS samples. Contact angle measurement; one of the simplest techniques to sense the change in surfaces chemistry\126,127 was used to detect any change in PDMS surface chemistry due to the change in base to crosslinking ratio. A deionized water droplet was used to measure surface contact angle over a clean PDMS surface with varied stiffnesses. In addition to changing the bulk properties of PDMS polymer due to changing crosslinking ratio, this test showed a significant change on PDMS surface wettability indicating a change in surface chemistry. In order to fully test the factors affecting cell adhesion, it was very important to quantify individual factors and
Avoid overlapping of the parameters that could result by changing the crosslinking ratio of PDMS, whereas both mechanical stiffness and surface wettability were changed. PDMS surfaces were modified to create a uniform monolayer with similar surface wettability regardless to the material stiffness. The contact angle measurements on modified PDMS surfaces proved the similarity in surface wettability with varied crosslinking ratios.

AFM indentation technique was used in this work to measure the elastic modulus of PDMS polymer of normal and modified surfaces. It was determined to be the best way to identify any change in the rigidity of modified thin surface layer because of its nanometer indentation depth. A nano-JKR model was applied in this part to avoid the measurement of adhesive force that is created between PDMS surface and the indenting tip.

Lastly, to identify the change in PDMS surface roughness, normal and modified PDMS surfaces were scanned by an Asylum MFP3D atomic force microscope to obtain surface topography information at all testing conditions. The different characterization approaches used in this work covered most of the parameters that could affect cell adhesion on PDMS surface; it was able to classify the parameters for good biomaterial model that can be used to enhance cell adhesion.

4.2 Experimental Work

4.2.1 Sample Preparation

A kit of Sylgard 184; silicone elastomer purchased from Dow Corning Corporation, was used in this work to prepare PDMS samples. PDMS elastomer base was mixed with the curing agent (crosslinker) with three different ratios (10:1, 30:1, and 50:1) to vary the PDMS stiffness. These ratios represent 9.00, 3.22, and 1.96 weight percent of crosslinker as described in the tables 3.1, 3.2, and 3.3. The standard ratio recommended by the manufacturer is 10:1. For the
PDMS formulations with higher amounts of cross linker (10:1 and 30:1), the components were mixed for 15 minutes, while 30 minutes of mixing was required in the case of low ratio of crosslinker (50:1) to have a homogeneous solution. The mixture was then degassed under vacuum until all air bubbles were removed. A specific volume of PDMS was poured on 25 mm glass cover slip and cured overnight at 65º C to obtain PDMS samples ready to use having 500 mm thickness.

Despite report to the contrary\textsuperscript{128}, varying the amount of crosslinker also alters the surface tension and changes the stiffness of the PDMS, thus at least two different factors that affect cell adhesion were changed, which would potentially confound the conclusions. For this reason, an approach to control the surface chemistry was adopted to decouple the effects of surface chemistry and bulk stiffness.

\textbf{4.2.2 Modified PDMS Surface}

For this purpose cured PDMS samples were subjected to oxygen plasma (Plasma Etch PE-50, 011810 1D-678, Carson City, NV) at 100 watts for 5 minutes to modify the surface chemistry. Immediately after plasma oxidation, the samples were exposed for 24 hours to vapor deposition of alkylsilaner (chlorodimethyloctylsilane-Sigma Aldrich) to assemble a uniform hydrophobic monolayer.

Plasma treatment results in the creation of a temporary silica like layer on the surface of PDMS, where low molecular weight chains diffuse to the PDMS surface and make a thin layer rich in oxygen and silicon\textsuperscript{129-131}. It has been reported that this layer has chemical, mechanical, and topography properties different than that of the bulk PDMS; it is a smooth layer of higher stiffness than normal PDMS. However, this change in surface properties recovers when those low molecular weight chains re-diffuse to the bulk. The surface properties of the PDMS surface
will then return to is the original state. Placing the treated PDMS samples in alkylsilane vapor after plasma treatment creates a covalent bond between the alkylsilane monolayer and the silica like PDMS surface layer preventing the diffusion based recovery\textsuperscript{132}, (figure 4.1).

\textbf{4.2.3 Measuring the Contact Angle of PDMS}

Basically; the contact angle is the angle made by a droplet of liquid resting on a plane straight solid surface (figure 4.2). This angle represents an energy balance between three different phases and is usually known as “three phase contact line”\textsuperscript{133}. If a liquid water droplet spreads on the top of the tested surface, a low contact angle (\(\theta\)) will result; indicating the wettability of this surface is favorable and then can be known as a hydrophilic surface. In contrast; if the water droplet beads on the tested surface it will generate a high contact angle (\(\theta\)) and the wettability of this surface is unfavorable and then can be known as a hydrophobic surface.

In fact, the contact angle created on the top of any material is a function of the surface tension of this material. This theory was developed in 1805 by Thomas Young\textsuperscript{134}, where he found that the angle made by liquid on the top of a flat surface is described by the mechanical balance of this droplet under the action of three interfacial tensions. This relation can be summarized by the following equation:

\[
\gamma_{lv} \cos \theta = \gamma_{sv} + \gamma_{sl}
\]

where; \(\gamma_{lv}\) is the liquid-vapor interfacial tensions, \(\gamma_{sv}\) is the solid-vapor interfacial tensions, and \(\gamma_{sl}\) is the solid-liquid interfacial tensions. It can be concluded from this equation that surfaces with high surface tension characterized by low wettability or a “hydrophilic surface”, while surfaces of low surface tension are characterized by low wettability or a “hydrophobic surface”.

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To measure water contact angles on PDMS in this work, a goniometer KSV CAM Optical Contact Angle and Pendant drop Sutface Tention software version 4.04 was used after calibration, and with Young / Laplace method to measure the static contact angle over normal and modified PDMS samples of different stiﬀnesses. A drop of deionized water of volume 0.9 – 2 µl was ditched on normal PDMS surface of different stiﬀness and the images of these droplets were captured for angle measurement.

4.2.4 Measuring the Elastic Modulus of Normal and Treated PDMS

The methods discussed in chapter 3 were used to accurately measure the bulk stiﬀness of the PDMS. These techniques do not operate at the scale of the surface modification using alkylsilane monolayers, thus we were concerned that if there were changes in the PDMS surface rigidity due to surface modification could not be detected. Moreover, cells “feel” the matrix on length scales in the nanometer to micrometer scale\textsuperscript{135-138}. Therefore, an AFM indentation technique was determined to be the best way to identify any change in the rigidity of modified thin surface layer because of its nanometer indentation depth. For accurate comparison, the elastic moduli of both normal and modified PDMS were measured with AFM.

For AFM indentation, the Nano-JKR model mentioned in the previous chapter was used, which is the most common model used to quantify the mechanical property of soft biological samples\textsuperscript{139,140}. In this test, a deflection-distance curve (DD curve) representing the tip-substrate interaction was recorded\textsuperscript{141,142}, (ﬁgure 4.3). Region A indicates a tip in its equilibrium state far from the surface. Region B is the approach zone, where the tip is moved toward the surface and starts to interact with it (jump-in). Region C is the region during indentation into the surface. Region D is the unloading curve. Regions E and F describe the stage where the tip breaks contact.
after adhesive forces are overcome by the elastic restoring forces of the cantilever and substrate (jump-off)\textsuperscript{141}.

Untreated silicon nitride cone tips (spring constant $K=3.36 \text{ N/m}$ and radius of 8 nm) were calibrated and used to indent normal and modified PDMS surfaces in air. The deflection-distance curve (DD curve) was recorded for 256 measurements that were repeated to create a force map over $20 \mu \text{m}^2$. The applied force was measured using equation (4.1)

$$F = \Delta Z \times K$$

where $\Delta Z$ is the jump-off deflection (vertical deflection), and $K$ is the spring constant.

Force-Displacement curve was formed to detect two important points: the applied force and the displacement of unloading plot at zero force ($P_0$ and $\delta_0$), and the applied force and displacement when the unloading curve reached the minimum force ($P_{\text{adh}}$ and $\delta_{\text{adh}}$)\textsuperscript{112,113}.

Finally, these points were used to measure the reduced Elastic Modulus as per the following equation (4-2)\textsuperscript{111}:

$$E_r = -0.95\frac{P_{\text{adh}}}{\sqrt{R}}\left(\delta_0 - \delta_{\text{adh}}\right)^{3/2}$$

here, $R$ is the spherical tip radius, the negative sign accounts for the measured negative pull-off force. Using the reduced modulus, $E_r$ and Poisson’s ratio $\nu$, the elastic modulus $E$ can be calculated as per equation 4.3:

$$E = \left(1-\nu^2\right)E_r$$

4.2.5 Measuring Surface Roughness

An Asylum MFP3D atomic force microscope (Asylum, USA) was used in this work to obtain surface topography scans of normal and modified PDMS using a $\mu$masch NSC 35 cantilever ($\mu$masch, DE). A mica surface was used to detect piezo response and the cantilever
spring constant following the thermal method\textsuperscript{143}. The spring constant was calculated to be 6.49 N/m with a resonant frequency of 186 kHz, and the scan measured over an area of 10 x 10 μm\textsuperscript{2} with scan rate of 2 Hz.

4.3 Results and Discussion

4.3.1 Contact Angle Measurement Results

For normal PDMS, it was found that increasing the crosslinker ratio from 50:1 to 10:1 results in decreasing the water contact angle by 30 °. These results indicate that there is a change in surface hydrophobicity that could affect protein adsorption and/or protein conformation and consequently will affect cell attachment. However the contact angle results for the modified PDMS showed that all samples with three different stiffnesses have almost the same surface contact angle (106 ° ± 1.55 °). These values are less hydrophobic when compared to the contact angle of normal PDMS samples of different stiffness. Table (4.1) summarizes the results of contact angle measurements of normal and modified PDMS with different stiffnesses together with an image of the water droplet.

Data of this table is blotted in the following curve (figure 4.4) as a contact angle versus crosslinker weight percentage to clarify the change of normal and modified PDMS contact angle as a function of crosslinker.

The change in normal PDMS surface hydrophobicity due to decreasing the amount of crosslinking could be related to the chemistry of crosslinker itself that might enhance the hydrophobicity of PDMS. As a result, the contact angle of normal PDMS samples was decreased by increasing the crosslinking ratio. Wang Z, Zhao Y, and Huang Z (2010) found in that there is a relation between surface tension of nano-scale material and the bulk elastic modulus\textsuperscript{144}. As
described above, the substrate contact angle is connected with the surface tension of the substrate.

Seghir and Arscott (2015) found a clear relationship between normal PDMS substrate elastic modulus and the surface contact angle. They found that increasing the base to elastomer weight ratio resulted in increasing the elastic modulus of PDMS and decreasing the contact angle of this surface. Seghir and Arscott established that the surface contact angle of PDMS proportionally decreases with increasing the crosslinking ratio\textsuperscript{145}. The results of Seghir and Arscott’s work are totally consistent with the results of this work.

In another study done by Eroshenko et al. (2013), it was shown that increasing the cross linker percentage of PDMS from 4.76 % to 16.6 % resulted in decreasing the surface contact angle by 10 degrees. It was also shown that the surface of stiff PDMS is less hydrophobic than that of soft PDMS\textsuperscript{146}.

The modification process of PDMS surfaces with different stiffnesses was utilized to develop an identical surface tension property with different base to elastomer weight ratios. And that will support to have clear assumption on the most important factors that might affect cell adhesion strength. The decrease in hydrophobicity of normal PDMS surface after plasma treatment was also reported by several studies\textsuperscript{147-151}.

Kim J et al. (2011) stated in their work that PDMS wettability can be controlled by oxygen/air plasma treatment, where this treatment resulted in a formation of hydroxyl groups that improve hydrogen bonding and therefore increase the overall PDMS surface free energy. As mentioned before, surfaces of high free surface energy are characterized by high wettability and low contact angle\textsuperscript{147}. These studies also reported that the reduction in modified PDMS surface contact angle after plasma treatment is not a stable condition, where this change in surface
property will be completed recovered by time. Hillborg and Gedde in (Hydrophobicity recovery of polydimethylsiloxane after exposure to corona discharges, 1998) demonstrated that hydrophobicity recovery of treated PDMS surface could be done by two different causes. The diffusion of low molecular chain of PDMS from the bulk of the polymer to the surface which will result in rebalancing the chemical composition of this surface is one possible cause, and/or the reorientation of side groups of the chain backbone on the surface, where the hydroxyl group (the most hydrophilic) will face the bulk material while the methyl group will face the surface could be the other cause. This sequence will clearly affect the surface wettability.

In order to control and establish the change in PDMS hydrophobicity after plasma treatment, studies showed that it is important to modify PDMS surface after this treatment. This is consistent with the results of this work, where the change on PDMS surface wettability was completely stable.

4.3.2 AFM Results

Modifying PDMS surface resulted in the creation of a thin layer with a different mechanical property, which required a nanoscale testing to sense the changes in these mechanical behaviors. Using AFM with Nano- JKR model ensured the measurement and determination of the elastic modulus of normal and modified PDMS surfaces and the detection of the change in PDMS stiffness after modification. However, the elastic modulus of the modified PDMS surface increased by 100% after treatment and the overall effect of changing the cross linker ratio still the same, where PDMS surfaces with higher amount of cross linker were stiffer than those samples of low cross linker ratio.

Results of measuring the elastic modulus of normal and modified PDMS surface are shown in figure (4.5) as a function of crosslinking weight percent.
As observed in the previous chapter, the elastic modulus of PDMS was increased from 0.08 MPa to 1.4 MPa as a result of increasing the crosslinker ratio from 50:1 (1.96 %, wt.) to 10:1 (9.00 %, wt.)

The results of this part also indicated that by modifying the PDMS surface a new surface layer having a different mechanical property was created, where the elastic modulus of modified PDMS surface was much higher than that for normal PDMS surface. Figure 4.6 represents a DD curve of normal and modified 50:1 PDMS. It is clear that there was a significant reduction in the adhesion force between the tested surface and the indenting tip. This indicated an increase in the mechanical stiffness of the tested sample. These results are consistent with previous studies related to the change in PDMS mechanical property due to plasma treatment, where PDMS surface became stiffer when subjected to oxygen plasma\textsuperscript{89,92,153}.

4.3.3 Results of Surface Roughness Measurements\textsuperscript{2}

In general, changing the base to elastomer weight ratio has no effect on PDMS surface roughness. The results of this measurement were ranged from 0.5 to 1.5 nm over an area of 100 \( \mu \text{m}^2 \). Table 4.2 shows surface topography images of normal and modified PDMS.

The same conception of surface roughness results was reported in previous studies, and it was concluded that changing the amount of crosslinker did not result in significant change of PDMS surface roughness that might affect cell response in biological applications\textsuperscript{154,155}. In general, cell activity can be controlled by changing surface roughness in the macroscale. At lower scales, cell activity will not be affected\textsuperscript{156,157}.

However, there are other studies that confirmed that focal adhesion controls cell adhesion and thus affect cell function in the range of 5-200 nm. This means that, any change in surface

\textsuperscript{2} Data of this this measurement has been collected by PhD candidate Michael C. Cross
roughness in this range will result in a change in cell adhesion$^{158,159}$. The change in surface roughness due to the change in crosslinking ratio and modification of PDMS surface was only 1 nm, this implies that this change in surface roughness of PDMS has no effect on cell adhesion.

### 4.4 Conclusion

There are many important points that can be concluded from the results of this chapter; surface contact angle of PDMS was affected by changing the crosslinking ratio; PDMS becomes more hydrophobic by decreasing the amount of crosslinker. Surface modification was an effective way to provide equivalent surface contact angle of different PDMS stiffnesses.

Bulk stiffness of PDMS can be controlled by changing base to elastomer weight ratio. In general; PDMS stiffness was increased by increasing the amount on crosslinker, while the surface stiffness can by doubled after surface modification.

Normal and modified PDMS surfaces were characterized with similar surface morphology which has no effect on cells activities.
Figure 4.1 Modified PDMS surface
Figure 4.2 Illustration of the contact angle created by liquid droplet on flat solid surface
Figure 4.3 Schematic represent deflection-displacement curve with its regions
### Table 4.1 Contact angle measurement of normal and modified PDMS

<table>
<thead>
<tr>
<th></th>
<th>10:1</th>
<th>30:1</th>
<th>50:1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Contact angle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(degree) Normal PDMS</td>
<td>111.4 ± 1.2</td>
<td>119.2 ± 2</td>
<td>132 ± 6</td>
</tr>
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<td><strong>Image of droplet</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>water Normal PDMS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Contact angle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(degree) Treated PDMS</td>
<td>104.4 ± 0.8</td>
<td>107.5 ± 1.2</td>
<td>106 ± 1.1</td>
</tr>
<tr>
<td><strong>Image of droplet</strong></td>
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<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>water Treated PDMS</td>
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</tbody>
</table>
Figure 4.4 The change in contact angle of normal and modified PDMS as a function of base to elastomer ratio (★ - p < 0.01).
Figure 4.5 Elastic Modulus measurement using AFM for normal and modified PDMS surface, $^{13}$

(★ - $p < 0.01$)

Figure 4.6 DD curve for normal (untreated) and modified (treated) 50:1 PDMS

$^{3}$ Data of this figure has been collected by PhD candidate Michael C. Cross
Table 4.2 Surface roughness results of normal and treated PDMS

<table>
<thead>
<tr>
<th></th>
<th>10:1</th>
<th>30:1</th>
<th>50:1</th>
<th>Color index nm</th>
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<tr>
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<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td></td>
</tr>
<tr>
<td>Modified PDMS</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td></td>
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</table>
CHAPTER 5: PROTEIN ADSORPTION AND CONFORMATION ON MODEL BIOMATERIAL MATRIX WITH TUNABLE PROPERTIES

5.1 Introduction

Proteins are the first elements that will interact with any biomaterial surfaces in vivo or in vitro biological conditions, and this interaction is considered to be a crucial process whereas it controls all subsequent reactions between an artificial surface and cells environment\textsuperscript{160}. For that reason, it was very essential to examine protein adsorption and conformation over all testing conditions of this dissertation work.

Fibronectin, which has a central role in cell adhesion and signaling, was used in this study as the adhesion mediator for cell enhancement to the biomaterial surface. In general, fibronectin can be found in two forms, as a soluble protein in blood plasma, and as insoluble protein in the connective tissue matrix such as that found in cell basement membrane\textsuperscript{161}. The functionality and the structure of fibronectin have been widely studied\textsuperscript{162-164} which made fibronectin an ideal protein to study cell response to a matrix coated biomaterial surface.

Proteins adsorbed at interfaces, a property that can be both a good advantage and a complicated process at the same time. The accumulation of proteins at a biomaterial surface has assisted the progress of varied biomedical applications, such as drug-delivery, immunological tests, and biosensors. In the biomaterial field, protein adsorption is much less desirable because it can stimulate contrary host responses such as blood coagulation and complement activation. In
contrast, cell adhesion to surfaces depends on the accessibility of specific protein-binding sites\textsuperscript{165}.

An enormous amount of research has been focused on protein adsorption phenomena on biomaterial surfaces. Exceptional focus was on the total amount of protein adsorbed, the kinetics of the adsorption process, the denaturing of adsorption, structure morphology, adsorbed layer thickness, and also on the protein conformation and orientation of protein molecules on the biomaterial surface. Basically, model proteins are used for adsorption studies, and these proteins were selected depending on several aspects including protein shape, stability, availability, and protein cost\textsuperscript{166}.

High performance liquid chromatography (HPLC), and other quantification techniques that involve coloring assays represent the simplest way to quantify protein adsorption, but all of these techniques required a sufficient large surface area to prevent protein sensitivity change which could affect the quantification accuracy. In addition, labeling assays could also have a conflicting effect on adsorbed protein structure\textsuperscript{167}. Measuring protein adsorption structure, orientation, and thickness can also be altered by drying\textsuperscript{168}.

Quartz crystal microbalance with dissipation (QCM –D) which is a unique and very sensitive technique; in nanogram scale, to a mass change was used in this study to inspect the change in fibronectin adsorption over normal and modified PDMS surfaces. QCM-D principles depend on sensing the change in oscillation frequency over Quartz crystal by piezoelectric, this change in frequency (\(\Delta f\)) is a result of mass change on the top of Quartz sensor. QCM-D also detects the change in surface dissipation (\(\Delta D\)) due to a change in damping characteristics of the sensor surface which is connected to the change in viscoelastic property of the sensor as a result of protein adsorbed on top of this surface\textsuperscript{169}. Sauerbrey (1959) found that mass changed on a
rigid surface is proportional to the change of the sensor frequency\textsuperscript{170}, and this change in frequency can be real time monitored with QCM-D. The change in surface dissipation after protein adsorption indicated a change in viscoelastic and hydration effects of the adsorbed layer of proteins\textsuperscript{171,172}

The procedure of using QCM-D to examine protein adsorption on biomaterial surface required coating quartz sensor with a thin layer of tested biomaterial (Nanoscale), and then running a buffer solution with specific flowing rate until having no change in frequency and dissipation; baseline in figure 5.1 region (A), followed by running a protein solution with certain concentration and flow rate as the change in frequency and dissipation are still monitored for a specific time or until the change on both sensor frequency and dissipation is negligible, region (B), and lastly the sensor should be rinsed with that same buffer solution, region (C).

In fact proteins are not penetrated through biomaterial, but they are accumulated and precipitated on the surface by electrostatic and hydrophobic interactions, followed by protein molecules spreading by conformational alterations\textsuperscript{3,4}. Cell-material interactions are governed by these proteins and their structural conformation. It was understandable that cells respond differently if the substrate properties were changed. Studies have stated that protein adsorption and conformation can be highly impacted by changing surface properties, and consequently cells adhesion and morphology will be affected. It has been proven the proteins prefer to adsorb over a hydrophobic surfaces rather than a hydrophilic surface with different conformational aspect\textsuperscript{173-176}. To investigate the effect of changing substrate rigidity on fibronectin conformation, Keselowsky et al procedure will be followed\textsuperscript{177}. In this experiment, a specific antibody for fibronectin cell binding is needed, thus a mouse anti human monoclonal antibody HFN 7.1 will be used to mimic cell binds affinity\textsuperscript{178,179}. This antibody binding to the cell binding domain of
fibronectin similar to α5β1 integrin, the affinity of HFN7.1 for cell binding domain is regulated by the conformation of the fibronectin which affect the relative arrangement of RGP and YRN motifs.

5.2 Experimental Work

5.2.1 Experimental Work for Protein Adsorption

5.2.1.1 Instrument and Sensor Coating

The Q-Sense E4 System was used in this work, which provides control working temperature between 15 to 65 C. Gold coated quartz sense with a frequency of 4.9 HZ was used, and this sensor was 14 mm in diameter (figure 5.2).

To create a PDMS thin uniform layer on top of the gold sensor, a spin coating device was used with a one-step spin program. A filtered mixture of 2% PDMS in Toluene was prepared to ensure having thin film\textsuperscript{180}. The coating process was applied for 15 seconds with a speed of 750 RPM. In order to get a uniform layer that covered the entire surface, a low acceleration rate was used (50 RPM/S). After that, the created layer of PDMS was cured in oven for overnight at 65° C, and then subjected for plasma treatment and saline coating in case to test protein adsorption on modified PDMS surface.

5.2.1.2 Cleaning Protocol

1- cleaning sensor prior to experiment: A cleaning solution was made by using ammonia (25%), Hydrogen peroxide (30%), and milliQ water with ratio of 1:1:5 . This solution was heated to 75 C before inserting the sensor. A teflon holder was used to hold the sensor in a stable position while the sensor was fully submerged in sufficient amount of cleaning solution for 5 minutes (figure 5.3). After that, the sensor was rinsed immediately with milliQ water then dried
with nitrogen gas. Then the sensor was placed in UV/O for 10 minutes to ensure removal of any organic contaminants from the top of the PDMS surface.

2- Cleaning the sensor after experiment: After testing the ability of reusing the sensor for another experiment, it was found that it is better to create a new PDMS surface for each experiment. For that reason, the used sensors were sonicated in toluene and milliQ water solution with a ratio of 1:1 for 10 minutes to remove the previous PDMS film, and then dried with Nitrogen gas.

3- Cleaning the chamber before running the experiment: A specific sensor was inserted in the chamber before running any experiments in order to clean the used chamber. A 2% Sodium dodecyl sulfate (SDS) in MilliQ water solution was pumped in the chamber with a flow rate of 100 µl/min, this process was stopped once the collecting running SDS solution was 2 ml or higher. Then a minimum of 6 ml of milliQ water was pumped in the chamber to rinse it. After that the attached sensor was taken from the chamber and dried using a Nitrogen gas. Then the clean coated sensor was attached in the clean chamber.

5.2.1.3 Working Conditions

The working temperature was fixed at 25 C. A buffer solution, in spicific Dulbecco’s phosphate buffered saline with Calcium and Magnesium (DPBS, Invitrogen; PBS +/-) was pumped in the chamber with a flow rate of 100 µl/min while the change in sensor frequency and dissipation was monitoring. Once there was no more change in these two parameters, the running solution was changed to protein solution (fibronectin 10 µg/mL in PBS +/-) for 30 minutes. After that, the buffer solution was running again. This procedure was followed for all coated sensors with different PDMS stiffness and for both treated and untreated conditions.
The change in dissipation was plotted versus the change in sensor frequency to compare the change in fibronectin adsorption on the different PDMS stiffness of normal and modified conditions (figure 5.4).

5.2.2 Experimental Work for Protein Conformation

In order to compare fibronectin conformation adsorbed on a normal and modified PDMS, PDMS layers were cured in Falcon 96 well plates. A sufficient amount of PDMS mixture; of different stiffness, was poured in the same way on two different plates of selected wells and then cured overnight at 65º C. In order to distinguish between normal and modified PDMS surfaces, one plate was subjected to plasma treatment for 5 minutes followed by an overnight saline vapor deposition. After that these plates were ready for protein conformation.

100 µl of ethanol was added in each well covered with PDMS to clean the tested surfaces. The ethanol was allowed to sit for 5 minutes on the PDMS and it was mechanically removed. 100 µl of Dulbecco’s phosphate buffered saline without Calcium nor Magnesium (DPBS, Invitrogen; PBS -/-) was added, allowed to sit for 5 minutes and it was mechanically removed. Solution of human plasma fibronectin (Gibco, Invitrogen) in PBS +/- was prepared with a concentration of 10 µg/ml. All PDMS surfaces were covered with a 100 µl of fibronectin solution for 30 minutes. Followed by another 100 µl of 1% bovine serum albumin (BSA, Fisher Scientific; 1% BSA) for 30 minutes, and then the 1% BSA was mechanically removed to add the primary antibody.

A blocking solution made of 1% BSA and 0.05% Tween 20 was used to dilute the primary antibody HFN7.1 (Developmental Studies Hybridoma Bank, Iowa City, IA) with a ratio of 1:10000. 100 µl of primary antibody was added to each well of PDMS for the two plates, and then these plates were incubated at 37º C for 1 hour. After removing the HFN7.1 solution, the
PDMS surfaces were rinsed 3 times with blocking solution 5 minutes at each time to ensure removing all unbounded antibody. A 100 µl of as goat anti-mouse conjugated with alkaline phosphatase secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was added to PDMS wells and incubated for 1 hour at 37º C. To remove all residual unbinding antibody, the PDMS surfaces were rinsed 3 times with blocking buffer for 5 minutes at each time. Lastly, a 200 µl of p-nitrophenylphosphate (pNPP) (Sigma Aldrich) was added as a substrate to each well and to other 3 empty wells to be negative control samples, then the plates were incubated at 37º C for 45 minutes. After that a microplate spectrophotometer (Biotek, Winooski, VT) was used to measure the adsorption at 405 nm (figure 5.5).

5.3 Results and Discussion

5.3.1 Results of Protein Adsorption

Since cells interactions with biomaterial surfaces can be mediated by pre-coating the surface of the substrate with human plasma fibronectin, fibronectin adsorption on normal and modified PDMS surfaces of different stiffness were investigated in this dissertation work. From protein adsorption experiment, the change in sensor frequency and dissipation due to the adsorbing fibronectin was recorded. The change in sensor frequency represents a change in the amount of adsorbed fibronectin, while the change in sensor dissipation represents a change in the viscoelasticity of the adsorbed layer. For clear comparison between the effect of PDMS stiffness and PDMS surface modification, a plot of the change of frequency vs. the change in dissipation for all tested surfaces was created, and plotted in figure 5.6 for normal (N) and modified (M) PDMS. Even though samples with n=3 were used in this test; figure 5.6 shows data for only one sample of each condition to avoid data overlapping. Table 5.1 provides the standard deviations of the change in sensor frequency and dissipation which were negligible.
It is obvious from these results that the amount of adsorbed fibronectin was almost the same on PDMS surfaces with different stiffness and surface conditions; whereas the change in sensor frequency was close for all testing conditions. For normal PDMS surface; only the softest PDMS (50:1) showed about 20% reduction in frequency change and this indicated that fibronectin adsorbed in less amount on 50:1 PDMS when compared with the other two PDMS stiffness.

For PDMS modified surfaces, similar frequency changes indicated similar adsorption amount of fibronectin and irrespective to the change in surface.

The slopes of all plots were in the range of 0.07 to 0.101, and considered to be similar slopes while statistically there was no significant difference between them. This similarity in the slope indicated that all testing conditions have the same change in dissipation associated with frequency change, and this can result in the viscoelastic property of adsorbed fibronectin layer was the same for all testing conditions. There was no significant change in sensor dissipation for all tested surfaces. Kristensen and his coworkers (2013) considered 1.4 E-6 in the dissipation change due to the adsorption of vitronectin on nanopatterned Surfaces of Ti as a low dissipation change\textsuperscript{181}. In addition, Aramesh and his coworkers (2015) found that the adsorption of two different proteins; bovine serum albumin and lysozyme, on nanodiamond surface were Considerably rigid since the change in surface dissipation were very small, and did not exceed 3E-6\textsuperscript{182}. In general, it has reported that for viscous protein adsorption, the change in sensor dissipation should not exceed 10 E-6\textsuperscript{183}. In this work the maximum dissipation changes in the range of 6 E-6.

The outcomes of this part are completely consistent with previous studies that reported similar results\textsuperscript{184-187}. Seo J and his coworkers (2013) used QCM-D to examined fibronectin
adsorption on PDMS surface with different stiffness, and they found that the bulk stiffness of PDMS has no effect on fibronectin adsorption on PDMS surfaces with different base to elastomer weight ratio. They reported not affect the amount of adsorbed fibronectin on normal PDMS surfaces of different stiffness\textsuperscript{184}.

Moreover, Brown XQ and his coworkers (2005) modified PDMS surfaces of different stiffness with a different treatment, and they found that there was not any significant change in the amount of fibronectin adsorbed on normal and modified PDMS surfaces\textsuperscript{185}.

Protein adsorption has been tested as a function of surface wettability over a wide range of surface contact angle for many surfaces of different functional groups. Arima Y and Iwata H (2007) used SPR angle shifts to measure the amount of adsorbed protein. These authors found that there was no significant change in the amount of adsorbed protein on surfaces with contact angle of 100º or more\textsuperscript{187}. The outcomes of Arima Y and Iwata H’s study are consistent with the results of this dissertation work.

5.3.2 Results of Protein Conformation

The accessibility of the cell binding domain was measured in this work to assess fibronectin functional activity on normal and modified PDMS surfaces of different base to crosslinking ratios. HNF7.1 antibody binding is an established technique for characterizing fibronectin conformation change because it mimics the affinity of cell binding via α\textsubscript{5}β\textsubscript{1} integrin to fibronectin on PDMS surfaces for different testing conditions. All data of this part was normalized to normal 10:1 PDMs after subtracting the results of negative control values from the other tested values (figure 5.7).

The results of HFN7.1 binding showed that changing the cross linking ratio affected fibronectin conformation for normal PDMS. However, these PDMS surfaces exhibited changes
in both stiffness and hydrophobicity. PDMS of higher crosslinking ratio which was less hydrophobic showed higher binding than the soft and more hydrophobic PDMS. For modified PDMS surfaces, it is very clear from the plotted data that HNF7.1 antibody was binding with fibronectin with the same affinity. The adsorption on 405 nm of the colored substrate was all in the same range for all different PDMS stiffness. Since modified PDMS surfaces have the same surface contact angle but different surface rigidity, these results indicated that fibronectin conformation is primarily affected by surface contact angle regardless of surface stiffness.

Anova test was performed using SigmaPlot 11.0 (Systat Software, San Jose, CA) to quantify the difference between protein conformations over different testing conditions. The statistical test with a sample number of 3 resulted in a significant difference between protein conformation over normal 10:1 and normal 30:1 PDMS with p-value of 0.006. Also there was a significant difference between fibronectin conformation over normal 10:1 and 50:1 with the same p-value; 0.006.

5.4 Conclusion

The experiment of protein adsorption done in this work using QCM-D gave strong evidence about factors affecting fibronectin adsorption on the top of normal and modified PDMS surfaces. Substrate stiffness has no significant effect on the amount of adsorbed fibronectin over normal and modified PDMS surfaces, while surface wettability can slightly affect protein adsorption. For the range of 104° -132°, these results showed that fibronectin adsorbed significantly with lower amount on more hydrophobic surface.

Surface wettability was also the major factor that controlled fibronectin conformation rather than surface stiffness of normal and modified PDMS. Binding affinity on the top of all surfaces of modified PDMS with different stiffness was in the same level, and consistent with the
contact angle degree. While for normal PDMS surfaces the binding affinity was varied by the change in surface contact angle, HNF7.1 antibody binding was enhanced on fibronectin on less hydrophobic surface and decreased on more hydrophobic surface.

Figure 5.1 Monitoring the change in sensor frequency and dissipation using QCM-D
Figure 5.2 QCM-D sensor size and shape

Figure 5.3 Cleaning sensor prior to experiment
Figure 5.4 QCM-D experiment setup
Figure 5.5 Protein conformation procedure.
Figure 5.6 Protein adsorption results on normal and modified PDMS

Table 5.1 Standard deviation values of the change in sensor frequency and dissipation

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta f$</th>
<th>$\Delta D$ E-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:1</td>
<td>1.828092</td>
<td>0.60587</td>
</tr>
<tr>
<td>30:1</td>
<td>3.527119984</td>
<td>0.060584228</td>
</tr>
<tr>
<td>50:1</td>
<td>1.71980157</td>
<td>0.29582711</td>
</tr>
<tr>
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<td>0.2703481</td>
</tr>
<tr>
<td>T30:1</td>
<td>1.882479362</td>
<td>1.571739821</td>
</tr>
<tr>
<td>T50:1</td>
<td>2.37834494</td>
<td>0.71261894</td>
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</tbody>
</table>
Figure 5.7 Protein conformation results on normal and modified PDMS of different stiffness

(★ - p < 0.01)
CHAPTER 6: CELL ADHESION AND MORPHOLOGY ON ELASTIC MATRICES

6.1 Introduction

Cells have the ability to sense the rigidity of the extracellular matrix (ECM) which directly affects the control of cellular functions in development, wound healing and malignant transformation\textsuperscript{188,189}. The assembly of specialized cells into tissues and organs ex vivo is the ultimate goal of tissue engineering. To achieve the construction of complex tissues, the adhesion and communication of cells, the smallest basic unit of each living system, with each other and with their surroundings must be regulated\textsuperscript{1}. The adhesion of cells with its surrounding protein scaffold, which is known as the ECM, is very important to provide a specific organization of these cells in the tissue. Moreover, cell adhesion is central to numerous basic cell functions, such as cell proliferation, migration, and differentiation\textsuperscript{2}.

In general, cells initially attach to the biomaterial surface weakly by binding a few receptors to specific sites on the ECM. After time these cells spread and assemble more binding sites in which further receptors will become involved in adhering the seeded cells to the biomaterial surface. As a result, cell adhesion to substrate surface is stabilized\textsuperscript{26}.

Cell adhesion is a complex multi-step process that is mediated by cell surface receptors recruited to ECM sites\textsuperscript{42}. This process progresses by coupling to the extracellular ligands followed by intracellular cooperation with the actin cytoskeleton and accumulation of structural and signaling proteins that create focal adhesions\textsuperscript{190}. Many studies demonstrated the role of focal adhesion distribution on cell adhesion strength and cell spreading area\textsuperscript{191-193}. These focal
adhesion sites are strengthened and stabilized by actin-myosin contractility that increases cell adhesion strength\textsuperscript{194,195}.

In this chapter, fibroblast cells were used to identify the effect of substrate stiffness and wettability on cell adhesion strength as a function of time. A spinning disk was used to apply laminar force over the attached cells for a specific time. Cell spreading area was also tested over a normal and modified PDMS samples with varied stiffness. Pharmacological agents that inhibit actin-myosin contractility were employed to identify the role of focal adhesions and cytoskeletal elements in the mechanotransduction involved in the sensing of the matrix stiffness.

6.2 Experimental Work

6.2.1 Cell Culture

Mouse embryonic fibroblast cells, NIH 3T3 bought from American Type Culture Collection (ATCC), were used in this research. These cells were cultured in complete cell growth media composed of Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) 1% penicillin, and 10% newborn calf serum (Invitrogen). All cells were incubated at 37°C and 5% CO2, and cells were passaged every other day using Trypsin/EDTA (Invitrogen) to release them. For all experiments, cells were used from passage 8 up to 23 passages, and each sample was seeded with the same cell density of 75 cell/ mm\textsuperscript{2}. Then those cells were used for cell adhesion, cell morphology, focal adhesion assay, and to test the inhibition of contractility experiments. PDMS surfaces were coated for 30 minutes with 10μg/ml fibronectin protein of followed by 30 minutes with a blocking buffer solution (1% BSA in PBS) to prevent serum protein adsorption.

6.2.2 Quantification of Cell Adhesion Strength

A spinning disk device, shown in figure 6.1(a), was used to measure mean cell adhesion strength in dyne/cm\textsuperscript{2} as the shear stress required for detaching 50% of cells adhered to normal
and modified PDMS samples. The device applies a range of detachment forces in a single experiment and is capable of detaching cells strongly adhered to a variety of surfaces \(^{39-41}\). This assay was carried out by mounting a coverslip with adhered cells to a rotating shaft that is submerged in the spinning buffer (2mM dextrose) of known density and viscosity and spun for 5 minutes, figure 6.1 (b). The shear stress is proportional to the radial distance from the center of the rotating disk and is given by\(^{196}\):

\[ \tau = 0.800 \ r \ \sqrt{\rho \mu \omega^3} \]

where \( \tau \) is the shear stress, \( r \) is the radial distance from the center of rotation, \( \rho \) is the fluid density, \( \mu \) is fluid viscosity, and \( \omega \) is rotational velocity. Cell adhesion strength was measured after 1, 4, 16 and 24 hours of attachment. Samples that were seeded for just one hour were spun at 3000 revolution per minutes (RPM), while the other samples which spent more attachment time were spun at 5000 RPM.

After spinning, the cells that remained attached after spinning were fixed in a 3.7% formaldehyde solution for 5 minutes, then permeabilized for 10 min (CSK–Triton X-100 buffer, and finally rinsed with 2 ml of blocking buffer (1% BSA in PBS) solution. The nuclei of the remaining cells were labeled with ethidiumhomodimer. Automated fluorescent microscopy (Nikon Instruments, Melville, NY) was used to count the attached cells in 61 fields as a function of their location; figure 6.1 (c), and then the number of attached cells was plotted as a function of related shear stress. A sigmoid fit was applied to the plot; figure 6.1 (d) and it was measured with the following equation\(^{196}\).

\[ f = f_0 / (1 + \exp \left[ b \ (\tau - \tau_{50}) \right]) \]

The fitting parameters are \( f_0 \), the fitted zero adherent stress fraction, \( b \), the sigmoid slope, and \( \tau_{50} \), the required shear stress to detach 50% of the attached cells.
6.2.3 Cell Morphology Characterization

Normal and modified PDMS samples of different stiffness were sterilized with ethanol for 5 minutes and then rinsed for 5 minutes with Dulbecco’s phosphate buffered saline with no Magnesium nor Calcium (PBS -/-, Invitrogen). A solution of 10 µg/ml of human fibronectin in PBS +/+ was used to precoat all the test samples with adhesion protein for 30 minutes, and followed by another 30 minutes with blocking buffer of 1% BSA. Cells were seeded at a density of 75 cell/ mm² over normal and modified PDMS surfaces with elastomer to crosslinker ratios of 10:1, 30:1, and 50:1, and then incubated for 24 hours. After that, cells were fixed for 5 minutes using 3.7% formaldehyde (Invitrogen), and then permeabilized for 10 minutes using 0.5% Triton X-100 in buffered saline. All the samples were stained using Ethidiumhomodimer diluted in blocking buffer for 45 minutes and then rinsed three times with DI water for 10 minutes each time. NIS-Elements software (Nikon) was used to analyze images and quantify morphological attributes.

6.2.4 Effect of Cell Contractility Inhibition on Cell Adhesion Strength

Rho-associated protein kinases, which facilitate actin-myosin contractility, were selectively inhibited using Y-27632. Mouse embryonic fibroblast cells were seeded on normal and modified PDMS samples with 10:1 and 50:1 base to elastomer ratio as described before, and incubated at 37º C for 24 hours. 10 µl of Y-27632 were added to all the tested samples then incubated again for 30 minutes at 37º C. Afterward these samples were placed into the spinning disk device to measure cell adhesion strength by applying a laminar force on the attached cells. After that cells were fixed, permeabilized, and stained with the same procedure previously explained. The cells that remained attach to PDMS surfaces were counted in different 61 spots as
a function of sample diameter. These numbers were plotted versus the applied shear stress to identify cell adhesion strength of these cells after inhibiting their contractility.

6.2.5 Focal Adhesion Assay

In order to compare the focal adhesion sites that connected cell cytoskeleton to the matrix and stabilized cell adhesions, 10:1 and 50:1 normal and modified PDMS surfaces were seeded with NIH 3T3 and then incubated for 24 hours at 37°C. 30 minutes before using these samples, 10 µl of Y-27632 were added to half of these samples to inhibit actin-myosin contractility and observe changes in the focal adhesion sites. All seeded samples were then rinsed with PBS +/- for 5 minutes before cells fixing and permeabilization of the cells as described previously to prepare them for immunofluorescence staining. Before adding the primary antibody, these samples were soaked in blocking buffer consisting of DPBS, 5% v/v serum, and 0.01% v/v NaN₃. Vinculin (Sigma-Aldrich) was used as primary antibody and it was diluted in the blocking buffer with a ratio of 1:100. These samples were incubated with Vinculin in the dark for 1 hour at room temperate. Then the samples were rinsed with 0.05% Tween 20 in DPBS for 5 minutes three times and one time with the blocking buffer for 5 minutes in the dark. Goat antimouse, IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used as secondary antibody, and all the tested samples were incubated with IgG diluted in blocking buffer, and conjugated Hoechst-33242 and rhodamine phalloidin (Invitrogen) in dark place for 1 hour. After that, all the tested samples were rinsed three times with 0.05% Tween 20 in DPBS for 5 minutes at each time. Lastly, the samples were rinsed with DI water for 5 minutes and then mounted on microscope slides.
6.3 Results and Discussion

6.3.1 Cell Adhesion Strength

Cell adhesion strength was measured over a range of attachment durations to characterize the evolution of adhesion strengthening and identify the steady state adhesion strength on these materials. All tested samples with different conditions showed the same trend of cell adhesion strength related to time, whereas cell adhesion was strengthening by time and dramatically increased until reaching a steady state of cell adhesion by 4 hours, figures 6.2 and 6.3.

For normal PDMS samples, cells were attached more strongly on stiff PDMS surfaces (10:1) than on the lower modulus surfaces (30:1 and 50:1). However, since these materials were already observed to also vary in hydrophobicity, it was difficult to decouple the effects of stiffness and wettability on adhesion strength (figure 6.2). Interestingly, it was observed that cell adhesion in the beginning was increasing with higher rate on 10:1 surface compared with other two tested surfaces. These results were statistically tested and showed a significant difference between the cells at steady state adhesion strength of 10:0 PDMS and the other to stiffness. Cells seeded over the stiffer surface (10:1 PDMS) were adhering strongly by ~30% than cells seeded over the softer PDMS (30:1 and 50:1). These are the first results to show a dependence of cell adhesion strength on matrix stiffness.

The resulting data of this part is consistent with a previous study made by Arima Y and Iwata H in 2007; they found that the adhesion of HUVEC and HeLa cells is strongly reliant on biomaterial wettability. Arima Y and Iwata H stated that HUVEC and HeLa cell adhesion was decreased by increasing the substrate hydrophobicity.\textsuperscript{187}

For modified PDMS surfaces, it was noticeable from the results that fibroblast cells were adhering with the same level of strength over the three different stiffness (figure 6.3).
Statistically, there was no significant difference between all tested modified surfaces regardless to the difference in substrate stiffness. However, since these tested modified surfaces were varied in stiffness but also had the same contact angle values, these results indicated that surface wettability may have dominated the difference in the cell adhesion strength of the unmodified PDMS rather than surface stiffness. Nevertheless, all adhesion strengths of cells over treated PDMS were higher in value than the adhesion strength of the same cells on normal PDMS which would indicate a role for stiffness as well because the modified PDMS had an increased surface stiffness due to the formation of permanent silica like layer.

A previous study tested osteoblast cell adhesion over a wide range of surface wettability on substrate coated with PDMS, and the results found a strong connection between cell adhesion and surface wettability, in which cell adhesion decreased intensely by increasing the contact angle up to 122 degree.197

6.3.2 Cell Morphology Results

Cell shape and morphological changes are important regulators of cell functionality.198 The collected data of fibroblast cell morphology over normal and modified PDMS with different stiffness represent three independent experiments (N=3) where five different position of each sample were imaged and analyzed using a Nikon Eclipse Ti-U fluorescence microscope (Nikon Instruments, Melville NY). A contrast threshold was used to create a binary mask for each image in order to count the number of cells and measure cell spreading area.

First, the number of cells attaching to the matrices varied with the surface type (Figure 6.4). For normal PDMS surfaces, 10:1 PDMS surface was covered by high number of fibroblast cells compared to the other PDMS surfaces; 30:1 and 50:1. Statistically; there was a significant change in cell numbers over normal PDMS surfaces, in which fibroblast cells number increased
by 70% when these cells were seeded on normal, stiff, and less hydrophobic PDMS surfaces. While cell number was almost in the same range when these cells were seeded on modified PDMS surfaces with varied stiffness, there was no any significant change in these cell number when statistical ANOVA test was applied. Many factors can affect this quantity, but it serves as a preliminary indicator of affinity for a particular surface and is often used prior to more robust quantitative assays for cell adhesion such as the spinning disk.

For normal PDMS surfaces, cells were spreading widely on stiffer and less hydrophobic surface (10:1) as opposed to soft and more hydrophobic surfaces; 30:1 and 50:1. ANOVA test was applied on cell spreading area over normal PDMS samples and a significant difference was found with p value less $< 0.01$. A previous study made by Lee and his coworkers in 2003 found that surface wettability can regulate cell spreading area, whereas osteoblast cells had lower spreading on hydrophobic substrate comparing with large cell spreading area found on hydrophilic surfaces\textsuperscript{199}. In contrast, cells were spreading generally with the same area on modified PDMS samples regardless of surface stiffness, and statistically there was no significant difference between fibroblast spreading area over modified PDMS samples of varied stiffness. Figure 6.6 shows 10X magnification images of fibroblast cells attached to normal and modified PDMS samples of different stiffness.

6.3.3 Focal Adhesion Testing

Vinculin, a mechanosensitive protein, was labeled to identify focal adhesion assembly, hoechst to stain the cell nuclei and rhodamine phalloidin to stain actin filaments in fibroblast cells attached for 24 hours on fibronectin-coated normal and modified PDMS of different stiffness. These PDMS samples were mounted over thin microscope slides, and an oil immersion objective of 60X magnification was used to image the samples.
Figure 6.7 shows the 60X images of 10:1 and 50:1 of normal and modified PDMS without inhibiting cell contractility. From these images, normal 50:1 PDMS showed the minimum existing of actin filament and focal adhesion sites which can be corresponding to the minimum cell spreading area measured in this work. Normal 10:1 PDMS showed slightly higher current actin filament and focal adhesion sites than normal 50:1 PDMS. For the images of modified PDMS samples that showed before the largest spreading area, a higher actin filament density was observed in addition to intense focal adhesion sites, and these images are consistent with the results of cell spreading area and cell adhesion. Many studies have reported the relationship between cell adhesion, their spreading area, and focal adhesion sites. Cell attachment to a biomaterial surface is initiated by integrin-ligand binding and is followed by the cell spreading widely to involve cytoskeletal components in this attachment and distribute the adhesion force over all the ECM creating focal adhesion sits and increasing cell adhesion.

The enhancement and maintenance of focal adhesions requires intracellular tension driven by actin-myosin contraction. Y-27632, a potent inhibitor of contractility, was added to 10:1 and 50:1 normal and modified PDMS 30 minutes before staining the attached cells with anti-vinculin antibodies, hoechst, and rhodamine phalloidin to detect focal adhesions, nuclei, and actin stress fibers. Y-27632 works to inhibit Rho-kinases that regulate actin-myosin contractility, thus it will inhibit the contractility of fibroblast cells which degrades focal adhesions and reduces adhesion strength. The 60X images of these tested samples are listed in figure 6.8. A reduction in spreading area over all testing conditions was observed, including the modified surfaces. Almost no existing of actin filament and focal adhesion sites in these images were observed; indicating a reduction in cell adhesion as well.
6.3.4 Cell Contractility Inhibition

Y-27632 was also used to inhibit cell contractility prior to measuring the strength of cell adhesion. Fibroblast cells were seeded for 24 hours on normal and modified 10:1 and 50:1. Y-27632 was added to each sample 30 minutes before spinning. Cells adhesion strength was measured by counting the attached cells as a function of applied shear stress. 3 independent samples of each condition were tested and the collecting data were plotted in figure 6.7 as a mean with maximum and minimum range. A cell adhesion strength at 24 hours for the same tested condition without inhibitor was also plotted in figure 6.7 in order to compare the change in fibroblast cell adhesion after inhibit cell contractility.

The result of this part of dissertation work was correlated with the result of cell spreading area of NIH 3T3 cells over PDMS surfaces and the results of testing focal adhesion sites. Previous studies demonstrated that cell microtubules system directly participate in cell spreading and consequently on cell adhesion strength\textsuperscript{204-206}.

There was a clear reduction in fibroblast cell adhesion strength over PDMS samples after inhibiting cell contractility. The minimum reduction of cell adhesion was observed for normal soft PDMS; 50:1, and it was only about 11\% less than cell adhesion without cell contractility inhibition. This result can be explained by the minimum spreading are detected of fibroblast cell on normal 50:1 PDMS samples, thus inhibiting contractility by the disruption of the microtubule system using Y-27632 has a slight effect on cell adhesion. About 20\% reduction in cell adhesion due to inhibit cell contractility was detected on stiff normal PDMS; 10:1, in which cell spreading area over this PDMS samples was significantly higher that fibroblast spreading area over normal 50:1 PDMS. The higher decrease in fibroblast cells over PDMS samples was observed on modified samples 10:1 and 50:1, whereas more than 35\% reduction of cell adhesion was detected.
due to cell contractility inhibition. The same result has been established in a previous study; in which it was found that cell adhesion can be enhanced by 30% without inhibiting cell contractility\textsuperscript{207}. The same results were found when these samples were seeded 24 hours with fibroblast cells with serum starving condition (data not shown).

6.4 Conclusion

All the results described in this chapter were consistent with each other to give a strong evidence on factors that can control cell adhesion. The cell adhesion assay of NIH 3T3 over normal and modified PDMS of varied stiffness resulted in an extreme effect of surface wettability on cell adhesion. Modified PDMS surfaces with different stiffness and the same surface wettability showed same level of cell adhesion, while cell adhesion strength varied by changing the contact angle on normal PDMs surfaces.

Cell spreading results were consistent with cell adhesion strength measurements. Cells appear to be controlled by surface contact angle rather than substrate stiffness, and spreading more on less hydrophobic surface rather than more hydrophobic surface. However, fibroblast cells on modified PDMS samples with different stiffness, spread over these surfaces with almost the same spreading area.

From focal adhesion assay, it was obvious that cells attached over modified PDMS surfaces characterized with advanced spreading area and higher adhesion strength, whereas actin filaments and focal adhesion sites were more existing on these surfaces regardless of the change in surface stiffness. These results are also consistent with cell adhesion measurement and cell spreading area, indicating that biomaterial wettability roles cell responding more as the rigidity of this surface.
Figure 6.1 (a) Spinning disk device, (b) how to establish the PDMS sample, (c) the distribution of cells after spinning, and (d) the sigmoid curve fit of the shear adhesion strength.
Figure 6.2 Cell adhesion results of normal PDMS
Figure 6.3 Cell adhesion results of modified PDMS
Figure 6.4 Cell numbers over normal and modified PDMS of different stiffness
Figure 6.5 Cell spreading area over normal and modified PDMS of different stiffness

(★ - p < 0.01).
Figure 6.6 Fibroblast cell attaches to normal and modified PDMS samples of different stiffness
<table>
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<tr>
<th>Nuclei Position</th>
<th>Actin filaments</th>
<th>Focal adhesion sites</th>
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</thead>
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<td>Normal 10:1 PDMS</td>
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<td><img src="image2" alt="Image" /></td>
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<tr>
<td>Modified 10:1 PDMS</td>
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<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>Normal 50:1 PDMS</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>Modified 50:1 PDMS</td>
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Figure 6.7 60X images of 10:1 and 50:1 of normal and modified PDMS
### Figure 6.8 60X images of 10:1 and 50:1 of normal and modified PDMS with inhibiting cell contractility
Figure 6.9 Comparison of cell adhesion after 24 hours of seeding with and without inhibiting contractility (★ p < 0.01).
CHAPTER 7: CONCLUSION AND FUTURE WORK

7.1 Conclusion

The overall objective of this work was to characterize a model biomaterial system and use it to investigate mechanotransduction events that regulate cell adhesion. We coated ECM onto tunable biomaterials with controllable stiffness and surface chemistry and applied quantitative assays for adhesion strength and morphological characteristics. This study aimed to, establish for the first time a connection between mechanotransduction of matrix stiffness and cell adhesion strength. Cell adhesion, which is a requirement of most cell functions including survival, is dependent on numerous intracellular and extracellular factors. The central hypothesis was that cell adhesion depends strongly on matrix mechanical properties. Four aims were developed in this work to test this central hypothesis. The successful accomplishment of the aims established procedures for modifying the bulk and surface mechanical properties and surface chemistry of a model matrix, established the best methods to characterize the mechanical properties, and employed these materials for investigating the dependence of cell adhesion strength on matrix stiffness.

The relationship between the mechanical properties of the silicone biomaterial and the extent of polymer crosslinking was identified by varying the base elastomer to crosslinker weight ratios to obtain a wide range of bulk stiffness. These mechanical testing methods were compared for measuring the elastic modulus of the prepared polymer, and a model fit was then used to
obtain a clear relationship between the elastic modulus of this biomaterial and the percentage of its crosslinker.

Since crosslinking had a slight effect on surface wettability, the PDMS surface was modified to keep the surface chemistry constant while varying the bulk mechanical stiffness, so the effects of mechanical and chemical properties on cell adhesion could be independently analyzed. For this purpose, chemical, mechanical, and topographic surface characterization methods were applied to confirm the achievement of this aim. The effect of matrix stiffness on protein adsorption and conformation was also examined. The function of integrin receptor binding and actin-myosin contraction in regulating matrix stiffness-regulated adhesion was quantified. Finally, the effect of substrate properties on cell adhesion and cell morphology using a stiffness-tunable silicone substrate was analyzed using mouse embryonic fibroblasts. Cell adhesion strength was quantified with a spinning disk device that applies a range of hydrodynamic shear forces to detach adherent cells, and cell spreading and morphology were analyzed with fluorescent microscopy.

The original and important findings presented in this dissertation can be summarized as:

- Bulk stiffness of PDMS depends non-linearly on the base elastomer to crosslinker weight ratio.
- PDMS stiffness is increased by increasing the amount of crosslinker.
- Nanoindentation with a nano-JKR model is the best testing method to measure the elastic modulus of PDMS surfaces because of the inherently large adhesion forces present.
- PDMS surface contact angle is affected by changing the crosslinking ratio; more crosslinker decreases hydrophobicity slightly.
Surface modification with vapor deposition of alkylsilane monolayers is an effective way to provide equivalent surface contact angle on different PDMS stiffnesses.

This surface modification increases stiffness locally at the surface by locking in the silica-rich layer.

Surface modification has no significant effect on the amount of adsorbed fibronectin on PDMS surfaces.

Substrate stiffness has no significant effect on the amount of adsorbed fibronectin over normal PDMS surfaces.

Fibronectin conformation was affected primarily by hydrophobicity and surface stiffness.

NIH 3T3 cell adhesion strength increased with surface stiffness, however, the increase may also be attributed to changes in fibronectin conformation.

Modified PDMS surfaces have a surface stiffness that is greater than the bulk stiffness.

Cell adhesion strength on modified PDMS was independent of stiffness.

Cells spreading more strongly correlated to surface contact angle rather than substrate stiffness.

From this study, the local surface stiffness and the contact angle of tested surface are the two main factors found to control cell response on normal and modified PDMS surfaces. Together these properties determine the matrix that cells interact with when contacting a protein layer coating on a synthetic biomaterial as in the case of an implanted device. These parameters are therefore inherently difficult to decouple^{49}.
In order to compare these two factors, and understand the correlation between them; all raw data of normal and modified PDMS elastic modulus (in logarithm scale) were plotted as a function of surface contact angle of these tested samples (figure 7.1). A Pearson correlation test was applied to statistically test the relation between surface wettability and surface stiffness. The result of this test is presented in table 7.1, and since the correlation factor is close to -1, a strong negative relationship between these two factors is indicated. In other words, an increase in surface stiffness is associated with a decrease in surface wettability in these materials, and this relationship is clearly present in the plot 7.1.

In addition, correlation testing was applied to compare the effects of these main factors on cell response and protein conformation. Figures 7.2 and 7.3 showed the raw data of cell adhesion strength as a function of surface stiffness (logarithmic scale), and as a function of surface wettability, respectively. The correlation test results are present in Table 7.2. The reduction in surface wettability results in an increase in cell adhesion strength, while increasing surface rigidity promotes cell adhesion. From this test it can be noticed that both factors are similarly correlated to cell adhesion strength since the correlation factors were almost the same absolute value. However, it is noted that >2 orders of magnitude range of elastic modulus produce the same adhesion strength variation as a much narrower range of contact angle.

The effects of elastic modulus and contact angle were also tested on protein conformation, and plotted in figures 7.4 and 7.5, while the correlation test results were listed in table 7.3. The correlation factor of surface rigidity with protein conformation was higher compared with the correlation factor of surface wettability and protein conformation, and this indicated that surface stiffness has a greater effect on protein conformation compared with surface wettability over this range of hydrophobicity.
Last, the effect of surface stiffness and wettability on cell spreading area was plotted in figures 7.6 and 7.7, and the correlation test results of this effect were listed in table 7.4. These correlation plots indicate that PDMS surface wettability has a slightly stronger influence on cell spreading area when compared with the PDMS stiffness.

As an overall conclusion, it can be reported in this dissertation work that biomaterial surface properties have a crucial effect on cell response. Substrate stiffness and surface wettability are considered as two of these important factors. In the case of silicone elastomer networks, these two factors are not absolutely independent of each other. However, irrespective of protein conformation changes which are known to affect cell adhesion, this is the first report on the quantification of the cell adhesion strength dependence on matrix stiffness.

7.2 Future Work

7.2.1 Chemical Analysis for Surface Composition

A key challenge in this work was the unexpected finding that the surface wettability varied with the change in crosslinking. We tried to overcome this by fixing the surface chemistry with a defined monolayer, but this also changed the stiffness in a thin layer at the surface. We would like to address this by analyzing the chemical composition of the surface of PDMS with carried stiffness. The exact formulation of the commercial PDMS used in this study is not known. X-ray photoelectron spectroscopy (XPS) is a sensitive technique that can be used to chemically analyze the normal and modified PDMS surfaces. Alternatively, we could formulate our own PDMS elastomer and crosslinking components.

7.2.2 Investigate Cell Adhesion of Other Cell Types

Fibroblasts are commonly used in cell adhesion studies, but we are specifically interested in endothelialization of biomaterial surfaces for regulated vascularization of implanted devices.
Therefore the effect of mechanotransduction on adhesion and spreading of endothelial cells is of great interest. In addition, the Adhesively nature of cancer cells in different microenvironments is central to the progression and metastasis of tumors.

7.2.3 Investigate the Role of Mechanotransduction in Differentiation

An important aspect of developing biomaterials that direct cell function is for the differentiation of stem cells. Since work by Engler et al (2006) indicates that phenotype specification in stem cell differentiation correlates to the stiffness of the tissue type\textsuperscript{77}, one appropriate target application of this material is the fabrication of skeletal muscle. As an initial screen for mechanotransduction effects in skeletal muscle engineering, C2C12 mouse muscle progenitors could be used in differentiation studies\textsuperscript{208-210}.
Figure 7.1 Correlation between elastic modulus and surface contact angle

Table 7.1 Statistic test result of surface stiffness and wettability correlation

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Figure 7.2 Correlation between cell adhesion strength and elastic modulus

Figure 7.3 Correlation between cell adhesion strength and contact angle

Table 7.2 Statistic test result of surface stiffness and wettability correlation with cell adhesion strength

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Figure 7.4 Correlation between protein conformation and elastic modulus

Figure 7.5 Correlation between protein conformation and contact angle

Table 7.3 Statistic test result of surface stiffness and wettability correlation with protein conformation

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Figure 7.6 Correlation between cell spreading and elastic modulus

Figure 7.7 Correlation between spreading area and contact angle

Table 7.4 Statistic test result of surface stiffness and wettability correlation with cell spreading area

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