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SUBSTRATE TOPOGRAPHY DESIGN AND FABRICATION FOR OSTEOBLAST AND DENTAL PULP STEM CELLS STUDIES

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SUBSTRATE TOPOGRAPHY DESIGN AND FABRICATION FOR OSTEOBLAST AND DENTAL PULP STEM CELLS STUDIES

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Materials Science and Engineering

by
Xue Chen
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Accepted by:
Dr. M.Kennedy, Committee Chair
Dr. D. Dean, Co-advisor
Dr. E. Skaar
Dr. J. Luo
ABSTRACT

Research for cell guidance based tissue engineering has rapidly grown due to the increasing interest in tissue engineering and reconstructive medicine applications, such as cranial reconstruction. Many research teams have begun the process of identifying what factors influence cell behavior (including cell growth, proliferation, alignment and spreading). Published studies have pointed to the influence of the cell medial pH, substrate stiffness, chemistry and topography. Conclusive results are often hard to identify since researchers often vary many of these factors simultaneously and it is hard to decouple individual factor influence. These reports also points out that the cellular response can be cell type dependent.

This work aims to identify the influence of substrate topography on cell response by designing a variety of substrate micropatterns with identical roughness, uniform stiffness and chemistry. These studies were designed primarily to give insight into dental stem cell response and features used were selected for their similarity to naturally occurring dental tissue. Using photolithography techniques developed for the semiconductor industries, Au micropatterned arrays with four feature shapes (lines, dots, holes and hexagons) were fabricated. The forty-eight unique micropatterns were produced with a range of feature heights (100, 500 and 1000 nm), widths (5, 10, 25 and 50 µm) and shapes (lines, dots, holes and hexagons). Subsequent processing (an additional 5 nm Au coating and 3 nm of 11-amino-1-undecanoth hydroxide) provided uniform roughness (RMS is 2 nm to 9 nm) and surface chemistry.
Micropatterns were characterized for uniformity, feature width and heights and surface roughness using atomic force microscopy, profilometry and optical microscopy.

To study cell response, two types of cells were utilized—mice 7F2 osteoblasts and porcine dental pulp cells. The mice 7F2 osteoblast cells were plated as a control, since there are already published studies characterizing this cell’s type response to microarrays of holes and lines. No published studies have been done to characterize the response of dental pulp stem cells. Each cell type was plated and characterized for cell density, alignment and spreading over three days. Initial results of the osteoblast cells confirmed earlier findings that the cells aligned on the anisotropic patterns (lines) and spread on the isotropic patterns (dots and holes). The dental pulp cells did not show any cell alignment or cell proliferation (as indicated by cell density) with the isotropic or anisotropic micropatterns.

Significance of the osteoblast and dental pulp cell normalized densities were analyzed with statistical software (SAS using procedure PROC GLM). This analysis showed that there were no significant effects in terms of geometry. However, it did indicate that there was significant variation between each repetition of cell plating and when repetition is taken into account, the feature height significantly influenced the cell density increase over three days.

Characterization of the micropatterns after the cell plating showed that the micropatterns could be used for multiple runs without significant degradation when
interlayers were used between the Au and Si substrates. However, handling techniques could produce scratches in the micropatterns and residual stresses could cause buckling.
DEDICATION

To those who made all of this possible,

for their endless encouragement and patience.
ACKNOWLEDGMENTS

First of all, I offer my sincerest gratitude to my parents, without their unconditional and endless love, none of this would’ve been possible.

I’d like to thank my advisor, Dr. M. Kennedy, who supported me with her patience and knowledge, while still allowing me the freedom to work in my own way. Without her encouragement and effort, this thesis, would not have been written or accomplished. Beside my advisor, I would like to thank my committee members: Dr. D. Dean (my co-advisor), Dr. E. Skaar, Dr. J. Luo, for their encouragement and insightful comments.

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on phase contrast and confocal microscope image processing, Mr. J. Holiday for sample holder preparation, and Mr. M. Fisher in the Nanofabrication Center in University of Minnesota for his guidance in the masks design and ordering process.
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CHAPTER ONE

INTRODUCTION TO DEVELOPING NOVEL MATERIAL SURFACES TO INFLUENCE CELL BEHAVIOR

Scientists have sought ways to attach, guide, and develop cells since the 1960s [1-3]. These experiments help in the study of cell regulation which helps to provide insight into how damaged tissue can repair and how to design biomaterials for tissue engineering applications [5]. In clinical practice, the design of biomaterial surfaces can help solve bio-compatibility, cytotoxicity, and cell death issues associated with faulty implants [6]. This thesis focuses on the design, creation, and implementation of substrates for cell studies.

To understand the importance of substrate topography to influence cell behavior, the following sections will discuss cell structures and cell types studied in this thesis, substrate topography, cell response to topography, and characterization methods.

1.1 Cell Structure

The cell is the smallest functional unit part of plants and animals [7]. Animal cells share the same overall structure (Figure 1.1) [4]. The basic structures of cells include: the cell membrane, cytoplasm, and nucleus. The cell membrane, or plasma membrane, is the flexible outer covering that separates the inside of the cell from surrounding environment and that helps maintain cell homeostasis. The membrane is made from phospholipids, a combination of hydrophilic phosphorus and hydrophobic lipids. The cytoplasm, including cytoskeleton, golgi apparatus,
mitochondria, and other organelles, is the collective term for the inside of the cell. The cytoplasm is responsible for a variety of cell activities. For instance, cytoskeleton is mainly for maintaining cells shape and movement. It is composed of microtubules, actins, microfilaments, and intermediate filaments. The genetic materials, known as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are contained in nucleus and mitochondria. The nucleus, which determines all cellular activities, is usually located near the center of the cell. Figure 1.1 shows the structure of a general animal cell.

![Figure 1.1 Schematic structure of an animal cell [4].](image)

Cells contact and react to their surroundings (body fluid, neighboring cells, extracellular matrices, and implantable biomaterials) by sending and receiving chemical and physical signals [8]. The cell adheres to substrate and the cytoskeleton gives cell physical support. For instance, membrane of epithelial cells has apophysis,
in nanometer dimension, to feel and adhere to substrates [9, 10]. Specifically, when cells in contact to various topography, lines, hole or dot, a series of transmembrane proteins lie along pattern edges cause cells to extend, deform and orientate in a certain way [11, 12]. It is reported that cells could also contact successfully without actin filaments and microtubules, which overthrew traditional view that actin and microtubules were the necessary conditions in cell alignment [21]. Other proteins, like vinculin, were found to guide cells go along edges of lines [9]. In summation, no matter what kind of protein found orientated along lines, cultured cells tend to move along the long axis direction.

1.2 Cell Type

There are two type of cells mainly discussed here. The first type of cell is 7f2 osteoblast. 7F2 osteoblast is a cloned cell line isolated from p53-/- mouse bone marrow. They express alkaline phosphatase (ALP) and mineralized bone matrix. Many researchers studied mice 7F2 osteoblasts due to their short growth time [13] significant expression of ALP (alkaline phosphatase), COL I (type I procollagen), and mineralized matrix [14, 15].

Dental pulp stem cells (DPSCs), one type of mesenchymal stem cell (MSC), express the STRO-1, CD73, CD90, and CD105 [16, 17]. DPSCs are the first stem cells isolated from dental pulp tissue of human [18]. They can be easily extracted from the third molar (“wisdom teeth”) of human. Studies have shown that both in vitro [19] and vivo [18], DPSCs have the potential to differentiation into dentin, which
is the main structure in teeth [20].

1.3 Substrate Topography

More and more studies are focus on how cells response to substrate topography [21-24]. Substrate topography includes a wide range of patterns created by various techniques. The widely investigated patterns include lines, dots, holes, nanoparticles, etc. The characterization of patterns include width/diameter, height, (see figure 1.2) surface roughness, morphology, etc.

Figure 1.2 Schematic of pattern size definitions. (a) Top view of pattern diameter/width. (b) Side view of pattern height.

All the patterns can be divided into two categories: isotropic and anisotropic patterns. Isotropic patterns are uniformity in all directions, like dots, holes, and well fabricated particles. Anisotropic patterns are directionally dependent, include lines, wedges, etc. So far, the height of finely created patterns can be controlled under 10 nm [24]. Similarly, the width of patterns can be controlled as small as 25 nm.
1.4 Cell Response to Topography

Back in the 1960’s, it was revealed that synthetic nano-sized features influenced embryonic and conjunctiva cells behavior [25, 26]. These nano-sized features were made by Langmuir-Blodgett techniques and were not uniform. Recently, another study showed that fibroblasts were sensitive to a height of 10 nm when width of lines was approximate 150 nm [27]. Fibroblasts and baby hamster kidney (BHK) cells, however, not respond to patterns with height less than 300 nm when feature width is greater than 5 µm in many studies [28-30]. The differences of cells (spreading area, alignment, orientation, etc.) on substrates with features less than 300 nm are very small. Wojciak-Stothard [31] observed that spreading area of macrophages on 44 nm deep lines was 50% that when the cells were plated on 282 nm deep ones. The width of groove was 10 µm here. It is the same case in the Curtis’s study [32]. In this study, feature size includes diameters of dot and hole, width of lines, and edge length of square.

Studies of interactions between a wide variety of cells and topographies showed that cultured cell response variously to anisotropic and isotropic patterns. In general, when contacted anisotropic topographies, like lines and ridges, cells tended to align along the long axis direction [18-20]. With regard to isotropic topographies (holes and dots), situations were much more complex.

Generally, when cells contact anisotropic patterns, depth is more significant than width in deciding cell alignment [33, 34]. Specifically, orientation of cells decreased
with increasing line width but increased with increasing lines depth [16, 22]. This was proved in Clark’s [35] study on alignment of BHK cells. This can be explained by size of pattern relative to it of cell. When the line width is large enough, the cells treat the shallow part of lines as plateaus instead of change in profile. Conversely, when the line width is small enough, cells tend to bridge over gap instead of traveling down to bottom. Walboomers’s study [36] also revealed that cells behaved more sensitively to the ratio of feature size with respect to depth.

When cells contact with isotropic topography (dots or holes), it hard to sum a constant cell behavior on isotropic topography regarding to various feature size, film thickness, surface roughness, etc. Macrophage proliferated less on 2 and 5μm nodes than on 8 um wells and flat surface [37]. Fibroblasts grew well on 2 and 5μm nodes but decrease intensity on wells with the same size. Furthermore, 10μm nodes and wells showed no difference from flat surface [38].

1.5 Substrate Cytotoxicity

In practice, a number of metal materials have been taken in to clinic trail and commercial use. Pure titanium, and various titanium alloys are the most investigated metallic dental implant [34, 39-42]. Ni alloys are another popular kind of implant currently. However, there is a concern on inflammation after implantation [43].

There were rare reports of adverse reaction of gold and its alloys. Karine tested the performance of osteoblasts cells adhesion on gold–palladium coating [44]. In a vitro test, osseointegration, the integration process between living bone and artificial
implants, is achieved high bone-to-implant contact (BIC%) as much as 36.5% and 19% in different test zone [45, 46].

1.6 Overview of Chapters and Objective of This Study

The objective of this thesis is to investigate a novel method to design and create substrate with wide aspect ratio micropatterns and implement fabricated substrates for cell studies on proliferation and deformation. Chapter Two will discuss the main characterization methods used in this study, including profilometry, AFM, fluorescent microscopy, and confocal microscopy. In Chapter Three, a detailed description of design, fabricate and characterization results of micropatterned substrate will be given. Chapter Four and Chapter Five will discuss response of osteoblasts and dental pulp stem cells to micropatterns, and investigate the difference. Finally, Chapter Six will summarize the conclusions of this study and predict the future work to further examine behavior of osteoblasts and dental pulp stem cells to different substrates.
1.7 References


3 A. S. G. Curtis and M. Varde *Control of cell behavior: topographical factors*, (1964)


CHAPTER TWO
CHARACTERIZATION TECHNOLOGY OF MICROPATTERNED SUBSTRATE AND CELL BEHAVIOR

As outlined in Chapter One, the topography of substrates used in cell study is extremely important. The material properties related to the substrate include surface roughness, residual stress, and pattern size. In this chapter, common techniques for use in micropatterned substrate and cell behavior characterizations will be reviewed. These include using profilometry (section 2.1) and atomic force microscopy (section 2.2) to study substrate (surface roughness, grain size, curvature, and topography), and monitor cells response by using fluorescence microscopy (section 2.3) and confocal microscopy (section 2.4).

2.1 Profilometry

Profilometry is a surface characterization technique that measures surface profile and roughness using a stylus or laser [2]. When a stylus is moved by a motor, the transducer connected to the stylus will transfer the slope of samples into the computer, and the height profile will be represented on screen, just as shown in Figure

![Fig 2.1 Schematic representation of profilometry.](image-url)
Normally, there is a ball-tip at one end of the stylus. The diameter of the ball used in this research is 12.5µm. Nevertheless, balls with smaller diameter contribute to higher resolution profile. Compared with atomic force microscopy (AFM), profilometer can only scan solid samples in air, while AFM can test both solid and liquid samples.

Compared with other techniques to obtain topographic image of samples, a profilometer is suitable for various materials without demanding of special sample preparation [3]. Moreover, it can scan larger area (15 cm X 15 cm) compared with AFM and get an image quickly. The scan speed is from 1µm /second to 25 mm/second.

There are two main error sources: the stylus tilt error and the stylus tip radius error [4]. The stylus tilt error introduced by either displacement or improper alignment. An effective way to test if the stylus was set up correctly is to calibrate the set up by scanning a standard, known size pattern (lines, for instance). The ball tip error is unavoidable as when ball touches curved sample surface, the height change of stylus isn’t the sample itself but the changes of touch point of ball. (Figure 2.2) Thus, as long as the slope of sample surface isn’t zero, the result contains error from ball tip.
2.2 Atomic Force Microscopy

Atomic Force Microscope (AFM) has significantly changed researcher’s understanding of surface structure and properties [5-7]. The application of AFM in the biology field of concentrates in biomaterials surface study [7-9] and interface between substrate and cells [10-12]. In this thesis, AFM was mainly applied to study substrate morphology, surface roughness, grain size, and interlay (amino group) between substrate and cells.

The unique contribution of AFM is the high resolution imaging of sample surface, which is based on its extra sharp tip [13-15]. In this way, scientists can study surface morphology, roughness, chemistry and mechanical (AFM indentation) properties down to nano scale [10, 16, 17].

The concept of AFM is converting z axis change of cantilever, into topographic image of sample. The cantilever tracks sample topography by the tip installed at one end. The main parts of an AFM are the laser, cantilever, and detector, as seen in Figure 2.3

Fig. 2.2. The tip shape of profilometer stylus can induce data error.
The tip, or cantilever, can deflect with force when scanning through sample. The deflection is sent to photodiode by a laser beam, which is shining at the tip of cantilever during scanning. The electrical signal received by photodiode is processed by computer and transferred into the morphology image. Meanwhile, the same signal is sent back to piezo to maintain a consistent force between sample and tip.

![Diagram of AFM](image)

Fig. 2.3 Schematic representation of AFM. A typical AFM is made up of three parts: the laser, cantilever and detector [1].

The tip, or cantilever, can deflect with force when scanning through sample. The deflection is sent to photodiode by a laser beam, which is shining at the tip of cantilever during scanning. The electrical signal received by photodiode is processed by computer and transferred into the morphology image. Meanwhile, the same signal is sent back to piezo to maintain a consistent force between sample and tip.

![Different modes of AFM](image)

Fig. 2.4 Different modes of AFM. (a) contact mode (b) non-contact mode (c) tapping mode.

Normally, there are 3 scanning modes: contact, non-contact and...
mode (Figure 2.4 a), the tip touches sample by maintaining a constant force. Nevertheless, this mode can create a high resolution image. Meanwhile, the sample surface can be changed by the shear force when tip is scanning. Therefore, this mode is suitable for hard and stable samples.

The next mode introduced here is non-contact mode, when a constant distance is maintained by electrical feedback loop. Instead of contacting sample surface, the tip is oscillated at a frequency which is higher than resonant frequency [18, 19]. The tip used here is placed in the attractive force region and collects force gradients information [20-22].

If the sample is soft and deforms from the forces during contact mode, tapping mode can be used. During testing, the tip “taps” the surface and moves horizontally. Figure 2.4 b illustrates how it works. This mode reduces damage to soft sample as it won’t change sample surface by shear force. For example, the tapping mode is suitable to collect topographic image of protein adhering to biomaterial.

2.3 **Fluorescence Microscopy**

Fluorescence microscopy was originally developed to study cellular structure [23]. As discussed in Chapter One section 1.1, most cellular parts are transparent and difficult to separate one from another using traditional optical microscopy. Fluorescent dyes make it is possible to label different parts of cells and imaging them by microscope. The basic procedure of imaging cells by fluorescence microscope includes: staining samples with specific dyes, illuminating them with excitation light
in a certain wavelength (UV, for instance), and monitoring samples with emitted longer wavelength light which can go through matched filter lens [24].

Another main contribution of fluorescence microscopy is making the ability to study dynamic behavior of living cells possible [25]. This function was realized with the discovery of a new protein, green fluorescent protein (GFP). Originally, GFP was founded in Aequorea Victoria, a jelly fish found in west coast of North America, and studied by Shimomura [26]. The mechanism of living cell staining is: GFP has the ability to insert its gene sequence into target protein complementary DNA (cDNA), as they share the same regulatory sequence. When DNA expresses target protein, GFP is expresses as well. All of this must be done in a living cell. Thus in this way, movement of living cells can be investigated across time.

Like other techniques, fluorescence microscope has its own limitations in dynamic cell characterization. First, most of the excitation light is toxic. As UV light is often used for sterilization purposes, the light may damage cell or even cause death. The second limitation is the dye itself, it will interact with cells in some way and change the cellular function [27]. At last, the fluorescence itself fades over time [28]. This situation won’t hurt cells but no longer allows the monitoring of fluorescence signal.

As this project concentrates on how cell interacts with biomaterials, fluorescence microscopy serves here to study cell adhesion to substrate. Cell adhesion involves adhesion protein binding to integrins (transmembrane protein) at
focal contact. The advantage of fluorescence microscopy to other approaches in analyzing cell adhesion, is it can provide information on adhesion strength [29, 30] and focal contact dynamics [31-34] as well. For example, as lifetime cell behavior investigation is possible, it is convenient to study how contact areas, proteins, etc. change with time.

2.4 Confocal Fluorescence Microscopy

Confocal fluorescence microscopy is a three dimension imaging method based on traditional fluorescence microscopy [35]. The applications of confocal fluorescence microscopy mainly focus in labeling cells [36, 37], antibodies [38], and reactive oxygen species [39].

The confocal microscopy idea was first published in 1957 by Minsky [40]. The principle of confocal microscopy is based on single plane image construction. A pinhole is inserted between sample and detector. When excitation light emits on the sample, light from focal plan could go through the pinhole. The smaller pinhole is, the less light from out-of-focus plan received. In lateral direction, images are collected during scanning line by line. After each 3 dimension block is obtained, reconstruction of those blocks will be processed by computer [41].

There are several components influencing quality of gained image, which is evaluated by spatial resolution. The spatial resolution R, defined by following equation:

\[ R = 1.4 \frac{n\lambda}{NA^2} \]
Where \(n\) is the object medium refraction index, \(\lambda\) is the excitation wavelength, and \(NA\) stand for numerical aperture \([42, 43]\). Normally, high numerical aperture lenses are preferred to get high resolution image. Otherwise, detailed information could be lost due to ultra high contrast. In this situation, low \(NA\) lenses are more proper. Excitation power is another issue affects image quality. When excitation power increases, less noisy will be collected. But if the power is too high, the fluorescence images become dark as photo quenching effect \([44]\).
2.5 References


23 The Lancet 230, 91-92 (1937).


40  M. Minsky microscopy apparatus, (1957)


42  M. G. L. Gustafsson, Current Opinion in Structural Biology 9, 627-628 (1999).


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3.1 Introduction to Micropatterns Fabrication

There are several different techniques to produce duplicated patterns, from traditional lithography technique to most recently cell printing, micro scale substrate patterns fabrication methods. This section will discuss the details of these techniques.

3.1.1 Soft Lithography

Soft lithography is a method covering a group of techniques using elastomeric material to create chemical structures, which is applied in tissue engineering since 1990’s [1, 2]. The two main categories in soft lithography are microcontact printing and microfluidic patterning [3]. The first step in microcontact printing is to prepare a layer of target molecular on model (usually made by poly(dimethylsiloxane)) which holds the desired pattern. Then closely contact the model with substrate and remove the model. The patterns will be left on substrate. About microfluidic patterning, the difference is the model is first brought close contact with substrate, and then the patterning solution is poured into channel of model.

So far, a number of groups have fabricated micropatterns by using soft lithography aimed in tissue engineering [4-6]. Delamarche fabricated lines with 3 µm and 10 µm width by using microfluidic patterning [7]. Anderson group used this method to fabricate three-dimensional structures [8].
It is flexible, low cost, and suitable for chemical adhesion. The challenge is to create complex substrate and stamp soft materials.

3.1.2 Photolithography

Photolithography is another way to produce patterns [9-12]. Geometric patterns recorded on a mask were transferred onto aiming substrate by UV illumination. It is suitable for large scale and duplicate pattern production, and tolerance with various substrates. Furthermore, precisely control size of pattern is relative easy. But the process is complex and expensive. Photolithography can produce both chemical and physical micropatterns. To produce chemical micropatterns, selected molecular, normally proteins, are arrayed in a certain shape. In physical patterning, surface with aimed topography is fabricated.

DNA array was made by Schena by using photolithography in 1995 [13]. Bhatia use this method for patterning 50 µm holes to culture fibroblasts [14]. He also use the same technique to produce 490 µm islands to study co-culture of fibroblasts and hepatocytes [15]. Recent study showed that 3D structure can also be produced by using photolithography. Lee and his co-workers studied human dermal fibroblasts on 3D hydro gel structure and found cells migration [16].

There is an improved technique of photolithography, plasma polymerization combined with photolithography [17, 18]. It shares the same principle with traditional photolithography. The difference is after photoresist is developed, coating substrate with plasma polymerized polymer and lifting rest photoresist, then
aimed patterns left. Favia cultured NCTC 2544 keratinocytes on polyethyleneoxide (PEO) polymers [19]. The advantage of plasma polymerization combined with photolithography is time efficient, replicable, and suitable for a great variety of substrates and large-area surface treatment. At the same time, it is challenging to produce durable plasma polymer layers.

3.1.3 Stencil Assisted Patterning

Stencil assisted patterning is a simple and low cost method to produce cellular patterns from 1967 [20]. The basic concept is contacting membrane (stencil) with through-holes with substrate, and seed cells on uncovered areas [21]. Stencil assisted patterning is suitable to any substrate material and non-flat surfaces, the absence of organic solvents also suitable for delicate molecules. To ensure an adequate sealing, technical skills are required to align the flexible stencil on the surface.

Folch used this method to fabricate holes to study rat hepatocytes proliferation [22]. Kim developed a novel method to produce low residual stress, reusable stencil [23]. More recently, Burckel fabricated 3D structure combined with lithography [24]. Although this method hasn’t been applied in tissue engineering yet, it is very promising to study cell behavior on substrate fabricated through this way.

3.1.4 Jet Printing

Jet printing cells is a recent technique that produce designed cell patterns [25-28]. The principle is to replace ink by cell suspension and print cells from modified inject
head. The other method to guide cell growth is to print collagens on substrate and then culture cells [29]. The advantage is very obvious that it is simple, flexible, and low cost. Meanwhile, the resolution is greatly limited due to the spot size. It is also unknown that if the heat during printing will influence cells behavior or not.

Fuller printed neurons cells on islands and letters made by collagen/poly-D-lysine (PDL) mixture, which is the cell-adhesive material [29]. Human fibrosarcoma cells division on printed substrate was successfully achieved by Saunders [30]. Boland successfully printed CaCl$_2$ into Naalginate solution to produce 3-d hollow structure [31]. The SEM image showed that the endothelial cells in that study attached both outside and inside of hollow structure.

In summation, the micropatterns required in this thesis should have solid physical structure, uniform topography, close tolerance, reusable in medium (liquid), and can be made easily in large scale. Photolithography was used in this study as it can precisely control pattern size, surface chemistry and uniform topography. The smallest feature in this study is 5µm, and solid physical patterns must be prepared as it is the part of the objective. Lithography and Stencil assisted patterning are only suitable to produce chemistry patterns. Jet printing technique is limited by its low resolution. It is a challenge to produce stable polymer interlayer in plasma aided photolithography. In sum, photolithography is the best way to fabricate designed patterns.
3.2 Substrate Matrix Design

Au film with three thicknesses of (100 nm, 500 nm, and 1000 nm) were deposited. Each thickness has four feature sizes: 5µm, 10µm, 25µm and 50µm. There are four kinds of patterns for each feature size: holes, dots, lines and hexagons. The hexagon is the novel feature that never been studied before. The reason why including hexagon in substrate design is it mimics the structure of hydroxyapatite which dental pulp stem cells grow on. The substrate matrix is shown as table 3.1.

<table>
<thead>
<tr>
<th>Feature size (µm)</th>
<th>Au film thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5µm</td>
<td>100 500 1000</td>
</tr>
<tr>
<td>10µm</td>
<td></td>
</tr>
<tr>
<td>25µm</td>
<td></td>
</tr>
<tr>
<td>50µm</td>
<td></td>
</tr>
</tbody>
</table>

3.3 Photolithography Masks Design

According to the substrate design, the four masks (Cr films on glass) designed using ICWIN were ordered from University of Minnesota Nanofabrication Center. Each mask contains four square arrays (4 mm * 4 mm) with the same feature size. Each array was composed by a periodic repeat of patterns: dot, hole, hexagon and line. The area density for 5µm, 10µm, 25µm and 50µm patterns are 19.625%, 19.625%, 16.23% and 50%, respectively. The image of masks and patterns are shown in Figure 3.1. Detailed mask design process is described in Appendix A.
Before film deposition, the three inches (100) Si wafers (Waferworld Co.) were cleaned using a two steps process. First, the wafers were cleaned by rising in aceton/IPA/deionized water/IPA/acetone/deionized water. Next, dried the wafers on hot plate at 100 ºC for 5 minutes, the wafers were cleaned in plasma cleaner (Harrick Plasma PDC-001) for 10 minutes.

Sputter deposition using a Lesker Unit was used to deposit the metallic films during all processing. Before depositing Au film onto the silicon wafers, a thin layer

Figure 3.1 Image of a mask. Image in the central is how mask looks like. The four bright squares in the middle are four patterns. The four images around the mask are magnified picture of how each patterns looks like. Patterns from the upper left corner to the upper right corner in counterclockwise are: holes, lines, dots and hexagons.

3.4 Au Film Deposition

Before film deposition, the three inches (100) Si wafers (Waferworld Co.) were cleaned using a two steps process. First, the wafers were cleaned by rising in aceton/IPA/deionized water/IPA/acetone/deionized water. Next, dried the wafers on hot plate at 100 ºC for 5 minutes, the wafers were cleaned in plasma cleaner (Harrick Plasma PDC-001) for 10 minutes.

Sputter deposition using a Lesker Unit was used to deposit the metallic films during all processing. Before depositing Au film onto the silicon wafers, a thin layer
of titanium (5 nm) was condensed for three minutes with DC power supply to enhance combining force between Au film and wafers. Argon was set to 0.150 ppm and power supply was set to 260 volt during sputtering. The chamber pressure was $1.5 \times 10^{-6}$ torr. Au film was deposited by RF power supply. Detailed process is outlined in Appendix B.

3.5 Process of Fabricating Micropatterns onto Au Film

Designed patterns with various feature size were fabricated onto Au film by photolithography technique is shown in Figure 3.2. First, clean wafers with acetone and dry them on hot plate at 100 ºC for 30 seconds. Next, coating wafers with positive photo resist (microposit s1813 photo resist, Shipley Company) on spinner, and followed by soft bake wafers at 100 ºC for 20 minutes on hot plate. Exposure was conducted on mask liner with ultraviolet (wavelength: 405 nm) and the exposure time of 2.5 seconds. After exposure, wafers with various patterns were developed in developer solution (microposit MF-312 developer Shipley Company) for 45 seconds. Put wafers in vacuum oven for 30 minutes at 150 ºC to before gold etch (Au etchant type TFA, transene company, Inc.). It took 10 -40 seconds to etch gold. The last step was remove remaining photo resist in stripper (Positive Photoresist Stripper R-10) for 30 minutes at 50 ºC. Detailed procedure is described in Appendix C.
Figure 3.2. Fabricate micropatterns by photolithography

In order to ensure uniform substrate chemistry, a second layer of 5 nm thick Au film was deposited on patterned wafer under the same sputtering condition as the previous layer.

3.6 Substrate Characterization

3.6.1 Film Thickness and Feature Size Measurement

A Dektak$^3$ (Veeco, Inc) profilometer was used to determine film thickness and feature size measurement. Four runs of thicknesses were measured on each type of wafer: twice on center of pattern arrays and twice on far from center edges of arrays. At the same time, feature size (diameter of hole, dot, and circumscribed circle of hexagon, half height width of ridge and groove for line characterization) data were
3.6.2 Curvature Measurement and Residual Stress Determination

Curvature measurements were conducted with Dektak\textsuperscript{3} (Veeco, Inc) profilometer. Prior to measurement, the tip of profilometer was calibrated by a standard sample with a 0.84 nm width line. The actual width was 0.8394 nm, and the difference was 0.0714%.

6 paths were run across the center of film. These 6 run were divided into 3 groups and each group contains 2 same run. To overcome the non uniform factored of film, there was a 60 degree angle between each pair. The scan scale is 30 mm, and scale speed is 20µm /s. After curvature measurement, Stoney’s equation [32] was applied to calculate the residual stress.

3.6.3 Substrate Roughness and Grain Size Measurement

Contact mode AFM (Veeco Co.) was applied to take Au film surface image and carry our substrate roughness and grain size measurement. All three film thicknesses (100 nm, 500 nm, 1000 nm) were checked. Two scan scale (500 nm and 1000 nm) and 3 spots for each size were carried out on each film thickness. Root mean squared (Rq) and average mean (Ra) were directly collected by computer. Grain sizes were calculated by line- cross section method. (ASTM, E112 – 96 (Reapproved 2004))
3.7 Results and Discussion

3.7.1 Au Film Thickness

Four thicknesses were measured on each type of wafer: twice on center of pattern arrays and twice on outer edges of arrays. It was shown that the thickness was uniform across pattern area. The variations between designed and actual film thickness were less than 6% in 500 nm and 1000 nm thick films, while the variations of 100 nm film was 28%.

Table 3.2. Actual film thickness measured by Dektak³.

<table>
<thead>
<tr>
<th>Designed film thickness (nm)</th>
<th>Measured Film Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Center(nm)</td>
</tr>
<tr>
<td>100</td>
<td>65.3</td>
</tr>
<tr>
<td>500</td>
<td>524</td>
</tr>
<tr>
<td>1000</td>
<td>960</td>
</tr>
</tbody>
</table>

3.7.2 Au Film Residual Stress

In order to estimate Au film residual stress, Stoney’s equation [32] was applied. A complete stress homogeneity and elastic isotropy were assumed within film and substrate. All curvatures of bare wafer before deposition were assumed 0.

Stoney’s equation was applied to calculate the residual stress:

$$\sigma = \frac{1}{6(1-\gamma_s)} \frac{E_s}{t_f} \left( \frac{1}{R_f} - \frac{1}{R_s} \right)$$  \hspace{1cm} (3.1)

where $\sigma$ is the residual stress, $E_s$ is Young’s modulus and $\gamma_s$ is Poisson’s ration. $T_s$ and $t_f$ are thickness of silicon substrate and Au film, $R_s$ and $R_f$ are radius of wafer and film, respectively. In this research, $E_s = 135$ GPa and $\gamma_s = 0.28$, $t_s = 375 \, \mu$m.
The results above showed that residual stress within film was increased with film thickness. In small scale, the stress results from defects inside material. With low adatoms mobility and shadowing effects, the accumulated defects are the source of void [33]. The adatoms mobility is a function of substrate temperature, substrate bias and sputtering pressure [34]. Au atom is relative heavy (the atomic mass is 196.96 u). Even under higher temperature, lower substrate bias and sputtering pressure, the mobility of Au atom is low.

Refers to other articles, residual stress of thin Au film coated on silicon wafers with various thicknesses differs from one to another. However, the differences are not distinguished. The thicknesses of Au films in Brennan’s study [35] ranged from 0.79µm to 2.54µm. The results showed that residual stress decreases with increasing film thickness, and the differences are very small in films above 1.75µm.

It is worth noting that when applying Stoney’s equation, a complete stress

<table>
<thead>
<tr>
<th>Film thickness (nm)</th>
<th>100</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measurement</td>
<td>R(f)(m)</td>
<td>σ(MPa)</td>
<td>R(f)(m)</td>
</tr>
<tr>
<td>1st</td>
<td>196</td>
<td>311.4</td>
<td>167</td>
</tr>
<tr>
<td>2nd</td>
<td>168</td>
<td>363.3</td>
<td>168</td>
</tr>
<tr>
<td>3rd</td>
<td>138</td>
<td>442.3</td>
<td>129</td>
</tr>
<tr>
<td>4th</td>
<td>146</td>
<td>418</td>
<td>118</td>
</tr>
<tr>
<td>5th</td>
<td>174</td>
<td>350.8</td>
<td>164</td>
</tr>
<tr>
<td>6th</td>
<td>127</td>
<td>480.6</td>
<td>119</td>
</tr>
<tr>
<td>Mean</td>
<td>158.2</td>
<td>394.4</td>
<td>144.2</td>
</tr>
<tr>
<td>STD.</td>
<td>25.7</td>
<td>63.3</td>
<td>24.6</td>
</tr>
</tbody>
</table>
homogeneity and elastic isotropy are assumed within film and substrate, or the method does not consider the elastic properties of the film. If consider the elastic mismatch, the following equation [32] can be applied:

$$\sigma = \frac{E_s}{1-\gamma_s} \frac{t_f^2}{6t_r} \left( 1 + \frac{E_r (1-\gamma_s) t_f^2}{E_s (1-\gamma_f) t_r^2} \right) \left( \frac{1}{R_f} - \frac{1}{R_s} \right)$$

(3.2)

The variables in above equation stand for the same meaning as indicated in formula (3.1).

3.7.3 Characterization of Film Roughness and Grain Size

Contact mode AFM (Veeco Co.) was applied to take Au film surface image and carry out substrate roughness and grain size measurement. All three film thicknesses (100 nm, 500 nm, 1000 nm) were characterized. Two scan scale (500 nm and 1000 nm) and three spots for each size were randomly chosen on each thickness film. Root mean square (Rq) and average mean (Ra) were directly calculated by computer.

Table 3.4. Average mean (Ra) of films with different AFM scan scales

<table>
<thead>
<tr>
<th>Film thickness (nm)</th>
<th>Scan scale</th>
<th>500 nm</th>
<th>1000nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (nm)</td>
<td>STD (nm)</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>4.1</td>
<td>0.5</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>9.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 3.5. Root mean square (Rq) of films with different AFM scan scales

<table>
<thead>
<tr>
<th>Film thickness (nm)</th>
<th>Scan scale</th>
<th>500 nm</th>
<th>1000nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (nm)</td>
<td>STD (nm)</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>5.2</td>
<td>0.7</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>11.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>
The phenomenon of increasing roughness with increasing scan area can be related to the dependency of the roughness on the spatial wavelength of the scanned area or the frequency [36]. In a small surface area, only the roughness of the “higher” frequencies is measured. When a larger surface area is scanned the roughness caused by additional lower frequencies also has to be taken into account. This results in a larger roughness value when a larger surface area is scanned.

Grain size was calculated by line- cross section method. (ASTM, E112 – 96 (Reapproved 2004)). Five pictures for each sample of each scan scale (500 nm and 1000 nm) were chosen. Results showed that the average grain size for 100 nm, 500 nm and 1000 nm thick films were 45.8 nm, 79.3 nm, and 100.3 nm. The standard deviation for those three thickness films were 1.4 nm, 2.9 nm, and 3.1 nm. The grain size increased with film thickness significantly.

Table 3.6. Grain size of Au films with different thicknesses

<table>
<thead>
<tr>
<th>Au film thickness (nm)</th>
<th>Grain size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean(nm)</td>
</tr>
<tr>
<td>100</td>
<td>45.8</td>
</tr>
<tr>
<td>500</td>
<td>79.3</td>
</tr>
<tr>
<td>1000</td>
<td>100.3</td>
</tr>
</tbody>
</table>

Images below were collected from AFM to illustrate grain size increased with film thickness. Figure 3.3 a showed the morphology of 100 nm thick film scanned by AFM. With deposition continuous, 500 nm thick film (Figure 3.3 b) showed uniform grains. In Figure 3.3 c, which is the 1000 nm thick film, the grains were rougher and larger.
3.7.4 Comparison Between Designed and Actual Pattern Size

The characterization of pattern size after photolithography showed that in all 48 patterns, 43 of them were fabricated with aimed sizes (difference between designed and actual pattern size was <5%). The patterns showed most variations compared with design were lines in 500 nm and 1000 nm height. When feature height (film thickness) went from 500 nm to 1µm, the half height width of ridge decreased more than 5% on each pattern size on masks (see Table 3.2). This is due to the time length of Au etching increases with Au thickness in order to clean all Au away. Meanwhile, due to the isotropic etching, Au was etched horizontally as well and over etching is unavoidable.

Figure 3.3 AFM scan of films with 1000 nm scan scale. (a) Scan image of 100 nm thick film. (b) Scan image of 500 nm thick film. (c) Scan image of 1000 nm thick film.
Table 3.7. Comparison between designed and actual pattern size measured by Detak$^3$.

<table>
<thead>
<tr>
<th>film thickness (nm)</th>
<th>designed size (µm)</th>
<th>actual size gained from measurement</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>diameter of patterns (µm)</td>
<td>hole</td>
<td>dot</td>
<td>hexagon</td>
<td>ridge</td>
<td>groove</td>
</tr>
<tr>
<td>100</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.1</td>
<td>5.0</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.1</td>
<td>9.9</td>
<td>9.8</td>
<td>10.0</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>25.8</td>
<td>25.1</td>
<td>24.7</td>
<td>24.0</td>
<td>26.0</td>
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</tr>
<tr>
<td></td>
<td>50</td>
<td>50.0</td>
<td>50.3</td>
<td>50.1</td>
<td>49.8</td>
<td>50.2</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>5.0</td>
<td>5.1</td>
<td>5.1</td>
<td>4.9</td>
<td>4.9</td>
<td>5.1</td>
<td></td>
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<tr>
<td></td>
<td>10</td>
<td>10.0</td>
<td>9.8</td>
<td>10.0</td>
<td>9.8</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>25.1</td>
<td>24.9</td>
<td>25.0</td>
<td>23.9</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50.2</td>
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<td>50.1</td>
<td>42.8</td>
<td>47.2</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>5.0</td>
<td>5.0</td>
<td>4.8</td>
<td>5.0</td>
<td>4.5</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.7</td>
<td>10.0</td>
<td>9.9</td>
<td>9.1</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
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<td>24.1</td>
<td>25.0</td>
<td>20.2</td>
<td>29.8</td>
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<td>47.2</td>
<td>49.3</td>
<td>41.3</td>
<td>58.7</td>
<td></td>
</tr>
</tbody>
</table>
3.8 References


CHAPTER FOUR

CHARACTERIZATION OF MICE 7F2 OSTEOBLAST RESPONSE TO MICROPATTERNS

4.1 Introduction to Osteoblast Response to Micropatterns

4.1.1 Osteoblasts and Bone Formation

Determining what external stimuli influence differentiation, ordering and growth of cells has been a major focus of research groups since the early 1980s [1-8]. Osteoblasts are bone tissue forming cells that stem from osteoprogenitor cells, which are found inside of bone marrow [9, 10]. Early studies showed that osteoblast cells responded to the stiffness of substrate stiffness [11, 12], surface chemistry [13, 14] and topography [15-18]. They respond by changing cell proliferation rate, protein expression, and shape deformation. Most of the studies have focused on osteoblasts, because they are the essential cells for bone tissue or dental implants to reconstruct themselves. During new bone tissue formation, osteoblasts differentiate directly from preosteoblasts and express type I collagen. At the same time, osteoblast mineralized the primary bone tissue with hydroxyapatite.

7F2 osteoblast is a cloned cell line isolated from p53/- mouse bone marrow. They express alkaline phosphatase (ALP) and mineralized bone matrix. Many researchers studied mice 7F2 osteoblasts due to their short growth time [19] significant expression of ALP, COL I (type I procollagen), and mineralized matrix [20, 21].
4.1.2 Osteoblasts Respond to Micropatterns Substrate

Osteoblasts have been deposited onto substrate materials with a wide range of stiffness values. The most popular substrates are Ti and its alloys (the stiffness is approximate 3.5-3.9 GPa) [11, 12], hydroxyapatite (the stiffness is approximate 0.2-5 kPa) [22] and sol-gel coated glass (the stiffness is approximate 2-80 kPa) [23-25]. A number of studies focus on how osteoblasts respond to stiffness changes because they are sensitive to their extracellular mechanical environment. Previous studies have shown that it is possible to control osteoblast differentiation by changing substrate stiffness [26-28]. On compliant collagen scaffold (serves as a template for tissue formation), low cell density but more osteogenic phenotype were found when compared to stiffer scaffold (1.37-1.75 KPa). When plated on substrate with different surface chemistry, osteoblasts on PEGDMA (poly(ethylene glycol) dimethacrylate) modified 2HEMA (2-hydroxyethyl methacrylate) copolymers became more differentiated on the 2HEMA surface, which is less stiff. On the PEGDMA (poly(ethylene glycol) dimethacrylate) –DEGDMA (diethylene glycol dimethacrylate) substrate, cells tend to differentiate to phenotype on the stiffer surface.

A second aspect of substrate to which osteoblasts respond is the surface topography. The topography here means fabricated patterns. Normally, there are two kinds of patterns: isotropic and anisotropic. An isotropic pattern has identical properties in all directions, like dot. On the other hand, an anisotropic pattern is directionally dependent, like lines. When deposited onto isotropic patterns (holes,
wells etc.), cells don’t show preference on shape deformation, which means no elongation on a certain direction [29]. While on anisotropic patterns, osteoblasts deform along the long axis in most cases [30]. Depth is another factor that affects cell response. Studies show that when depth is smaller than 30 nm, cells cannot tell the difference [31]. When depth increases, the cell alignment shows up and the percentage of aligned cells increases as well. At the same time, width has been shown not to influence cells as much as depth.

Surface roughness is another factor should be taken into concern when talking about cell substrate integration. Roughness is different from micropatterns. Micropatterns are designed feature that made by a carefully controlled procedure. While for roughness, it is less controlled and only can be estimated from previous experience before substrate fabrication. In a study where there were no micropatterns, osteoblasts had decreased cell adhesion and increase proliferation on mirror polished Ti₆Al₄V when compared to no polished Ti₆Al₄V. The arithmetic mean roughness of polished Ti₆Al₄V was 160 nm [32].

There is no strict scientific definition of cell alignment currently. The widely acceptance standard is if long axis of cells stretched along infinite direction of anisotropic substrate features [33-35]. Cells contact and respond to their contacting environment by the proteins, mainly collagen fibers, in the extra cell membrane. The cell response is controlled by the signaling pathways, which are initiated by transmembrane proteins. When contacting the substrate, cells tend to contact where
the signaling pathways can be activated easily. On anisotropic substrate, like lines, cells deform by initiating Wnt signaling pathway (a network of proteins) along the edges of these features [34].

4.1.3 Objective of Osteoblasts Study

The objective of this study is to investigate how osteoblasts and dental pulp stem cells respond to micropatterns. Using similar patterns to previous researchers, we should see trends in cell density, spreading, etc. This study will use mice osteoblast cells and look of their response over five days on micropatterns. There are two reasons for the use of five days instead of three days, which is most commonly used in most of studies described in Chapter One. First, in order to ensure that there was enough time for cells to deform (e.g., align to lines) longer culture period was preferred. The second reason is that there are few studies that provide information of cells response to substrate beyond three days. Most of the studies show increasing in cell density in the first three days. While theoretically, there should be a time point when cells density decreases either due to cell apoptosis.

4.2 Experimental Methods

4.2.1 Substrate Fabrication and Preparation

There were two steps to prepare substrate. The first step was producing replicable and controlled engineered micropatterns. As outlined in Chapter Three, the technique used in this thesis to fabricate micropatterns was traditional photolithography. The second step was applying amine group as interlayer between
substrate and cells. Amine group serves here as substance that is necessary for cells culture in similar studies [36-39].

Before cell culture, substrates were rinsed with steps of acetone/IPA/deionized water/IPA/acetone/deionized water. After the substrates were dried on hot plate at 100°C for five minutes, the substrates cleaned in plasma cleaner (Harrick Plasma PDC-001) for ten minutes. This was followed with 30 minutes UV sterilization in biology safety cabinet (bio-hood) (Fisher Scientific Inc. Model 785). 5 ml 11-amino-1-undecanoth hydroxide amino group was pipetted onto wafer to enhance cell adhesion by pipette aid.

4.2.2 Experimental Timeline

Osteoblasts were plated on each pattern with a density of 5,000 cells/cm² by counting using a hemocytometer. They were given 30 minutes before adding medium. The cells were allowed one day to stabilize before changing the medium. The day when plating cells was considered Day 0.

There are overall three runs of experiments. The first run of experiments was a one day cell culture to determine how cells spread after seeded on patterns for one day. The second run of experiment was conducted over five days. This experiment was aimed to investigate how cells proliferation rate, process of alignment, and migration across five days culture. In the third run of experiment, three wafers with significant trend shown in the previous experiment were chosen to build a statistic model.

The experimental timeline and characterization techniques at each time point
were shown in Table 4.1.

Table 4.1 Experimental Timeline and Characterization

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Characterization Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Plating cells.</td>
</tr>
<tr>
<td>Day 1</td>
<td>Staining and imaging cells to determine density, alignment and migration.</td>
</tr>
<tr>
<td>Day 3</td>
<td>Staining and imaging cells to determine density and alignment.</td>
</tr>
<tr>
<td>Day 5</td>
<td>Staining and imaging cells to determine density, alignment and migration.</td>
</tr>
</tbody>
</table>

4.2.3 Cell Culture, Staining and Fixation

7F2 Osteoblast cells (ATCC, CRL-12557) from mice bone marrow were seeded to the patterned wafer with a density of 5,000 cells/cm². The plating density was determined by counting cells in hemocytometer, a cell counting chamber to determine cell density in suspension. The cells were cultured in medium consisting of 80% MEM alpha (dipeptide L-Alanyl-L-Glutamine), 5% Penicillin- Streptomycin, and 15% Fetal Bovine Serum (FBS). The cell culture was maintained over a period of 5 days in an incubator (37 °C, 5% CO₂), and medium was replaced every other day. Detailed culture protocol is described in Appendix D.

Using the same method as Spyrou’s study [40], the day cells were plated was designed as day 0. During culture, cells were live stained using Nile Blue (Alfa Aesar Co.) at Day 1, which is the 24 hrs after plating, Day 3 and Day 5. Nile Blue (Alfa Aesar Co.) with concentration of 3 ug/ml was added into PBS rinsed cells.
Right after staining, the cells were kept in an incubator (37 °C, 5% CO₂) for 30 minutes. During this time, the nucleus of cells interacted with Nile Blue and were stained blue. After that, the cells were removed from the incubator and monitored live stained osteoblast under phase contrast microscopy to determine cell density. The detailed technique to determine cell density will be addressed in the following section. After imaging, cells were rinsed with PBS twice to remove Nile Blue, and then pipetting media in petri dish and stored in incubator.

At Day 5, the cells were fixed using 4 % Paraformaldehyde (PFA). Before fixing, the PFA was warmed up using a water bath (Cole-Parmer) to 37°C. Pipetted media out of petri dish and add enough 4% PFA to cover cells. Stored cells in 4% PFA for exactly ten minutes, and then cells were rinsed in Phosphate-buffered Saline (PBS) twice for 15 minutes each.

4.2.4. Characterization of Cell Density

Cell density was measured by counting cells on optical images at Day 1, Day 3 and Day 5. To find cell density, cells were counted on each image and divided by image area (2.16 mm²). In order to track cell density and shape on different patterns, phase contrast microscopy (Olympus BX60) was applied to image cells after staining. In order to reach statistic confidence, six images of each pattern were taken covering approximately 80% of whole pattern area. Average cell density of all images was used to produce the mean and standard deviation.

During experiment, some wafers were damaged during usage. For those
patterns which had scratches, the area was recalculated. The first method used to calculate the area was dividing the scratched area with complex shape into triangle, rectangle and square. Measured these basic shapes by ImageJ and got the area. The second method was counting missing patterns, and timed the known area of each pattern. Table 4.2 is the Area of scratches on lines and holes with 100 nm height, 25μm width.

Table 4.2 Area of scratches on lines and holes with 100 nm height, 25μm width.

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Area of Scratches Calculated By Method 1</th>
<th>Area of Scratches Calculated By Method 2</th>
<th>Mean Area of Scratches</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line</td>
<td>0.569 mm²</td>
<td>0.575 mm²</td>
<td>0.572 mm²</td>
</tr>
<tr>
<td>Hole</td>
<td>0.627 mm²</td>
<td>0.622 mm²</td>
<td>0.625 mm²</td>
</tr>
</tbody>
</table>

4.2.5. Characterization of Cell Alignment

To study single cell alignment, images were taken by Differential Interference Contrast (DIC) microscopy (Olympus BX60) and analyzed by ImageJ. DIC microscopy is widely used to obtain enhanced contrast of cells [41]. Although there’s no established standard to determine cell alignment, researchers often use different ways to evaluate cell alignment [42-45]. The widely applied method is to measure ten degrees away from long axis of micropattern [46]. Assuming that the angle that cells randomly spread is 45 degree. If long axis of a cell is ten degree away from long axis of lines, in this study, the cell aligns with micropatterns. ImageJ was used to determine the angle. The steps are: drawing the angle between cell and line, and the “measure” function will calculate the angle. Figure 4.1 shows
how to measure angle by in ImageJ.

![Figure 4.1 Measurement of angle between cell and line by using ImageJ.](image)

4.2.6. Characterization of Cell Migration

In order to identify the initial area where cells spread, a one day experiment was conducted under the same condition (the same cell line, seeded density, media and protocol). Optical images of overall wafer (patterns as well as space between them) were taken by Nikon AZ100 microscopy. At Day 5, cells were fixed with 4% paraformaldehyde in PBS. Optical images of overall four patterns on the same wafer were taken in one picture by Nikon AZ100. In this way, both the patterns and the spaces between them could be recorded.

4.2.7. Statistic Analysis

Linear model analysis was applied to study if cell density related to pattern type, width, height, culture time and experiment times by using SAS program. The significance was judged by using $p=0.05$.

4.3 Results and Discussion

In this section, the results obtained from these experiments will be analyzed.
We will discuss the change of cell density, cell alignment, cell migration, and the statistics to determine if there is any trend on cell density depending on culture time, pattern size (width or height), and geometries will be addressed.

4.3.1 Cell Density

As there was a large cell density variation on Day 1 (refer to Figure 4.2), normalization based on cell density of Day 1 was applied here to assist future analysis. The normalization includes both mean value and standard deviation of cell density. The applied method was dividing mean value and standard deviation of cell density on Day 5 by mean value of that on Day 1.

Fig. 4.2 Significant variation of cell density on Day 1 of all patterns.

Cell density from Day 1 to Day 5 was also studied. The data was collected in Figure 4.3-4.5. It is very interesting that the trends including increasing, decreasing and no significant change of cell density were all observed. In Figure 4.3, cell density increased from Day 1 to Day 3. In Figure 4.4, cell density decreased over five days. Meanwhile, Figure 4.5 shows no general trend on cell density was observed. Each plot contains patterns with the same depth and same feature size.
(diameters of dots, holes and hexagons, width of lines). These are three plots containing all trends in cell density in 12 samples. Due to space limitation, plots of all wafers are collected in Appendix E.

The decreasing of the cell density over time was not expected as shown in Figure 4.4. As according to previous study, cell density should increase or no change, at least. From the pictures, there’s no debris of dead cells which means most of cells are still alive on Day 5. The decreasing density on Day 5 can be explained in cell migration and will be addressed later.

Fig. 4.3 Cell density on Day 1, 3 5 of 100 nm thick film with 5 µm patterns. (a) Non normalized data. (b) Normalized data. Cell Density increase from Day 1 to Day 3.

Fig. 4.4 Cell density on Day 1, 3 5 of 100 nm thick film with 50 µm patterns. (a) Non normalized data. (b) Normalized data. Cell density decreased over time.
4.3.2 Cell Alignment

Cell alignment was only observed on lines, and this was coordinate with other’s studies [30, 47, 48]. After Day 1, cell alignment with the long axis of all 1µm high lines was observed. At Day 3, cells aligned with all 1µm and 500 nm high lines besides the line with 25µm width and 500 nm height. At Day 5, lines with 5µm width and 100 nm height showed cell alignment as well. From aspect ratio aspect, which is the ratio of height and width, all lines with aspect ratio equal or bigger than 1:20 induced cell alignment in this study. Bigger aspect ratio means deeper and thinner lines, which provided a more significant contrast of morphology for cells.

Fig. 4.5 Cell density on Day 1, 3 5 of 500 nm thick film with 5 µm patterns. (a) Non normalized data. (b) Normalized data. No general trend was observed.

4.3.2 Cell Alignment

Cell alignment was only observed on lines, and this was coordinate with other’s studies [30, 47, 48]. After Day 1, cell alignment with the long axis of all 1µm high lines was observed. At Day 3, cells aligned with all 1µm and 500 nm high lines besides the line with 25µm width and 500 nm height. At Day 5, lines with 5µm width and 100 nm height showed cell alignment as well. From aspect ratio aspect, which is the ratio of height and width, all lines with aspect ratio equal or bigger than 1:20 induced cell alignment in this study. Bigger aspect ratio means deeper and thinner lines, which provided a more significant contrast of morphology for cells.
Figure 4.6 and 4.7 show that whether cell deformed along the long axis of the line or not. On the other three patterns, dots, holes, and hexagons, cells didn’t show significant deformation preference.

4.3.3 Comparison between Cell Density and Images Taken after Five Days Culture

As data indicated, there was a decrease of cell density on Day 5. As no dead
cell debris was found from optical image, there might be cell migration off pattern or the dead cells was washed away during medium change. In order to determine the reason, comparison between calculated cell density and optical image was collect in Table 4.3.

Table 4.3 Summary of calculated cell density and overall image of patterns.

<table>
<thead>
<tr>
<th>Thickness</th>
<th>Feature Size</th>
<th>Aspect Ratio</th>
<th>Migration Seen on Wafer</th>
<th>Cell Counts indicate death or migration</th>
<th>Alignment (A) or Spread out (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 nm</td>
<td>5 µm</td>
<td>1:50</td>
<td>Yes</td>
<td>No</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>10 µm</td>
<td>1:100</td>
<td>No</td>
<td>Yes</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>25 µm</td>
<td>1:250</td>
<td>No</td>
<td>No</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>50 µm</td>
<td>1:500</td>
<td>No</td>
<td>Yes</td>
<td>S</td>
</tr>
<tr>
<td>500 nm</td>
<td>5 µm</td>
<td>1:10</td>
<td>Yes</td>
<td>Yes</td>
<td>A</td>
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<td>10 µm</td>
<td>1:20</td>
<td>Yes</td>
<td>Yes</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>25 µm</td>
<td>1:50</td>
<td>No</td>
<td>Yes</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>50 µm</td>
<td>1:100</td>
<td>Yes</td>
<td>Yes</td>
<td>A</td>
</tr>
<tr>
<td>1000 nm</td>
<td>5 µm</td>
<td>1:5</td>
<td>No</td>
<td>Yes</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>10 µm</td>
<td>1:10</td>
<td>Yes</td>
<td>No</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>25 µm</td>
<td>1:25</td>
<td>Yes</td>
<td>Yes</td>
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</tr>
<tr>
<td></td>
<td>50 µm</td>
<td>1:50</td>
<td>No</td>
<td>Yes</td>
<td>A</td>
</tr>
</tbody>
</table>
From the table 4.4, there are four wafers (500 nm 5µm, 500 nm 10µm, 500 nm 50µm, and 1µm 25µm), whose cell density calculation and optical image both show cell migration or not. The density decrease on Day 5 can be explained by the cell migration seen on Day 5. Cell density was only calculated by the number of cells per unit area on patterns. Cells migrate to spaces between patterns decrease cell density. The wafer with 100 nm height and 25µm width patterns didn’t show migration both from optical image or cell calculation. The data wasn’t reliable as there are scratches on pattern during usage. The data of rest seven wafers show opposite results between cell count and optical image. There are several reasons for that. For the wafer with 100 nm height and 5µm width patterns, the data are not reliable as there was damages to wafers during usage. For wafers whose images didn’t show migration while cell count implied migration, the disagreement was because dead cells might have been washed away during media change.

After Day 1, cells didn’t migrate and stayed where they were seeded. This can be seen from figure 4.8 (a). After five days culture, sample shows mass migration of cells is represented as Figure 4.8 (b). Other samples didn’t show cell migration after five days culture, just like Figure 4.8 (c).
Fig. 4.8 Optical image of the patterns show the cells spread on Day 1 and Day 5. (a) Cells spread on patterns at Day 1 after seeded. No migration was observed. (b) Cells migrated on patterns after five days culture. (c) Cells didn’t migrate on patterns after five days culture.
Cell delamination from wafer was observed after three weeks since cells were fixed as seen in Figure 4.9. This was because wafer with fixed cells was preserved in PBS, which had a counter reaction on cell adhesion. Figure 4.9 a and b illustrate cell delamination on the same wafer. During culture, mass cells migration was observed. After three weeks, the same sample showed cell delamination.

4.3.4. Statistical Analysis

Statistical analysis was applied to evaluate how normalized cell density related to different pattern type, width, height, growth time and experiment receptions (first or second). A general linear model with significance of $p=0.05$ was used.

The results showed that on Day 3, cell density had no statistically significant relation with pattern type, width or height. But cell density increased with
experiment receptions: the cell density in second run of experiment was statistically higher than the first run. This may due to experiment error, location of wafer when cultured in incubator (the back of incubator is warmer than front), etc. More trials of experiments are needed to reach statistically confidence.
4.4 Reference


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CHAPTER FIVE

CHARACTERIZATION OF PORCINE DENTAL PULP STEM CELLS RESPONSE TO MICROPATTERENS

5.1 Introduction of Dental Pulp Stem Cells Interaction with Biomaterials

Exploring new methods in tissue engineering, especially aimed in teeth reconstruction, has drawn a lot of interests in recent years [1-3]. Traditional techniques to treat damaged teeth, no matter it is caused by periodontal disease or trauma, include repair or replacement with artificial materials [4-6]. It is well known that the concerns on artificial implantation are mainly risks of inflammation, implantation degradation, infection, etc. [7-9]. The teeth regeneration with self tissue is a prospective trend with the help of tissue engineering.

Dental pulp stem cells (DPSCs) are one type of mesenchymal stem cell (MSC), as they express the STRO-1, CD73, CD90, and CD105 [11, 12]. DPSCs are the first stem cells isolated from human dental pulp tissue [13]. They can be easily extracted from the third molar (“wisdom teeth”) of human. Studies have shown that both in vitro [14] and vivo [13], DPSCs have the potential to differentiation into dentin, which is the main structure in teeth [15]. Figure 5.1 shows schematic structure of human tooth.
Fig. 5.1 Structure of human tooth [10]. A: enamel, B: dentin, C: dental pulp, D: gum, E: periosteum, F: jaw.

composition [16-20]. There are several non-collagenous proteins (osteocalcin, osteonectin, alkaline phosphatase, bone sialoprotein, etc.) presenting both in bone and dentin tissue. In mineralization process of dentin and bone tissues, the same mechanism (matrix-mediated mechanisms), morphology, and composition mineralized matrix were found. Chapter Four mainly described how osteoblasts respond to micropatterns. The osteoblasts served in this thesis as control experiment.

The substrates used to plate DPSCs in *vitro* include collagen scaffold [21], hydrogel [22], and hydroxyapatite [23, 24]. So far, there are rare studies on how dental pulp stem cells behave on micropatterned Au film. Additionally, we produced hexagon, which is a unique pattern that hasn’t been studied. The purpose of producing hexagon is to mimic the shape of hydroxyapatite in dentin, the tissue where
DPSCs form new dentin-pulp tissue. At the same time, we will study how DPSCs respond to line, dot and holes in terms of proliferation rate, cell alignment and migration.

5.2 Experiment Design

5.2.1 Substrate Fabrication and Preparation

The substrates used in this study are the same as used in 7F2 osteoblasts. The substrates are micropatterned Au film with four kinds of geometries (lines, dots, holes and hexagons). The geometries were designed with the combination of three different heights (100 nm, 500 nm and 1000 nm) and four different widths (5 µm, 10 µm, 25 µm and 50 µm). The fabrication procedure was described in Chapter Three.

Before depositing DPSCs, the substrates were cleaned and sterilized using the same protocol described in Chapter Four. Briefly, the substrates were first cleaned by aceton/IPA/dionized water, and then dried on hot plate. Using UV light sterilize the substrates for 30 mins,

5.2.2 Porcine Dental Pulp Cell Isolation and Primary Culture

Porcine dental pulp stem cells (pDPSCs) were used in this study because the sources of human dental pulp stem cells are limited. The procedure to obtain pDPSCs includes extracting porcine molars and getting pulp tissue, breaking down pulp tissue and isolating pDPSCs.

The jaw which had porcine molars was from Snow Creek meat processing facility, Seneca, SC. The molars were extracted in the Godley-Snell Animal Research
Facility, Clemson University. The first step involved exposing the roots of the molars by cleaning away the gum and alveolar bone around the molars. The molars were extracted by dental forceps and elevator and put them in sterile Hank's Buffered Salt Solution (HBSS) with 2% antibiotic/antimycotic (anti/anti solution; penicillin/streptomycin/amphotericin B) directly. To minimize contamination of molars, rinse their outer surfaces with 70% ethanol and sterile HBSS with 1% anti/anti solution. As the ends of roots were broken during molar extraction, make sure they were not in contact with 70% ethanol and sterile HBSS with 1% anti/anti solution. Those solutions could decrease the activity of pulp tissue. The pulp chamber was broken by cracking the roots. Carefully pull out the pulp tissue from chamber and transfer it to a sterile petri dish. Rinse pulp tissue by sterile HBSS with 1% anti/anti solution three times. To isolate dental pulp cells, digest the pulp tissue with 3 mg/mL collagenase II for 1 hour at 37°C and the cell suspension was ready. Filter the cell suspensions by a 70-µm cell strainer and centrifuged at 1200 rpm for 10 minutes to get cell pellets. Resuspend the pellets and culture the cells in T-25 flasks with Dental Pulp Cells Growth Media (DPCGM). The DPCGM consists of 15% Fetal Bovine Serum (FBS), 1% 100 M L-ascorbic acid 2-phosphate, 1% 200 mM L-glutamine, 1% 100 U/mL penicillin and 100 g/mL streptomycin, and 82% MEM alpha (dipeptide L-Alanyl-L-Glutamine). The primary cells were labeled as Passage One.
5.2.3 Cell Culture, Staining and Fixation

Passage One pDPSCs were cultured approximately seven days to grow to Passage two and stored in liquid Nitrogen. Before depositing on to micropatterns, Passage Two pDPSCs were thawed and cultured for five days to become Passage Three.

To reach identical experiment condition, the protocols to seed, culture, stain, and fix cells were the same as osteoblast as described in Chapter Four, section 4.2.3.

5.2.4 Characterization of Cell Density, Alignment and Migration

The methods to analysis pDPSCs density, alignment and migration were the same as analyzing osteoblasts, and they were outlined in Chapter Four. Basically, the analysis of cell density, alignment and migration was based on optical images processing. Cell density was obtained by averaging density of each image captured by Olympus BX60. At the same time, images of cell alignment were captured as well and the angle between cells and pattern (lines) was processed by ImageJ. By comparing cell spreading on Day 1 and Day 5, the result of cell migration can be obtained.

The detailed procedure to get cell density, alignment and migration was described in Chapter Four, section 4.2.4 to 4.2.6.

5.3 Results and Discussion

In this section, the result obtained from experiment will be analyzed. In summation, this section will discuss if there was any change of cell density, cell
alignment determined by optical images.

5.3.1 Cell Density

Cell density from Day 1 to Day 3 was studied. Each plot contains patterns with the same depth and same feature size (diameters of dots, holes and hexagons, width of lines). Figure 5.2-5.10 are normalized cell density of pDPSCs.

Fig. 5.2 Normalized pDPSCs density of 100 nm thick film with 10 µm patterns. No general trend was observed.

Fig. 5.3 Normalized pDPSCs density of 100 nm thick film with 50 µm patterns. No general trend was observed.

Fig. 5.4 Normalized pDPSCs density of 500 nm thick film with 5 µm patterns. No general trend was observed.

Fig. 5.5 Normalized pDPSCs density of 500 nm thick film with 10 µm patterns. No general trend was observed.
In summation, cell density did not change on Day 3 as compared with Day 1.

Fig. 5.6 Normalized pDPSCs density of 500 nm thick film with 25 μm patterns. No general trend was observed.

Fig. 5.7 Normalized pDPSCs density of 500 nm thick film with 50 μm patterns. No general trend was observed.

Fig. 5.8 Normalized pDPSCs density of 1000 nm thick film with 5 μm patterns. Cell density decreased.

Fig. 5.9 Normalized pDPSCs density of 1000 nm thick film with 10 μm patterns. Cell density decreased.

Fig. 5.10 Normalized pDPSCs density of 1000 nm thick film with 50 μm patterns. Cell density decreased.

In summation, cell density did not change on Day 3 as compared with Day 1.
except on the 1000 nm thick film samples. The pDPSCs density was significantly lower than that of osteoblasts. This was not a surprise as pDPSCs are more sensitive to an environment, like media pH, growth temperature, etc. On the other hand, the freezing and thawing process didn’t decrease pDPSCs bioactivity. To identify if the freezing and thawing processes influenced cells proliferation, pDPSCs from the same cell source used in this study were also cultured in T-75 flask at the same time from Day 0 after freezing and thawing. It took six days for pDPSCs cultured in T-75 to grow into another generation, which is the same time as fresh cells to grow from Passage Two to Passage Three.

5.3.2 Cell Alignment and Migration

No obvious cell alignment or cell migration was observed. The reason is the cell density is too low to observe cell migration. More trials of the experiments are needed to increase cell density and to identify if the cells can migrate or not.
5.4 References


CHAPTER SIX

CONCLUSIONS AND FUTURE WORK

6.1 Au Film and Micropattens Fabrication

As the work described in Chapter Three, three thicknesses Au films and all forty-eight unique patterns were designed and fabricated. Characterizations of Au film by using AFM and profilometer showed surface roughness, residual stress, and grain size increased with film thickness. Results of micropattern characterizations revealed that the custom photolithography technique could successfully fabricate designed feature with aimed size and uniform topography. Further study on micropatterns topography led to the conclusion that micropatterns topography didn’t change after applying amino group.

6.2 Osteoblast

Chapter Four described how studies on osteoblasts were conducted and results obtained. Au micropatters showed no cytotoxicity to osteoblast as the cells were alive over five days culture. Image analysis showed that osteoblasts align along anisotropic pattern (lines) and migrate during experiment. SAS statistical analysis revealed that osteoblast cell density of the second reception was significantly higher than the first time.

6.3 Dental Pulp Stem Cells

Studies relating to dental pulp stem cells were contained in Chapter Five. Cell density of all 1000 nm height features decreased over 3 days culture while other
samples didn’t show overall trend. As the cell density was too low to analyze, no cell alignment and migration were found. But the living cells confirmed that Au micropatterns can be used as novel biomaterial as it was not toxic to cells.

6.4 Future Work

In the future, three aspects of work should be done. The first is to improve sample handling methods to protect micropatterns from scratches. For the study on osteoblast, more receptions of experiment are needed to reach statistical significant results on interactions between cell density and parameters (geometry, width, height, and culture time). For dental pulp stem cells study, the primary effort is to increase cell density. The potential methods include increasing seeded cell density from 5000 cell/cm$^2$ to 10,000 cell/cm$^2$, add growth factors in growth media, etc.
APPENDIX A

Photolithography Mask Design

The masks used to produce micropattens in photolithography were designed by the ICWIN program and ordered from the University of Minnesota. Four masks were designed with feature sizes of 5 µm, 10 µm, 25 µm and 50 µm.

ICWIN is a computer assisted graphic program invented by RSA Data Security Inc. All four patterns of the same size (5 µm, 10 µm, 25 µm and 50 µm) were designed in the same mask (see Figure A.1). All pattern design process was started by Bonnie Zimmerman.

After finishing all the designs, fill out masks order form and sent it to Nanofabrication Center of University of Minnesota. The masks were made of glass plate, which was coated with a thin layer of chromium film. The film was non-transparent and contains designed patterns. If the tolerance was about 10 µm, then printing features on transparent foil by ink-jet printer was also acceptable.
Figure A.1 Image of a mask. Image in the center is what the whole mask looks like. The four bright squares in the middle are the four patterns. The four images around the mask are magnified pictures of what each pattern looks like. In counterclockwise, the patterns from the upper left corner to the upper right corner are: holes, lines, dots and hexagons.
Magnetron Sputtering

Magnetron sputtering is a consequence procedure in which atoms and secondary electrons in target are ejected due to bump of energetic ions in plasma, and then target atoms condense on substrate. Meanwhile, secondary electrons play an important role to maintain plasma as they are confined in a dense plasma region near target and constantly create argon ions by bump [1-3].

The sputtering system used in this experiment is Kurt J. Lesker RF Sputtering Unit (Kurt J. Lesker Co.). It is composed of mechanical pump, cryo pump, Hi-vacuum pump, and chamber. Basically, there are two steps to conduct magnetron sputtering process: pumping down to desired pressure and sputtering. Desired pressure, normally high vacuum, is to ensure initiate argon plasma. Following are the specific steps to carry out this experiment.

Before system was turned on, make sure all valve, power supply, and gas were off. Check oil level, gas pressure, and cooling water system. The first step was set nitrogen and compressed air to less than 5 psi and 80 psi each. Power supply, vacuum gauge controller and mechanical pump were turned on after that. Cryo pump was purged with nitrogen and pump down to 100 mtorr, followed by leak check. Ensure all system run in good condition. Let cryo pump operate for a while till the temperature inside less than 15 K.

Wafers were cleaned with steps of acetone /IPA /dionzied water / IPA/ acetone/
deionized water. Dried wafers on 100 ºC hot plate for five minutes, and cleaned it in plasma cleaner for ten minutes.

Sample loading was the next step. Valve connecting chamber and mechanical pump should be turned off first. Vent chamber with nitrogen until atmosphere pressure, and then opened chamber. Loading wafers carefully and swiftly. Chamber was closed and valve was turned on again. Pump down to 50 mtorr and check leak.

Hi-vacuum valve and ion gauge were turned on after leak check. Plasma was started till pressure down to 1 *10^-6 torr. Adjust argon to 0.150 ppm, and set power supply to 260 volt. After plasma was ignited, target was cleaned by pre-sputtering with cover closed for 3 minutes. Next, cover was opened and stepper motor was set on. A thin layer of Ti was deposit first for 3 minutes with DC power supply to enhance combining force between Au film and wafers. After that, DC power supply was turned off and pressure inside chamber was increased to 1.5 *10^-6 torr. Au film was deposit by RF power supply.

After sputtered to desired time, ensure shutter, power supply, gas and Hi-vacuum pump closed. Vent chamber and unloaded samples. Shut down power supplies, cryo pump, cooling water and gas. Make sure all valves were closed and shut down all system.
References


APPENDIX C

Photolithography Process at Clemson University

Photolithography produces micropatterns by exposing substrate to ultraviolet light to remove certain part of substrate according to mask [1-3]. The procedure set up in this thesis includes wafer cleaning, photo resist coating, exposure and developing, Au etching and photo resist removing.

The photolithography procedure began with wafer cleaning. Wafers were coated with acetone by spinning for ten seconds, and then immediately putting wafers on hot plate at 100 °C for 5 minutes to evaporated acetone.

The next step was to coat wafers with photo resist by spinning. Mount wafer on vacuum spinner and sprayed photo resist (microposit s1813 photo resist, Shipley Company). Span wafer in 1000 rpm for 5 seconds and then 5000 rpm for 30 seconds. After spinning, wafers were transferred to 100 °C hot plate and soft baked for 20 minutes. Cooled wafers in air for another ten minutes on ware.

Before exposure, ultraviolet bulb in mask liner should be warmed up for 20 minutes. Exposed cooled wafers in ultraviolet light for 2.5 seconds. Rinsed exposed wafers in developer (microposit MF-312 developer Shipley Company) for 45 seconds to remove developed photo resist. Rinsed wafers three times in deionised water and dried them with Nitrogen gun. In order to ensure photo resist has been corrected exposed, optical microscope was used to monitor developed patterns. A good pattern should have clean edge, uniform design and mirror surface substrate.
After checking under microscope, hard baked wafers in 150 °C vacuum oven for 30 minutes.

After wafers were cooled in air for 10 minutes, Au film was etched in enchant (Au enchant type TFA, transene company, Inc.) for 20 seconds. Wafers were rinsed in deionized water for three times and dried in Nitrogen gun. Wafers were monitored with optical microscope again to protect them from being over etched.

The last step was removing photo resist. Rinsed the rest photo resist in photo resist striper (Positive Photoresist Stripper R-10) for 30 minutes at 50 °C in water bath. The rest photo resist striper was removed by rinsing wafers in running deionized water for five minutes, and dried wafers with Nitrogen gun.
Figure C.1. Photolithography process flow
References


APENDIX D

Cell Culture Protocol

Before cell culture, substrates were rinsed with ordered step of acetone, IPA, deionized water, IPA, acetone, and deionized water. After drying on hot plate at 100 °C, the substrates were cleaned in plasma cleaner for 10 minutes. 5 ml 11-amino-1-undecanoth hydroxide amine group was pipetted onto substrates to enhance cell adhesion. To remove extra amino group, the substrates was rinsed with ethonal and deionized (DI) water for 5 minutes each.

7F2 Osteoblast cells (ATCC, CRL-12557) from mice bone marrow were seeded onto micropatterns after detached from T-75 culture flask with 5mL 0.25% Trypsin and EDTA. After detached, cell suspension was centrifuged for 5 minutes at a speed of 1000 rpm. Break cell pellet with 1 ml culture media. In order to obtain cell density in suspension, hemocytometer was used to count cells. After counting, osteoblasts were seeded on to micropatterns with a density of 5000 cells/cm². Culture media was added onto micropatterns after cells were plated for 30 minutes. The culture media was consisted of 84% MEM alpha (dipeptide L-Alanyl-L-Glutamine), 1% pen/strep (Penicillin- Streptomycin), and 15% FBS (Fetal bovine serum). Cell culture was maintained over a period of 5 days in incubator (37 °C, 5% CO₂), and media was replaced every other day.
APPENDIX E

Osteoblasts Cell Density on Day 1, Day 3 and Day 5

Cell density from day 1 to day 5 was studied and compared between samples. Both non-normalized and normalized data were investigated. Figure 1 to figure 12 are data of all the 12 wafers.

Fig. E.1 Cell density on day 1, 3 5 of 100 nm thick film with 5 µm patterns. (a) Non normalized data. (b) Normalized data. Cell Density increase from day 1 to day 3.

Fig. E. 2 Cell density on day 1, 3 5 of 100 nm thick film with 10 µm patterns. (a) Non normalized data. (b) Normalized data. No general trend observed.
Fig. E.3 Cell density on day 1, 3, 5 of 100 nm thick film with 25 µm patterns. (a) Non normalized data. (b) Normalized data. No general trend observed.

Fig. E.4 Cell density on day 1, 3, 5 of 100 nm thick film with 50 µm patterns. (a) Non normalized data. (b) Normalized data. Cell density decreased over time.

Fig. E.5 Cell density on day 1, 3, 5 of 500 nm thick film with 5 µm patterns. (a) Non normalized data. (b) Normalized data. No general trend observed.
Fig. E.6 Cell density on day 1, 3, 5 of 500 nm thick film with 10 µm patterns. (a) Non normalized data. (b) Normalized data. Cell density decreased over time.

Fig. E.7 Cell density on day 1, 3, 5 of 500 nm thick film with 25 µm patterns. (a) Non normalized data. (b) Normalized data. Cell density decreased over time.

Fig. E.8 Cell density on day 1, 3, 5 of 500 nm thick film with 50 µm patterns. (a) Non normalized data. (b) Normalized data. No general trend observed.
Fig. E. 9 Cell density on day 1, 3, 5 of 1000 nm thick film with 5 µm patterns. (a) Non normalized data. (b) Normalized data. Cell density decreased over time.

Fig. E.10 Cell density on day 1, 3, 5 of 1000 nm thick film with 10 µm patterns. (a) Non normalized data. (b) Normalized data. No general trend observed.

Fig. E.11 Cell density on day 1, 3, 5 of 1000 nm thick film with 25 µm patterns. (a) Non normalized data. (b) Normalized data. Cell density decreased with time.
Fig. E.12 Cell density on day 1, 3, 5 of 1000 nm thick film with 50 µm patterns. (a) Non-normalized data. (b) Normalized data. No general trend observed.
APPENDIX F

Design of Statistical Analysis Program

SAS program was applied to analyze osteoblasts density in Chapter Four. General linear model was tested by using the proc glm function.

There were no interactions between cell density and pattern type, width and height. But the cell density is significantly higher in the second run of experiment.

Following is the SAS code:

```
DATA Density;
INPUT height $ geo $ density rep;
DATALINES;
100 Line 3.418 2
100 Line 0.424 1
100 Dot 2.101 2
100 Dot 0.426 1
100 Hole 7.436 2
100 Hole 0.292 1
100 Hexagon 4.109 2
100 Hexagon 0.872 1
```
PROC GLM;

Class height geo;

Model celldensity=height/geo;

Run;

Quit;