CONTROLLING CELL ADESION USING NANOPATTERNS AND APPLICATIONS

DISSERTATION

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Engineering

by

Elena Iris Liang

Dissertation Committee:
Professor Albert F. Yee, Chair
Professor Wendy F. Liu
Professor M. Cristina Kenney

2017
DEDICATION

I dedicate this thesis to my parents, Lung K. Liang and Shi-hwa Yuan—they have been supportive throughout my PhD and always pushed me to do my best. I also dedicate my thesis to my faculty advisor and mentor, Prof. Albert F. Yee, for his direction on this work and for putting his full trust in me to build the lab for biomaterials research. Finally, I dedicate this thesis to Dr. Roger Steinert, my co-mentor, who passed away in June 2017. The artificial cornea project was only possible because of his contributions. I am honored to have the privilege to work with such a renowned ophthalmologist.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>VI</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>VIII</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>IX</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>X</td>
</tr>
<tr>
<td>ABSTRACT OF THE DISSERTATION</td>
<td>XI</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION – CELL ADHESION TO SURFACES</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Cell adhesion to the extracellular matrix</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Methods to control adhesion</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1 Chemical modifications to material surface</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2 Topographical changes to material surface</td>
<td>6</td>
</tr>
<tr>
<td>1.3 Application: Artificial Cornea</td>
<td>8</td>
</tr>
<tr>
<td>1.3.1 Current art</td>
<td>9</td>
</tr>
<tr>
<td>1.3.2 Design of New Artificial Cornea</td>
<td>12</td>
</tr>
<tr>
<td>1.4 Significance</td>
<td>14</td>
</tr>
<tr>
<td>1.5 Overview of Dissertation</td>
<td>15</td>
</tr>
<tr>
<td>CHAPTER 2 FABRICATION AND CHARACTERIZATION OF NANOPATTERNS</td>
<td>17</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>17</td>
</tr>
<tr>
<td>2.2 Methods</td>
<td>20</td>
</tr>
<tr>
<td>2.2.1 Substrate preparation</td>
<td>20</td>
</tr>
<tr>
<td>2.2.2 Nanopattern Mold Preparation</td>
<td>21</td>
</tr>
<tr>
<td>2.2.3 Fabrication</td>
<td>22</td>
</tr>
<tr>
<td>2.3.4 Characterization</td>
<td>23</td>
</tr>
<tr>
<td>2.4.3.1 Scanning Electron Microscopy</td>
<td>23</td>
</tr>
<tr>
<td>2.4.3.2 Atomic Force Microscopy</td>
<td>23</td>
</tr>
<tr>
<td>2.4.3.3 Ultraviolet-Visible Spectroscopy</td>
<td>24</td>
</tr>
<tr>
<td>2.3 Results and Discussion</td>
<td>24</td>
</tr>
<tr>
<td>2.3.1 Validation of FDTS coating</td>
<td>24</td>
</tr>
<tr>
<td>2.3.2 SEM Characterization</td>
<td>25</td>
</tr>
<tr>
<td>2.3.3 AFM Characterization</td>
<td>28</td>
</tr>
<tr>
<td>2.3.5 UV-Visible Spectroscopy Characterization</td>
<td>29</td>
</tr>
<tr>
<td>2.3.6 Fabrication by Double-Imprinting</td>
<td>31</td>
</tr>
<tr>
<td>2.4 Summary</td>
<td>33</td>
</tr>
</tbody>
</table>
CHAPTER 3 MAMMALIAN CELL ADHESION ON NANOPATTERNS .................. 34

3.1 Introduction .................................................................................................................. 34
3.2 Methods .......................................................................................................................... 36
    3.2.1 Nanostructure fabrication ..................................................................................... 36
    3.2.2 Cell culture .......................................................................................................... 36
    3.2.2 Cell count, viability, and proliferation analysis ..................................................... 37
    3.2.3 Scanning electron microscopy ............................................................................. 38
    3.2.4 Statistical Analysis .............................................................................................. 38
3.3 Results and Discussion .................................................................................................. 39
    3.3.1 Adhesion and viability on nanopatterned surfaces .............................................. 39
    3.3.2 Proliferation on nanopatterned surfaces ............................................................. 42
    3.3.3 Cell morphology and interaction on nanopatterns .............................................. 44
3.4 Summary ....................................................................................................................... 47

CHAPTER 4 CORRELATION OF FOCAL ADHESION ASSEMBLY AND
DISASSEMBLY WITH CELL MIGRATION ON NANOPATTERNS .................. 48

4.1 Introduction .................................................................................................................... 48
4.2 Materials and Methods .................................................................................................. 49
    4.2.1 Cell lines and reagents ....................................................................................... 49
    4.2.2 Fabrication of Nanostructures ............................................................................. 50
    4.2.3 Sample Preparation ............................................................................................. 50
    4.2.4 Imaging ................................................................................................................ 51
    4.2.5 Cell Migration Analysis ....................................................................................... 52
    4.2.6 Paxillin Aggregation Analysis ............................................................................. 53
    4.2.7 Adhesion Size Measurement ............................................................................. 55
    4.2.8 Statistical Analysis .............................................................................................. 55
4.3 Results and Discussion ................................................................................................. 55
    4.3.1 Cell Migration on Nanostructured Surfaces ...................................................... 55
    4.3.2 Paxillin Aggregation on Nanostructured Surfaces ............................................. 61
4.4 Summary ....................................................................................................................... 67

CHAPTER 5 MEASURING MAMMALIAN CELL ADHESION ON NANOPATTERNS
UNDER SHEAR FLOW ................................................................. 69

5.1 Introduction .................................................................................................................... 69
5.2 Methods .......................................................................................................................... 71
    5.2.1 Sample preparation ............................................................................................. 71
    5.2.2 Cell culture .......................................................................................................... 71
LIST OF FIGURES

Figure 1.1 Schematic of focal adhesion complex of a cell ......................................................... 3
Figure 1.2 Schematic of Rho protein activation-deactivation cycle ........................................... 3
Figure 1.3 Proposed artificial cornea .......................................................................................... 14
Figure 2.1 Schematic of nanoimprint lithography (NIL) flow process ..................................... 18
Figure 2.2 Plot of spin-film thickness relationship used to determine spin-coating conditions for nanoimprinting ................................................................. 21
Figure 2.3 Evaluation of FDTS coating validation and contamination ....................................... 25
Figure 2.4 Nanoimprinted line gratings in PMMA .................................................................... 27
Figure 2.5 Nanoimprinted PMMA pillars ................................................................................... 27
Figure 2.6. Representative 3D profiles of AFM scans of nanolines .......................................... 28
Figure 2.7 Representaive 3D profiles of AFM scans of nanopillars ......................................... 29
Figure 2.8 Optical transmittance of nanoimprinted PMMA films .............................................. 31
Figure 2.9 Fabrication of square-topped pillars ........................................................................ 32
Figure 3.1 Method of examining cell adhesion on nanostructured surfaces ............................ 37
Figure 3.2 Cell viability on the nanostructured surfaces after 24 hours .................................... 40
Figure 3.3 Cell adhesion on the nanostructured surfaces after 24 hours ................................. 41
Figure 3.4 Cell proliferation on nanostructured surfaces ......................................................... 43
Figure 3.5 SEM micrographs of cells on nanostructured surfaces ............................................. 45
Figure 3.6. Distribution of elongation ratios of cells on nanopatterns ...................................... 46
Figure 4.1 Migration on nanostructured surfaces ...................................................................... 57
Figure 4.2 Migratory direction of cells on nanopatterns .......................................................... 60
Figure 4.3. Brightness of monomeric GFP ................................................................................. 63
Figure 4.4 Paxillin aggregation states of adhesions in cells on nanopatterns .......................... 64
Figure 4.5 Area of disassembling adhesions of cells on nanopatterns .................................... 66
Figure 4.6 Summary of findings correlating migration with paxillin aggregation in adhesions ... 68
Figure 5.1 Parallel plate rheometer setup for shear stress application .................................... 73
Figure 5.2 Adhesion on nanopatterned surface before shear.......................................................... 75
Figure 5.3 Adhesion on nanopatterned surfaces under varying shear rates................................. 77
Figure 5.4 Percentage of cells remaining after 90/s and 300/s shear rates .................................. 79
Figure 6.1 Schematic of vibrationally resonant sum-frequency generation (VR-SFG) spectroscopy using ultrashort visible and mid-infrared pulses ........................................... 86
Figure 6.2 Schematic of nanopatterns and SFG polarization direction ........................................ 89
Figure 6.3 SFG spectral intensities of CH₂/CH₃ groups on L825 nanolines and P700 nanopillars made of PMMA measured with ppp and ssp polarization configurations ......................... 91
Figure 6.4 SFG spectral intensities C=O stretching mode on L825 nanolines and P700 nanopillars made of PMMA measured with ppp and ssp polarization configurations ........ 91
Figure 6.5 SFG spectral intensities of C=O stretching mode on L416 nanolines and P300 nanopillars made of PMMA measured with ppp and ssp polarization configuration .......... 93
Figure 6.6 Schematic layout of VR-SFG experimental setup to probe hydrated biomacromolecules on a nanopatterned polymer film ................................................................. 95
Figure 7.1 Proposed migration experiment to mimic movement in cornea ................................. 101
Figure 7.2 Schematic of double imprinting method with two topographies in proximity to one another on the same PMMA surface ................................................................. 102
Figure 7.3 Preliminary data of NAD (yellow) and SHG (magenta, backward-scatter) signals of keratocytes 2 weeks post-treatment of various agents ................................................. 104
Figure 7.4 Preliminary data of collagen thickness ........................................................................ 105
LIST OF TABLES

Table 1.1 Comparison of current keratoprostheses with design criteria ........................................ 13
Table 2.1 Measured dimensions of nanoimprinted features and respective stamps .......................... 26
Table 2.2 Summary of Nanostructures .......................................................................................... 33
Table 5.1 Shear rate at 50% remaining .......................................................................................... 84
I would like to acknowledge my faculty adviser and mentor, Prof. Albert F. Yee. He is the most supportive adviser that anyone could ask for. He not only taught me to become a better scientist, but how to be an effective teacher and mentor. He always believed in me when I doubted myself. I would also like to thank my co-mentor, Dr. Roger F. Steinert. Dr. Steinert’s vision for a new keratoprosthesis kept my graduate work running, and brought clinical significance to my thesis work. He provided necessary funds to allow me to start my work with Prof. Yee. I appreciate that Dr. Steinert took time from his busy schedule to meet and discuss my progress. Additionally, I acknowledge my thesis committee members Prof. Wendy F. Liu and Dr. M. Cristina Kenney for their instrumental feedback.

None of the work presented in this thesis would have been possible without the help of my labmates in the Yee Group. Mary Nora Dickson, co-author on the bactericidal nanopatterns paper, was a great teammate who led the efforts to create artificial cornea prototypes and for continuing bacteria studies on the nanopatterns; the artificial cornea project would not have progressed to the point of performing in vivo studies without her. Emma Mah, co-author on work evaluating cell migration and focal adhesion formation on the nanopatterns, took many of the images used for analysis, and her contributions brought the innovation aspect to the work. Rachel Rosenzweig provided feedback in communicating to audiences unfamiliar with the work. Susan Wu assisted in the initial fabrication of the nanopatterns, which included long hours in the clean room, and with data analysis. Additionally, I was very fortunate to have dedicated undergraduates to assist with my work: Sara Heedy was instrumental in gathering data for my last experiments; Junming Cai developed a platform for measuring single cells; Sydney Tea was very helpful and reliable in data analysis; and Anisha Hegde and Naomi Thomson performed several preliminary experiments to help optimize experimental parameters.

I would also like to thank my collaborators: Prof. Michelle Digman, who introduced Numbers and Brightness analysis, as well as resources offered through the Laboratory for Fluorescence Dynamics; Dr. Marjan Farid, who established the surgical procedure for the prototypes; Dr. Kate Xie and Dr. Priscilla Vu, ophthalmology residents who initiated the animal studies; and Dr. Paul Blaze and Russell Baxter for fabricating the physical artificial cornea prototypes. Additionally, I would like to acknowledge Prof. Szu Wang for allowing me to use her lab’s inverted microscope for imaging and liquid nitrogen dewar for storing cells; Prof. Ali Mohraz and Max Kaganyuk for allowing me to use the lab’s rheometer for my shear experiments; Dr. Jian-Guo Zheng, the director of the Irvine Materials Research Institute; Jake Hes of the Bio-Integrated Organic Nanofabrication facility; and the staff members from the BME and CBEMS offices.

I am grateful for the friends I made the past several years in both BME and CBEMS, and especially my roommates in PV 7402. I am also thankful for David Li, my partner and sounding board when I needed scientific advice or emotional support. And of course, I would not have been able to persist in graduate school without the support of my brother and parents.

I like to acknowledge my funding sources: multiple Teaching Assistantships through the BME and CBEMS departments, the Institute for Clinical and Translational Sciences, the Public Impact Distinguished Fellowship, the Science Communications Fellowship, the School of Engineering Travel Grant, the Association of Graduate Students travel grants, and the Materials Research Society via the Graduate Student Silver Award in Spring 2016.
CURRICULUM VITAE
Elena Iris Liang

EDUCATION
2009
Bachelors of Science in Bioengineering
University of California, Berkeley

2014
Masters of Science in Biomedical Engineering
University of California, Irvine

2017
Doctorate of Philosophy in Biomedical Engineering
University of California, Irvine

FIELD OF STUDY
Biomedical Engineering; Biomaterials; Nanofabrication

PUBLICATIONS
Liang EI, Mah EJ, Yee AF, Digman MA. Correlation of Focal Adhesion Assembly and Disassembly with Cell Migration on Nanotopography. Integrative Biology, 2017; 9, 145-155.


ABSTRACT OF THE DISSERTATION

Controlling Cell Adhesion with Nanopatterns and Applications

By Elena Iris Liang

Doctor of Philosophy in Biomedical Engineering

University of California, Irvine, 2017

Professor Albert F. Yee, Chair

The ability to control cell adhesion on material surfaces is critical to the performance and compatibility of medical implants in the body. One such application that would benefit from controlled adhesion is an artificial cornea, an implant for replacing the anterior portion of the eye. For a successful implant, we need to simultaneously promote cell adhesion on the periphery of the implant to improve integration, while limiting adhesion in the central region to provide clear vision.

To address this challenge, we used nanoimprint lithography to create arrays of precisely defined nanopatterns on the scale of 100-500 nm on surfaces of polymer films. We postulated that nanoline patterns resembling the natural collagen fibers in the cornea would encourage adhesion, and that the transparent nanopillars would discourage cell adhesion on polymer films. We designed experiments to evaluate the degree of adhesion on the nanopatterned polymer surfaces, which confirmed our postulates. Using fluorescence correlation spectroscopy, we found that nanotopography influenced cell motility by inducing protein reorganization in cell adhesions. Studies under conditions of fluid flow induced shear confirmed that nanoline patterns were better than flat surfaces at encouraging cell adhesion, as significantly more cells remained on nanolines at the highest shear rate applied.
Results described in this thesis demonstrate the feasibility of using nanotopography to control the degree of cell adhesion. As for the artificial cornea, future in vitro studies with corneal keratocytes are suggested to evaluate corneal wound healing before ultimately conducting in vivo studies to evaluate the efficacy of our proposed device. This thesis shows that implementing nanotopography could lead to improved implantable medical devices and scaffolds for tissue-engineered constructs by providing greater spatial control of cell adhesion even under kinematic conditions without the need to chemically modify material surfaces.
Chapter 1 Introduction – Cell Adhesion to Surfaces

Cell interactions with material surfaces are critical to the performance of medical implants and systems immersed in aqueous environments. There are many factors that can lead to failure of an implant. First, proteins need to adsorb to the surfaces of implants in order to initiate cell adhesion. Protein adsorption usually occurs shortly after implantation, within minutes. However, the body would react to an implant as a foreign body, leading to a recruitment of inflammatory cells to the implant surface. This elicits a response to literally wall off the implant from the rest of the body by forming scar tissue; otherwise, the target cells would find their way to the implant surface. Therefore, the greater challenge is ensuring the compatibility of the material surface with the cells. If cells do not grow well, they will start to die off, resulting in necrosis and consequently, a failed implant. In contrast, cells may like the surface too much that too many cells proliferate on the surface, leading to tissue overgrowth. Thus, the ability to control cell adhesion is critical for medical implants. There is consequently interest in modifying device surfaces through chemical or physical means. However, in order to decide how to modify material surfaces, it is important to first understand how cells adhere naturally to the extracellular matrix (ECM) so that surfaces can be designed to mimic the natural environment.

1.1 Cell adhesion to the extracellular matrix

Cells adhere to the ECM through focal adhesions, which are large multi-protein complexes that link the cytoskeleton inside the cell to ECM proteins. Focal adhesions first form when integrins, which are transmembrane proteins serving as the physical link between the cell
and the ECM, bind to recognizable protein domains of the ECM. Upon binding to the ECM, integrins undergo a conformational change, revealing binding sites to the actin cytoskeleton and other signaling proteins that are involved in the formation of focal adhesions. Focal adhesions also include talin, a protein that binds directly to the actin filaments of the cytoskeleton, and vinculin, a protein that reinforces that binding of actin by binding with $\alpha$-actinin, the actin-crosslinking protein (Figure 1.1). The size of focal adhesions not only depends on the number of integrins clustered together, but also the ECM substrate properties. It was found that fibroblasts cultured on firm, stiff substrates formed more stable focal adhesions while fibroblasts on flexible substrates were more motile. Upon integrin binding, focal adhesion kinase (FAK), a tyrosine kinase, is activated by tyrosine phosphorylation and recruited by talin, which binds to the integrin $\beta$ subunit, or paxillin, which binds to one type of integrin $\alpha$ subunit. Clustered FAK molecules cross-phosphorylate with each other, creating a docking site for members of the Src family of tyrosine kinases, paxillin, and vinculin to bind (Figure 1.1). In addition, FAK activates Rho-GTPases Rac and Cdc42, inducing actin polymerization in the cytoskeleton.

The Rho protein family of GTPases, a subclass of the Ras superfamily, plays active roles in many cell functions, including proliferation, differentiation, and migration. Three members of the Rho family, Rac, Rho, and Cdc42, regulate the signaling pathway between cell membrane receptors and the actin cytoskeleton. Rac1 activation leads to actin polymerization assembly near the front periphery of the cell, leading to the development of the lamellipodia. Cdc42 activation mediates the formation of filopodia, which are actin-rich protrusions from the lamellipodia. Lastly, RhoA activation leads to the assembly of actin-myosin contractile filaments near the back of the cell. The Rho proteins serve as molecular switches to control such processes, cycling between an active conformation (GTP-bound) and an inactive state (GDP-bound) (Figure 1.2).
Two proteins regulate Rho activity: guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP). GEFs activate Rho by facilitating the release of GDP and replacement with GTP. GAPs inhibit Rho by hydrolyzing GTP to GDP.\(^3\)

**Figure 1.1 Schematic of focal adhesion complex of a cell.** Focal adhesions are found at the cell membrane-extracellular matrix interface. Important proteins involved in the complex are shown, including integrins, focal adhesion kinase (FAK), talin and paxillin. Reprinted with permission from Nature Publishing Group. Adapted from Mitra et al.\(^7\)

**Figure 1.2 Schematic of Rho protein activation-deactivation cycle.** Reprinted with permission from Nature Publishing Group. Adapted from Etienne-manneville.\(^3\)
Interestingly, the mechanism for bacteria adhesion to surfaces is similar to that for eukaryotic cells. Bacterial adhesion is one major concern with biomedical implants and is worthy of investigation, as bacteria compete with mammalian cells to adhere to surfaces. Bacteria have pili or fimbria (fimbria are pili that are used for adhesion), which are filamentous surface structures that protrude from the bacterial outer membrane to probe the surface for ECM molecules (fimbria are equivalent to filopodia of eukaryotic cells). At the tip of each fimbria are adhesins, which binds to specific ECM molecules, similar to integrins of eukaryotic cells. Initial adhesion to surfaces is important for the formation of bacterial colonies, which will eventually aggregate to form a biofilm. The growth of the biofilm eventually creates an impenetrable shield of extracellular polysaccharides (EPS), which prevents antibiotics from reaching individual bacterium. If part of the biofilm breaks off, bacteria could reach other areas inside a patient, initiating the spread of infection.

1.2 Methods to control adhesion

1.2.1 Chemical modifications to material surface

Changing surface chemistry would control protein adsorption on material surfaces, which would affect how and where cells would adhere. There are several methods of chemically modifying material surfaces.

One method is chemical graft modification, which involves covalently bonding the proteins or monomers to the material surface, altering the surface chemistry while avoiding detachment of proteins or monomers. In this approach, surfaces are first activated with reactive groups, such as hydroxyl and amino groups, before adding on the desired chemical functionality. However, grafted proteins lose their mobility on the surface, and they may be presented in...
unfamiliar conformations to cells. Additionally, the process could leave toxic monomer residues on the material surface.\textsuperscript{1}

A more refined method to address chemical grafting issues is self-assembled monolayers (SAMs).\textsuperscript{1,13} The monolayers consist of molecules composed of a surface attachment group, long hydrocarbon chain, and a functional head group. The functional head group is designed to either alter hydrophobicity or reactivity of the material. Attachment groups, such as silanes, have strong interactions with the surfaces. Once the molecules gather on the surface, the hydrocarbon chains would arrange to achieve tight packing with each other. As a result, SAMs are chemically stable. With SAM molecules, a wide variety of chemical groups can be achieved by modifying attachment and functional group. Thus, it is possible to simultaneously encourage or discourage protein adsorption. However, similar to plasma treatment, surface functionality may not last very long. Likewise, implantation of implants coated with SAMs would need to occur shortly after coating.

Another method is plasma modification, which utilizes gaseous species, which are highly excited ions, free radicals, molecules, and atoms, to functionalize surfaces.\textsuperscript{1,14} An electric potential is applied to the gas in a chamber. Electrons produced collide with molecules to form gaseous species that would react on the sample’s surface. Plasma treatment functionalizes material surfaces with the addition of hydroxyl groups (using oxygen plasma), or amine groups (using nitrogen plasma). This changes the surface charge and the hydrophobicity of the surface for improved protein adsorption. This method is also good for binding another material to the treated surface. However, this functionalization also remains active for a limited period of time, which means implantation needs to occur very shortly after the surface treatment.
Yet another method that spatially controls protein adsorption to surfaces is micro- and nano-contact printing. Micro- and nano-contact printing are extensively used to transfer ECM proteins onto the substrate.\textsuperscript{15–19} A stamp, often made of polydimethylsiloxane (PDMS), with the desired features is coated with an ECM protein, biomolecule, or SAMs. These species are then stamped onto the region of interest. This technique has also been used to print onto the tops of microstructures.\textsuperscript{20} However, this method produces a two-dimensional (2D) surface and does not simulate a three-dimensional (3D) in vivo environment.

1.2.2 Topographical changes to material surface

Due to the drawbacks of chemical modification methods, another direction in surface modifications is changing the roughness and topography. These physical modifications introduce an extra dimension that better simulates the three-dimensional in vivo environment. There has been increasing interest in designing novel nanostructured surfaces, given the evidence that the ECM consists of many nanoscale features.\textsuperscript{21–23} Microfabrication techniques have made these physical modifications possible. For example, photolithography has been widely used in the semiconductor and electronics industry to make micron-sized features. This technique uses UV-light to etch features in photosensitive polymers called photoresists, which are normally spin-coated on silicon wafers. It is limited by the wavelength of light used in the process; thus, it is difficult to get precise nanofeatures.\textsuperscript{21}

Fortunately, other techniques are available for fabricating nanofeatures, especially with polymers. Etching using chemicals or plasma is the simplest method for creating uniform surface roughness on material; however, the distribution of features may be variable. If chemical etching is used, residual chemicals could remain on the surface if not thoroughly cleaned. Though plasma etching alleviates the issues with chemical etching, the nanofeatures made are not always
reproducible.\textsuperscript{21,24} There are methods that are good for fabricating one type of geometry. For example, colloid lithography is great for creating hexagonally packed features. In contrast, electrospinning is a great method for creating polymeric nanofibers; it is the method of choice for tissue engineering as it is thought to produce topography that closely resembles the natural ECM.\textsuperscript{21} Thus, lithography-based methods are more desirable for fabricating regular surface nanostructures.

Electron-beam (e-beam) lithography is a precise technique for creating nanoscale features. A beam of electrons is used to scan the surface to form features into a sacrificial resist layer that is later washed off from the desired material. The resolution of this technique depends on the size of the electron beam used to form feature. However, because of the short wavelength of electrons, the resolution of this technique is well below 10 nm.\textsuperscript{24,25} While the resolution is ideal for fabricating precise nanostructures, the process is very expensive and very slow, especially when going down to 10-nm size features, as features are made one at a time. Ion beam lithography is a similar technique, but uses a beam of ions to form features. Polymer resists are more sensitive to ions, but similar to e-beam lithography, features are made one at a time. Thus, neither method is ideal for large-scale manufacturing.

Another approach is nanoimprint lithography (NIL), an industrial process used to transfer features from a mold to a polymer film over large surface areas under specific force and temperature condition.\textsuperscript{26–28} NIL is able to create well-defined, nanoscale features down to the scale of tens of nanometers, which cannot be made using common microfabrication techniques. NIL is uniquely capable of forming high aspect ratio structures. More importantly, these nanostructures can be formed on surfaces of glassy polymers. Other variations have NIL have been developed, including UV-NIL,\textsuperscript{29} which utilizes UV-light irradiation to cure the material.
after imprinting features with a transparent mold, and reversal imprinting, a technique performed at reduced temperature and pressure needed to imprint nanofeatures;\textsuperscript{30,31} additionally, this method is useful for imprinting features on surfaces of curved geometries.\textsuperscript{32} With these advantages, NIL was the fabrication method of choice for the current work.

1.3 Application: Artificial Cornea

One application that would benefit from controlled cell adhesion is an artificial cornea. The cornea, the outermost, transparent part of the eye, serves as structural support for the tear film and protects the eye from debris or outside organisms. Most importantly, the cornea is responsible for two-thirds of the focusing power of light into the eye by refracting light rays towards the lens and ultimately, the retina. The epithelium, the outermost layer, protects the cornea and the rest of the eye from outside debris. In the native cornea, the optical clarity of the cornea depends on the integrity of the corneal epithelium, which is attached to the epithelium covering the sclera and the internal part of the eyelid.\textsuperscript{33} The endothelium serves to maintain the relative dehydrated state of the stroma by active transport.\textsuperscript{34} The stroma, which is 90 percent of the cornea, consists of 200 parallel 1-2\,µm thick sheets of lamellae of collagen fibrils, about 36 nm in diameter. The organization of the stroma is essential for light scattering into the eye. Between the lamellae sheets are keratocytes or corneal fibroblasts. Keratocytes are responsible for producing crystallins, proteins thought to contribute to the cornea’s transparency, and keratan sulfate, which aids in the hydration of the cornea.\textsuperscript{34,35}

Corneal disease leading to scarring from infection, trauma and inherited diseases is a common cause of monocular and bilateral blindness and is a leading cause of non-retinal related blindness, second only to cataract and afflicting an estimated 2.8 million individuals worldwide.\textsuperscript{36,37} The epidemiology of corneal blindness is complicated since it encompasses
injuries as well as infectious and inflammatory eye diseases that cause corneal scarring. The prevalence of corneal blindness is more evident in developing countries. In many regions in Africa, corneal disease is the most common cause of monocular blindness. Furthermore, the incidence of childhood cornea-related visual loss is 20 times greater in high-risk groups in Africa and Asia than in industrialized countries.\(^{36}\)

Currently the only available treatment option is corneal transplantation: replacement of the damaged cornea with a cornea from a human donor. Access to corneal transplantation in both developed and undeveloped countries is limited due to lack of donors, access to eye banks and trained surgeons.\(^{36}\) Not every country has an eye-banking program, and very few export corneas globally. It has been estimated that for every 70 corneas needed worldwide, only one is available.\(^{37}\) Additionally, in the developing world where most corneal blindness occurs, transplantation is rarely an option due to barriers in donor tissue collection and storage. In the United States, about 76,000 corneal transplants were performed in 2014, of which over 6,000 were performed for treatment of mechanical or chemical corneal injuries.\(^{38}\) More importantly, 20% of these grafted corneas or over 15,000 surgeries in 2014 were for repeat transplants due to graft rejection. Younger patients would be expected to undergo multiple transplants over the course of their lives. While transplantations in the United States have been successful, these facts indicate that there is a major unmet need for a device that can restore corneal transparency and functional vision without using donor corneal tissue.

1.3.1 Current art

Currently, the only keratoprosthesis, or artificial cornea, in clinical use is the Boston Keratoprosthesis (K-Pro).\(^{39}\) A donor corneal graft is placed between front and back plates, both made from poly(methyl methacrylate) (PMMA). The front plate is customized to include the
appropriate dioptic powers while the back plate contains eight holes that are 1.3 mm in diameter. After locking the graft-KPro assembly together, it is sutured into the corneal opening and covered by a soft contact lens.\textsuperscript{40} Clinical studies have shown that this construct has restored partial vision in patients and can be retained in the eye for years,\textsuperscript{41,42} but with ongoing risks for infection and extrusion, requiring intensive ongoing monitoring by an ophthalmologist and use of antibiotics and a bandage soft contact lens. The Boston K-Pro requires a donor cornea, which is problematic if there are no donor corneas available. Additionally, a common complication resulting from the implantation of the Boston K-Pro is retroprosthetic membrane formation (RPM), which means the growth of unwanted cells behind the posterior optic surface.\textsuperscript{42,43} An RPM requires another surgical procedure to remove and may recur. Infection and extrusion of the K-Pro are other common problems and can result in loss of the eye.

An alternative implantable device is the AlphaCor\textsuperscript{TM}, a keratoprosthesis made of poly(2-hydroxyethyl methacrylate) (pHEMA), a soft hydrogel.\textsuperscript{44,45} This device consists of a transparent central optical zone, and a peripheral porous sponge skirt that is fused to the central zone. The porous skirt is intended to encourage in-growth of host stromal fibroblasts, promoting biointegration of the device. The success rate of AlphaCor\textsuperscript{TM} is low and use is limited. The major complication with the AlphaCor\textsuperscript{TM} is stromal melt.\textsuperscript{44,45} Stromal melt is thought to occur when immune mediators and collagenase attack the exposed stroma during the implant operation. Another potential reason is low glucose permeability of the device, a consequence of its low water content.\textsuperscript{46} To prevent stromal melt, patients have to take medication to discourage the occurrence of stromal melt for the rest of their lives. Additionally, the AlphaCor\textsuperscript{TM} suffers from the accumulation of protein deposits. Moreover, pHEMA is susceptible to staining and discoloration, which can affect visual acuity.\textsuperscript{45} These complications indicate that the device
construct and material properties are exceedingly important to the design of an artificial cornea. In addition, a solution is needed to prevent stromal melt. Several possibilities include promotion of stromal fibroblast growth or inhibition of immune cells that may be attacking the stroma.

Another synthetic artificial cornea has been designed which utilizes a photolithographically patterned hydrogel construct. The core of the device is made of a poly(ethylene glycol)-poly(acrylic acid) (PEG-PAA; water content: 85%) hydrogel network synthesized using two steps of UV-initiated free radical polymerization. The skirt, fused to the optic of the device by a polymerization reaction, is made of poly(hydroxyethyl acrylate) (PHEA) punctuated by a radial array of micropores (60 or 120 µm diameter; spaced 10–20 µm apart along 1–2 degrees of radial separation). In attempts to promote cell adhesion on the device, the surface is chemically modified with collagen Type I. The researchers have shown the PEG-PAA hydrogel network to be optically transparent (96% transparency) with a high tensile strength (1.1 MPa). A huge drawback for this device is that the fabrication of the device is time-consuming, involving multiple UV polymerizations steps. Additionally, though this device prevents protein accumulation on its surface, it discourages cell adhesion as well. Therefore, the hydrogel surface needs to be chemically modified to encourage cell adhesion.

A different synthetic artificial cornea is the ArtCornea®, recently developed at the Fraunhofer Institute for Applied Polymer Research in Germany. The surfaces of the optic (anterior and posterior) of the ArtCornea® are treated in an attempt to prevent cell adhesion or extracellular matrix protein deposition, and thus, the optic is transparent. Additionally, the optic anterior is made to allow tear film formation. The haptic or peripheral region of the device is porous and coated with fibronectin in order to encourage cell growth. This device is currently in clinical trials in Germany. However, the ArtCornea® also requires chemical modification to
promote cell adhesion on the haptic. While this may initially be effective, it does not provide a long-term solution. In general, chemical modification of a device surface could also make FDA-approval more challenging.

An additional consideration is infection. While infections that occur in patients with the Boston K-Pro and the AlphaCor™ are well-known, the photolithographically patterned hydrogel artificial cornea, and the ArtCornea® are all made of hydrogels; yet, there have been studies indicating that there can be significant bacterial growth on the surfaces of hydrogels. Therefore, we believe that demonstration of bacterial resistance of the surface of an artificial cornea should be an integral part of its design and validation.

1.3.2 Design of New Artificial Cornea

To address this unmet need, we propose to develop a completely synthetic artificial corneal device or keratoprosthesis that does not require any donor tissue. We developed criteria for an ideal keratoprosthesis based on input from collaborating ophthalmologists: (1) is made of an ophthalmic material; (2) does not use chemicals to modify the material; (3) is transparent; (4) is inherently against infections; and (5) can control cell adhesion. We evaluated the current keratoprostheses described in the previous section to see how well they match the five design criteria. As observed in Table 1.1, each device achieved three out of the five criteria.
Table 1.1 Comparison of current keratoprostheses with design criteria

<table>
<thead>
<tr>
<th></th>
<th>Boston KPro</th>
<th>AlphaCor</th>
<th>Patterned hydrogel</th>
<th>ArtCornea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ophthalmologic polymer</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>No chemical modifications</td>
<td>✔️</td>
<td>✔️</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transparent</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>Inherent against infections</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controlled cell adhesion</td>
<td></td>
<td>✔️</td>
<td>✔️</td>
<td></td>
</tr>
</tbody>
</table>

The proposed artificial cornea from our group will use physical surface topography to allow cells to distinguish between the central optic zone and the peripheral skirt (Figure 1.3). The surface of the skirt will consist of nanoline gratings to encourage cell elongation, alignment, and growth on the surface, making it easier for the implanted device to integrate with the host body. The surface of the optic zone will be comprised of nanopillars. Based on results from a previous study in our lab, we hypothesize that the pillar structures will prevent cells from adhering, creating a clear and transparent region to let light enter so that the patient can see clearly with this device.
Figure 1.3 Proposed artificial cornea. The design includes a central optic zone, and a thinner skirt containing through holes for suture placement. The skirt will have nanoline gratings to promote cell adhesion and growth to integrate with host tissue, while the optic zone will have imprinted nanopillars to prevent cell and bacterial adhesion. Figure by Rachel Rosenzweig.

1.4 Significance

There is growing evidence of nanotopographical cues dictating various types of cell behavior. However, not many studies have examined the effect of nanotopography on cell adhesion or investigate the degree of cell adhesion on these surfaces. We have previously shown that polymer pillar structures made by nanoimprint lithography have the ability to kill bacteria. In this work, we found that the array of nanostructures with the smallest diameter and spacing were the most effective at killing *Escherichia coli.*\(^{51}\) This work was the first published study that showed synthetic polymer nanostructures had bactericidal properties, and has led to other studies in our group to examine the killing mechanism of the structures, and the structures’ effect on biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis.* Most recently, our group has begun to study the potential ability to inhibit the growth of fungi that cause fungal keratitis. However, if these nanopillar structures are to be implemented to the surfaces of medical implants, it is important to ensure that the nanopillars are not detrimental to the patients’ own cells. Thus, the objective of this thesis was to determine if there is differential cell adhesion to
nanotopographies and see if these nanotopographies could be utilized for the proposed artificial cornea, and eventually other medical implants.

1.5 Overview of Dissertation

First, I describe the use of nanoimprint lithography to fabricate well-defined nanotopographies on thin PMMA films (Chapter 2). Next, I present my examination of whether the fabricated nanopatterns would encourage or discourage cell adhesion (Chapter 3). The experiments confirm that different patterns would influence the degree of cell adhesion on polymer surfaces. This raised the question of how the cells were influenced by the nanotopographies, and led to the examination of the cells’ focal adhesions using fluorescence correlation spectroscopy, a collaborative study with Prof. Michelle Digman (Chapter 4). This particular study was the first report of how nanotopography influences cell motility and focal adhesion protein reorganization with single molecular detection.

With evidence of the dynamics of focal adhesions on nanotopography, the next question was to determine whether the focal adhesions could indicate the degree of cell adhesion on surfaces. This led to another study that examined cell adhesion under shear flow in attempts to measure the adhesion strength on the nanotopography (Chapter 5). Preliminary results demonstrate the importance of topographical orientation with respect to shear force, and further supported my hypothesis that it is possible to use nanotopography to control cell adhesion to material surfaces. These results also suggested that polymer chains might orient differently on the surface of nanostructures. To investigate this potential difference, I collaborated with Prof. Nien-hui Ge to identify the surface molecular moieties on the nanostructures with a surface-sensitive technique called sum frequency generation spectroscopy (Chapter 6). Findings from the experiments described in this thesis demonstrate the feasibility of using nanotopography to
encourage or discourage cell adhesion. Future *in vitro* studies with corneal keratocytes are suggested to evaluate corneal wound healing with regards to the artificial cornea before ultimately conducting in vivo studies to evaluate the efficacy of our proposed artificial cornea (Chapter 7). This thesis will show that implementing nanotopography could lead to improved implantable medical devices and scaffolds for tissue-engineered constructs by providing greater spatial control of cell adhesion without the need to chemically modify the surface.
Chapter 2 Fabrication and Characterization of Nanopatterns

2.1 Introduction

Nanoimprint lithography (NIL) is an industrial process used to transfer features from a mold to a polymer film over large surface areas under specific force and temperature conditions.\textsuperscript{26-28} NIL can be used to create well-defined, nanoscale features down to the tens of nanometers. As mentioned in Chapter 1, the objective of this work is to find nanopatterns that would control cell adhesion. To encourage adhesion, I took inspiration to the natural cornea, which consists of densely packed sheets of collagen fibers. Corneal cells naturally adhere to these fibers, which are about 50 to 200 nm in diameter and packed with a high degree of orientation order with each other. To prevent too many cells from adhering, I took inspiration from wings of the cicada, which have the ability to cleanse itself from dirt, water, and microbes due to small nanocone structures on its surface. Additionally, these structures were found to kill bacteria,\textsuperscript{52,53} which would be another benefit for the artificial cornea and other implants. In my thesis work, I took inspiration from these observations and fabricated structures that mimic these from nature on the surfaces of a synthetic polymer.

The specific NIL technique used was thermal imprinting, or hot embossing NIL, to fabricate nanopatterns on the surfaces of thermoplastic polymers, including poly(methyl methacrylate) (PMMA). PMMA was chosen because it is transparent to visible light, and is less brittle than polystyrene, another transparent polymer; this is desirable for the synthetic artificial cornea implant. In this process, the temperature is elevated to melt the polymer so that the polymer could flow into the recessed features of the mold (Figure 2.1B). The polymer melt flow is accomplished by applying pressure (MPa). Pressure is applied at the elevated temperature for a
given amount of time before cooling the assembly down to room temperature. Afterwards, the molded polymer piece is released from the mold, leaving the nanopatterns on the polymer surface (Figure 2.1A).

![Figure 2.1](image)

**Figure 2.1 Schematic of nanoimprint lithography (NIL) flow process.** (A) Steps of NIL process. A mold with an anti-stiction coating is pressed into polymer film, and imprinted on the softened polymer for about 10 minutes under a specific temperature and pressure. Low surface energy aided in releasing the mold from the underlying polymer film. (B) Schematic correlation between temperature and viscosity over time during NIL. (After Mary Nora Dickson.) (C) The effects of temperature on the modulus of a thermoplastic polymer like PMMA. Ideal imprinting temperature would be near the tail end of the rubbery regime.
To determine the right conditions to use for imprinting, it is important to understand what is happening to the polymer at the molecular level. Three variables influence the success of a nanoimprint: temperature, pressure, and time. Temperature contributes the energy needed for changes in the configuration of polymer chains in the condensed state. At room temperature, prior to imprinting, the polymer chains are mostly immobile because they are kinetically trapped in the amorphous, glassy state. In a glassy state, the modulus of PMMA is about 2.8-3.7 GPa, depending on the temperature. At a specific temperature, known as the glass transition temperature, the polymer chains are provided energy to slide with respect to the other chains when a stress is applied; this temperature for PMMA is about 105 °C. Because of this chain mobility, the modulus and viscosity of the polymer decreases by order of magnitude towards a rubbery state. As the temperature increases, the increased thermal energy overcomes the enthalpic forces between adjacent polymer chains to allow overall chain motion and reorientation (Figure 2.1C). Eventually, at a temperature significantly higher than the glass transition temperature, referred to as the melt temperature, the polymer turns into a melt. In this state, the modulus is significantly lower and the polymer chains can freely slip past one another.

With this knowledge, the ideal printing temperature would be at the transition between a rubbery polymer state and the polymer melt. While temperature is the printing variable that would significantly change the polymer modulus and viscosity, the pressure applied to the mold and the amount of time the pressure was applied to the mold at the elevated temperature provide the motive force to push the polymer chains into the negative mold features.

In this chapter, I describe our use of nanoimprint lithography to fabricate well-defined and reproducible polymeric nanostructures, such as lines and pillars of several different 2-D geometries and periodicities. The quality of the nanoimprinted structures was evaluated by
scanning electron microscopy (SEM) and atomic force microscopy (AFM). I expected, knowing about the advantages of NIL, the nanostructures to closely mirror dimensions in their respective negative molds.

2.2 Methods

2.2.1 Substrate preparation

Thin films of poly(methyl methacrylate) (PMMA) (120 000 MW, Sigma Aldrich) were prepared on glass cover slips (Fisherbrand, 22 x 22 mm, #2 thickness). PMMA solution in toluene (5% by weight) was spin-coated on a film-casting spinner (Laurell) on the glass substrates at 600 rpm for 45 seconds to create a flat PMMA coating. This spin speed was chosen based on the spin-thickness curve from preliminary spin-coating experiments (Figure 2.2); the film thickness was measured using a profilometer (Dektak XT). After spin casting, the PMMA-coated glass substrate was placed on a hot plate at 100°C for at least 5 minutes to allow the PMMA to anneal and remove excess solvent from the film. Thereafter the assembly was left to cool on a surface at room temperature.
Figure 2.2 Plot of spin-film thickness relationship used to determine spin-coating conditions for nanoimprinting. Data represents mean ± standard deviation of four samples. The linear relationship does not hold beyond 800 rpm. This is due to the adhesive effect of the substrate when the film is too thin.

2.2.2 Nanopattern Mold Preparation

Silicon nanoline and nanohole molds (Lightsmyth, 12.5 x 12.5 mm and 8 x 8.3 mm, respectively) made by electron-beam lithography (EBL) were used for imprinting the line patterns and two of the pillar patterns (P700, P600) on the PMMA film. Prior to any processing, silicon molds were cleaned with piranha solution (3:1 sulfuric acid: hydrogen peroxide). Nickel molds (Temicon, Germany) were used for the next smaller pillars (P300). These molds were made using interference lithography.55

Prior to imprinting, an anti-stiction coating, specifically perfluorodecyltrichlorosilane (FDTS), was applied to the molds by molecular vapor deposition (MVD-100, Applied Microstructures). Canisters holding water and the FDTS precursor (Gelest, Inc.) were heated to 100 °C and 55 °C, respectively. Inside the chamber of the MVD, which was warmed to 35 °C and pumped down to 0.130 Torr, the molds were first treated with oxygen plasma at 200W for one minute to clean the mold surface. Next, the molds were exposed to water vapor for two
minutes to create hydroxyl groups for the subsequent silane treatment. After purging the chamber with nitrogen, the molds were exposed to FDTS precursor for 30 minutes; only a finite amount of vapor (0.5 Torr within 2 minutes) for was released into the chamber. This water-FDTS cycle was repeated for a total of 6 cycles. Contact angle measurements were used to validate the effectiveness of the anti-stiction coating when the contact angle was greater than 90 degrees.

The negative molds of the two smallest pillars (P300, P200) were made of polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning). First, a thin layer of hardened PDMS (hPDMS) formulation adapted from Kang et al. was dropcast on the master mold. The master mold for the P300 pillars was the nickel mold previously mentioned, while the cicada wing was the master mold for the P200 pillars. This thin hPDMS layer was bonded to a PDMS backing at 110 °C for 2 hours to form a composite stamp. Previous examination under scanning electron microscopy (SEM) by Mary Nora Dickson revealed nanoholes in the hPDMS-PDMS composite. These hPDMS-PDMS composite molds did not require the FDTS anti-stiction coating to fabricate the corresponding pillars; however, the silane coating could still be helpful for even smaller features.

2.2.3 Fabrication

The majority of the nanopatterns were fabricated on the PMMA films using a nanoimprinter (Jenoptik, Hex03) in the Bio-organic Nanofabrication Facility (BION), following the process shown in Figure 2.1A. For optimal imprinting results, inside the nanoimprinter, the PMMA film (120,000 MW) was heated from 50 °C to 170 °C, which is at least 60 °C above the glass transition temperature of PMMA ($T_g = 105$ °C), and the FDTS-coated mold was pressed down against the PMMA film. To fabricate the nanolines, 2 MPa of pressure was applied for 10 minutes. To fabricate the nanopillars, 5 MPa pressure was applied for 10 minutes. Afterwards,
the mold was cooled to 50 °C over three minutes. Five minutes after the imprinting at room temperature (25 °C), the mold was released from the glass-supported PMMA film, resulting in nanostructures on the PMMA film. To fabricate the smaller nanopillars (P300, P200), the temperature was increased to 185 °C to increase the flow of PMMA into the smaller holes of the negative mold of the respective pillars. Additionally, a pressure of about 2.2 MPa was applied to the hPDMS composite mold for 20 minutes in another imprinting press (Tetrahedron).

2.3.4 Characterization

2.4.3.1 Scanning Electron Microscopy

Nanoimprinted PMMA surfaces were examined using scanning electron microscopy (SEM) to assess how successful nanoimprinting was in creating the structures on the PMMA surface. Before examination, PMMA samples were coated with a 1-2 nm layer of iridium with the sputter coater (South Bay Technologies). The imprinted nanostructures were examined using scanning electron microscopes: the FEI Quanta 3D (5 kV, 3.3 pA current) or the FEI Magellan (2-3 kV), which has an immersion lens for increased resolution of the smaller nanofeatures. All SEM characterization was performed at the Irvine Materials Research Institute (IMRI; formerly called Laboratory for Electron and X-ray Instrumentation (LEXI)).

2.4.3.2 Atomic Force Microscopy

Nanoimprinted PMMA surfaces were examined using atomic force microscopy (AFM) to assess the heights of the nanopatterns. Height measurements were made with the atomic force microscope (NTEGRA, NT-MDT Spectrum Instruments) under tapping mode. Silicon tips (< 5 nm tip diameter, AppNano) were used for these measurements. Height data were obtained from AFM scans using the NT-MDT program software or Gywddion.\textsuperscript{57}
2.4.3.3 Ultraviolet-Visible Spectroscopy

The transparency of the nanoimprinted PMMA surfaces was evaluated by measuring the transmission coefficient of visible light of the various imprinted materials using ultraviolet-visible spectroscopy (UV/Vis) using the Lambda 950 UV/Vis Spectrophotometer (PerkinElmer). The measurements would also gauge how well the device works for peripheral vision by testing the transparency of the nanostructured surfaces under varying angles of incidence light. Inside the spectrometer, the nanolines, P300, and P200 were placed on a sample holder with a 10 mm long slit, while the P700 and P600 pillared pattern were placed in a customized holder for measurements with the integrating sphere. Optical transparency was determined by calculating the percentage of visible light wavelengths that are transmitted through the nanoimprinted PMMA films. Nanopatterned surfaces that have at least 92% transparency, which is how much visible light that PMMA transmits, will be considered as optically transparent.

2.3 Results and Discussion

2.3.1 Validation of FDTS coating

To determine if there was FDTS on the silicon surface, one can perform contact angle measurements. The FDTS-coated substrate is expected to be more hydrophobic; hence, the contact angle should be greater than an untreated silicon surface. This was indeed observed, as shown in Figure 2.3. This large difference between the silicon control (green) and the silicon coated with six cycles of FDTS (violet) is evidence that the FDTS coating is effective.

Additionally, we evaluated whether the FDTS would contaminate the PMMA surface. PMMA prints 1 and 2 (orange and red, respectively in Figure 2.3) are flat imprints made with the FDTS-coated silicon. Contact angles of these flat imprints were in the same range as the contact
angle of an untreated PMMA film: 84 degrees vs. 81 degrees, respectively. The difference is within the normal range of measurement uncertainties, indicating the PMMA nanoimprinted structures was not contaminated by the FDTS silane coating on the stamps, since contact angles measurements are excellent indicators of surface contamination.\textsuperscript{58}

![Figure 2.3 Evaluation of FDTS coating validation and contamination. Data provided by courtesy of Susan Wu.](image)

2.3.2 SEM Characterization

Nanostructures were first characterized with SEM to evaluate the quality of the imprinted structures. It is necessary to examine the samples under low voltage, low current conditions to prevent damage to the polymer. Three nanoline gratings (Figure 2.4) and four nanopillar patterns (Figure 2.5) were fabricated using the nanoimprinting conditions previously described.

Dimensional measurements from SEM micrographs were performed using the measurement tool in the SEM software and ImageJ. Micrographs have a horizontal field width as low as 2.98 μm for measuring the dimensions of lines and pillars structures of the first and last samples. The dimensions of four stamp molds and their respective imprinted patterns are listed in Table 2.1. We found that the line width and periodicity of the imprinted line gratings were
similar to the corresponding dimensions of the silicon stamp gratings. Likewise, we found that the diameter and periodicity of both imprinted round pillar structures were similar to those of the respective silicon molds containing nanoholes. The decrease in observed dimensions is possibly due to the image contrast of the edges, which may cause the holes and trenches in the molds to appear smaller and the pillars and line gratings larger. Notwithstanding this uncertainty, the imprinted structures appear to be faithful reproductions of the molds. This indicates that nanoimprint lithography is a reliable and consistent technique for reproducing structures of approximately the same dimensions as the mold features.

Table 2.1 Measured dimensions of nanoimprinted features and respective stamps

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Imprint Dimensions (nm)</th>
<th>Stamp Dimensions (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Width</td>
<td>Pitch</td>
</tr>
<tr>
<td>L416</td>
<td>225 ± 10</td>
<td>411 ± 16</td>
</tr>
<tr>
<td></td>
<td>(n=12)</td>
<td>(n=12)</td>
</tr>
<tr>
<td>L278</td>
<td>134 ± 8</td>
<td>272 ± 9</td>
</tr>
<tr>
<td></td>
<td>(n=14)</td>
<td>(n=14)</td>
</tr>
<tr>
<td>P700</td>
<td>267 ± 11</td>
<td>692 ± 24</td>
</tr>
<tr>
<td></td>
<td>(n=18)</td>
<td>(n=14)</td>
</tr>
<tr>
<td>P600</td>
<td>215 ± 23</td>
<td>595 ± 18</td>
</tr>
<tr>
<td></td>
<td>(n=13)</td>
<td>(n=7)</td>
</tr>
</tbody>
</table>
Figure 2.4 Nanoimprinted line gratings in PMMA. Scale of nanoimprinted lines = 1 µm. Nanolines served to mimic collagen fibrils (bottom right). Scale = 0.2 µm. Image of collagen fibrils reprinted with permission from Nature Publishing Group.

Figure 2.5 Nanoimprinted PMMA pillars. P700, P600 pillars were taken at a 45° tilt, while P300, P200 were taken at a 30° tilt. P300 was imaged by Susan Wu. Scale = 1 µm.
2.3.3 AFM Characterization

Nanostructures were also characterized using AFM to determine the heights of the features. The z-height was first calibrated using a 21 nm high line grating spaced approximately 3 µm apart. This grating is traceable to a secondary NIST standard. The calibration ensured that measured heights were accurate readings. A cantilever tip with a radius of curvature of 6 nm was used in order for the entire tip to reach as close to the bottom of the features as possible. Shown below are representative three-dimensional profiles of the nanoimprinted line gratings (Figure 2.6) and nanoimprinted pillars (Figure 2.7). The heights of each pattern are shown in Table 2.2.

![Representative 3D profiles of AFM scans of nanolines](image)

**Figure 2.6. Representative 3D profiles of AFM scans of nanolines**

In general, the heights of the nanoimprinted structures are within 10 percent of the respective depths of the negative molds. This validated the printing conditions used to make the
structures. The expected height was not fully achieved, possibly because the cantilever tip was not able to completely reach the bottom of the features, as evidenced by the rounded bottom of the cantilever trace in AFM scans. Sharper tips with radius of 1-2 nm could be used instead, but those tips were not used because they wear quickly and would need to be exchanged for new ones very often. Height data could also be obtained from SEM micrographs. The height of the structures can be calculated with trigonometry, given the tilt angle of the stage with respect to the detector and the apparent height of the structures in the micrographs.

Figure 2.7 Representative 3D profiles of AFM scans of nanopillars. Scans were taken by Nicolas Vollereaux (A), Luis Rodriguez (B) and Mary Nora Dickson (C, D).

2.3.5 UV-Visible Spectroscopy Characterization

Transparency is an important design requirement for the artificial cornea, as the patient needs to be able to see through the implant. Fortunately, PMMA is transparent and is already
used in many ophthalmic devices, including the intraocular lens to treat patients with cataracts, and the Boston-KPro. However, validation of transparency was still needed to ensure that the imprinted nanostructures would not affect vision, especially in the central optic zone. From the SEM and AFM characterizations, the features are found to be indeed smaller than the wavelength range of visible light. I expected that even with the nanostructures, the PMMA surface would have about the same transmittance as a flat PMMA surface. Using a UV-Vis spectrometer, I measured the transmittance of the imprinted PMMA thin films on glass (#2 thickness) in the wavelength range of visible light (400 – 700 nm).

Figure 2.8A shows that the transmittances of the two smallest pillars (P300, P200) were just as high as the flat PMMA control (prepared following the protocol in Section 2.2.1). Similarly, the transmittance of the smallest nanoline pattern (L278) was about the same as the flat PMMA. Data of the nanolines in Figure 2.8B were oriented 90 degrees with respect to the incidence light. These findings are similar to those from previous work that investigated the reflectivity of various nanostructures, including those on cicada wings, from which the P200 pillars were directly replicated. As Cai et al. described, rays of certain incidence angles bend in the array of structures that are smaller than the wavelength of light, allowing the transmittance of the incidence rays rather than reflecting from the surface.

Interestingly, the P600 and the L416 nanopatterns did not transmit consistently across the visible light spectrum, even though the features are smaller than the wavelengths of light. However, the spacing between the P600 pillars was on average 342 nm (n=11). The spacing may be large enough for the incidence rays to stay trapped between the pillars. Yet, the spacing for the L416 nanolines was on average 165 nm (n=12). The transmittance could also depend on the orientation of the lines during measurements, as light may diffract along the trenches of the
nanoline gratings. Thus, having data of lines at varying orientations would provide an overall assessment of the transmittance of the nanoline gratings.

**Figure 2.8 Optical transmittance of nanoimprinted PMMA films.** Nanopillars (A) and the two smaller nanolines (B) were evaluated using the Lambda 950 UV-Vis spectrophotometer, courtesy of Dr. Matthew Law. Transmittance was measured in the wavelengths of visible light.

2.3.6 Fabrication by Double-Imprinting

Thus far, I have fabricated nanostructures using negative molds. However, this restricts the types of structures I could fabricate. Thus, my colleague Susan Wu and I developed a double imprinting process to fabricate pillars with a square cross-sectional area—the resulting structures are referred as square pillars. To fabricate the square pillars, we first imprinted a line grating
pattern using the lines molds, then rotated the first imprint by 90 degrees, and printed a line pattern a second time (Figure 2.8A), thus creating a grid pattern on the polymer surface similar to that described in Wang et al.\textsuperscript{63} The first imprint was fabricated following the usual printing conditions for the nanolines. For the second imprint, the line mold was oriented perpendicular to the lines from the first imprint. Fabrication of the square pillars was challenging in that we had to make sure that the lines from the first step did not melt during the second imprinting step. To make sure the first imprint did not lose its shape during the second imprinting step, the temperature of the second imprinting was 30 °C lower than that in the first. We succeeded in imprinting square pillars with side lengths of approximately 200 nm and a periodicity of approximately 420 nm (Figure 2.8B) and another student imprinted larger square pillars (Figure 2.8C). My colleague Mary Nora Dickson described the use of the same process to vary the spacing between pillar structures without changing the dimensions of the pillars.\textsuperscript{64}

Figure 2.9 Fabrication of square-topped pillars. (A) The first imprint was fabricated following the normal printing procedure. For the second imprint, the line mold was placed perpendicular to the direction of the first imprint. The double imprinting process resulted in square pillars. Two examples are 200 nm wide square pillars, imaged at a 45° tilt (B), and 424 nm wide square pillars (C), which were imprinted by Patrick Lo. Scales = 1 µm.
2.4 Summary

In this chapter, I describe techniques for the fabrication of nanostructures that were inspired by two natural topographies: linear gratings of collagen fibrils in the natural cornea to potentially encourage cell adhesion, and nanocone-shaped pillars found on the surfaces of cicada wings. Dimensions of the structures are summarized in Table 2.2.

Table 2.2 Summary of Nanostructures

<table>
<thead>
<tr>
<th>Patterns</th>
<th>Width (nm)</th>
<th>Pitch (nm)</th>
<th>Height (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L825</td>
<td>432</td>
<td>825</td>
<td>182</td>
</tr>
<tr>
<td>L416</td>
<td>225</td>
<td>411</td>
<td>115</td>
</tr>
<tr>
<td>L278</td>
<td>134</td>
<td>272</td>
<td>94</td>
</tr>
<tr>
<td>P700</td>
<td>267</td>
<td>692</td>
<td>300</td>
</tr>
<tr>
<td>P600</td>
<td>215</td>
<td>595</td>
<td>300</td>
</tr>
<tr>
<td>P300</td>
<td>190</td>
<td>320</td>
<td>325</td>
</tr>
<tr>
<td>P200</td>
<td>Cap: 60</td>
<td>170</td>
<td>225</td>
</tr>
<tr>
<td></td>
<td>Base: 120</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P300, P200 characterized by Mary Nora Dickson

These topographies have the potential to encourage or discourage cell adhesion. Using nanoimprint lithography, I have fabricated three nanoline gratings and four nanopillar patterns that have dimensions that are comparable to the dimensions of the respective negative mold features. I describe in the next chapter investigations on whether these nanopatterns would influence cell adhesion to surfaces.
Chapter 3 Mammalian Cell Adhesion on Nanopatterns

3.1 Introduction

There are many examples of nanotopography in nature, including insect wings, gecko feet, and plant leaves. In animals, tissues exhibit many chemical and topographical cues that a cell interacts with. This natural nanoscale topography has significant effects on cell behavior. The most common topography used to examine cell behavior is line gratings, as they are easier to fabricate. Many studies show that several cell types are influenced by nanoline topographies. Nanoline gratings were found to cause smooth muscle cells to elongate and align to the lines; simultaneously, however, cell proliferation on such surfaces was significantly reduced.\textsuperscript{65} McWhorter \textit{et al.} and Luu \textit{et al.} showed a correlation of elongated cell shape and a pro-healing macrophage phenotype.\textsuperscript{66,67} In a study examining contact guidance, ridge patterns with sub-100 nm feature dimensions (as small as 70 nm) were able to induce elongation of corneal epithelial cells and alignment along those patterns.\textsuperscript{68,69} Similarly, keratocytes, corneal fibroblasts, and corneal myofibroblasts aligned and elongated with line grating with pitches ranging from 400 to 4000 nm; pitch was defined as the sum of the groove and ridge widths.\textsuperscript{70} These cells were not affected by patterns of submicron pitch; the smallest pitch on which changes in alignment and elongation were observed was 800 nm for keratocytes and fibroblasts, and 1200 nm for myofibroblasts.\textsuperscript{70} A follow-up study showed that these line grating patterns, specifically 1400nm-pitch lines, stabilized the corneal fibroblast phenotype, reducing the differentiation to myofibroblasts.\textsuperscript{71}

Fewer researchers have examined the effects of other geometries, such as nanopillars to and nanopores, on cell behavior. Nanopores have been observed in the natural basement
membrane, while nanopillars or nanocolumns have been found on insect wings and the lotus leaf for self-cleaning purposes. Dalby et al. first observed increased response from fibroblasts on 13 nm-high islands, and later found changes in fibroblast morphology when the cells were seeded on 160-nm high nanocolumns. Hu et al. showed that nanopillars of widely varying aspect ratios and surface energies had strong effects on cell morphology, discouraging cell spreading of human dermal fibroblasts. Kong et al. discovered that human embryonic stem cells grown on nanopillar structures had a significantly reduced number of focal adhesions per cell and exhibited increased cell motility on the nanopillar structures. Recently, Jeon et al. found that nanocrater-patterned silicon surface repel cells by altering the size and distribution of the cells’ focal adhesion, leading to changes in morphology and more directional migration. These nanocraters disrupted the formation of mature focal adhesions, resulting in directed migration towards regions with increased planar area. Similarly, Chapman et al. showed nanoporous gold selectively decreased adhesion and surface coverage of astrocytes while maintaining high neuron count. Importantly, the chemical nature of the material surface does not appear to be as significant as the topography of the surface.

All these studies indicate that micro- and nanotopography can influence cell behavior and suggest that it may be possible to control cell adhesion and proliferation through surface topography. However, the majority of the studies have not examined nanotopographical influence on cell adhesion. Researchers assumed if cells are elongating and aligning with linear ridges, then the cells are adherent to the surface. In this chapter, I describe how I evaluated cell adhesion on the nanopatterned surfaces previously described, to determine which nanopatterns are more effective in encouraging or discouraging cell adhesion. I hypothesized that nanoline gratings would encourage cell adhesion while nanopillars would discourage cell adhesion.
3.2 Methods

3.2.1 Nanostructure fabrication

Nanostructures were fabricated on thin films of poly(methyl methacrylate) (PMMA). PMMA was spin coated on glass coverslips (#2, 22 x 22 mm) that were pretreated with UV-ozone for 5 minutes and then coated with a coupling agent, 3-aminopropyltriethoxysilane (APTES), in deionized water (1 volume %) to facilitate PMMA adhesion to the glass. PMMA (M.W. = 120 kDa, Sigma-Aldrich, Milwaukee, WI) was dissolved in toluene (5 weight %) and spin-coated on glass coverslips at 600 rpm for 45 seconds. Films were annealed on a hot plate at 100 °C for 5 minutes to remove residual solvent. Nanostructures were fabricated by nanoimprint lithography following protocols previously described. In one set of adhesion experiments, nanoimprinted structures included one line pattern, designated as “L825” (periodicity = 825 nm, depth = 200 nm, duty cycle = 50%) as a reference to compare current results with literature, and three types of pillars, designated as “P700,” “P600,” and “P300,” shown in order of decreasing periodicity. Another set of experiments included an additional line pattern designated as “L416” (periodicity = 416 nm, depth = 100 nm, duty cycle = 50%). Nanostructures were characterized by scanning electron microscopy (SEM) (FEI Quanta 3D, 5 kV) and atomic force microscopy (NT-MDT Integra).

3.2.2 Cell culture

Figure 3.1 summarizes how cell adhesion experiments were performed. Samples, placed in 6-well plates, were sterilized with UV light for 5 minutes. Samples were then rinsed with sterile 1X phosphate buffered saline (PBS) to clean off remaining debris. Nanopatterns were coated with fibronectin (2 µg/ml) for 1 hour at room temperature. After incubation, surfaces
were rinsed with 1X PBS. NIH 3T3 fibroblasts (ATCC CRL-1658) were seeded on the nanopatterns and flat PMMA control surface. Cells were incubated at 37 °C, 5% CO₂ for 4-6 hours. After incubation, media was replaced with fresh media to remove non-adhering cells so that the only cells that remained on the surfaces were ones that fully adhered. Well plates were gently swirled ten times. Incubation at 37 °C, 5% CO₂ was continued for a total time of 24 hours. For proliferation studies, cells were incubated up to 48 hours and 72 hours. Media in the wells were replaced with fresh media after imaging cells at the 24-hour and 48-hour time points.

Figure 3.1 Method of examining cell adhesion on nanostructured surfaces

3.2.2 Cell count, viability, and proliferation analysis

Three to five randomly selected regions, approximately 2 sq. mm, on each pattern were imaged on the Olympus IX51 inverted microscope with a 4X objective. Cells were counted with ImageJ software using the Cell Counter plugin. The number of cells in each image was divided by the total area of the image to get cells per sq. mm. The number of cells per area representing each sample is the average number per area of the three to five regions.

Viability of cells on the nanostructured surfaces was assessed using Live/Dead® Viability/Cytotoxicity Kit for mammalian cells (ThermoFisher Scientific, Carlsbad, CA). The staining solution was prepared following the manufacturer’s recommended dilutions for calcein-AM, used for live cell detection, and ethidium homodimer, used to detect dead cells. Samples were incubated in the staining solution at room temperature for 20 minutes. Afterwards, samples
were rinsed with 1X DPBS containing calcium and magnesium ions after incubation, and then examined under fluorescence using a standard green filter set to image live cells and a standard red filter set to image dead cells. Live and dead cell populations were counted with ImageJ software using the Analyze Particles function. Viability was calculated by dividing the number of live cells by the total number of cells, the sum of live and dead cell counts. Total cell count data from the viability studies were included in the 24-hour cell counts.

Proliferation rate after 24 hours was determined from the cell count data. Cell counts were taken at three time points: 24 hours, 48 hours and 72 hours. The number of cells on the surfaces after 48 hours and 72 hours was obtained as previously described. The percentage increase in cell count was determined from the 24 to 48 hour time points, and 48 to 72 hour time points.

3.2.3 Scanning electron microscopy

Cells were fixed in 2.5% glutaraldehyde for one hour. Afterwards, the samples were subjected to a series of ethanol dehydration (50-100% ethanol) steps. Samples were then rinsed with hexamethylsilazaine for about 2 minutes, and left in the fume hood to air-dry. Prior to imaging under scanning electron microscopy (SEM), samples were sputter-coated with iridium (2-5 nm) (South Bay Technologies). Samples were examined under the high-vacuum mode at 2-5 kV (FEI Quanta 3D, Irvine Materials Research Institute).

3.2.4 Statistical Analysis

Statistical significance for all data was determined using the Student’s t-test (two-sample, unequal variance) in Microsoft Excel. A p-value of 0.05 or less was defined as statistically significant. Unless specified, the data represented the mean ± the standard error of the mean
(S.E.M.) for at least three trials, each done in duplicate or triplicate. Viability evaluation included three independent experimental trials with three samples for each pattern in each trial. Proliferation determination included three independent experimental trials with two samples for each pattern in each trial. The mean proliferation is the average of six total samples. Density distribution plot for cell elongation ratio was prepared using the ggplot2 package in R software to observe the variance among samples.

3.3 Results and Discussion

3.3.1 Adhesion and viability on nanopatterned surfaces

A live-dead assay was performed to determine if mammalian cells would survive on the nanopatterns, especially on the nanopillars, as the pillars have been shown to kill bacteria.\(^{51}\) From the live-dead staining assay, I observed viable 3T3 fibroblasts on all types of surfaces (Figure 3.2). In the fluorescence images, there were many green live cells, while there were very few, if any, red dead cells. The average viability of the flat control surface was 96 percent. The viability on the other surfaces were greater than 90 percent: 98 percent on P700 pillars, 93 percent on P600 pillars, 97 percent on P300 pillars, and 97 percent on L825 lines. This is encouraging, as it is not ideal for the patient’s cells to die on the surfaces. A preliminary experiment also showed that cells remained viable on the surfaces after 72 hours. To ensure long-term viability, one could perform a live-dead staining assay of cells after growing on surfaces for up to one week.
Figure 3.2 Cell viability on the nanostructured surfaces after 24 hours. Overall the cells are viable on all surfaces, even on the P300 nanopillars, which we previously found to kill bacteria.

In my initial evaluation of adhesion on the nanostructured surfaces, there were six experimental trials, done in duplicate. However, there was inconsistent cell seeding on the surfaces for the first three experiments. To reduce any effects due to varying cell seeding densities, data from those first three experiments were not included in the data analysis. The seeding densities between the last three trials were different from one another. In order to still compare data from those trials, I normalized the average number of cells per area on the nanostructured surfaces to the average number of cells per area on the flat control surface. To utilize results from the viability experiments, I added the number of live and dead cells to obtain the total number of cells in the image area. The average total number of cells per area on each nanopattern was normalized as mentioned earlier.

Normalizing to the number of cells on the flat control surface, I observed decreased adhesion on the nanopillared surfaces (Figure 3.3). There was a 20-30% decrease of adhered cells on the three nanopillars evaluated after 24 hours (n=6 trials). The P700 pillared surface was more effective in decreasing adhesion compared to P600 and P300 (p<0.05 vs. flat). By contrast, there was an approximate 13% increase in adherent cells on the L825 nanoline gratings (n=10
trials), and a 17% increase on the L416 nanolines (n=3 trials). Both nanoline gratings seem to encourage cell adhesion, though the larger line pattern appeared more effective.

Figure 3.3 Cell adhesion on the nanostructured surfaces after 24 hours. Fewer cells per sq. mm are observed on the P700 nanopillared surfaces compared to flat PMMA; *p<0.05. A trend of increased adhesion is observed on the nanolines. Data represent the mean ± S.E.M. for at least three trials, done in duplicate or triplicate.

Findings from the two experiments show a trend towards increased number of cells/mm$^2$ on the nanoline gratings and decreased number of cells/mm$^2$ on the nanopillars. However, the difference may not have been as clearly noticeable after 24 hours of adhesion, as 24 hours may have been too long, which provided cells ample time to adapt on surfaces. To better understand and evaluate cell adhesion, one would need to evaluate within 2-4 hours after seeding cells on surfaces, in the initial period of adhering to surfaces. Nonetheless, adhesion findings after 24 hours provided evidence that nanotopography could be used to either encourage or discourage adhesion.


3.3.2 Proliferation on nanopatterned surfaces

Since any medical implant will be in a patient for more than 24 hours, I examined the cell proliferation on the nanostructured surfaces. Figure 3.4A shows the increase in the number of cells/mm$^2$ on the nanostructure surfaces over 72 hours. The cell count on the flat control surface increased by 467% compared to the cell count after 24 hours. This is expected since the doubling time for NIH 3T3 fibroblasts is 18 hours. The increase was possibly higher than 400% since the second 24-hour proliferation rate was higher than the first 24-hour rate (Figure 3.4B). The number of cells on the P700, P600, and P300 pillared surfaces increased by 430%, 500%, and 400%, respectively. Again, the increase in cell population was supported by the 24-hour proliferation rate. Interestingly, the second 24-hour proliferation rate for cells on the P300 pillars was lower than that same rate for cells on flat PMMA, which may explain a lower cell population on the P300 pillars. The results suggest that the P300 pillars were more effective in controlling proliferation. Surprisingly, the lowest increase in cell population after 72 hours was observed on the L825 nanolines (380% increase). This smaller increase may be because the nanoline pattern was close to being completely covered with cells, as NIH 3T3 fibroblasts are contact-inhibited cells. The 24-hour proliferation rates on L825 lines were similar to the rates on the P300 pillars.
Figure 3.4 Cell proliferation on nanostructured surfaces. L825 had the highest number of cells compared to the other surfaces, while P700 pillars had the lowest cell population (A). The increase in cell count was calculated after 24-hour intervals (B).

Though the proliferation rate on the L825 nanolines was not as high as for the flat surface, a strategy to control proliferation on surfaces is still necessary to prevent the overgrowth of cells. For the artificial cornea implant, it is possible for keratocytes to differentiate into myofibroblasts, potentially leading to retroprosthetic membrane formation. As a way to control adhesion, a surface could be fabricated to include line grooves and pillars to simultaneously encourage and discourage adhesion. Though the methodology for calculating
proliferation from the cell count data is valid, a more controlled approach to evaluating cell proliferation is to use a bromodeoxyuridine (BrdU) incorporation assay. In this assay, BrdU, an analog of thymidine, is added to a cell culture, and incorporated in the DNA of dividing cells. Using various DNA detection methods, one can count the number of cells with BrdU to determine cell proliferation. However, more samples would be required to evaluate proliferation at the longer time points; one could not evaluate proliferation using the same samples at different time points since cells may not survive long shortly after BrdU incorporation.\textsuperscript{80} Preliminary testing is needed to establish the optimal concentration for long time-span experiments.

3.3.3 Cell morphology and interaction on nanopatterns

Morphologies of 3T3 fibroblasts on the nanopatterns are shown in Figure 3.5. The fibroblasts on flat PMMA surface have characteristic spindle-shaped morphology. Cells on nanoline gratings were also spindle-shaped. Additionally, they tended to align with the line gratings and elongate, as observed in previous studies.\textsuperscript{65,68,81} Cell elongation was characterized by calculating the elongation ratio, defined as the ratio of the longest length to the width of a cell. As seen in Figure 3.6, the distribution of the elongation ratios of cells on the L825 nanolines was broader, since there were more cells with higher elongation ratios in comparison to cells on a flat surface. In contrast, the majority of cells on the nanopillared surfaces exhibited a rounded shape and appears more spread-out. However, the cells on the smaller, closely spaced P300 nanopillars were more elongated than those on the P700 and P600 nanopillars, as shown in an increase in elongation ratio. In fact, the elongation ratio distribution on the P300 pillars was similar to the distribution on the L825 lines. This may indicate that the dimensions of the P300 pillars are close the minimum size and spacing that would deter cell adhesion.
Figure 3.5 SEM micrographs of cells on nanostructured surfaces. Cells on flat PMMA and L825 lines are spindle-shaped. Cells rounded on larger nanopillars. Scale = 10 μm at low magnification. Cell interaction with nanostructures observed at higher magnification. Scale = 1 μm.
Figure 3.6. Distribution of elongation ratios of cells on nanopatterns. Cells on P700 and P600 pillars exhibit a slightly smaller elongation distribution compared to the flat PMMA control, while cells on the L825 nanoline pattern could exhibit larger ratios. Data collected from at least 100 cells for each nanopattern across three experimental trials.

While imaging the interface between the nanopatterns and cells, I found that the cells were not merely sitting on top of the nanopillars. It appears that the filopodia of the cells were pulling the nanopillars towards the cell bodies, consistent with previous observations. The right column of Figure 3.5 shows the cells interacting with the nanopatterns themselves, which is astounding, considering that the nanostructures are one thousand times smaller than the cells. This led to the question of how the cells were interacting with the nanostructures. We decided to investigate the cells’ focal adhesions, which are protein complexes serving as anchoring points of how cells adhere to the extracellular matrix in vivo.
3.4 Summary

We aimed to determine if nanotopography could indeed affect how cells adhere to surfaces. I first evaluated cell adhesion in a static state using NIH 3T3 fibroblasts to compare results from the literature. I found evidence of the nanostructured surfaces affecting the number of cells adhering to those surfaces. There was a decrease in adhesion on the nanopillared surfaces compared to the flat control surface, while there was an increase in adhesion on the nanoline gratings. Additionally, there were changes in proliferation rate and cell morphology, and other indicators for differences in adhesion on the nanostructured surfaces. These findings encouraged me to determine how cells would be adhering differently on the nanostructured surfaces, leading to a study examining focal adhesions of cells on the nanostructured surfaces.
Chapter 4 Correlation of Focal Adhesion Assembly and Disassembly with Cell Migration on Nanopatterns

4.1 Introduction

Mesenchymal cell migration is regulated through focal adhesions (FAs) and can be modulated by their microenvironment, including changes in surface topography. During migration, cell polarization causes new adhesions to form at the leading edge while mature stable adhesions disassemble at the trailing edge. Nanotopography is most appropriate for modulating cell behavior through individual adhesions. For example, cells on nanolines migrate and form adhesions parallel to the features while those on nanopillars form smaller adhesions and migrate omnidirectionally. Many of these studies examine the role of focal adhesions (FAs)—protein complexes that regulate adhesion and migration—during migration by staining for FA proteins; however, this method does not capture the dynamics of proteins of the FAs. Thus, examination at the single-protein scale in live cells is important to understand how the formation of adhesions on these patterns will affect cell migration.

Advances in fluorescence correlation spectroscopy (FCS) enable spatiotemporal detection of single molecules. Using the same data set, the molecular brightness of individual molecules can be calculated by photon counting histogram analysis within the diffraction-limited volume (~250 nm). To obtain a pixel by pixel protein aggregation map of the FA protein paxillin diffusing within the cell, we use an image analysis technique, Number and Molecular Brightness (N&B).

---

1 This work is published in Integr. Biol., 2017, 9, 145. DOI: 10.1039/c6ib00193a
We further compared migration behavior, paxillin aggregation ratios, and adhesion size of NIH 3T3 fibroblasts plated on a flat surface, a nanoline pattern, and nanopillars of varying width and periodicity. We found that cell motility is highly sensitive to particular types of nanotopography. Specifically, nanopillar PMMA surfaces, which possess a six-fold symmetry, guided cells to migrate in specific orientations. In addition, cells on the smallest nanopillars (P200 and P300) exhibited the highest motility but had the lowest population of disassembling clusters of protein (~2 paxillin/cluster). Cells on pillars spaced 380 nm apart (P600) exhibited the largest population of higher order protein aggregates (~3-5 paxillin/cluster) and concurrently larger disassembly adhesions. These results provide a deeper understanding of the dynamics of adhesion formation and disassembly during migration. This is the first report on molecular scale dynamics of adhesions influenced by nanostructures, and the correlation of assembly and disassembly of adhesions to cell migration on nanostructured surfaces.

4.2 Materials and Methods

4.2.1 Cell lines and reagents

NIH 3T3 fibroblasts (ATCC, CRL-1658) were cultured in high-glucose Dulbecco’s modified Eagle’s Medium (Life Technologies, Rockville, MD) containing 10% (v/v) fetal bovine serum (FBS, Life Technologies, Rockville, MD), 1% (v/v) nonessential amino acids, and 1% penicillin-streptomycin (Life Technologies, Rockville, MD) and maintained at 5% CO₂ and 37°C. Cells were washed with 1X Dulbecco’s Phosphate Buffer Saline (Life Technologies, Rockville, MD) and detached with 1X Trypsin in EDTA (Life Technologies, Rockville, MD) and plated on various surfaces coated with 1 µg/ml fibronectin (Sigma, Aldrich, Milwaukee, WI) for experiments. For migration experiments, 100,000 cells were seeded on the nanostructured
surfaces. For paxillin experiments, cells were seeded into a 6-well culture plate overnight and transfected with 1 µg monomer EGFP or paxillin-EGFP DNA plasmids (both were generous gifts from Rick Horwitz, University of Virginia) with Lipofectamine 2000 (Life Technologies, Rockville, MD) following the manufacturer’s protocol. Transfected cells were then transferred to the nanostructured surfaces and incubated for 1-2 hours at 37°C in 5% CO₂ before conducting imaging experiments.

4.2.2 Fabrication of Nanostructures

Nanostructures were fabricated on thin films of poly(methyl methacrylate) (PMMA) on glass coverslips (#1.5, 22 x 22 mm) that were pretreated with UV-ozone for 5 minutes and then coated with 3-aminopropyltriethoxysilane (APTES) in deionized water (1 volume %) to facilitate PMMA adhesion to the glass. PMMA (M.W. = 120 kDa, Sigma-Aldrich, Milwaukee, WI) was dissolved in toluene (5 weight %) and spin-coated on glass coverslips at 600 rpm for 45 seconds. Films were heated on a hot plate at 100°C for 5 minutes to remove residual solvent. Nanostructures were fabricated by nanoimprint lithography following protocols described in Chapter 2. Nanoimprinted structures included one line pattern, designated as “L825” (periodicity = 825 nm, depth = 187 nm, duty cycle = 50%) as a reference, and four types of pillars, designated as “P700,” “P600,” “P300,” and “P200,” shown in order of decreasing periodicity. Nanostructures were characterized by scanning electron microscopy (SEM) (FEI Quanta 3D, 5 kV) and atomic force microscopy (NT-MDT Integra).

4.2.3 Sample Preparation

Custom-made sample dishes were prepared for experiments. A 15-mm cork borer was heated over an open flame to 100°C and then immediately pressed into the center of a 35-mm
culture dish (Corning, Midland, MI). The edges of the hole were sanded down until smooth. The nanostructured PMMA-on-glass coverslips were mounted to the bottom of the dishes with clear silicone adhesive (Corning, Midland, MI) so that the nanostructured region was placed in the center of the opening. The mounted samples were dried overnight before use. At the time of cell culture, the samples were UV-sterilized for 5 minutes. Nanostructured surfaces were coated with 1 µg/mL of fibronectin (Sigma-Aldrich, St. Louis, MO) for 1 hour at 37°C before cell seeding.

4.2.4 Imaging

A Zeiss LSM 710 Axio-Observer inverted microscope was used for migration experiments and the paxillin aggregation measurements for flat, L825, P700, P600 and P300 surfaces. Time-lapse imaging migration experiments were performed using an EC Plan-Neofluar 20X/0.5 M27 air objective with the Zeiss LSM 710. Images are 512 by 512 pixels at 8-bit depth. The pixel dwell time was set to 1.58 µs with one-minute intervals for 12 hours using the transmission pathway. Cells were kept under incubation at 37 °C and 5% CO₂ for the entire duration of the experiment.

For aggregation experiments, cells were imaged using a C-Apochromat 40X/1.2NA Korr M27 water immersion objective. Cells were excited with a 488-nm argon laser. An excitation dichroic was used to send the laser beam to the sample. The emission pathway consisted of a diffraction grating to disperse the emitted light and two prisms were used to select the emission band for each photomultiplier tube (PMT). 500-550nm was used to collect the EGFP signal. Image acquisition was performed with a 12.5 µs per pixel dwell time as a continuous time series of 100 frames (size = 256x256 pixels). Images were taken at 12-bit depth. Cells were kept under incubation chamber set to 37 °C and 5% CO₂ while imaging.
Paxillin aggregation for cells on P200 was measured using the Olympus FluoView FV1000 and a 60X/1.2 NA water immersion objective (Olympus, PA). Cells were excited with a 488-nm argon laser. The emission was collected with a BA505-605 bandpass filter. Images (256x256 pixels) were collected at 10 µs per pixel dwell time for 100 consecutive frames.

4.2.5 Cell Migration Analysis

Images collected for the migration experiments were analyzed using the Manual Tracking plugin in Fiji (ImageJ) software. The positions of the cells were tracked for each surface in each experiment by following the nucleus of the cells. Cells included in tracking were those present in the first time point so that all cells were exposed to the same initial condition. Tracking of the cells was cut off at 700 frames (approximately 12 hours) since this was the longest time that a majority of the cells remained in the imaging frame without dividing or undergoing apoptosis. Migration experiments were performed on three separate occasions per surface. The migration path of each cell was plotted in MATLAB using coordinates collected from Manual Tracking. The first coordinates of each path were set at the origin, and the remaining coordinates were adjusted using the first coordinates as the reference. The radius of trajectory was calculated by taking the distance between the last coordinates and the origin. The immobility of cells was determined by counting the number of zero velocities over the recorded migration period. The angle of each cell migration trajectory was measured using the SimFCS program, developed at the Laboratory for Fluorescence Dynamics (http://www.lfd.uci.edu), by determining the angle of the best-fit line of the each trajectory from 0 degrees. The distribution of angles for each surface pattern was displayed on angle histogram plots using Matlab.
4.2.6 Paxillin Aggregation Analysis

N&B analysis was performed to assess paxillin aggregation using the SimFCS program. Cells expressing paxillin-GFP were seeded on each fibronectin-coated surface with or without the nanostructures and incubated for 2 hours under specified culture conditions before imaging the adhesions. Imaging soon after cell attachment ensures that the cells are in an active state and accurate N&B measurements are captured. For the N&B analysis, images of adhesions of at least ten cells were de-trended using a moving Gaussian average. The molecular brightness of cells transfected with monomeric GFP was first assessed to obtain the brightness of paxillin monomers. This value was then used to calculate the brightness of paxillin dimers and higher order aggregates by multiplying by a factor of 2 for dimers, and 3 or greater for higher order aggregates. Details of the mathematics and analysis process have been previously described.\textsuperscript{97}

A brief description of the N&B analysis is given here. 100 frames were collected over time so that each pixel in the image contains intensity fluctuations of the proteins as a function of time. The average intensity (first moment) of each pixel $\langle k \rangle$ was calculated along with its variance $\sigma^2$ (second moment). The first and second moments were calculated with Equations 4.1 and 4.2, respectively, where $K$ is the total number of frames collected, $k_i$ is the intensity of pixel $i$ collected over time.

$$\langle k \rangle = \frac{\sum_i k_i}{K} \quad (4.1)$$

$$\sigma^2 = \frac{\sum_i (k_i - \langle k \rangle)^2}{K} \quad (4.2)$$

During the analysis, variance, a combination of the occupation number ($\sigma_n^2$) and additional contributions from the detector such as shot noise or count statistics ($\sigma_d^2$), were
calculated. These two parameters are a function of the true molecular brightness, $\varepsilon$, and the average number of molecules illuminated within the focal volume, $n$ (Equations 4.3-4.5).

$$\sigma_n^2 = \varepsilon^2 n$$  \hspace{1cm} (4.3)

$$\sigma_d^2 = \varepsilon n$$  \hspace{1cm} (4.4)

$$<k> = \varepsilon n$$  \hspace{1cm} (4.5)

The apparent brightness ($B$) for each pixel was defined as the ratio of the variance and average intensity. This was also used to calculate the apparent number of particles ($N$) as a fraction of the total intensity. Both Equations 4.6 and 4.7 below show the mathematical formula for $B$ and $N$. By rewriting Equations 4.6 and 4.7, the values of $n$ and $\varepsilon$ were easily calculated, as shown in Equations 4.8 and 4.9.

$$B = \frac{\sigma^2}{<k>} = \frac{\sigma_n^2}{<k>} + \frac{\sigma_d^2}{<k>} = \frac{\varepsilon^2 n}{\varepsilon n} + \frac{\varepsilon n}{\varepsilon n} = \varepsilon + 1$$  \hspace{1cm} (4.6)

$$N = \frac{<k>^2}{\sigma^2} = \frac{\varepsilon n}{\varepsilon + 1}$$  \hspace{1cm} (4.7)

$$n = \frac{<k>^2}{\sigma^2 - <k>}$$  \hspace{1cm} (4.8)

$$\varepsilon = \frac{\sigma^2 - <k>}{<k>}$$  \hspace{1cm} (4.9)

For images with immobile fractions, the above expressions did not hold since there would not be any temporal fluctuations. Thus, the apparent molecular brightness would equal 1 ($B=1$). The ratio of these two terms was used to isolate the immobile fraction of cell images, denoted as $B=\sigma^2/<k>$. If the pixel had immobile and mobile components, $B$ would be between 1 and the value obtained from the mobile fraction.
4.2.7 Adhesion Size Measurement

The adhesions containing dimers and higher order aggregates are found by comparing the confocal images with the corresponding brightness map. The area of those adhesions was acquired using the freehand selection tool in ImageJ to trace the individual adhesions from the confocal microscopy images used in N&B analysis. The areas of these adhesions were determined using the ImageJ measure function.

4.2.8 Statistical Analysis

Unless stated otherwise, data represents as the mean ± standard error of the mean (S.E.M.). Statistical significance for migration and paxillin aggregation data was determined using the Student’s t-test (two-sample, unequal variance) in Excel.

4.3 Results and Discussion

4.3.1 Cell Migration on Nanostructured Surfaces

In order to observe cell migration behavior on our nanostructures, we took time-lapse images of cells seeded on the different surfaces at one-minute intervals over a 12-hour period. The positions of cells on each surface were tracked for the full 12 hours. Cells included in the analysis were ones present from the first frame to the ending time point (frame 700). We excluded cells that migrated out of the frame of view, divided, or died before the 12-hour period from analysis. For all experiments, we coated the PMMA surface with fibronectin, and we assumed that PMMA surfaces behaved as a rigid material.

First, we calculated the average radius of each migration path. This represents the distance from the original position to the furthest point. Radial plots of the cell trajectories on the surfaces show that cells on L825 (Figure 4.1B) generally migrated along the underlying line...
structures within an average net radius of 150 µm, while cells on the nanopillars (Figure 4.1C-F; P700, P600, P300, P200, respectively) migrated in orientations that are guided by the geometric arrangement of the pillars. Cells on flat surfaces (Figure 4.1A) migrated in random directions within an average net radius of 112 µm. More significantly, cells on P300 and P200 nanopillars traveled within an average net radius of 154 and 153 µm, respectively (p<0.01 for P300 vs. flat surfaces, p<0.05 for P200 vs. flat surfaces).

It is interesting to determine if these nanostructures affected cell motility. To this end, we determined the migration speed (Figure 4.1G) and the total migration distance traveled of each cell on the nanostructured surfaces (Figure 4.1H). We defined the total migration distance as the sum of the distances traveled at each time point. We found that cells on the P700 and P600 nanopillared surfaces traveled similar total distances (372 and 336 µm, respectively) over 12 hours compared to cells on flat surfaces (387 µm). We found also that cells on flat and P700 surfaces migrated at similar average speeds (0.55 and 0.57 µm/min, respectively), while those on P600 surfaces migrated at a slightly slower average speed (0.48 µm/min). By comparison, cells on P300 and P200 surfaces were the most motile with an average total distance traveled of 472 and 483 µm, respectively. Interestingly, cells on the P200 pillars migrated on the surface at a higher average speed than those on the other surfaces, especially the P300 pillars (0.77 vs. 0.59 µm/min; p<0.01). Upon closer examination of the speed data, we found that cells on P300 pillars had more periods of rest compared to cells on the P200 pillars. This suggests that cells on P200 were moving more often than cells on P300 during the recorded migration time (data not shown). Over the 700-minute period, cells on P300 were immobile for on average 82% of the time, while cells on P200 were immobile for on average 71% of the time. This significant difference (p<0.01 by Student’s t-test) may imply that adhesions formed on P200 pillars were less stable than
adhesions formed on the P300 pillars, as suggested by Kong et al.\textsuperscript{75} The instability could be due to the size of the adhesions, which we discuss later. This would explain the discrepancy between average speed versus the total distance traveled by cells on P300 and P200 pillars.

Figure 4.1 Migration on nanostructured surfaces. Trajectories of 3T3 cells over 12 hours on (A) flat surface, n=54 cells; (B) L825 lines, n=63; (C) P700 pillars, n=70; (D) P600 pillars, n=72; (E) P300 pillars, n=32; and (F) P200 pillars, n=36. The axes display coordinate values in µm. Black circle in plots represent the net radius of migration trajectories of cells on each surface. (G) Average migration speed of cells. The values below each pattern represent mean ± standard error of the mean (S.E.M.) Statistical significance was assessed using the Student’s t-test: *: p<0.05 compared to P700; **: p<0.01 compared to other surfaces. (H) Total distance traveled on nanostructured surfaces over 12 hours. **: p<0.01 compared to L825; #: p<0.01 compared to Flat, P700, P600.

Cells on L825 surfaces traveled the shortest total distance on average (229 µm). The average speed of these cells was also significantly lower than the speed of cells on the other surfaces (0.36 µm/min; p<0.01). In addition, cells were immobile for 89% of the time. These
results should be contrasted with findings in other studies that show cells on nanolines (width = 350 nm to 6 µm, spacing = 70 nm to 4 µm) where it is reported that they travel farther than those on flat surfaces.\textsuperscript{99–101} However, our results are similar to those of Ferrari \textit{et al.}, in which cells on nanolines of similar size as L825 traveled a shorter distance over time compared to cells on flat surfaces.\textsuperscript{88} This suggests that the size of the line gratings may affect the formation of the adhesions and, as suggested by Ferrari \textit{et al.}, cytoskeletal structures that are needed in the correct orientation optimal for migration. In addition, cells on the L825 patterns initially take time to elongate before motion of the cell body is observed. This may be why we observe less cell motility on the nanolines within the time frame of tracking.

Migration trajectories of cells showed possible directional migration that correlated with the topography. To determine if a topographical correlation does exist, we measured the migration direction on each surface using an algorithm that determined the best-fit angle of each trajectory with respect to a reference line on the surface pattern that we define as the 0 degree direction. The choice of the reference direction is detailed as follows. The range of the measured angles was between -90 to 90 degrees. We defined the migration direction of the cells on the line surface as the absolute difference between the measured cell angle and the nanoline pattern (at either 0 or 90 degrees), as illustrated in Figure 4.2A. In our calculations, we took migration directions along the same orientation as the lines to be 0 degrees.

On the P700, P600 and P300 pillared surfaces, we defined the trajectory angles of the cells on the pillars as the absolute difference of the measured cell angle and the closest 60-degree increment axis (-60, 0 or 60 degrees), which was defined as the reference direction in Figure 4.2B. The entire pillared surface is arranged in a hexagonal array, which has a six-fold symmetry; hence, the axes at -60, 0 or 60 degrees have the same symmetry and cells could travel
in the proximity of any of these axes if presence of the pillars guided their migrations. In our calculations, migration directions along these axes of symmetry were defined as 0 degrees. Since the 0-degree axis corresponded to the directions with the highest density of pillars, it could correlate with a directional preference for cell migration. Alternatively, if cells were exactly half way between two axes, the difference between the symmetry and migration direction would be 30 degrees. This would indicate that cells on the nanopillars were migrating along the path with the lowest density of pillars. On the P200 pillared surfaces, there was no long-range order, in contrast to the other pillared surfaces. However, there was still short-range order,\textsuperscript{102} which provided six-fold symmetry locally. We confirmed the presence of this short-range order by performing a Fast Fourier Transform of an SEM image of the P200 pillars. We found that the P200 pillars apparently could provide the same guidance for cell migration as the other pillars despite the lack of long-range order.

The angular histograms show that cell migration was indeed guided by the symmetry of the underlying nanostructures. On a flat surface (Figure 4.2C), the histogram was spread between 0 and 90 degrees, indicating that there is no preferential direction for migration. On the L825 nanolines (Figure 4.2D), the majority of cells migrated at directions close to 0 degrees. This indicates that most cells migrated in the same direction as the lines, consistent with previous studies, as is expected.\textsuperscript{65,68,70} On the P700 and P600 pillars (Figure 4.2E, F, respectively), half of the cells migrated at directions close to 0 degrees—along one of the hexagonal axes. On the P300 and P200 pillars (Figure 4.2G, H, respectively), a higher proportion of cells migrated at directions close to 0 degrees—along one of the hexagonal axes—compared to cells on the larger pillars. This indicates that the smaller pillars were more effective at guiding cells to migrate along the paths with the highest density of pillars.
Figure 4.2 Migratory direction of cells on nanopatterns. (A) Schematic showing reference frame of migration direction on nanolines. (B) Schematic showing reference frame of migration direction on nanopillars. (C) Angular histogram for migration direction on the flat surface indicates random migration. (D) Distribution of migration direction on the L825 lines shows a large proportion of cells migrating between 0° and 15°, indicating migration along the nanolines. (E-H) Plots for nanopillars indicate that all cells migrated at angles between 0° and 30°, indicating preferential migration in directions with the highest density of nanopillars.

The foregoing demonstrated that the differences in motility on the pillars can be attributed to pillar size and spacing. Previous studies have shown that when integrin proteins are separated more than 73 nm apart, they are not able to dimerize and form the FA complex. In turn, the actin cytoskeleton is also unable to sustain the FAs. Accordingly, we fabricated
nanostructures with inter-pillar spacings greater than 73 nm to study the effect of potentially unstable adhesions. In particular, the P700 and P600 pillars had similar diameters and spacings; the difference in spacing between the two surfaces is only 45 nm. Similarly, the P200 and P300 pillars differed in spacing by 50 nm, which could explain why the cells traveled similarly on these respective sets of pillared surfaces.

In addition, three to five integrin heterodimers are necessary in order for the FA complex to form. The diameters of the P200 and P300 pillars were 70 nm and 190 nm, respectively, which could cause very small adhesions to form (about 15-30 integrin proteins). In fact, we found that cells on the P300 and P200 pillars have the smallest adhesion area compared to those on the other patterns, which we discuss later in this paper. These small adhesions would more easily disassemble during migration. These results suggested that the high motility on P300 and P200 was due to an increase in adhesion assembly and disassembly. This suggests to us that there were changes in protein dynamics caused by the underlying surface morphology that could affect the assembly and disassembly of the adhesions. However, these dynamics could not be observed by simply imaging adhesions. In the next section, we describe how we utilized the N&B analysis to examine the assembly and disassembly aggregation dynamics of the adhesions of cells on the various nanostructures at the molecular level.

4.3.2 Paxillin Aggregation on Nanostructured Surfaces

The process of adhesion assembly and disassembly is dynamic. Adaptor proteins such as paxillin and many others bind to partners with tight affinity rendering conformational changes that trigger other proteins to associate at the focal region. We showed that this process of assembly occurs with monomeric proteins (i.e. paxillin, vinculin and FAK), but not with preassembled adhesion aggregates. In order for migration to occur, these adhesive
complexes must dissociate with high temporal dynamics. It is not feasible for the adhesion to disassemble one protein at a time. Instead, we speculate that the cells utilize the protease identified as calpain to cleave focal adhesion proteins such as talin, which tethers integrin to the cytoskeleton structure.\textsuperscript{109} This cleavage gives rise to conformational instability for the adhesive proteins. Large aggregates begin to dissociate and are quickly returned to their monomeric conformation to be recycled or disposed of.\textsuperscript{106} This process presents a unique opportunity to study regions of cellular migration by detecting and quantifying the size of these protein dissociation aggregates using Number and Brightness (N&B) analysis, a powerful tool that graphically quantifies the aggregation states of diffusing proteins of adhesions in living cells.

We transfected cells with the paxillin-EGFP vector prior to each experiment, after which we performed brightness analysis to quantify the aggregation states of paxillin at the adhesions. The average intensity of paxillin-EGFP was detected in the confocal images of the adhesions at the trailing edges of cells on the different surfaces. However, the average intensity images would not indicate the composition of paxillin molecules in the fluorescent regions; thus, we calculated the brightness of the fluorescence intensity by detecting the fluctuations of the fluorescent paxillin molecules over time to calculate its molecular brightness and translating this values to its aggregation state.\textsuperscript{97} A graph was generated to show brightness versus intensity of every pixel in the original image. The molecular brightness of monomeric EGFP (mEGFP) was used as a calibration to calculate the molecular brightness of a monomer. The average intensity of mEGFP in Figure 4.3A showed a gradient of mEGFP in the cell: a higher concentration at the center of the cell (red) and less at the edges (blue). However, the brightness map of the same cell (Figure 4.3B) showed a uniform coloring of the cell (red) since the cell was only expressing monomeric versions of EGFP. Therefore, the brightness was found to be independent of concentration. Once
the apparent brightness of mEGFP was found, we used it to calculate the molecular brightness of a dimer by doubling its value (a two-fold increase). The molecular brightness of higher order aggregates was determined in the same manner (three-fold or higher increase). For our results, we combined the population of trimers, tetramers, and 5-mers into one tier. In Figure 4.4B, red, green, and blue pixels was used to map out the location of monomers, dimers (~2 paxillin proteins/cluster), and higher order aggregates (~3-5 paxillin proteins/cluster), respectively. Pixels of the image were colored according to the corresponding cursors (Figure 4.4A).

**Figure 4.3. Brightness of monomeric GFP.** (A) Average intensity of monomeric EGFP (mEGFP) in a cell only expressing mEGFP on a flat surface. Scale = 5 µm. (B) Brightness map of the same cell. Red pixels highlight monomer EGFP, while yellow pixels highlight background. The brightness value was used to calculate the brightness of all the aggregate tiers. (C) Brightness of every pixel of image versus intensity generated from N&B analysis.

Based on the definition of molecular brightness, immobile protein aggregates would have an apparent molecular brightness equal to 1. For example, the average intensity image of the cell on flat surfaces showed that there are many adhesions present. These adhesions were stable, i.e., they do not assemble or disassemble very quickly (within ~3-minutes) and thus, we assign to them a brightness of 1, indicated in red on the brightness map. We quantified the number of pixels highlighted by each cursor in the brightness versus intensity plot to give the ratio of dimers to monomers (D:M) or higher order aggregates to monomers (H:M). During these measurements, the observed cells appeared to retract; thus, we could quantify the disassembling
adhesions with high accuracy. Based on previous data that paxillin molecules in disassembling adhesions cluster as aggregates, we expected highly motile cells to have a larger population of aggregates due to increased adhesion disassembly.

**Figure 4.4 Paxillin aggregation states of adhesions in cells on nanopatterns.** (A) Average fluorescence intensity of the trailing edges of 3T3 fibroblasts (scale = 5µm) and their corresponding brightness maps. Colors correspond to pixels of monomers (red), dimers (green) and higher order aggregates (blue) of diffusing paxillin. (B) Plot of the brightness of every pixel in an image versus its intensity generated from the N&B analysis. This exemplary plot is of cells on P600 pillars. (C) Ratio of dimers to monomers (D:M; green) and higher order aggregates to monomers (H:M; blue) in cells on nanostructured surfaces. Cells on P600 pillars (n=16 cells) exhibited the largest D:M and H:M ratios, indicating that the adhesions in those cells have a larger population of higher order aggregates. Cells on the other pillars (P700: n = 11; P300: n = 15; P200: n = 10) had smaller ratios compared to those on flat (n=12) and L825 lines (n=16). *: p<0.05 for D:M ratios; **: p<0.01 for D:M ratios; +: p<0.05 for H:M ratios; ++: p<0.01 for H:M ratios. For P200: **: p<0.01 for D:M ratios compared to all other surfaces; +: p<0.05 for H:M ratios compared to flat and P700; ++: p<0.01 for H:M ratios compared to P600, P300, L825.

With the N&B analysis, we distinguished between the molecular differences in the adhesions of cells that exhibit similar motility behavior. We show the D:M and H:M ratios in cells on all surfaces in Figure 4.4C. We found that D:M and H:M ratios in cells on flat and L825 lines were similar to one another. The D:M and H:M ratios for cells on the four nanopillar
patterns showed the highest ratio in cells on P600 pillars (0.12 D:M ratio, 0.02 H:M ratio), indicating that the adhesions in those cells had a larger population of higher order aggregates. However, cells on this surface were not the most motile. In fact, cells were the most motile on the P200 pillars, but contrary to our initial hypothesis, we found that the adhesions in these cells consisted mostly of monomers. We note that the size of the adhesions also contributes to the rate of adhesion disassembly during cell migration. Since the adhesions in cells on P600 nanopillars had the greatest percentage of dimers and higher order aggregates, these cells would have large adhesions. In turn, there are more aggregates to detach for full disassembly of the large adhesions, resulting in decreased cell motility on those pillars, as observed in Figure 4.1H.

Kim and Wirtz have shown that there is an optimal adhesion area that yields a peak migration distance, and if these are very large (>2.5 µm²), then migration distance will also decrease due to the stability of the adhesion. Smaller, less stable nascent adhesions are more predominant in motile cells, while stable, mature adhesions are found in cells that adhere well to a surface. We hypothesized that adhesions formed on nanopillars probably will be smaller than adhesions formed on flat surfaces, and that these smaller adhesions should disassemble at an increased rate. To investigate this, we measured the size of the disassembling adhesions examined with the N&B analysis (Figure 4.5A) using the freehand selection tool in ImageJ to trace the individual areas of the adhesions (Figure 4.5B). We specifically measured the area of each adhesion containing clusters of paxillin dimers or higher order aggregates localized at the retracting edges of cells.
Figure 4.5 Area of disassembling adhesions of cells on nanopatterns. (A) Brightness map of trailing edge of a cell on P700 pillars. Adhesions involved in disassembly contain greater populations of dimers and higher order aggregates of paxillin. (B) Adhesion area of these adhesions was measured by using the free selection tool in ImageJ. Flat: n=152 adhesions, P700: n=258, P600: n=408, P300: n=71, P200: n=192, L825: n=299. (C) The largest adhesions were found in cells on the P700 and P600 pillared surfaces compared to flat and L825 lines. The smallest adhesions were in cells on the P200 pillars. **: p<0.01 for P700, P600 compared to flat and L825, and for P300 compare to P700, P600; ++: p<0.01 for P200 compare to all patterns.

We found that on average, adhesions of cells on P700 and P600 surfaces (~1.0 µm² for both) had the largest area compared to the other surfaces (Figure 4.5C). In contrast, cells on P200 surfaces had, on average, the smallest adhesion area (~0.34 µm²; p<0.01 compared to all surfaces). Cells on the P200 also exhibited the highest motility and had smaller ratios of H:M. Cells on L825 lines exhibited smaller adhesions (~0.65 µm²) than those in cells on the P700 and P600, yet they traveled the least total distance on the nanolines. This discrepancy could be explained by differences in feature size or the time for the cell to orientate with the underlying structures.
These observations, taken together, suggest that cell migration on pillared surfaces depended on the ability to form mature adhesions, which requires time. Cells on larger nanopillars (P600) had a greater population of dimers and higher order aggregates while cells on smaller nanopillars (P300, P200) had fewer aggregates. This suggests that adhesions on P200 were more dynamic, which correlates to high cell motility on the P200 surfaces. Additionally, given the fact that adhesion size can modulate migration, it is also possible that the clusters of proteins that dissemble are correlated with adhesion size, thereby regulating their migration accordingly. In the case of the P200 surfaces, we found that there were fewer integrin protein clusters, forming very small adhesions, which allowed easier adhesion disassembly. We also found that cells on nanolines elongated and migrated parallel to the structures, which led to adhesion formation along the nanolines. The results indicate that the differences in the adhesion dynamics governing migration led to changes in the adhesions themselves, which are known to play an important role.

4.4 Summary

We have shown that physical nanotopography can modulate adhesion assembly and disassembly and migration of cells on polymer surfaces by changing the dimensions and geometry of nanostructures to similar dimensions of the adhesions, and that changes in adhesion dynamics influenced by the nanostructures affected cell migration. We found that cells on smaller nanopillars spaced 100-130 nm apart (P200 and P300, respectively) were more motile compared to cells on larger nanopillars (Figure 4.6), the nanoline grating reference, and the flat surface. At the molecular level, we found that cells on nanopillars spaced 380 nm apart (P600) had the largest population of higher order paxillin aggregates, and this population decreased with decreasing pillar size (Figure 4.6). This correlated well with the size of the adhesions; cells on
larger nanopillars had the largest adhesions while those on the P200 pillars had the smallest adhesions. Moreover, we found that the cells migrated largely along the axes of symmetry of the hexagonal lattice of the nanopillars, which have the highest density of nanopillars of each pattern. We conclude that adhesion formation is essential for migration; yet, higher order paxillin aggregates provide the greatest temporal stability. The total distance traveled and paxillin aggregation population results suggest that the cells were able to adapt to the changes in topography up to a minimum threshold by modulating adhesion assembly and disassembly. By manipulating cell behavior at the molecular level solely through topographical cues, we can selectively control cell adhesion on the surfaces of biomedical devices and control their functionality.

Figure 4.6 Summary of findings correlating migration with paxillin aggregation in adhesions.
Chapter 5 Measuring Mammalian Cell Adhesion on Nanopatterns Under Shear Flow

5.1 Introduction

We can observe difference in adhesion on nanostructured surfaces by observing the number of cells on the surface over a period of time, cell migration, and cell shape; however, these observations do not inform the degree of cell adhesion. In designing medical implants, it is important to know the adhesion strength of cells on surfaces. It is often assumed that cells that do not detach after rinsing surfaces had adhered well. This may be an oversimplification. In this chapter I describe how the patterns affect cell adhesion under shear flow.

Methods for quantitatively assessing cell adhesion are divided into single cell studies and population studies. Single cell adhesion assessment methods include: micropipette aspiration,\textsuperscript{111} atomic force microscopy,\textsuperscript{112} and more recently, force spectroscopy.\textsuperscript{113,114} Examining adhesion of single cells is useful for probing potential mechanisms of adhesion, and differences between individual cells are considered in measurements. However, many measurements of a large number of cells would be required to achieve statistical significance, which would be time-consuming. Additionally, instrument setup is costly and often customized. Hence, methods measuring the adhesion of a population of cells are ideal for preliminary investigations for adhesion.

Nowadays, a common method of assessing adhesion is microfluidics to apply shear across cells in microchannel flow chambers.\textsuperscript{115–117} Experimental design with microfluidic devices is relatively simple: after incubating cells in the microfluidic device, fluid is pumped into the device to apply shear across cells. Real-time visualization of cell detachment would be easier.
In addition, flow geometry can be varied more easily by changing the designs of devices. However, if the pump rate is already at its highest, the microchannels would need to be narrow in order to achieve higher shear rates for detachment. For example, the device used by Christ et al. had channels 57 µm high at a pump flow rate of 950 ml/hr, which was the highest rate used in their experiment. As the diameter of the channels gets closer to the size of cells, cells would perturb shear flow such that it would deviate from a fully developed parabolic profile. As a result, the actual shear rate that cells in the above experiment were subjected to is unknown.

Another method for assessing adhesion at the population level is using a spinning disc to apply a shear force across cells. An advantage of using a spinning disc is the ease of changing the gap between the disc and the surface with cells. One can set the gap to be very large compared to the size of cells such that the wall effects at the disc and sample are negligible. This would reduce potential artifacts in the system, and also overcome the uncertainty of the actual shear rate in the vicinity of cells. Ideally, there should be little, if any, deformation of the disc interface, so the shear rate imposed on cells can be determined using equations for a rotational rheometer. The established shear models for a rotational rheometer provides a well-defined shear rate on cells provided that the gap is large compared to the cell height. For this reason, I used a rotational rheometer to apply shear across cells adhered to nanostructured surfaces to determine the shear rate needed to detach cells from the nanostructures.

Based on results from the previous two chapters, I hypothesize that the shear rate to detach cells on nanopillared surfaces would be lower than that of flat surfaces, while a higher shear rate would be required to detach cells on nanoline gratings. Additionally, I hypothesize that orienting lines perpendicular to shear will provide better adhesion than orienting lines parallel to shear stress. From an adhesion model on microline grooves presented by Fujita et al., I expect
more focal adhesions to form on perpendicular lines. This is similar to how geckos adhere well to surfaces—they have spatula-shaped microstructures called setae at the bottom of their feet. I predict that more force would be required to detach the many focal adhesions formed on orthogonal lines compared to the force required to detach the fewer focal adhesions formed on parallel lines.

5.2 Methods

5.2.1 Sample preparation

PMMA thin-films were spin-coated on large glass cover slips (45mm x 50mm, #2 thickness) using the same spin-coating conditions previously described. Nanopatterns were imprinted on the PMMA thin films using previously described printing conditions in Chapter 2. The center of the negative stamps was placed 12.5-mm away from the center of the substrate (Figure 5.1). The following nanopatterns were included in the study: flat PMMA control (spin-coated on the glass substrate, as described in Chapter 2), P300 pillars, a pattern that we previously found to effectively affect paxillin aggregation in focal adhesions, and L416 and L825 nanoline gratings. The latter line grating was previously used as a reference in earlier adhesion studies. Once imprinted, glass substrates with nanopatterns were glued down to the bottom of 100 mm-diameter flat polystyrene petri dishes using a cyanoacrylate adhesive (Loctite 4011, Henkel).

5.2.2 Cell culture

After letting the glue cure, samples were sterilized with UV light for 5 minutes. They were then rinsed with sterile 1X PBS to clean off remaining debris. Nanopatterns were coated with fibronectin (10 µg/ml) for 1 hour at room temperature. After incubation, surfaces were
rinsed with 1X PBS. To ensure that cells did not adhere anywhere else on the PMMA substrate, the entire PMMA surface was coated with sterile-filtered 2% bovine serum albumin (BSA) for one hour at room temperature to prevent non-specific protein binding to the surfaces. After this second incubation, surfaces were rinsed one more time with 1X PBS. Approximately 300,000 cells were seeded on each sample dish containing 10 ml of DMEM + 10% FBS. Cells were incubated on the surfaces at 37 °C, 5% CO₂ for two hours. After 2 hours, media in dishes were replaced with fresh media.

5.2.3 Rheometer to apply shear

Samples were placed on the stationary bottom plate of a rotational rheometer (AR-G2, TA Instruments). Because the range of shear rates to detach cells was unknown, I chose to use a parallel plate geometry (40mm diameter, stainless steel), shown in Figure 5.1, over a cone-and-plate geometry. In a parallel plate geometry the shear rate varies linearly with the distance from the center of rotation, while in a cone and plate geometry the shear rate is ideally constant throughout. The gap distance between the top plate and the pattern with cells was kept constant (0.5 mm) and filled with serum-free DMEM medium before spinning. This gap distance was chosen because the gap was large enough to not disrupt cells while applying shear, since the 3T3 fibroblasts were only about 10-20 µm in size. Additionally, the viscosity profiles of the medium were most consistent with a 0.5 mm gap, as opposed to 0.1 or 0.3 mm gap, which I found to produce erratic results in the present set up. Maintaining a constant gap is important as we change the spin speed of the parallel plate. Prior to each run, a piece of silicon wafer of 0.5 mm thickness was placed between the two plates as a shim to ensure that this gap is consistent across each sample.
Figure 5.1 Parallel plate rheometer setup for shear stress application. Patterns were nanoimprinted in one region on a PMMA-coated large glass coverslip. This large coverslip was glued down to the bottom of a large petri dish. Parallel and orthogonal nanoline orientations with respect to shear flow (depicted by black arrow) are shown as imprinted on the PMMA film (bottom left). Patterns were coated with fibronectin followed by bovine serum albumin (BSA). Cells were seeded on the pattern prior to applying shear with the rheometer.

Once the gap distance was set, we performed experiments at two spin speeds for 2 minutes each, first at 3 rpm, and then at 10 rpm. After each spin, I imaged the surfaces to determine the number of cells remaining on them. The percentage of remaining cells under varying shear rates was calculated using the initial count of adhesion. Equation 5.1, the shear rate for the parallel plate rheometer is shown:

$$ \dot{\gamma} = \frac{\Omega \times r}{h} $$  

(5.1)

In Equation 5.1, \(\Omega\) is the rotational speed of the parallel plate, \(r\) is the radial distance from the center of the plate, and \(h\) is the gap distance between the rotating top plate and the sample surface. Cells were counted in 4 regions, each approximately 2 mm\(^2\) in area, at 3 radii: 10 mm, 12.5 mm and 15 mm. I assumed that shear rate would increase proportionately between 3 rad/s and 10 rad/s in accordance with the parallel plate geometry.
5.2.4 Data analysis

For each sample, four regions were imaged at 10 mm, 12.5 mm and 15 mm from the center, where nanopatterns were positioned, prior to shear application across the surfaces. After each spin cycle the same four regions were imaged. The average number of cells for each shear rate from the 4 regions was first calculated. The average cell count per sq. mm for each shear rate was normalized to the initial cell count per sq. mm, which are designated as shear rate of 0/s, to yield the fraction of cells remaining on the surfaces. The initial cell count data before spinning was normalized to the average cell count per sq. mm for the corresponding surface to obtain the fraction of cells remaining. Box plots of fraction remaining were prepared using the ggplot2 package in R software to observe the variance among samples. The band inside the box represents the median fraction remaining. The median is used to determine the shear rate at which 50 percent of cells have detached.\(^{121}\) Statistical significance of the percentage of cells remaining on the nanopatterns under 90/s and 300/s shear rates was determined using the Student’s t-test (two-tailed, unpaired) using Excel.

5.3 Results

5.3.1 Initial adhesion on surfaces

After 2-3 hours of incubation, differences in the number of cells adhered on the nanostructured surfaces became observable. Cell count data showed that there were more cells adhering on the line patterns parallel to the shear direction (Figure 5.2). By contrast, the number of adhering cells on lines aligned orthogonal to the flow direction and the P300 pillars were slightly lower compared to the flat control. The difference in adhesion could also be due to molecular orientation of PMMA as a result of spin-coating the PMMA solution on the large glass
coverslip. It was assumed that the surface chemistry on the surface was the same since the flat control and the nanopatterns were coated with fibronectin. However, it may be possible that on the spin-coated PMMA film, there is more polymer chain orientation further away from the center of the glass substrate. This orientation is expected to be higher with a larger substrate. Experiments using a surface specific spectroscopy technique, described in more detail in Chapter 6, would test this hypothesis.

**Figure 5.2 Adhesion on nanopatterned surface before shear.** Even before applying shear across the surfaces, cells appear to have a preference in nanoline orientation. “P” indicates lines oriented parallel with spin direction, while “Or” indicates lines oriented perpendicular or orthogonal to spin direction.

### 5.3.2 Adhesion on nanopillared surfaces under shear

Figure 5.3A shows a trend of decreased percentage of cells remaining on flat surfaces with increasing shear rates; black line in the boxes indicates the median of the fraction remaining. The shear rate to detach 50 percent of cells was between 90/s and 200/s. The
discrepancy between the two shear rates may be related to the radial position. Shear rate of 90/s was measured at a radius of 15 mm at the angular speed of 3 rad/s, while shear rate of 200/s rate was measured at a 10 mm radius at an angular speed of 10 rad/s. I assumed there was a linear trend of adhesion with respect to spin speed, and therefore, shear rate. However, I observed a greater fraction of cells remaining under 200/s compared to the fraction remaining under 90/s. It appears that the position of cells with respect to the center of the disc may be contributing more to the cell adhesion data. In addition, the same sample was used to assess adhesion at the two spin speeds. For future experiments, using completely different samples at the two spin speeds may resolve this discrepancy.

Figure 5.3B shows the trend of cells remaining on the P300 pillared surfaces. The trend is similar to that on flat surfaces; however, the spread of the data is larger, especially at shear rates of 75/s and 90/s. This could be the transition point of the biphasic model for adhesion. The shear rate to detach 50 % of cells was at 75/s, which is slightly lower, compared to that for flat surfaces. This would appear to be consistent with the preliminary data which suggest that cells on P300 pillars might require a lower shear rate to detach cells compared to those on a flat surface. However, I emphasize that the observations on P300 pillars have not been statistically validated.
Figure 5.3 Adhesion on nanopatterned surfaces under varying shear rates. Data include 4-5 samples per shear rate on each surface. Shear rates at three different radii (10, 12.5, 15 mm) for 3 rad/s spin speed were 60, 75, and 90 1/s, while shear rates at the same radii produced by 10 rad/s spin speed were 200, 250, and 300 1/s.
5.3.4 Adhesion on nanolined surfaces under shear

A different trend for cell detachment was observed on the L416 nanolines (width = 208 nm). For both line patterns, more cells remain attached to the line grooves with increasing shear rate than on flat. In addition, the 50% detachment shear rate for the lines was greater than that for the flat surface. On L416 lines oriented orthogonal to flow (L416-Or), the shear rate for 50% detachment was 250/s (Figure 5.3C). In comparison, the median fraction of cells remaining on L416 lines parallel to shear (L416-P) after a shear rate of 300/s was greater than 50% (Figure 5.3D), indicating a higher shear rate would be required to detach 50% of cells when the flow is parallel to L416. The statistical variance on the L416-P lines was relatively small, indicating that the majority of cells adhere similarly on this surface.

In contrast, the larger lines (L825, line width = 432 nm) appear to be more effective against shear when oriented orthogonal to shear. On those lines (L825-Or), the detachment shear rate would be greater than 300/s, as a fraction greater than 0.5 remain after 300/s. In contrast, the shear rate to achieve 50% detachment on L825 lines parallel to shear (L825-P) would be between 90/s and 200/s, similar to the flat surface detachment shear rate. Additionally, there is a greater variance of cells remaining on these lines, observed by the height of the box plots in Figures 5.3E, F. This is especially significant for the L825-P lines. The large variance suggests that the cells were ambivalent with regards to adhesion on the L825 lines.

To help visualization of statistical significance, I evaluated the fraction remaining on the surfaces after shear rates of 90/s and 300/s; these were the highest shear rates from 3 rad/s and 10 rad/s spin speeds, respectively. As seen in Figure 5.4, L416 lines parallel to shear flow had a significantly greater number of cells remaining on the surfaces compared to the flat surface. It appeared that the flat surface was actually the least effective surface against shear, contradicting
our hypothesis that the nanopillars would be the ideal pattern for decreasing adhesion. However, only one nanopillar pattern was tested. The P200 pillars, which were the better nanopattern to induced migration (see Chapter 4), may reduce adhesion more effectively than the P300 pillars, and potentially the flat surface as well. Additionally, the cell incubation time on the surfaces could also be adjusted.

![Graph showing percentage of cells remaining after 90/s and 300/s shear rates.](image)

**Figure 5.4 Percentage of cells remaining after 90/s and 300/s shear rates.** Data represents mean ± standard error of the mean. Statistical significance determined using Student’s t-test: *p<0.05 with flat, **p<0.01 with flat.

5.4 Discussion

The objective of this study was to measure the adhesion strength of cells on nanopatterned surfaces to provide a quantitative test of the hypothesis that nanopatterns would encourage or discourage adhesion. To this end choosing the right system for measuring cell adhesion was important. It was most appropriate to start off with population measurement methods, as single-cell measurements would be time-consuming, and the appropriate force to apply to cells was unknown. The use of a rotational rheometer is a well-established method of measuring adhesion of a population of cells with well-defined models for calculating shear rates.
and shear stress. Additionally, the advantage of using a parallel plate was the ability to test a range of shear rates at a single angular speed for cell detachment. Because the artificial cornea implant will be subject to shear force from eye blinking, I first evaluated cell adhesion at high shear rates in an attempt to reach the shear rate range of blinking (4000-28000/s).\textsuperscript{125}

This proved to be a more difficult task than anticipated. Though the highest spin speed of the rheometer is 300 rad/s, I could only spin up to 100 rad/s without the media exuding outside the polystyrene petri dish that held the nanopatterns. Thus, according to Equation 1, the gap between the parallel plate and the sample has to be smaller in order to achieve higher shear rates. However, decreasing the gap distance to 0.1 mm, which was need to achieve 10,000 per second at 10 mm away from the center, resulted in instability as evidenced by large fluctuations in the torque signal. We then increased the gap distance to 0.3 mm, which was enough to reach a shear rate of 1000/s. I found that only a low fraction of cell remained after applying such a high shear rates on the surfaces, which meant that none of the cells would remain adherent in the central optic of the artificial cornea device. Nevertheless, I was interested to search for the transition rate proposed in the biphasic model for cell adhesion.\textsuperscript{120,121} As a result, I evaluated adhesion under lower shear rates. I was once again limited by the spin speed of the parallel plate geometry. Spinning the plate too slowly, at 1 rad/s, resulted in inconsistent data for media viscosity. Thus, I used only spin speeds of 3 rad/s and 10 rad/s to evaluate adhesion on the nanopatterned surfaces under lower shear rates.

I first hypothesized that the shear rate for cell detachment would be less on P300 pillared surfaces compared to a flat surface, and that the shear rate for cell detachment would be greater on the nanoline gratings. Results from the study provide evidence supporting the hypothesis. On the flat surface, the shear rate to detach 50 percent of cells was between 90/s and 200/s. In
contrast, the shear rate to detach the same fraction on the P300 pillars was 75/s, and the detachment shear rates on the lines were at least 200/s. Between the two nanoline gratings, L416 lines, which have a smaller line width and spacing, were more effective against cell detachment when shearing across the lines. This finding is consistent with the findings by Karuri et al., who also found 400 nm pitch line grooves were the most effective against a shear stress of 80 Pa.\textsuperscript{116} I even observed this difference in my experiments using an even lower stress of 0.3 Pa (calculated using viscosity = 1x10^{-3} Pa*s, shear rate = 300/s). I speculate that the increased adhesion of cells on the L416 lines compared to L825 lines may be because there are more edges available for cells to form focal adhesions. It is interesting to note that the size scale of the L416 lines is similar to that of the topography of tissues, such as the basement membrane.\textsuperscript{68,116}

These observations lead me into my second hypothesis: cells on nanolines oriented orthogonal to shear would adhere better on surface compared to cells on the same lines oriented parallel, or in the same direction, to shear. I did observe differences in adhesion on the nanoline gratings when they were oriented parallel or orthogonal to the direction of shear. Interestingly, parallel line orientation was effective for adhesion on the L416 lines, while the orthogonal line orientation was more effective for adhesion on the L825 lines. When comparing between the two nanolines, the difference in cell adhesion was more apparent when the lines were oriented parallel to flow direction. These findings are in contrast to those of Karuri et al., who claimed that line directionality with respect to flow in their flow chamber did not affect experimental outcomes. However, cells in that study were adhered to surfaces for 24 hours before attempts to detach the cells. As suggested in Chapter 3, most adhesions on cells have stabilized by 24 hours. The differences in adhesion on the lines in the two different orientations may come down the interaction of the cells with the lines in first one or two hours after seeding. Additionally, the
depth of their line grooves is considerably deeper than either of the line patterns I used in the experiment. Even though the widths of the line ridges are similar, the deeper grooves provide more wall surface area for cells to adhere.

An important question that still remains to be answered is whether the nanostructures were still standing after experiencing various shear rates. After conducting experiments, iridescence was observed in the nanopatterned area, indicating the presence of the nanostructures. Additionally, the nanostructures have low height-to-width aspect ratios (0.5 for the nanolines, 1.5 for the nanopillars), which makes the structures less susceptible to damage from shear flow. This also implies nanostructures on the artificial cornea would survive shear force from eye blinking. Characterizing the structures under SEM would provide further validation. SEM evaluation requires complete removal the nanopatterns from the petri dish, but this task was more difficult than anticipated. Solvents that are normally used to remove cyanoacrylate did not penetrate between the glass substrate with the polymer nanopatterns and the petri dish. Fortunately, the only the edges of the glass substrate were glued. By breaking the substrate from the center, the patterned area can be carefully removed from the petri dish.

The findings so far provide a good basis for further experiments for measuring the adhesion of cells on the nanopatterned surfaces. Now that I know the shear rates to detach 50% of the cell population on each type of surface, I can apply the same uniform shear rate across cells on all the nanopatterned surfaces. This can be accomplished by changing the spinning disc of the rheometer from a parallel plate to a cone-and-plate geometry. Another improvement to better mimic the eye environment is to use artificial tears with about the same viscosity as that of the tear film (approximately 1 mPa*s). Other studies have used PBS instead of culture media in spinning disc and microfluidic systems. However, PBS not containing calcium and magnesium
ions cause cells to detach from surfaces more readily, which could potentially confound results from the proposed experiments. Results from these experiments would be validated by comparing shear forces of the spinning disc to detach cells with shear forces of individual cells. An ongoing experiment being conducted in our laboratory is to use a microcantilever device compatible with an inverted microscope to measure the detachment force of single cells on patterned surfaces.

5.5 Summary

I aimed to measure the adhesion strength needed to detach cells from the different nanostructured surfaces. After considering the options available, we used a rheometer with a parallel plate geometry to test a range of shear rates across adherent cells. Findings revealed that the shear rates to detach 50 % of cells vary with nanopatterns, summarized in Table 5.1. I found that the detachment shear rate was lower on the P300 pillars compared to the flat surface (75/s vs. 90-200/s). By contrast, the detachment shear rate was higher on nanolines compared to the flat surface. In particular, the detachment shear rate was higher on 208 nm wide lines parallel to shear (L416-P), and on 432 nm wide lines orthogonal to shear (L825-Or); both shear rates would be greater than 300/s. Comparing the nanolines, the variance in fraction remaining for L416 nanolines was less than the variance in the fraction for L825 nanolines. This suggests that the L416 nanolines may encourage adhesion better than the L825 nanolines. Additionally, the directionality of nanoline features with respect to shear force affects the ease of detachment of cells. By comparing the percentage of cells remaining at shear rates 90/s and 300/s, I found that the L416 nanolines parallel to shear flow significantly improved adhesion on PMMA surfaces.
Table 5.1 Shear rate at 50% remaining

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Shear rate at 50% detachment (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat</td>
<td>90-200</td>
</tr>
<tr>
<td>P300</td>
<td>75</td>
</tr>
<tr>
<td>L416-Or</td>
<td>300+</td>
</tr>
<tr>
<td>L416-P</td>
<td>250</td>
</tr>
<tr>
<td>L825-Or</td>
<td>300+</td>
</tr>
<tr>
<td>L825-P</td>
<td>90-200</td>
</tr>
</tbody>
</table>

These findings shed light on which features could be used to improve adhesion under shear on implant surfaces, and as a result, improved \textit{in vivo} integration. Future experiments planned include measuring the adhesion strength of individual cells adhering on the nanopatterned surfaces using a custom-built microcantilever device, and investigating the cell’s focal adhesions as it experiences different shear forces while adhering to the nanopatterns. These experiments will validate current findings of this preliminary investigation and provide a better indication of how well cells adhere to nanopatterned surfaces.
Chapter 6 Investigation of Molecular Moieties on Surfaces of Polymer Nanopatterns

6.1 Introduction

In Chapter 5, I described the observation that more cells were adhered to the nanolines oriented parallel to shear compared to the nanolines oriented orthogonal to shear flow; but, this was before I even applied shear stress across the nanopatterned surfaces. I suggested that on the spin-coated PMMA film, there is possibly more polymer chain orientation further away from the spin axis, especially on larger substrates. This suggests that the surface chemistry that cells are exposed to may vary with different nanopatterns due to differences in polymer chain orientation. To gain insight into the molecular basis of surface chemistry and physics, I needed to use experimental techniques with the necessary surface selectivity and chemical sensitivity. Scanning probing techniques, such as scanning electron microscopy and atomic force microscopy are very useful for observing three-dimensional topography and morphology of surfaces with nanometer resolution. However, these methods are incapable of giving information on different functional groups on the top molecular layer of a surface. Attenuated total reflection FTIR (ATR-FTIR) is a common method to evaluate chemical species, but this method can only give information averaged over the first few hundred nanometers beneath the surface, and hence also cannot provide information on molecular structure of the top surface layer.

Another technique to scan the molecular moieties is Sum Frequency Generation Spectroscopy (SFG). The main advantage of SFG over scanning probing techniques is the capability of distinguishing different functional groups on the top few layers of the surface; hence, it can provide detailed information on what effect would arise due to other materials, liquids, or biological species that come into contact with the material under investigation without
being confounded by signals of molecules beyond the surface. One specific form of SFG is Vibrationally Resonant SFG Spectroscopy (VR-SFG). In VR-SFG spectroscopy, vibrational spectra of chemical species at the interface are acquired through measurement of the second-order nonlinear polarization induced by intense ultrashort (about 6 ps or less) visible and tunable mid-infrared (mid-IR) pulses, as shown in Figure 6.1.

![Diagram of vibrationally resonant sum-frequency generation (VR-SFG) spectroscopy using ultrashort visible and mid-infrared pulses.](Image)

**Figure 6.1 Schematic of vibrationally resonant sum-frequency generation (VR-SFG) spectroscopy using ultrashort visible and mid-infrared pulses.** Figure by Dr. Hiroaki Maekawa.

The surface selectivity of VR-SFG spectroscopy originates from the symmetry of the environment where molecules exist under the electric dipole approximation. The sensitivity of specific chemical species is achieved through vibrational resonant enhancement of SFG signal. Vibrational modes on the surfaces and interfaces that are both infrared and Raman active can generate strong SFG signals when the peak frequency of the incident mid-IR pulse is in resonance with the fundamental transitions between \( v = 0 \) and 1 vibrational states. While the peak frequency, intensity, and line width of vibrational bands in FTIR and Raman spectra are quite sensitive to distinct functional groups, molecular species, and microscopic chemical environments around the vibrational modes in the bulk, VR-SFG spectral signatures sensitively reflect molecular properties on the surface and interfaces. In addition, molecular conformation
and orientation, such as the angle between a bond axis and the surface normal, can be determined based on the polarization dependence of VR-SFG spectra.\textsuperscript{56,127}

VR-SFG has been employed in the last three decades to study a wide range of surfaces and interfaces.\textsuperscript{126–135} This unique technique has also been utilized in the field of polymer science,\textsuperscript{136–141} and revealed dominant chemical species and structure between air/polymer,\textsuperscript{142} water/polymer,\textsuperscript{143,144} metal/polymer,\textsuperscript{145} and buried polymer/polymer interfaces.\textsuperscript{146,147} In particular, this method was utilized to gain deep insights into the nature of molecular species on the surface of PMMA films in air and water.\textsuperscript{142,148–150} Surface reorganization of PMMA films caused by temperature,\textsuperscript{151} pressure,\textsuperscript{152} and polymer blend composition\textsuperscript{146} was investigated by the same technique as well. Most VR-SFG studies on PMMA interfaces focused on the CH\textsubscript{2}/CH\textsubscript{3} symmetric and asymmetric modes in the frequency range from 2800 to 3000 cm\textsuperscript{-1}. Only a few studies explored the carbonyl stretching mode as a reporter for polymer conformation analysis,\textsuperscript{153} or as an indicator for surface hydrogen bonding status by monitoring the redshift of C=O frequency.\textsuperscript{144,148}

Most surfaces and interfaces studied by VR-SFG spectroscopy in the past were azimuthally isotropic and planar. However, applicability of the method is not restricted by the surface morphology. For example, VR-SFG has been applied to probe self-assembled monolayers of carbon monoxide molecules prepared on platinum nanoparticle arrays,\textsuperscript{154} and alkylthiol molecules on the surface of metallic nanoparticles\textsuperscript{155,156} and nanopillars arrays.\textsuperscript{157} These studies clearly indicate that SFG signals from the molecules on a nanostructured surface can be measured and their polarization dependence will reflect the local structure of adsorbed molecules. Accordingly, I hypothesized that VR-SFG spectra of PMMA nanoline and nanopillar array will show different spectral signatures from those of a spin-coated flat PMMA
film, thus reflecting the changes of chemical composition and side chain / backbone polymer structure that could be induced on the top and side surfaces of the nanostructures.

In this chapter, I describe the collaboration I conducted with Dr. Hiroaki Maekawa and Prof. Nien-hui Ge to investigate the SFG signals of the nanostructures. On the nanolines, we aimed to determine whether extra SFG signal arises from the top surfaces of the lines or at the edges, i.e., regions where two surfaces meet. We hypothesize that if it is the former, increasing the density of the lines would not change the signal since the net amount of surface area would not change; if the latter, increasing line density would increase the signal because there would be a higher amount of line edges. On the nanopillars, we hypothesize that we will observe similar effects, because the density of pillar edges would increase with decreasing pillar diameter. As mentioned in Chapter 2, the heights of the lines were 100-200 nm, while the heights of the nanopillars were 300 nm. Measurement of the nanopatterns was possible since the heights were within the technique’s sensitivity.

6.2 Methods

6.2.1 Sample preparation

Thin films of PMMA (120,000 Da) dissolved in toluene (5 w.t.%) were spin-coated on gold-coated glass slides (EMF Corporation) at 600 rpm for 45 seconds, as described in Chapter 2. A thin titanium layer (50 angstroms thick) was used between the glass substrate and gold to provide adhesion between the two materials. Two nanoline patterns (L825, L416) and two nanopillar patterns (P700, P300) were included in this experiment. L825 lines and P700 pillars are shown in Figure 6.2A, B.
6.2.2 VR-SFG spectroscopy

VR-SFG spectroscopy experiments were performed by Dr. Hiroaki Maekawa, a Specialist working with Prof. Nien-Hui Ge, using the group’s home-built multiplex SFG spectrometer with femtosecond mid-IR pulse laser tunable from 3200 to 1450 cm\(^{-1}\). The polarization of the generated SFG signal, spectrally narrowed 800nm pulse (served as the “visible” pulse), and tunable mid-IR pulse can be controlled independently (Figure 6.1).

Besides measuring SFG signal intensity, the experimental setup can also perform phase sensitive SFG measurements by heterodyne detection with a local oscillator field. We focused on two frequency regions: one from 2800 to 3000 cm\(^{-1}\) for CH\(_3\) and CH\(_2\) stretching modes, and the other from 1700 to 1800 cm\(^{-1}\) for the C=O stretching mode, taking advantage of the presence of the carbonyl group, which should be particularly sensitive to the surface environment. The SFG
spectra of PMMA nanopillar and nanoline arrays were compared with that of a spin-coated flat PMMA film on a gold covered substrate to detect the functional groups on the surfaces of the nanopatterns and the conformations of their groups. We have performed preliminary studies on PMMA nanostructures using two different polarization configurations: \( ppp \) (\( p \) polarized sum frequency light, \( p \) polarized visible light, \( p \) polarized IR light), and \( ssp \) (\( s \) polarized sum frequency light, \( s \) polarized visible light, \( p \) polarized IR light (Figure 6.2C).

### 6.3 Results

#### 6.3.1 SFG spectra of L825 and P700 nanopatterns

Measurements were first made on the larger nanolines L825 and larger nanopillars P700. The spectra of the C=O stretching mode (Figure 6.4) are stronger than the spectra of the CH\(_2\)/CH\(_3\) modes (Figure 6.3). It is also intriguing that the spectral intensities of C=O modes in the P700 nanopillar array are much higher than those in the planar film and nanoline array. Generally, the SFG intensity depends on the number of functional groups on the surface, the molecular hyperpolarizability of vibrational mode, and orientation of the bond axis to the surface normal and its distribution.\(^{127}\) These experimental results show that these physical values are strongly affected by the morphology of nanostructured PMMA, suggesting that there may be an overabundance of the C=O stretching modes on the surface of the larger nanopillars compared to the flat spin-coated PMMA film. Additionally, it appears the line orientation results in a difference in surface chemistry presentation, as the spectra for lines parallel to the SFG signal (L825-X) were different from lines oriented perpendicular to the signal (L825-Y). Another set of measurements on these specific nanopatterns should be performed to verify these results.
Figure 6.3 SFG spectral intensities of CH$_2$/CH$_3$ groups on L825 nanolines and P700 nanopillars made of PMMA measured with ppp and ssp polarization configurations.

Figure 6.4 SFG spectral intensities C=O stretching mode on L825 nanolines and P700 nanopillars made of PMMA measured with ppp and ssp polarization configurations.
6.3.2 SFG spectra of L416 and P300 nanopatterns

Measurements were also performed on smaller nanolines L416 and nanopillars P300. The width and spacing between the structures are about two times smaller than L825 lines and P700, respectively. In previous chapters, I described the finding that the smaller nanopatterns were more effective in affecting cell behavior. Cells on the smaller nanopillars migrated a greater distance on the nanopatterned surface, as I described in Chapter 4. Likewise, I found that more cells adhered to the surface of the smaller nanolines under shear conditions, as described in Chapter 5. This led us to hypothesize that the chemical moieties on the surfaces of the smaller nanopatterns may be different than those on a flat surface. As with the L825 and P700 nanopatterns, we examined the L416 and P300 patterns from 1700 to 1800 cm$^{-1}$ for C=O stretching mode (Figure 6.5). Unlike the previous experiment, the spectral intensities for all the surfaces we examined were in the same range. Interestingly, we found that the spectra for P300 were almost identical to the spectra of the flat PMMA film. This may suggest that surface molecular moieties of the P300 pillars and the flat PMMA are similar. However, this apparent similarity may have been observed because the dimensions of the P300 pillars are smaller than the spatial resolution of the lasers used. The spectra of the L416 lines appear similar to the spectra of the larger L825 lines, except for a second peak at 1740 cm$^{-1}$. Additionally, in contrast with the L825 lines, the line orientation during the measurements was not a factor for the L416 lines, as the spectra appear similar—the only difference was the SFG intensity.
6.4 Discussion

The SFG results suggest that surface molecular moieties are dependent on the geometry, size and in the case of the nanolines, the orientation of the line structures. From the spectra, there appears to be an overabundance of carbonyl groups on the larger nanopatterns, as the SFG intensity for those patterns were much higher than the SFG intensity on the flat surface. However, the intensities of the SFG signals from the smaller nanopatterns appear to be similar to the flat PMMA surface. This suggests that the surface chemistry of the smaller PMMA nanopatterns is not much different from the flat PMMA. These findings indicate that the size of the structures in the nanopatterns may dictate which surface cues would influence cell behavior. The SFG spectra on the larger nanopatterns were very different from the flat surface, suggesting that cell behavior on these patterns may change, in part, due to surface chemistry. In contrast, the
similar SFG signal intensity between a flat surface and the smaller nanopatterns suggest that differences in cell behavior on these nanopatterns may be more strongly influenced by physical topographical cues. These results suggest that we may have found a size range in which physical cues are more influential than chemical cues. Ongoing detailed data analysis together with computational modeling will allow us to elaborate on the three-dimensional nanostructure dependence of SFG spectra. Surface chemistry presentation may affect protein adsorption on the nanopatterend surfaces, especially in cell culture studies. A future experiment could be conducted to study the molecular conformations of the fibronectin on the nanopillar surfaces using VR-SFG, and the configuration of the fibronectin on these surfaces using AFM in tapping mode.

Once we know what chemical species are on the surfaces of the nanopatterns, we can determine whether ECM proteins adsorb differently on them, and if so, why. SFG spectroscopy has been employed to study the ECM under a layer of rat embryonic fibroblast cells adhered on fibronectin-coated gold surfaces in the CH stretching region. Similar SFG spectra were observed from samples with cells and those without cells, indicating that SFG spectroscopy is capable of probing the ECM layer in between a solid substrate and cells.

When VR-SFG is applied to study biomacromolecules interacting with a polymer surface, it is desirable to keep them hydrated in a solution with controlled pH. Figure 6.6 shows a schematic of our setup for this experiment. We will need to fabricate a sample cell composed of a CaF$_2$ prism similar to the chamber described by Verreault et al. The prism will be covered with a nanostructured polymer film and a reservoir of protein solution. Prior to VR-SFG, the samples will be coated with protein solutions for up to one hour at 37 °C. We will test varying concentrations of each protein solution on the nanopatterns.
Figure 6.6 Schematic layout of VR-SFG experimental setup to probe hydrated biomacromolecules on a nanopatterned polymer film. The prism will be covered with a nanostructured polymer film and a reservoir of protein solution. Figure by Dr. Hiroaki Maekawa.

Incident visible and mid-IR pulses through the prism are spatially overlapped at the interface between the polymer layer and the solution to measure amide-I VR-SFG spectra of adsorbed biological molecules. The amide-I mode is well-known as a good probe of peptide and protein structure, since the backbone conformation and contents of different secondary structures are sensitively reflected in the peak frequency and intensity of the vibrational band.\textsuperscript{160,161} The usefulness of the amide-I band to investigate the surface molecular structure of biomacromolecules has also been proven by many studies of VR-SFG in the past.\textsuperscript{162–165} The amide-I VR-SFG band typically have a peak frequency between 1600 and 1700 cm\textsuperscript{-1}, while that of the carbonyl band of a spin-coated PMMA film contacting with water was observed above 1700 cm\textsuperscript{-1}.\textsuperscript{148,153} Therefore, we will be able to measure amide-I SFG spectra without it being distorted by the C=O band of the PMMA layer after dispersion of the generated SFG signal by a monochromator.

6.5 Summary

I collaborated with Dr. Maekawa to evaluate the surface chemistry of the nanopatterns using vibrationally resonant sum frequency generation (VR-SFG) spectroscopy. The
experimental results show that there are significant differences in the SFG spectra, especially of the C=O stretching mode, between not only nanoline gratings and nanopillars, but also between nanopatterns of the same geometry of different sizes. The much higher SFG intensity of the C=O spectra of the L825 nanolines and P700 nanopillars indicate a greater abundance of carbonyl groups on the top surface of the PMMA nanopatterns compared to the surface of flat PMMA. Interestingly, the intensities of the SFG signals from the smaller L416 nanolines and P300 nanopillars were in the same range of the intensity as flat PMMA, suggesting that the surface chemistries of the three surfaces are similar. These findings support the overall hypothesis that physical nanopatterns has a major influence over how cells adhere or migrate on those surfaces. Additionally, these findings suggest that further studies to examine conformation of adsorbed ECM proteins on the nanopatterned surfaces should be fruitful, and could shed more light on the preliminary results described here.
Chapter 7 Key Implications and Future Work

7.1 Summary of Key Findings

The main objective of the current thesis was to determine if we can use fabricated nanopatterns to control how mammalian cells adhere to surfaces, as selective cell adhesion contributes to the function and longevity of implanted medical devices. This is based on the hypothesis that the nanopatterns could induce changes in how the focal adhesions in a cell assemble and disassemble on surfaces, both spatially and temporally. Consequently, we set out to develop methods for measuring cell adhesion to surfaces. These goals were realized by the design of a completely synthetic artificial cornea that simultaneously encourages cell adhesion on the peripheral skirt of the implant to improve implant integration, and deters adhesion in the central optic zone for clear vision through the implant.

First, I evaluated cell adhesion on the nanopatterned surfaces using 3T3 mouse fibroblasts (Chapter 3). I hypothesized that compared to the number of cells on a flat surface, there would be fewer cells adhering on the nanopillars, and more cells on the nanolined surfaces. After seeding and incubating cells on the surfaces for 24 hours, I found a trend of increased cell adhesion on nanoline gratings; the smaller L416 line pattern (208 nm line width) being slightly more effective than the larger L825 line pattern. More interestingly, I observed a trend of decreased adhesion on the nanopillared surfaces. Normalizing to the number of cells on the flat control surface, I observed a 20-30% decrease of adhered cells on the three nanopillared surfaces evaluated; the decrease on the larger nanopillars, P700, was statistically significant. Results from these experiments showed that cells adhere differently on the nanopatterns, and led me to wonder why there were differences in adhesion on the nanopatterns.
I hypothesized that the different adhesion behavior was due to how the focal adhesions of cells were forming and disassembling on the surfaces. I therefore evaluated (Chapter 4) cell migration on nanostructured surfaces in vitro using time-lapse microscopy, and examined for potential differences in paxillin aggregation in the focal adhesions of cells on the different nanopatterns using the Number and Molecular Brightness imaging analysis, a method developed by Prof. Michelle Digman to distinguish the number of molecules that are responsible for the fluorescence intensity in confocal micrographs. I found there was increased cell motility on nanopillars spaced 100-130 nm apart over a 12-hour period, evidenced by the total distance travelled, while cell motility on nanolines was the lowest compared to the other surfaces. On average, cells on the smaller pillar surfaces also migrated the fastest, while cells on the lines migrated the slowest. Additionally, the smallest nanopillars (100 nm spacing) gave rise to a low population of disassembling adhesion clusters of ~2 paxillin proteins whereas the larger nanopillars (380 nm spacing) gave rise to a much larger population of larger disassembling clusters of ~3–5 paxillin proteins. This study was the first report describing how nanotopography influences cell motility and protein reorganization in adhesions with single molecular detection.

These findings motivated the study to investigate how much force is needed to detach cells from the nanostructured surfaces (Chapter 5). Similar to the adhesion experiments in Chapter 3, and extrapolating from those results, I hypothesized that cells would adhere better on the nanoline patterns compared to the flat surface or the nanopillars under the influence of an external force. I evaluated adhesion on the nanostructured surfaces at various shear rates using a parallel plate rheometer to induce shear across the cells attached to surfaces. I estimated the minimum shear rate to detach 50% of cells on the surfaces; the lowest shear rate was for the
P300 pillars; however, these results need to be statistically validated. I also learned that the orientation of the nanolines with respect to shear flow affects likelihood for cell detachment. I found that the smaller lines L416 oriented parallel to flow was the most effective pattern against shear flow. The fraction remaining after a shear rate of 90 per second was about 70% higher than the fraction at the same rate on a flat surface (p<0.01). At an even higher shear rate, the difference between the two fractions was statistically significant (p<0.05). This would be an important design consideration when applying these structures to medical device surfaces, especially if the device would experience shear force in vivo. Having found the range of shear rates that is optimal for studying cell adhesion under dynamic conditions, I also conclude that one could use cone and plate rather than parallel plate geometry to produce constant shear rate across the nanopatterns, and compare results with detachment shear force of a single cell.

Results from the shear experiment suggested that the side groups of polymer chains might orient differently on the surface of nanopatterns. I hypothesized that another reason for the differences in cell adhesion on the nanopatterns is differences in surface chemistry, which would affect ECM protein adsorption to the nanopatterns. In need of a surface-sensitive method to examine chemical moieties on my surfaces, I collaborated with Dr. Hiroaki Maekawa and Prof. Nien-hui Ge to identify the surface molecular moieties on the nanopatterns using vibrationally resonant sum frequency generation spectroscopy (VR-SFG) (Chapter 6). The SFG spectra appeared to show an overabundance of carbonyl groups on the larger nanopatterns, as the SFG intensity for those patterns were much higher than the SFG intensity on the flat surface, while the intensities of the SFG signals from the smaller nanopatterns appear to be similar to the flat PMMA surface, which suggests that the surface chemistry of the smaller PMMA nanopatterns is similar from flat PMMA. These findings indicate that the size of the structures in the
nanopatterns may dictate which surface cues would influence cell behavior. More interestingly, we may have found a size range in which physical cues are more influential than chemical cues.

These studies show that we can take advantage of nanopatterns to control cell adhesion to surfaces, especially those of medical implants. More importantly, findings from this thesis validate the use of nanopatterns in the proposed artificial cornea (Figure 1.3) to fulfill the design requirements listed in Table 1.1. In the central optic zone, the P200 and possibly the P300 nanopillars would be the nanopattern of choice. An array of these structures would keep the implant transparent while killing bacteria, and increasing cell migration. The central optic zone would also stay clear due to eye blinking, which would prevent too many cells from clumping on the surface of the optic zone. In the peripheral skirt, the L416 nanolines oriented in the same direction as the shear from blinking, would be the best nanopattern to encourage cell adhesion and prevent the cells from detaching, increasing the chances of the implant staying secured in the corneal stroma. Now that I have identified the optimal nanopatterns for encouraging and discouraging adhesion of 3T3 fibroblasts, other researchers can plan future studies using corneal keratocytes to examine corneal wound healing on the nanopatterns.

7.2 Future Work

7.2.1 Migration of keratocytes to topographies

We previously assessed cell migration on different nanotopographies by seeding cells on fibronectin-coated nanostructured surfaces, then observing migration trajectories after one hour incubation on the surface. However, this experimental design was not intended for assessing migration of cells to the nanopatterns in the corneal stroma. Instead, cells will remain stagnant in
the stromal tissue until the artificial cornea is implanted. Thus, a more appropriate experiment would be to observe migration of the cells towards nanopatterned surfaces.

To prepare the surfaces, the nanopatterns will need to be imprinted on a larger substrate, such as the glass cover slide used for the rheometer adhesion experiments. After coating with a collagen solution (97% collagen I, 3% collagen III), a hollow divider device can be used to temporarily separate the flat region from the nanopattern. The inside area of the divider will cover the flat region (Figure 7.1). Primary keratocytes, obtained from donated rabbit or human corneas following a previously established protocol, will be seeded on the flat region inside the hollow divider. After incubation, non-adhering cells will be removed from the sample, and the hollow divider will be removed to provide keratocytes access to the nanopattern. Time-lapse microscopy will be performed to observe potential migration of cells from the flat region to the nanopattern. If there is a nanoline pattern, we expect cells to migrate towards the line pattern. If there is a nanopillar pattern, we expect cells to primarily remain in the flat region.

![Figure 7.1 Proposed migration experiment to mimic movement in cornea](image)

**Figure 7.1 Proposed migration experiment to mimic movement in cornea.** Cells are seeded in the flat region inside the center of a hollow cylindrical divider (A). When the divider is removed, cells will be able to freely migrate towards the line or pillar pattern. Expected results are more cells migrating towards the nanoline pattern, while very few cells would be found on the nanopillar pattern (B).

Ultimately, one could include nanoline and nanopillar patterns on the same surface. Ideally a custom-designed stamp would be fabricated using photolithography. There are two
low-cost alternative fabrication methods. One method is to imprint the line and pillar pattern simultaneously, as depicted in Figure 7.1. Another method is to imprint twice: the first print will be a large area of lines; the second print will be made by using a line stamp of smaller area and placing it orthogonal to the first line print, creating square-shaped nanopillars (Figure 7.2). We would expect to observe similar migration results to the experiments with only one pattern with the flat control surface.

![Figure 7.2 Schematic of double imprinting method with two topographies in proximity to one another on the same PMMA surface. A line pattern will surround a square pillar pattern in the center as a result from this imprinting method.](image)

### 7.2.2 Effect of line topographies on keratocyte differentiation in vitro

With the line topographies, we will first evaluate the effects of the lines on keratocyte differentiation. We will first use primary rabbit keratocytes for experiments. Prior to cell seeding, the line patterns and flat control will be coated with collagen solution (97% collagen I, 3% collagen III) for one minute. Cells will be incubated on the lined surfaces for up to 7 days.

To determine whether keratocytes differentiated into myofibroblasts, we can evaluate the gene expression of the cells for six genes that have previously been used to identify keratocyte phenotype: Keratocan (KERA), prostaglandin D2 synthase (PTGDS), N-acetylglucosamine-6-O sulfotransferase (CHST6), beta1,3-N-acetylglucosaminyl-tansferase-7 (B3GNT7), aldehyde
dehydrogenase 3A1 (ALDH), and aquaporin 1 (AQP1) using quantitative polymerase chain reaction (q-PCR). We will also evaluate expression of markers for myofibroblast: α-smooth muscle actin (α-SMA), collagen I, and collagen III using q-PCR. All experiments will be repeated three times for a total of three biological replicates. Statistical difference between the flat control and the line topographies will be determined using a one-way ANOVA test.

In addition, we will examine the morphology of the keratocytes, which should exhibit stellate morphology; if the keratocytes differentiated into fibroblasts, the cells will appear elongated. We will calculate the percentage of cells exhibiting the stellate versus elongated morphologies. We will also perform immunofluorescence staining for α-SMA and phalloidin. The experiments will be repeated using primary human keratocytes. Similarly, the morphology experiments will be repeated three times for a total of three biological replicates. Statistical difference between the flat control and the line topographies will be determined using a one-way ANOVA test.

7.2.3 Effect of line topographies on ECM organization in vitro

To examine collagen organization on the line topographies, we will use second harmonic generation (SHG) microscopy. SHG is useful for noninvasively imaging biological structures that lack inversion symmetry, with the advantages including increased penetration depth, enhanced axial depth discrimination and reduced photodamage. The SHG microscopy can provide collagen images in situ, with high resolution and more details of collagen organization and distribution. It has been used in quantitatively establishing collagen fibril orientation and studying 3-D collagen organization.
Figure 7.3 Preliminary data of NAD (yellow) and SHG (magenta, backward-scat ter) signals of keratocytes 2 weeks post-treatment of various agents. These include mitomycin C (MMC) (C,D), TGF-beta (E, F), and the combination of MMC and TGF-beta (G, H). Morphology and collagen network changed with each treatment. Keratocytes grown in serum-free medium was used as a control (A, B). Scale bar = 100µm. Data provided with permission from Dr. James Jester.

The line topographies and flat control will be coated with collagen solution as described above. Primary rabbit or human keratocytes from cadaver corneas will be seeded on the patterns, and incubated for 2 weeks. Collagen organization on the patterns will be examined after each
day. We will evaluate collagen arrangement (Figure 7.3), collagen content, and thickness of collagen (Figure 7.4) from the SHG images.

![Figure 7.4 Preliminary data of collagen thickness](image)

**Figure 7.4 Preliminary data of collagen thickness.** Thickness was analyzed by depth code at 2 weeks post-treatment of MMC (B), TGF (C) and a combination of MMC and TGF (D). Thickness was compared with collagen thickness produced by cells in serum-free medium (A). Scale bar = 100µm. Data provided with permission from Dr. James Jester.

7.2.5 Implant Safety in Rabbits

Nonrodent animals, such as rabbits, dogs, pigs and primates, are common animal models used for ocular safety testing. Rabbits are indeed the most common model, given the large size of the eye and cornea compared to rodents such as rats and mice. Rabbits have a similar sized corneal diameter, 12 mm, to that of the human cornea and have a corneal epithelial thickness (47 µm) and corneal stromal thickness (331 µm) comparable to that of human (50 µm and 465 µm, respectively). The wound healing and irritation response of the rabbit cornea has also been extensively studied at both a cellular and molecular level. The rabbit eye is also adaptable for in vivo evaluation using OCT, slit-lamp biomicroscopy and in vivo confocal microscopy, allowing repeat evaluation of the same animal. However, it should be noted that
the rabbit is thought to over predict human responses,\textsuperscript{189,190} and the biomechanical structure of the rabbit cornea is considerably more compliant than that of the human.\textsuperscript{191–194} While dogs have a slightly thicker cornea compare to humans (600 µm), pigs tend to have a thicker corneal epithelium and stroma (>1 mm) making them less desirable models.\textsuperscript{195} Additionally, little is known regarding the wound healing response in these animal models. Non-human primates have the closest corneal structure to human, but experiments with this model are usually limited in number due to humane reasons and restricted to locations that can appropriately handle these animals. Based on these considerations, we have chosen to evaluate the safety of our keratoprosthetic device using a rabbit model.

Nanotextured prototypes will be fabricated following a recently established procedure.\textsuperscript{32} These prototypes will be implanted in live rabbits using surgical procedure established by collaborators in the ophthalmology department at UC Irvine’s School of Medicine to identify implant survival and corneal wound healing. Rabbits will be followed for 84 days (3 months). Rabbits will be evaluated post-operatively at 10 time points. At each time point the condition of the eyes will be scored for conjunctival injection, conjunctival discharge, limbal vasculature, corneal opacity, extent of corneal opacity, aqueous cells, aqueous flare, iris vasculature, and posterior synechiae using a clinical grading system as reported by McDonald and Shadduck in 1977. In addition, \textit{in vivo} confocal microscopy analysis of changes in corneal epithelial thickness, stromal thickness and haze as well as pentacam/OCT measurement of corneal thickness and IOP will be recorded. The percent change from baseline for each of these measurements will then be statistically analyzed using either parametric or non-parametric statistic as necessary and difference between devices assessed by repeat measures ANOVA. At the end of 3 months, rabbits will be euthanized, and corneas with devices will be explanted.
Surrounding corneal tissue will be analyzed using immunocytochemistry and histology to assess corneal fibrosis, neovascularization, inflammation and presence of matrix metalloproteases (MMPs), specifically MMP-2 and MMP-9. Implants will be assessed using scanning electron microscopy to observe the adherence of corneal tissue.

7.3 Conclusions

In this thesis, I have shown strong evidence of the ability of nanotopography to affect cell adhesion. I observed a trend of increased cell adhesion on nanoline gratings, and a trend of decreased cell adhesion on nanopillared surfaces. Additionally, using a unique imaging analysis method, I have shown that nanotopography influences protein aggregation changes inside focal adhesions of cells and correlated these findings with cell migration on the surfaces. Lastly, I have found that 208 nm-wide nanoline pattern oriented parallel to shear flow was most effective in preventing cells from detaching under high shear rates. At the time of this writing, our prototypes implanted in two rabbits have shown encouraging results.

These findings provide insight into choosing the optimal array of nanostructures to apply to medical implant surfaces to modulate cell adhesion. The artificial cornea is not the only implant that would benefit from controlled cell adhesion on the surface of the implant. Any implant that depends on tissue integration to succeed would benefit from topography. For example, tooth and hip implants with topography could improve osseointegration. Vascular stents need a way to reduce aggregation of platelets or endothelial cells at the walls; nanotopography tailored to these implants may provide the answer. I hope that this work will lead to improvements in 2-D and 3-D platforms mimicking in vivo cell adhesion, and inspire more research evaluating molecular mechanisms governing differences in adhesion on nanotopographies.
References


31. Tan, L., Kong, Y. P., Pang, S. W., Yee, A. F. & Tan, L. Imprinting of polymer at low


64. Dickson, M. N. Towards a Scalable, Biomimetic, Antibacterial Coating. (2017). at <http://www.escholarship.org/uc/item/4br3n017>


103. Li, J.-R., Shi, L., Deng, Z., Lo, S. H. & Liu, G. Nanostructures of designed geometry and...


116.


119.


120.


121.


122.


123.


124.


125.


126.


127.


128.


129.


130.


155. Pluchery, O., Humbert, C., Valamanesh, M., Lacaze, E. & Busson, B. Enhanced detection of thiophenol adsorbed on gold nanoparticles by SFG and DFG nonlinear optical


183. Reinstein, D. Z., Archer, T. J., Gobbe, M., Silverman, R. H. & Coleman, D. J. Epithelial


