DEVELOPING SURFACE CHEMISTRY STRATEGIES TO STUDY CELL ADHESION, MIGRATION AND STEM CELL DIFFERENTIATION

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ABSTRACT

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Developing Surface Chemistry Strategies to Study Cell Adhesion, Migration and Stem Cell Differentiation

(Under the direction of Prof. Muhammad Yousaf)

The ability to precisely control the interactions between mammalian cells and materials at the molecular level is crucial to understanding the fundamental chemical nature of how the local environment influences cellular behavior, as well as for developing new biomaterials for a range of biotechnological and tissue engineering applications. In this work, we have developed a quantitative electroactive microarray strategy that can present a variety of ligands with precise control over ligand density on gold substrates to study cell adhesion and stem cell differentiation. We found that both the ligand composition and ligand density influence the rate of adipogenic differentiation from hMSCs. We also incorporated a simple microcontact printing technique to pattern cells on gold substrates to study how the cell population, surface adhesion area, and pattern geometry combine to influence stem cell differentiation. Furthermore, we transferred our sophisticated chemoselective immobilization strategy onto different materials including carbon nanotubes (CNTs), indium tin oxide (ITO), and gold nanorods. These tailored materials provide great platforms for studying the surface effects on cellular behaviors such as cell adhesion, migration and stem cell differentiation.
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# TABLE OF CONTENTS

List of Tables .................................................................................................................. viii
List of Figures .................................................................................................................. ix

## CHAPTER:

1. Introduction: Building Surface Systems to Study Cell Adhesion, Migration and Stem Cell Differentiation

   1.1 Cell Adhesion, Migration and Stem Cell Differentiation .................................. 1
       1.1.1 Cell Adhesion ......................................................................................... 1
       1.1.2 Cell Migration ....................................................................................... 3
       1.1.3 Stem Cell Differentiation ....................................................................... 3

   1.2 Building Surface System for Cell Studies ......................................................... 9
       1.2.1 Self-Assembled Monolayers (SAMs) ....................................................... 9
       1.2.2 SAMs of Alkanethiolates on Gold .............................................................. 10
       1.2.3 Design of the Surface System .................................................................. 13

   1.3 REFERENCES .................................................................................................. 14

2. Development of Surface Microarray Strategy to Study Stem Cell Differentiation

   2.1 Introduction ....................................................................................................... 19
   2.2 Results and Discussion ....................................................................................... 22
List of Tables

Table 3-1. List of oxyamine-tethered small molecules used to generate quantitative ligand density microarrays for studying hMSC differentiation .......................... 46

Table 4-1. Comparison of pattern geometry versus pattern area for the data shown in Figures 4-3 and 4-5 ............................................................................. 71
List of Figures

| Figure 1-1 | Scheme of stem cell differentiation and tissue development | 5 |
| Figure 1-2 | Scheme of self-assembled monolayer (SAM) of alkanethiolates on gold surface | 12 |
| Figure 2-1 | Schematic showing the fabrication of a self-assembled monolayer array (SAM-array) on a gold substrate | 21 |
| Figure 2-2 | Electrochemical reaction and characterization of SAM-arrays | 23 |
| Figure 2-3 | Applying SAM-arrays to hMSCs differentiation | 25 |
| Figure 2-4 | A three-dimensional plot comparing differentiation rate and SAM compositions to ligand density and differentiation time | 28 |
| Figure 3-1 | Strategy to transfer a range of mixed alkanethiol solutions (each solution may contain more than one alkanethiol) from a microplate to a bare gold substrate via microarray printing to generate a corresponding array of self-assembled monolayers | 38 |
| Figure 3-2 | Strategy to develop quantitative and electroactive ligand density spot arrays | 40 |
| Figure 3-3 | Strategy to generate quantitative, chemoselective, and electroactive ligand density spot microarrays | 43 |
| Figure 3-4 | Application of the ligand density microarray to study the rate of hMSCs differentiation | 45 |
| Figure 3-5 | Representative micrographs showing adipogenic differentiation of hMSCs | 47 |
| Figure 3-6 | A three-dimensional plot comparing differentiation rate versus ligand composition versus ligand density | 50 |
| Figure 3-7 | Representative gene expression comparison of adipogenic differentiation rate | 51 |
| Figure 4-1 | Applying µCP to study of hMSCs differentiation | 62 |
Micrographs of mesenchymal stem cells differentiated to adipocytes on circularly patterned SAMs with varying diameters (top row) and durations (left column).

(Top) Three-dimensional plot comparing differentiation rate versus time versus cell circular pattern size. From the data, there is no significant change in the differentiation rate by increasing the circular pattern size and therefore increasing the cell population. The data were normalized to 1.0 for fully differentiated cells after 10 days. (Bottom) Gel comparison example of the rate of adipogenic differentiation via gene expression for 220 μm circles. An adipose-specific gene, lipoprotein lipase (Lpl), and a control gene, β2-microglobulin (β2mg), were used to ensure equal loading of the DNA. hMSCs were cultured until confluent and then induced to adipose and monitored. Total RNA was extracted and analyzed by reverse transcription PCR. Lane 1, control cells; lanes 2-6, days of induced differentiation.

Representative micrographs of mesenchymal stem cells differentiated to adipocytes on varying geometrical patterns and durations.

Three-dimensional plot comparing differentiation rate versus time versus geometry.

Scheme of tailoring gold nanorods with an electroactive chemoselective immobilization strategy.

Electrochemical characterization on gold nanorods.

Scanning electron micrograph images of cells on gold nanorods.

Comparison of phase contrast images of mesenchymal stem cells on flat gold and nanorods surfaces.

Strategy to chemoselectively tailor single wall carbon nanotubes (CNT) by combining π-π stacking and electroactive immobilization.

Electrochemical characterization of ligand immobilization to the electroactive hydroquinone carbon nanotube (H2Q-CNT).

A microfluidic strategy to pattern hexadecanethiol (C10SH), CNTs and RGD-CNTs on gold surfaces for spatially controlled biospecific studies of cell adhesion and cell migration.

Scanning electron micrographs (SEM) of patterned CNTs and fibroblast cells.
Figure 6-5. Representative fluorescent micrographs of 3T3 Swiss Albino mouse fibroblasts adhered to $C_{16}$SH SAM, CNTs and RGD-CNTs…………… 106

Figure 6-6. Comparison of cell migration rate on the tailored surfaces……………… 108

Figure 7-1. Scheme of renewable tailored electroactive Indium Tin Oxide (ITO) surface……………………………………………………………………… 123

Figure 7-2. Electrochemical characterization and control of the interfacial immobilization and release of ligands on ITO surfaces by cyclic voltammetry (CV) ………………………………………………………………………… 125

Figure 7-3. Comparison of ITO and gold surfaces robustness to generate renewable and re-usable surfaces for multiple rounds of ligand immobilization and release…………………………………………………………………… 127

Figure 7-4. Comparison of fluorescent micrographs of live-cell GFP-actin transfected Rat 2 fibroblasts on ITO and gold surfaces presenting the biospecific RGD peptide……………………………………………………… 130
CHAPTER 1  
Introduction: Building Surface Systems to Study Cell Adhesion, Migration and Stem Cell Differentiation

The ultimate goal of this work is to explore and build ideal surface environments for supporting and controlling cell behaviors, including cell adhesion, cell migration, and stem cell differentiation. Since my research involves two significant aspects: building surface systems and studying cell behaviors via the manufactured surface systems, this chapter will be divided into two sections. In the first section, background information and the significance of studying cell behavior will be addressed. In the second section, background and the design of ideal surface systems to investigate cell behavior will be discussed.

1.1 Cell Adhesion, Migration, and Stem Cell Differentiation

1.1.1  Cell Adhesion

Most mammalian cells must adhere to an underlying matrix in order to carry out normal metabolism, proliferation and differentiation. The biological matrix that serves this role comprises a collection of insoluble proteins and glycoaminoglycans that are collectively referred to as the extracellular matrix (ECM).\(^1\) In addition to maintaining the organization and mechanical properties of tissue, the ECM directly interacts with cell surface receptors and regulates cell behaviors. These receptor-ligand interactions are critical to maintaining cellular function and enables cells to respond appropriately to their surrounding environment.
As the ECM provides the physical microenvironment in which cells live, the primary function of ECM is to mediate the adhesion of cells. If detached from the ECM, most cells initiate programmed apoptosis that results in their death. In addition, adhesion contributes to processes such as malignant transformation, inflammation, hemostasis, and immune recognition.

Cell adhesions is mediated by a number of transmembrane receptors, including the integrins, immunoglobulin supergene family, cadherins, selectins, CD44-related molecules, and transmembrane proteoglycans. However, the adhesion of most cells to ECM is mediated by integrins. All integrins discovered are heterodimeric receptors composed of at least one β and one α subunit. To date, there are 18 α subunits and 8 β subunits found in mammalian cells, and there are 24 heterodimeric proteins identified.

The adhesions include several subgroups such as focal adhesion (also termed focal contact), fibrillar adhesion, focal complex and podosome. The focal adhesion is considered to represent the general types of interaction between cytoskeletal proteins, integrins, and the substratum. Since focal adhesions can be easily visualized by light microscopy, it is also a convenient model for investigating integrin-cytoskeleton dynamics.

While most peptide sequences responsible for integrin-receptor recognition are still unknown, the short peptide sequence Arg-Gly-Asp-Ser (RGDS) was identified as a binding motif in several ECM components, including fibronectin, fibrinogen, vitronectin, laminin, and some collagens. It was later discovered that the minimum sequence necessary to promote cell adhesion was RGD, which can be recognized by almost half of the known integrin receptors.
1.1.2 Cell Migration

Cell migration contributes to many processes, including embryonic development, wound healing and the immune response. In general, cells undergo directional migration via a complex series of events. First, the cell surface receptors recognize and adhere to components of the ECM. Then, the cell must polarize and extend protrusions in the direction of migration according to external and internal signals. The protrusions are usually lamellipodia or filopodia, which are driven by actin polymerization and stabilized by adhering to ECM or adjacent cells via transmembrane receptors linked to the actin cytoskeleton. Finally, the cell can begin migration by forming extensions of the leading edge while releasing contacts in the rear of the cell body. There are many factors that influence this process, thereby creating an intricate network of interactions. As a consequence, the exact mechanism of directional cell migration is still an area of active study.

1.1.3 Stem Cell Differentiation

Stem Cells

Stem cells are unique cell populations that have two crucial characteristics: self-replication and differentiation. Stem cells play an essential role in the development and maturity of many organ systems including the central nervous, respiratory, cardiovascular, hematologic, immunologic, and endocrine systems. The ability to differentiate into specialized cell lineages makes stem cells a very important resource for many cell-based therapies. According to the potential of differentiation, stem cells can be divided into several types, including totipotent stem cells such as zygote (capable of forming the embryo and the trophoblast of the placenta), pluripotent stem cells such as embryonic stem cells (capable of
differentiating into almost all cells that arise from the three germ layers), multipotent stem cells and oligopotent stem cells (most tissue based stem cells, capable of producing a limited range of differentiated cell lineages appropriate to their location), and unipotent stem cells such as the epidermal stem cells and the spermatogonial cells of the testis (only able to generate one cell type).
Figure 1-1. Scheme of stem cell differentiation and tissue development.
As shown in Figure 1-1, all the lineages in body are derived from fertilized egg (zygote). After fertilization for approximately 3 days, the zygote will divide into multiple cells and form morula. After reaching the 16-cell stage, the morula will start differentiating into cells that will eventually become either the blastocyst's inner cell mass or outer trophoblasts. Fertilized eggs and morula are considered totipotent and capable of differentiating into all types of cells in the body. After cell division for around 5 days, the blastocyst is formed. Blastocyst contains pluripotent stem cells that can give rise to any fetal or adult cell type except a fetal or adult animal since they lack the potential to contribute to extraembryonic tissue, such as the placenta.

Between 12 and 14 days after fertilization, the embryo begins to form germ layers, the endoderm, ectoderm and mesoderm, which will eventually develop into the three major tissue types found in adults. The endoderm, the innermost layer, gives rise to the lining of the digestive and respiratory tracts, and the glands such as the pancreas and liver. The ectoderm, the outermost layer, will develop into the central nervous system, hair, fingernails and the epidermis of the skin. The mesoderm, the middle layer, is most diverse and it will eventually yield muscles, gonads, cartilage, and the circulatory system, to name a few.

In this work, studies are based on human mesenchymal stem cells (hMSCs) which are derived from mesoderm. This topic will be described in detail in the following section.

Due to their unique abilities, stem cells offer great potential in regenerative medicine for treating diseases such as diabetes, heart disease, and Parkinson's disease. However, there is still a lot of unclear information and much work to be accomplished to better our understanding on how to use these cells for cell-based therapies.
Differentiation, Dedifferentiation and Transdifferentiation

In classic embryology, differentiation is considered a unidirectional pathway. For example, when primitive endoderm becomes committed to forming liver, it is not able to dedifferentiate or transdifferentiate into another tissue type. Therefore, stem cell differentiation used to be considered irreversible. However, studies in recent years suggested that differentiation is not a one-direction pathway. Instead, differentiation, dedifferentiation and transdifferentiation can all occur under certain conditions.

Differentiation is the process by which a less specialized cell becomes a more specialized cell type. As introduced in the previous section, differentiation occurs numerous times from a single zygote to a complex system of tissues and cell types in order to develop into a multicellular organism. Dedifferentiation is a process often seen in more basal life forms, such as worms and amphibians, in which a partially or terminally differentiated cell reverts to an earlier developmental stage, usually as part of a regenerative process. Dedifferentiation is a process often seen in more basal life forms, such as worms and amphibians, in which a partially or terminally differentiated cell reverts to an earlier developmental stage, usually as part of a regenerative process. Transdifferentiation is the process that a committed cell type changes its differentiation pathway and develops into another differentiation lineage. For instance, phenotype switches between differentiated osteoblasts and adipocytes have been reported in previous studies, even at the single cell level. In this work, stem cell differentiation will be the focus of study.

While differentiating, stem cells receive signals from inside and outside cells that trigger each stem of the differentiation process. The internal signals are triggered when activating certain genes, which are interspersed across long strands of DNA and carry coded instructions for all cellular structures and functions. The external signals can be chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in their microenvironment. Therefore, it is very important to have a molecularly well-defined model...
surface system that can mimic the microenvironment and present specific biomolecules in order to study and control cell behaviors.

*Human Mesenchymal Stem Cells (hMSCs)*

The middle embryonic layer, mesoderm, gives rise to all of the body’s skeletal elements. The term, mesenchyme, is derived from the Greek meaning “middle” (meso) and refers to the ability of mesenchymatous cells to spread and migrate in early embryonic development between the ectoderm and endoderm layers. Human mesenchymal stem cells (hMSCs) are multipotent stem cells capable of differentiating into several lineages including bone, cartilage, fat, tendon, muscle, and marrow stroma. Although hMSCs are found in many regions of human body, they are most often and conveniently isolated from bone marrow, and can be induced to differentiate exclusively into the chondrocytic, osteocytic, or adipocytic lineages. While chondrocytic and osteocytic lineages are widely used as important resources for reconstruction and transplantation of cartilage and bone tissue, adipocytic lineage has also been suggested to be a potential source of adipose tissue for fat tissue engineering therapies, such as breast and facial reconstructions. From a health and social perspective, the failure to control the amount and function of adipose tissue results in obesity that may initiate or act synergistically with other factors to cause more serious health issues. Moreover, the adipogenic differentiation can be easily identified and observed due to the obvious increase of lipid vacuoles during differentiation process. Therefore, adipogenic differentiation was chosen as the first target of our study and it will be the focus of this work.
1.2 Building Surface System for Cell Studies

As introduced in a previous section, cells must adhere to the underlying ECM and receive complex molecular signals from the microenvironment in order to undergo fundamental biological processes. Therefore, understanding and exploring the interactions between cells and their surface microenvironment is very important and will impact a broad range of research communities including medicine and cell-based biotechnologies. In the past few years, the surface chemistry community has integrated many strategies to control the interface between cells and a supporting scaffold. In doing so, tailored substrates that aim to mimic the ECM and induce cellular behaviors have been generated.\textsuperscript{54,55}

Among many types of substrates, ranging from planar surfaces (glass or silicon slabs supporting thin films of metal, metal foils, single crystals) to highly curved nanostructures (colloids, nanocrystals, nanorods, nanotubes), self-assembled monolayers (SAMs) of alkanethiolates on gold remain the ideal model platform due to a number of factors.\textsuperscript{56-58}

1.2.1 Self-Assembled Monolayers (SAMs)

Although metals or metal oxides tend to adsorb adventitious organic materials in the ambient environment to lower the surface free energy,\textsuperscript{59} the adsorbed adventitious organic materials cannot form well defined monolayer and do not present specific chemical functionalities and physical properties.

Self-assembled monolayers (SAMs) are highly ordered (crystalline or semicrystalline) monomolecular films formed by the spontaneous adsorption and organization of surfactant molecules on a solid substrate.\textsuperscript{56-58} SAMs are the most elementary form of a nanometer-scale organic thin-film material, typically with a thickness of 1-3 nm. The molecule that forms
SAM has a chemical functionality and a specific head group that can bind onto metals, metal oxides or semiconductors. Depending on the molecular components of SAMs, the atomic composition and assembling structure of SAMs can be determined. This characteristic makes SAMs convenient, synthetically flexible, and simply controllable system with which to tailor the interfacial properties of metals, metal oxides, and semiconductors. There are a number of different monolayers including monolayers of fatty acids, organosulfur adsorbates on metal, alkyl monolayers on silicon, long-chain organic acids on metal oxides. However, the most widely studied SAMs to date are monolayers of alkanethiolates on gold.

### 1.2.2 SAMs of Alkanethiolates on Gold

SAMs of alkanethiolates on planar gold are usually considered as a molecularly well defined surface and ideal model platform as shown in Figure 1-2. The wide usage of this surface system is due to a number of advantages.56-62 (1) Due to the high affinity of gold for thiols, SAMs of alkanethiols can be easily formed on gold without undergoing any unusual reactions. Particularly, Long-chain alkanethiolates can efficiently form densely packed, well-ordered, and trans-extended monolayers on gold (111) surfaces. (2) Since alkanethiols can be easily synthesized to contain specific chemical functionality, various SAMs can be generated to tune the chemical properties of gold surface. By mixing different alkanethiols, mixed monolayer can be formed as well. (3) Gold is easy to obtain and gold coated-substrates can be easily fabricated by physical vapor deposition, sputtering, or electrodeposition. (4) Gold is reasonably inert. It does not oxidize at temperature under its melting point and does not react with most chemicals. (5) SAMs/gold can be easily patterned by a combination of lithographic tools and chemical etchants. (6) Thin films of gold are common substrates used for a number
of existing spectroscopies and analytical techniques, such as SPR, SEM, optical microscopy, cyclic voltammetry, etc. (7) Gold is biocompatible, which is the key feature to make it wildly used as a platform in cell biology studies. SAMs on gold are stable for weeks in media for cell culture.
Figure 1-2. Scheme of self-assembled monolayer (SAM) of alkanethiolates on gold surface.

\[
\text{RSH} + \text{Au}(0)_{\text{n}} \rightarrow \text{RS}^-\text{Au}(1)\text{Au}(0)_{\text{n-1}} + \frac{1}{2} \text{H}_2
\]
1.2.3 Surface System Design

As previously noted, alkanethiols are synthetically flexible. Theoretically, any functionalized alkanethiol can be introduced into the monolayer, thus tuning the SAM properties. By preparing two or more alkanethiols, mixed SAMs can be fabricated on gold surface. Since the maximum coverage of alkanethiolates on gold is usually considered constant (~4.5 × 10^{14} molecules/cm^2),\textsuperscript{56-58} the surface composition of SAMs can be precisely controlled by adjusting the mixing ratio of alkanethiols. This provides great potential for generating surfaces with controllable physical, chemical, and biological properties.

In order to characterize the surface composition of SAMs and obtain a predictable relationship between surface density and mixing ratio in solution, electrochemical characterization has been employed to the conductive gold substrate in this work. By introducing redox active alkanethiols to SAMs, we can use cyclic voltammetry (CV) to monitor and quantify the surface density of the redox active molecules and thus, analyze the surface composition of mixed SAMs. This technique will be described in detail in the following chapters.
1.3 REFERENCES


(33) Garry, D. J.; Olson, E. N. Cell 2006, 127, 1101-1104.


CHAPTER 2

Development of Surface Microarray Strategy to Study Stem Cell Differentiation

2.1 Introduction

Self-assembled monolayers (SAMs) of alkanethiolates on gold have been increasingly used as well-defined, model substrates for studying cell behaviors, such as adhesion, migration, proliferation, polarization and stem cell differentiation.\textsuperscript{1-10} Additionally, SAMs have served as a platform to probe and investigate cell signaling effects and signal transduction.\textsuperscript{11} Alkanethiols are synthetically flexible and can be modified with a variety of functional groups\textsuperscript{12} and can be specifically tailored to mimic the dynamic cell microenvironment. Although SAMs are important materials for cell biology studies, their use in tissue culturing applications have been limited due to the low throughput of standard SAM fabrication and difficulty of incorporating multiple ligands onto one substrate,\textsuperscript{13} with poor control of ligand density.

We aimed to develop a high-throughput methodology that allows for the presentation of a variety of tailored SAMs with controlled composition and spatial distribution. Using microarray technology, cell-surface interactions are able to be screened in parallel. To date, microarray technology has shown to be a powerful tool for conducting high-throughput analyses of gene expression, DNA sequence, proteomics, tissue engineering and drug discovery.\textsuperscript{14-18} In a previous study, Langer and coworkers developed a system in which
copolymer arrays on pretreated glass were generated to study cell-polymer interactions and determine the most suitable polymer scaffold for stem cell differentiation.\textsuperscript{19-21}

Herein, we have integrated microarray technology with SAMs on gold to develop a well-defined model substrate for high-throughput investigation of cell-surface interactions. This system is able to control the composition, ligand density, and spatial distribution of SAMs on the surface to probe the chemical effects on stem cell differentiation. With the ability to mimic the microenvironment of cells, this platform serves as a potential model system for surveying different cellular behavior for a range of ligands with precise control of ligand density.
Figure 2-1. Schematic showing the fabrication of a self-assembled monolayer array (SAM-array) on a gold substrate. (a) Generation of SAM-array. A Spotbot2 microarrayer was used to transfer-print alkanethiol solutions onto a bare gold substrate, allowing the alkanethiols to form an arrayed SAM. Followed by backfilling with a solution of tetra(ethylene glycol)-terminated alkanethiol (EG₄C₁₁SH), the remaining regions surrounding the SAMs-array can be made inert to non-specific protein adsorption and cell adhesion. (b) Cartoon showing four series of a SAM-array. By transfer-printing mixed solutions from a 384-well microplate of H₂QC₁₁SH and HOĆ₁₁SH, FcC₁₁SH and HOĆ₁₁SH, different electroactive SAM density compositions can be generated but with the same spot size (10%-100% H₂Q group and 10%-100% Fc group in the monolayer). Oxidizing SAMs containing hydroquinone (H₂Q) or ferrocene (Fc) converts the SAMs with the same density to the corresponding quinine (Q) or Ferrocenium (Fc⁺).
2.2 Results and Discussion

To prepare a SAM-array, mixtures of various alkanethiolates contained in each well of a 384-well microplate were transferred onto a bare gold substrate at programmed positions by a spotbot2 microarrayer (Figure 2-1a). Upon spotting, the mixed alkanethiols efficiently adsorb and self-assemble onto the gold surface, similar to the SAM formation process for micro-contact printing. After rapid evaporation of the solvent, the substrate is thoroughly washed with ethanol and backfilled with tetra(ethylene glycol)-terminated alkanethiol (EG₄C₁₁SH, 1mM, 12 h). The ethylene glycol group is widely used to resist non-specific adhesion of protein and cells to SAM surfaces. Human mesenchymal stem cells (hMSCs) were then seeded onto the entire substrate, adhering only to the patterned regions with adhesive characteristics. Therefore, this SAMs-array substrate is capable of evaluating various complex surface chemistry effects on cellular behavior, such as cell adhesion, cell polarization and stem cell differentiation.

Since the gold is conductive, redox active molecules, 2-(11-mercaptopoundecyl) hydroquinone (H₂QC₁₁SH) and 11-ferrocenylundecanethiol (FcC₁₁SH) were synthesized and used in this study to control the surface composition and measure the precise density of the SAM-array. Mixing H₂QC₁₁SH or FcC₁₁SH with 11-mercapto-1-undecanol (HOC₁₁SH) in different ratios (consistent total concentration of 1mM) in each well of a 384-well microplate creates two series of mixed-alkanethiol solutions.
Figure 2-2. Electrochemical reaction and characterization of SAM-arrays. (a) The SAMs presenting H₂Q or Fc are redox active and can allow reversible oxidation and reduction. (b) Cyclic voltammetry (CV) can be used to analyze the SAM-arrays generated by this microarray strategy. By integrating the area underneath each peak in the CV for each SAM-array, the surface density of the electroactive moiety can be determined. A plot of the integrated peaks was used to determine the relationship between the solution composition of H₂Q ($\chi_{\text{solution}}$) and the surface composition of H₂Q ($\chi_{\text{surface}}$). The linear correlation indicated that the solution concentration of H₂Q matches the surface density of H₂Q and is also the case for Fc SAM arrays.
Upon transferring these solutions to the bare gold substrate a mixed alkanethiolate spot SAM is formed. This method can be used to prepare SAM-arrays with different densities but the same spot size on the gold surface, as shown in Figure 2-1b. Because the H$_2$Q and Fc are electroactive and can be characterized by cyclic voltammetry (CV), the amount of electroactive group presented in the SAM-array can be quantitatively determined (Figure 2-2). Upon oxidation, SAMs displaying hydroquinone (H$_2$Q) or ferrocene (Fc) can be converted to SAMs presenting quinone (Q) or ferrocenium (Fc$^+$), respectively.

As shown in Figure 2-2a, SAMs presenting H$_2$Q or Fc can undergo a reversible oxidation and reduction process. By tracing and analyzing the signal from CV, the surface density of the 40 redox active molecules (Γ$_{\text{H}_2\text{Q}}$ and Γ$_{\text{Fc}}$) can be precisely quantified according to the equation $Q = nF\Gamma$. We first compared the analyzed surface composition with the solution composition and plotting $\chi_{\text{surface}}$ versus $\chi_{\text{solution}}$ as shown in Figure 2-2b, where $\chi_{\text{surface}}$ represents the ratio of the redox active molecule on the mixed SAM-array, and $\chi_{\text{solution}}$ represents the ratio of redox active molecule in the mixed alkanethiol solutions. A linear correlation was then found, indicating that the H$_2$Q or Fc density within the spots of the SAM-array matched the H$_2$Q or Fc concentration in the mixed alkanethiols solutions in the wells of the 384-well microplate. This discovery concludes that the surface composition of the SAM-array can be controlled by the composition of the mixed alkanethiol solutions. This gives rise to the opportunity to introduce a variety of molecules and generate a library of SAM-arrays for future high-throughput, parallel studies of cell-surface interactions on one substrate.
Figure 2-3. Applying SAM-arrays to hMSCs differentiation. After fabrication of a substrate composing of a SAM-array, hMSCs are cultured and adhere only to the micro-patterned regions. Simultaneous induction to adipogenic differentiation then follows. Patterned cells are fixed after different time periods and stained by Oil Red O and Harris Hematoxylin (lipid vacuoles and nucleus, respectively). Micrographs displaying hMSCs on a SAM-array before and after differentiation are shown. The scale bar represents 100 μm.
The SAM-array system was used to study surface chemistry effects on hMSC differentiation as a representative application. HMSCs are multipotent stem cells with the capability of differentiating into several lineages including adipocytes, osteocytes and chondrocytes. As a result, increasing studies of hMSCs differentiation have been launched in recent years due to the wide-range of applications of hMSCs in transplantation, repair of bone, cartilage and adipose tissue. Although it’s known that the surface environment plays a crucial role in stem cell differentiation, mimicking the surface environment and developing model substrates for studies of cell-surface interactions are challenging and time-consuming. Therefore, we designed this well-defined, model system of SAM-arrays to perform high-throughput screening of cell behavior in various surface microenvironments simultaneously (Figure 2-3).

After the SAM-array is fabricated, hMSCs are seeded to the entire substrate, resulting in confined cell adhesion to the patterned regions. The surrounding regions are composed of the inert, ethylene glycol terminated alkanethiols, which resist nonspecific cell attachment. All cell patterns, with varying underlying surface chemistries, were maintained and studied at the same physiological conditions. In this work, the differentiation of the hMSCs to an adipogenic lineage was investigated. A standard Oil Red O staining procedure was used to analyze the differentiation process. As shown in Figure 2-3, differentiated adipocyte cells contain characteristic lipid vacuoles which are stained red. Control hMSCs patterned show no red vacuoles with the same staining procedure, while differentiated cell patterns show red lipid vacuoles indicating the generation of adipocyte cells.

Based on our observation, hMSCs can differentiate on most adhesive SAMs, but the rate of differentiation varies. After 1 day, no differentiation was observed on any of the SAM
array surfaces. After 10 days, almost all cells were differentiated on all tested surfaces. After 5 days, many variations of the extent of differentiation were observed on the different surface chemistries. To determine any subtle differences of stem cell differentiation, we developed a quantification program to measure the amount of red lipid vacuoles to analyze the amount of differentiation. The relative differentiation rate versus time and surface composition are plotted in Figure 2-4. The data shows a clear dependence of differentiation rate on the underlying SAM density composition. For example, after 5 days at 20% ligand density the ferrocenium (Fc⁺) spot has many more differentiated cells than the ferrocene (Fc), hydroquinone (HQ) or quinone (Q) spots. However after 8 days at 20% ligand density the cells on the ferrocene (Fc) spots increase their rate of differentiation significantly while the hydroquinone (HQ) and quinone (Q) adhered cells do not.
Figure 2-4. A three-dimensional plot comparing differentiation rate and SAM compositions to ligand density and differentiation time. The data was normalized to 1.0 for cells differentiated after 10 days.
2.3 Conclusion

We report a novel SAM-array methodology that can generate a variety of functionalized alkanethiolas onto a gold substrate for chemical and biological studies. By testing two electroactive molecules as representative surface molecules, we found that complex SAM-array compositions can be achieved by transfer-printing via microarray technology. The CV characterization shows control over the ligand density on the surface and provides an opportunity for future studies probing ligand density effects on cell behavior. Using this microarray platform, parallel studies of multiplex cell-surface interactions can be accomplished at molecular level. By applying our system to adipogenic differentiation of hMSCs, we showed that stem cell differentiation can be influenced and potentially controlled on a non-natural surface environment for future tissue engineering applications. The electroactive quinone (Q) molecule has been shown to react efficiently with oxyamine tethered ligands to generate a stable oxime linkage. By combining the SAM array strategy with a ligand immobilization strategy to the quinone molecules a ligand density SAM microarray can be generated with a range of oxyamine tethered ligands. Future studies will focus on creating a library of ligands for ligand density SAM-arrays to study adipogenic differentiation and other cell behavior phenomena such as adhesion and migration.

2.4 Materials and Methods

Preparation of Monolayers

All gold substrates were prepared by electron-beam deposition of titanium (3 nm) and then gold (12 nm) on glass cover slips (7.5 cm × 2.5 cm). All gold coated glass substrates were cut into 1 cm² pieces and washed with absolute ethanol. The substrates were immersed
in an ethanolic solution containing the alkanethiols (1 mM) for 12 hours, and then cleaned
with ethanol prior to each experiment.

Electrochemical Measurements

All electrochemical experiments were performed using a BAS 100B/W
Electrochemical Analyzer (Bioanalytical Systems, Inc., West Lafayette, IN).
Electrochemistry on SAMs was performed in 1 M HClO₄, using a platinum wire as the
counter electrode, Ag/AgCl as reference, and the gold SAM substrate as the working
electrode. All cyclic voltammograms were recorded at a scan rate of 50 mV/s.

Cell Culture

The 3T3-Swiss albino cells (Tissue Culture Facility, UNC at Chapel Hill) were cultured
in Dulbecco’s Modified Eagle’s Medium (Sigma) supplemented with 10 % bovine calf serum
(Hyclone) and 1 % penicillin/streptomycin (100 units of penicillin/100 µg of streptomycin
per mL, Gibco) at 37 °C and 5 % CO₂. To detach cells from the culture flask, cells were
rinsed with phosphate-buffered saline twice (PBS, sigma) and 0.05 % trypsin /0.53 mM
EDTA (Gibco) was added. After incubating for 5 minutes, cells were resuspended in serum-
free medium and centrifuged at 1000 rpm for 5 minutes to remove trypsin. Cells were
resuspended in serum-free medium and added onto the substrates for the experiments.
2.5 REFERENCES


3.1 Introduction

Stem cells possess the ability to self-replicate to give rise to identical daughter cells and they can also undergo a complex differentiation process to generate new cell lineages.\textsuperscript{1-3} While stem cells hold much promise as an unlimited source of cells for transplantation therapies, and for treating numerous cancers and diseases, the precise control of the differentiation process is challenging and little is known about the complex interplay of the multitude of crucial factors ranging from signaling molecules to the cell microenvironment that influences stem cell differentiation.\textsuperscript{4} For example, stem cells use cell surface receptors to receive important signals from the extracellular environment in order to initiate differentiation.\textsuperscript{5-8} A major issue in using stem cells as therapies is the ability to control their interactions with man made materials. Modulating their growth and differentiation behavior on or within these materials, which are used as scaffolds for implant devices and as delivery vectors, will be important for a range of biotechnologies and therapeutics.\textsuperscript{9-15}

While the chemical nature of the interaction between the cell surface receptors and the extracellular environment is complex and unclear, it is highly possible that the surface conditions and properties of the material to which the stem cells adhere would have an influence on the stem cell differentiation process. Therefore, investigation of the material-
stem cell interaction and how this association can be manipulated is essential to discover new features of stem cell differentiation that can ultimately be utilized to build therapeutic devices or delivery systems. The ability to simultaneously survey different surface conditions in a high-throughput way to assess the factors that influence stem cell differentiation would facilitate our understanding of the material-stem cell interaction and potentially generate new biomaterials. In a landmark study, Langer and co-workers showed that the modulation of stem cell differentiation could be accomplished by modifying the macroscopic properties of the supporting materials. In their study, they used microarray technology to determine the most suitable polymer blends that cause stem cell differentiation for tissue engineering applications.16-20

We believe, to elucidate and further investigate the chemical nature of the surface effects on stem cell differentiation, a general high-throughput, multiplex, and quantitative model surface system that meets the following criteria is required. (1) The surface composition can be defined at the molecular level, thus analytical techniques can be used to tune and characterize the surface properties. (2) A general surface immobilization strategy to install a library of ligands/molecules in arrays where the amount and therefore surface density is measurable and controllable. (3) The surface must be biocompatible and inert to nonspecific protein and cell adhesion. This allows for the correct interpretation of ligand-receptor mediated interactions, that is, biospecific associations where the only interaction between cell and material is a receptor-ligand mediated interaction (no nonspecific surface interactions).

To achieve a molecular level investigation of surface properties that might influence stem cell differentiation, we have developed a multiplex and quantitative microarray strategy.
to study a range of surface effects on stem cell differentiation. This novel surface strategy is compatible with a powerful synthetic immobilization technique, which results in the capability of immobilizing a variety of molecules onto the surface quantitatively to create various surface properties in a high-throughput microarray format. The surface is also conductive and therefore electrochemistry can be performed on the substrate to precisely characterize the ligand density presented on the surface. This provides an opportunity for quantitatively determining the interplay of several surface properties such as surface roughness, hydrophobicity, chemical functionality, and specific ligand-receptor interactions as potential factors in stimulating stem cell differentiation. Based on this model substrate, the role of specific surface properties on stem cell differentiation can be studied at the molecular level.

Our surface chemistry methodology is based on self-assembled monolayers (SAMs) of alkanethiolates on gold. SAMs of alkanethiolates on gold are molecularly well-defined and synthetically flexible and therefore can be modified with a variety of functional groups, which determine the characteristics of the substrate. Importantly, SAMs are compatible with tissue culture conditions and optical and fluorescence microscopy. Furthermore, the conductive feature of gold substrates enables the use of several analytical surface spectroscopy techniques including mass spectrometry, SPR (surface plasmon resonance), XPS (X-ray photoelectron spectroscopy), STM (scanning tunneling microscopy), and cyclic voltammetry (CV) to characterize interfacial associations.

Microarray technology has revolutionized basic science and biotechnology, is recognized as a very powerful high-throughput experimental tool for screening a variety of conditions, and has been applied to material science, tissue engineering, proteomics, and drug
discovery.\textsuperscript{25} By using standard microarray technology many microliter-scale and even nanoliter-scale chemical reactions can be performed rapidly with spatial control on a single substrate. For cell-based assays, each of the spotted regions can be used for an independent analysis of a certain material-cell interaction. To create a variety of surface chemistries on one substrate and to precisely quantify the amount of ligands on each spot and then determine their influence on the rate of stem cell differentiation, we developed a multiplex analytical biotechnology that combines electroactive SAMs and microarray technology.

### 3.2 Results and Discussion

Using a spotbot2 microarrayer, we printed mixed alkanethiol solutions in various ratios directly onto a bare gold substrate. By using a 384-well microplate with different ratios of mixed alkanethiolates in each well, a range of surface chemistries can be installed onto one gold surface (Figure 3-1). Upon spotting, the alkanethiols immediately adsorb to gold and efficiently selfassemble on the surface. Since small volumes are printed (less than 1 \(\mu\)L), the solvent (ethanol) rapidly evaporates resulting in further concentrating the alkanethiol solutions. The substrate is then washed thoroughly with ethanol and backfilled by immersion into an ethanolic solution of tetra(ethylene glycol) terminated alkanethiol (EG\textsubscript{4}C\textsubscript{11}SH) for 12 h. The ethylene glycol group is known to resist nonspecific protein adsorption and cell attachment.\textsuperscript{26,27} Upon cell seeding, the cells only attach to the spotted regions if the underlying surface chemistry supports adhesion. In this way, the ability to test many different combinations of surface chemistries on cellular behaviors such as cell attachment and differentiation can be performed.
Figure 3-1. Strategy to transfer a range of mixed alkanethiol solutions (each solution may contain more than one alkanethiol) from a microplate to a bare gold substrate via microarray printing to generate a corresponding array of self-assembled monolayers. (A) A 384-well-microplate contains a number of different ratios of mixed alkanethiols. (B) The pins of a spotbot microarrayer will pick-up a specific solution from the microplate and transfer print the solution onto the bare gold substrate at programmed positions, allowing the alkanethiols to form ordered SAMs. Each color represents a unique ratio or combination of SAM composition. (C) A solution of tetra(ethylene glycol) terminated alkanethiol (EG₄C₁₁SH) was then used to backfill the remaining regions to render the surface inert to nonspecific protein adsorption and cell attachment. Based on this strategy, the gold substrate is composed of regions that present varying surface chemistries to study cell–material interactions (arrayed regions) and regions that do not allow cell adhesion.
To precisely control and characterize the surface composition, we printed, via a microarrayer, different ratios of mixed alkanethiol solutions containing 2-(11-mercaptoundecyl) hydroquinone (H$_2$Q) and 11-mercapto-1-undecanol (HOC$_{11}$SH) onto bare gold surfaces. Because the H$_2$Q molecule is electroactive, the absolute surface density of the H$_2$Q within the SAM spots can be quantitatively determined by using cyclic voltammetry (CV) and compared directly to the concentration of H$_2$Q solution transferred from the original microwell.$^{28}$ Mixing H$_2$Q with 11-mercapto-1-undecanol (HOC$_{11}$SH) in different ratios creates a series of mixed alkanethiol concentrations that can be transferred onto the gold substrate to generate SAMs of the same spot size but with different amounts of H$_2$Q on the surface (Figure 3-2).
Figure 3-2. Strategy to develop quantitative and electroactive ligand density spot arrays. (A) Examples of SAM spot arrays of mixed H$_2$QC$_{11}$SH and HOC$_{11}$SH. By printing mixed ratios of solutions of H$_2$QC$_{11}$SH and HOC$_{11}$SH, different electroactive SAM compositions can be generated (examples show 10%, 40%, and 100% of H$_2$Q) and characterized. (B) The H$_2$Q group is redox active and can be reversibly oxidized and reduced to the quinone (Q) form. (C) The density of the H$_2$Q on the surface in each array can be determined by integrating the area underneath each peak in the cyclic voltammogram for each H$_2$Q density array generated. (D) A plot of the integrated peaks for each H$_2$Q surface density was used to determine the
relationship between the solution composition of H$_2$Q ($\chi$H$_2$Q solution) and the surface density of H$_2$Q ($\chi$H$_2$Q surface). The straight line shows the solution concentration of H$_2$Q matches the surface density of H$_2$Q with an r value of 0.96. (E) Strategy showing the transfer of mixed solution of H$_2$Q- and −OH-terminated alkanethiols to form the corresponding H$_2$Q- and −OH-terminated SAMs.

We used electrochemistry to quantitatively correlate the relationship between the concentration of H$_2$Q in the mixed alkanethiol solutions from the 384-well microplate with the surface density of H$_2$Q generated on gold via microarray transfer printing. SAMs presenting hydroquinone groups can undergo a reversible 2 electron, 2 proton, oxidation and reduction process in aqueous conditions, as shown in the cyclic voltammogram (CV) in Figure 3-2c.$^{29,30}$ The surface density of the redox active H$_2$Q molecule ($\Gamma_{H2O}$) can be precisely determined by integration of the CV peaks to determine the total charge Q, and using the simple equation $Q = nFA\Gamma$ (where $Q =$ total charge, $n =$ number of electrons, $F =$ Faraday constant (96500 C), $A =$ surface reaction area [(spot size) $\times$ (number of spots)] and $\Gamma =$ H$_2$Q density on surface (molecules/µm$^2$)).$^{31}$ The surface density of H$_2$Q can then be directly correlated with the spotting solution concentration of H$_2$Q by plotting $\chi_{H2O}$ (surface) versus $\chi_{H2O}$ (solution) (Figure 3-2D ($\chi_{H2O}$ surface represents the ratio of the redox active H$_2$Q molecule on the mixed SAMs surface, and $\chi_{H2O}$ solution represents the ratio of H$_2$Q in the microwell solution. The slope is linear, indicating that the H$_2$Q density within the spots on the SAM surface is in accord with the spotting solution H$_2$Q concentration. This unique feature allows for the transferring of a mixed alkanethiol solution from a microplate (via a microarrayer) to generate a mixed SAM surface spot that can be characterized (via electrochemistry) precisely because the H$_2$Q is electroactive (Figure 3-2E).$^{32}$ To prepare surface microarrays presenting a range of ligands with different densities for stem cell
differentiation studies, we used the electroactive H\textsubscript{2}Q molecule not only as a quantitative read-out of surface density but also as a quantitative chemoselective immobilization strategy (Figure 3-3). We have shown previously that the H\textsubscript{2}Q group can be oxidized to the quinone (Q) group, which can selectively react in high yield with a number of functional groups (cyclopentadiene, hydrazide, and hydroxylamine).\textsuperscript{28,33,34} This important feature permits chemoselective immobilization of a library of molecules with differing functional groups and provides a powerful method for tailoring surfaces for a range of applications. In this study, we incorporated an immobilization strategy based on the reaction between Q and oxyamine-tethered ligands (R-ONH\textsubscript{2}) to form an interfacial oxime conjugate. This reaction is rapid and stable under physiological conditions.\textsuperscript{35-43} Introducing the oxyamine (-ONH\textsubscript{2}) group into a range of molecules is straightforward and allows for the generation of libraries of compounds that can be precisely arrayed on these electroactive surfaces.\textsuperscript{31,36} A unique feature of this system is that the oxime conjugate is also redox active with diagnostic peaks in the cyclic voltammogram allowing for a sensitive probe to monitor the extent of the interfacial reaction in situ and as a quantitative determination of amount of immobilized ligand to the surface. By integrating the oxime peaks and comparing it with the original H2Q peaks, the extent of the immobilization can be quantitatively monitored and the surface density of the immobilized ligand can be precisely controlled.\textsuperscript{31} This quantitative electroactive immobilization strategy provides a general strategy to immobilize a range of ligands with precise control of density of each ligand to study a range of cell behaviors.\textsuperscript{36}
Figure 3-3. Strategy to generate quantitative, chemoselective, and electroactive ligand density spot microarrays. (A) The H$_2$Q group can be oxidized to the quinone group which can chemoselectively react with oxyamine tethered ligands (RONH$_2$, where R can be any ligand, small molecule or biomolecule) to generate an interfacial oxime conjugate. The oxime is also redox active but with a distinct cyclic voltammogram that allows for the precise monitoring and quantification of ligand immobilization. (B) Cyclic voltammograms showing the diagnostic peaks that characterize the hydroquinone to quinone redox couple and the oxime product. By integrating the peak area of the oxime conjugate from the cyclic voltammogram the yield of interfacial reaction and therefore surface density of ligand can be determined.
To study the role of ligand density on stem cell differentiation, we synthesized a small library of oxyamine tethered ligands to generate an electroactive microarray presenting a range of molecules with different surface densities (Table 3-1). We first created a substrate presenting various H$_2$Q densities in many spots (Figure 3-4). We then oxidized the surface to generate the Q and then arrayed different oxyamine tethered ligands (R-ONH$_2$) to each spot. Cyclic voltammetry was used to show the quinones reacted completely to provide the corresponding oxime, indicating the ligands are immobilized at the same density as the original hydroquinone (For example, a 10% H$_2$Q spot density is oxidized to generate the Q at 10%; when an oxyamine tethered ligand (R-ONH$_2$) is reacted to completion as indicated by the shift in the cyclic voltammetry signal to the oxime product, 10% of the ligand is now presented on the spot). Stem cells were then seeded onto the ligand density microarray substrate and only adhered to the spot regions that supported adhesion. Once the cell array is formed, induction medium was added to the entire array to induce stem cell differentiation. Therefore, the rate of stem cell differentiation can be monitored over time as a function of the underlying ligand composition and ligand density (Figure 3-5).
Figure 3-4. Application of the ligand density microarray to study the rate of hMSCs differentiation. (A) Schematic describing the generation of stem cell arrays to study differentiation as a function of ligand composition and ligand density. (b) A 4× micrograph of hMSCs patterned on a ligand density array. To determine differentiation the cells are stained with Oil Red O, which selectively targets lipid vacuoles to indicate adipocyte cells and Harris Hematoxylin which targets the nucleus and shows blue; (c) 20× micrograph of control hMSCs patterned with no differentiation; (d) 20× micrograph of a pattern of fully differentiated adipocyte cells. The rate of differentiation is influenced by the ligand properties and ligand density. All images were taken by phase contrast microscopy.
Table 3-1. List of Oxyamine-Tethered Small Molecules Used To Generate Quantitative Ligand Density Microarrays for Studying hMSC Differentiation

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Figure 3-5. (Left) Representative micrographs showing adipogenic differentiation of hMSCs on different ligand density surfaces after 5 days. Each column shows micrographs of hMSCs differentiating to adipocytes on a particular surface ligand at varying ligand densities. Each row shows the same ligand density but varying ligand composition on hMSCs differentiation. The first and second columns show hMSCs on hydroquinone (H2Q) and quinone (Q) surfaces, respectively. Shown in the third and fourth columns are hMSCs on immobilized carboxylic acid (−COOH (5)) and methoxy (−OCH3 (3)) presenting surfaces. (Right) Representative micrographs showing adipogenic differentiation of hMSCs on surfaces presenting the same density (40%) at varying durations. Each column shows hMSCs on surfaces with 40% ligand density, including hydroquinone, quinone, (−COOH), and (−OCH3) group, respectively. Cells were stained with Oil Red O and Harris Hematoxylin. Images were taken by phase contrast microscopy.
In one application, we applied the electroactive ligand density microarray strategy to human mesenchymal stem cells (hMSCs) to study the role of ligand composition and ligand density on rate of differentiation. Human mesenchymal stem cells, as multipotent stem cells, have the ability to differentiate into several lineages including adipocyte, osteocyte and chondrocyte cells.\textsuperscript{44,45} hMSCs are increasingly being used in therapeutic applications for bone, cartilage and adipose transplantation and repair. Unlike embryonic stem cells, hMSCs are more amenable to controlled differentiation and can be readily induced to produce relatively pure differentiated cells. Because of ethical concerns regarding embryonic stem cell research and the ease of manipulation of hMSCs, many studies concerning hMSCs have been performed in recent years. The ability to precisely control stem cell differentiation into the corresponding lineage is crucial for developing new biomaterials for a range of therapies.

To study the effect of ligand composition and ligand density on hMSC differentiation, we used a visible dye to analyze specific differentiated lineage. To distinguish which cell lineage is derived from mesenchymal stem cell differentiation, specific marker dyes have been developed. For adipogenic differentiation, Oil Red O and Harris Hematoxylin are used as the specific marker stains. The generation of many lipid vacuoles is characteristic of adipocytes and can be specifically targeted and therefore visualized by Oil Red O staining. Nuclei are stained blue by Harris Hematoxylin (Figure 3-4).\textsuperscript{46,47} A sample of micrographs showing adipocyte generation versus ligand composition and ligand density and time is shown in Figure 3-5.

To determine the differences in stem cell differentiation rate on the varying ligand density microarray surfaces, we developed a new quantification method that measures differentiation rate versus ligand density. This strategy is based on measuring the ratio of red
pixels to the total number of pixels within the cell patterns and can precisely determine the subtle differentiation differences at any time point on any transparent surface without damaging the sample. Conventional quantification strategies rely on manually counting the number of differentiated cells after weeks or measuring fluorescence absorbance of cell elution or measuring gene expression. Using this quantification method, even subtle differences in stem cell differentiation can be measured on the gold substrate. On the basis of this analysis, a 3D plot of stem cell differentiation versus time versus ligand density was generated and averaged for over 15 experiments (Figure 3-6, Table 3-1). The plot clearly shows that the ligand composition is important but the ligand density also has a dramatic influence on the rate of differentiation. For example, carboxylic acid terminated oxyamine ligand (5) differentiates to a greater extent at higher ligand density than at low ligand density. For thiol terminated ligand (6) the stem cells differentiate at lower ligand density compared to higher ligand density. We only show a sample of the differentiation data in Figure 3-6 (5 day time point). At various durations the differentiation profile changes based on ligand composition and ligand density. At 10 days, approximately all cells on the ligand density spot arrays are fully differentiated (normalized to 1.0, see methods section). As a comparison to the quantitation method used to generate the 3D plot, we also examined the gene expression profiles of the adipocyte markers lipoprotein lipase (Lpl) and peroxisome proliferators-activated receptor gamma 2 (PPARγ2) (Figure 3-7). We observed that the marker genes were turned on at high levels at approximately 5 days and stayed at a nearly constant level afterward during the duration of the differentiation study. However, some expression was observed at earlier time points, indicating the gene expression profile is complementary but not as sensitive as the Oil Red O staining analysis.
Figure 3-6. A three-dimensional plot comparing differentiation rate versus ligand composition versus ligand density. The data were normalized to 1.0 for fully differentiated cells after 10 days. There is a clear dependence on differentiation rate and ligand composition as well as ligand density. The representative plot shows data for 5 days differentiation.
Figure 3-7. Representative gene expression comparison of adipogenic differentiation rate. An adipose-specific gene, lipoprotein lipase (Lpl) and peroxisome proliferators-activated receptor gamma 2 (PPARγ2), and a control gene, β2-microglobulin (β2mg), were used to ensure equal loading of the DNA. hMSC’s were cultured on varying surface ligand compositions and ligand densities and then induced to adipose and monitored. Total RNA was extracted and analyzed by reverse transcription PCR. Lane 1, control cells; lanes 2–6, days of induced differentiation.
3.3 Conclusion

This report shows for the first time the development of a quantitative ligand density microarray that can immobilize a variety of molecules for a range of cell biological and biochemical studies. This analytical biotechnology strategy is based on the transfer printing of an electroactive hydroquinone alkanethiol that can be oxidized to a quinone for subsequent ligand conjugation. All surface bound molecules are redox active and therefore provide a sensitive in situ probe to monitor and characterize the interfacial reaction. We applied this method to develop a unique microarray to study the effect of ligand composition and ligand density on stem cell differentiation. We observed that the density of ligands influences the rate of hMSC differentiation to adipocytes. This feature of ligand density is often overlooked when developing biomaterials for stem cell therapies due to the difficulty in preparing materials where the relationship between ligand density and cellular behavior is molecularly controlled. The electroactive microarray strategy is general and can be used to prepare a wide range of microarrays for a variety of biointerfacial studies including cell-based assays to enzymology platforms. Since the substrates are conductive they may be used in conjunction with surface plasmon resonance technology to measure small molecule or protein binding and mass spectrometry to identify protein partners to the presented ligands. Furthermore, as an additional feature, since the ligand bound molecule is also electroactive the reaction can be reversed to selectively release the immobilized ligands to regenerate the H$_2$Q, which allows for a renewable microarray platform.$^{29,30,49}$ Finally, interfacing with microfluidic technology will allow for the discrete delivery of reagents to select regions of the surface for a range of systems biology approaches to study a variety of signaling pathways.$^{50-58}$
3.4 Materials and Methods

Microarray Printing

Microscope glass slides were cleaned by 1:1 mixed solution of hydrogen peroxide and sulfuric acid for 4 h (Caution! Piranha solutions react explosively with trace quantities of organics), followed by washing with distilled water and 200 proof ethanol. After drying with a stream of N₂, a 5 nm adhesion layer of titanium followed by 20 nm of gold was evaporated onto the glass slides. Different alkanethiol solutions (total 1 mM in ethanol) were mixed in varying ratios, filled in designated positions of the 384-well microplate, and then printed in a programmed array format on the gold-coated microscope glass substrate by a spotbot2 microarrayer, which allows for transference of the alkanethiols to programmed positions on the gold substrate. The substrate is then thoroughly washed with ethanol and immersed into a 1 mM ethanol solution of tetra(ethylene glycol) terminated alkanethiol for 12 h, rendering the remaining surface inert to nonspecific protein adsorption and cell attachment.

Electrochemical Characterization

Alkanethiols terminated with the hydroquinone group (H₂QC₁₁SH) and the tetra(ethylene glycol) group (EG₄C₁₁SH) were prepared as previously described.¹⁹ By microarray printing mixed ratios of solutions of H₂QC₁₁SH (H₂Q) and HO⁻C₁₁SH (−OH), varying electroactive SAM compositions can be generated. Cyclic voltammetry (CV) was used to quantitatively follow the oxidation and reduction process on the surface. All electrochemical experiments were performed using a Bioanalytical Systems CV-100 W potentiostat. Cyclic voltammetry (CV) on SAMs was performed in PBS (pH 7.4), using a platinum wire as the counterelectrode, Ag/AgCl as the reference electrode, and the
gold/SAM substrate as the working electrode. All cyclic voltammograms were scanned at 50 mV/s.

**Cell Culture**

Human mesenchymal stem cells (hMSCs), basic medium, growth medium, and differentiation medium were obtained from Lonza. hMSCs were cultured as instructed by the vendor. After cells were washed with PBS and trypsinized for 3–5 min, they were centrifuged in serum containing medium and followed with gentle resuspending in serum-free medium. The cells were then seeded onto the substrates containing a ligand density array and then incubated at 37 °C in a humidified atmosphere of 5% CO₂ overnight. Adipogenic differentiation was induced by induction medium and kept by induction/maintenance cycles as described in the Lonza protocol.

**Immunohistochemistry**

The substrates were washed by PBS and fixed in 4% formaldehyde for 30 min, followed with sterile water and 60% isopropyl alcohol for 2–5 min. Samples were then stained by Oil Red O for 5 min followed by Harris Hematoxylin for 1 min.

**RT-PCR Analysis**

Human mesenchymal stem cells (hMSCs) were induced to adipogenic differentiation for varying durations. Total RNA was then extracted by RNA isolation kits (Qiagen). A 1 μg portion of total RNA was converted to cDNA using AMV reverse transcriptase and random hexamer primers (Promega). The resulting cDNA was used in PCR with the following primer,
Lpl (sense 5′-GAGATTTCCTGATGCGACC-3′, antisense 5′-CTGCAAATGAGACACTTTC-3′), PPARγ2 (sense 5′-GCTGTTATGGTGAACCTCTG-3′, antisense 5′-ATAAGGTGGATGCAGGCTC-3′), β2mg (sense 5′-ACCCCCACTGAAAAAGATGA-3′, antisense 5′-GCATCTTCAAACCTCCATGAT-3′), at annealing temperatures of 52, 55, and 53 °C, respectively. Amplification reactions were carried out for 1 min through 30 cycles, and the reaction products were subjected to 1% agarose gel electrophoresis. The reaction products are 276bp (Lpl), 351bp (PPARγ2), and 116bp (β2mg), respectively.

Quantification of Adipogenic Differentiation

Quantification by Matlab program is based on measuring the ratio of red pixels to the total number of pixels within the cell pattern. Data from 10 days were used as a frame of reference and normalized to 1.0, which indicates complete differentiation. Data were obtained by this method for various durations (1−10 days) of differentiation to determine the differentiation rate on the ligand density microarray.
3.5 REFERENCES


CHAPTER 4

Study of Spatial and Geometric Effects on Stem Cell Differentiation

4.1 Introduction

Stem cells have the potential to differentiate into different cell lineages with various functions.1,2 Because of this unique ability, stem cells are a promising source for cell transplantation therapies. In particular, human mesenchymal stem cells (hMSCs), as multipotent stem cells, have the ability to differentiate into several lineages including adipocyte, osteocyte, and chondrocyte cells. HMSCs are increasingly being used in therapeutic applications for bone, cartilage, and fat transplantation and repair.3,4 Unlike embryonic stem cells, hMSCs are more amenable to controlled differentiation and can be readily induced to produce relatively pure differentiated cells. Because of ethical concerns regarding embryonic stem cell research and the ease of manipulation of hMSCs, many studies concerning hMSCs have been performed in recent years.3,5 The precise control of stem cell differentiation into the corresponding lineage is crucial for several therapies. For example, adipocyte cells are the major component of adipose tissue, which is important for energy storage and for cushioning organs and insulating the body. Therefore, adipogenic differentiation from hMSCs has been suggested to be a potential source of adipose tissue for fat tissue engineering therapies, such as breast and facial reconstructions.6-9 From a health and social perspective, the failure to control the amount and function of adipose tissue results
in obesity that may initiate or act synergistically with other factors to cause more serious health issues. To study adipocyte formation and the differentiation rate from hMSCs, a multidisciplinary coordinated effort ranging from mechanical force studies, RNAi screens, small-molecule screens, and genetic manipulations must be used to probe the parameters that influence these complex processes. Although controlling stem cell differentiation is under intense investigation, little is known about the complex interplay of the many crucial factors that influence this process.  

Recent studies using model surfaces show that the surface microenvironment in which certain stem cells grow plays a key role in initiating and controlling differentiation. For example, by changing the elasticity of the substrate, the polymer material to which the cells adhere or the number of cells on the surface all influence the differentiation process.  

Herein, we develop a general methodology to create a patterned surface array that allows for the study of how the cell population, surface adhesion area, and pattern geometry combine to influence stem cell differentiation. By employing soft lithography, patterned hydrophobic alkanethiol self-assembled monolayers (SAMs) can be formed on a thin gold-coated glass substrate to control the cell adhesion area and population and geometry parameters. Because of the transparency of the substrate, sophisticated microscopy techniques can be used to quantify various cellular behaviors precisely, including stem cell lineage differentiation.

4.2 Results and Discussion

To determine the role of cell population and the effect of cell-cell interactions on adipogenic differentiation, an elastomeric stamp made of polydimethylsiloxane (PDMS) was
Figure 4-1. (a) Scheme for generating different cell patterns on surfaces to study the role of pattern geometry and cell adhesive area (cell population) on differentiation. Microcontact printing (μCP) is used to pattern a hydrophobic alkanethiol (C₁₆SH) to the gold surface. The remaining bare gold region is backfilled with a tetra(ethylene glycol)-terminated alkanethiol (EG₄C₁₁SH) to generate a patterned cell adhesive and inert surface. hMSCs are seeded onto the entire surface but will adhere only to the hydrophobic patterns. (b) Differentiation can be induced, and the role of pattern size, cell population, and cell adhesive area in the stem cell differentiation rate can be measured. Phase contrast microscopy and marker staining allows the quantification of the differentiation rate from hMSCs to adipocytes. Scale bars represent 145 (top) and 60 μm (bottom), respectively, in micrographs shown in b.
used to pattern hydrophobic alkanethiols onto a gold substrate and therefore control the cell adhesion area, population, and geometry.

As shown in Figure 4-1a, after inking with a solution of 1-hexadecanethiol (C\textsubscript{16}SH), the PDMS stamp transfers the 1-hexadecanethiol onto a gold substrate to form a hydrophobic SAM. A solution of a tetra(ethylene glycol)-terminated alkanethiol (1mM in ethanol, EG\textsubscript{4}C\textsubscript{11}SH) was then used to backfill the remaining bare regions of gold to generate cell adhesive and inert regions on the surface. The ethylene(glycol) alkanethiol is known to resist nonspecific protein adsorption and cell attachment\textsuperscript{14}. After seeding hMSCs onto the substrate, cells adhere only to the patterned region with the C\textsubscript{16}SH monolayer. By designing the stamp, spatially controlled cell patterns of different sizes can be created.

To distinguish which cell lineage is derived from mesenchymal stem cell differentiation, specific marker dyes have been developed. For adipogenic differentiation, Oil Red O and Harris Hematoxylin are used as the specific marker stains\textsuperscript{15,16}. The generation of many lipid vacuoles is characteristic of adipocytes and can be specifically targeted and therefore visualized by Oil Red O staining. Nuclei are stained blue by Harris Hematoxylin.

As shown in Figure 4-1b, after hMSCs are patterned on surfaces, an induction medium is used to induce differentiation. After varying durations, the cells are stained and analyzed for differentiation. Because the marker stains are visible dyes and the substrates are transparent, micrographs were obtained by standard phase-contrast microscopy.

We first used the methodology to correlate the adipogenic differentiation rate with the cell adhesive area and therefore the cell population. Circle patterns with different diameters were microcontact printed to control the number of cells in each pattern. Figure 4-2 shows representative images of hMSCs on differently sized circle patterns versus the rate of
differentiation to adipocyte cells. An evaluation of differentiation based on staining with Oil Red O and hematoxylin shows no differentiation on any size of circular pattern after 1 day, but most cells on the patterns show significant differentiation after 5 days and almost all cells fully differentiate to adipocytes after 10 days. The critical duration for differentiation on these surfaces is around 5 days, during which we can observe minor differences in the differentiation rate depending on pattern size and therefore cell population. Interestingly, irrespective of circular pattern size the cells differentiated much faster on C_{16}SH surfaces (8-10 days) than on tissue culture plates (12-14 days), showing that the surface chemistry is critical for cell differentiation.
<table>
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<tr>
<th>Diameter (µm)</th>
<th>106 µm</th>
<th>220 µm</th>
<th>284 µm</th>
<th>470 µm</th>
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<tr>
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Figure 4-2. Micrographs of mesenchymal stem cells differentiated to adipocytes on circularly patterned SAMs with varying diameters (top row) and durations (left column). Cells were stained with Oil Red O, which specifically targets the lipid vacuoles in adipocytes. The lipid vacuoles are stained red, and the nuclei are stained blue by a Harris Hematoxylin dye.
To determine any subtle differences in stem cell differentiation on the varying patterned surfaces, we developed a new quantification method that measures differentiation rate versus pattern size (Figure 4-3). This strategy is based on measuring the ratio of red pixels to the total number of pixels within the cell patterns and can precisely determine the subtle differentiation differences at any time point on any transparent surface without damaging the sample.
Figure 4-3. (Top) Three-dimensional plot comparing differentiation rate versus time versus cell circular pattern size. From the data, there is no significant change in the differentiation rate by increasing the circular pattern size and therefore increasing the cell population. The data were normalized to 1.0 for fully differentiated cells after 10 days. (Bottom) Gel comparison example of the rate of adipogenic differentiation via gene expression for 220 μm circles. An adipose-specific gene, lipoprotein lipase (Lpl), and a control gene, β2-microglobulin (β2mg), were used to ensure equal loading of the DNA. hMSCs were cultured until confluent and then induced to adipose and monitored. Total RNA was extracted and analyzed by reverse transcription PCR. Lane 1, control cells; lanes 2-6, days of induced differentiation.
Figure 4-4. Representative micrographs of mesenchymal stem cells differentiated to adipocytes on varying geometrical patterns and durations. Cells are stained by Oil Red O and Harris Hemotoxylin, and images are obtained by phase-contrast microscopy.
Conventional quantification strategies rely on manually counting the number of differentiated cells after weeks or measuring the fluorescence absorbance of cell elution or gene expression. Using this quantification method, even subtle differences in stem cell differentiation can be measured on the gold substrate. On the basis of this analysis, there is only a slight change in the differentiation rate for different cell pattern sizes (cell adhesive area). Thus, changing the cell population has no dramatic effect on the adipogenic differentiation rate. Interestingly, even after the cell adhesive area is increased 20-fold (still circular patterns), there is only a marginal change in the differentiation rate.

Because the cell population has no dramatic effect on hMSCs differentiation, we performed a separate study where we correlated the pattern area (cell population) with pattern geometry to determine if the shape of the pattern influences the differentiation rate (Figure 4-4). Whereas cell shape has been shown to have a major effect on biological processes such as adhesion, migration, and proliferation, the data regarding specific geometrical effects on cell differentiation behavior remains unknown and mostly unexplored. We fabricated an array of SAM patterns with various geometries, including octagon, pentagon, right triangle, square, trapezoid, and triangle, and then added hMSCs and analyzed the adipogenic differentiation rate. Surprisingly, we found that there were clear differences in the rate of differentiation depending on geometrical shape. For example, the pentagon and right triangle have very different differentiation rates at 5 and 8 days. Even the two different triangle shapes with almost the same surface area and therefore population have different differentiation rates at 5 and 8 days. To rule out the possibility that the geometric area is influencing differentiation and not the geometry itself, we calculated the area of each pattern for comparison (Table 4-1). From Table 4-1, the square and octagon shapes have
approximately the same surface area \((7.3 \times 10^4 \, \mu\text{m}^2)\) but have very different differentiation rates. By analyzing the data in Table 4-1, it is clear that a geometrical influence is causing a change in the adipogenic differentiation rate and not the area of the pattern. However, decreasing the size of the pattern and therefore decreasing the cell population to a few cells does not lead to cell differentiation because a certain minimum density of cells is required for adipogenic differentiation.\(^{21}\)
Table 4-1. Comparison of Pattern Geometry versus Pattern Area for the Data Shown in Figures 4-3 and 4-5.

<table>
<thead>
<tr>
<th>Pattern Shape</th>
<th>Area (*10^4 μm^2)</th>
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<tbody>
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<td>Octagon</td>
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</tr>
<tr>
<td>Triangle</td>
<td>3.3</td>
</tr>
<tr>
<td>Trapezoid</td>
<td>5.3</td>
</tr>
<tr>
<td>Square</td>
<td>7.4</td>
</tr>
<tr>
<td>RT-Triangle</td>
<td>3.2</td>
</tr>
<tr>
<td>Pentagon</td>
<td>4.9</td>
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<tr>
<td>Circle 1 (106 μm)</td>
<td>0.88</td>
</tr>
<tr>
<td>Circle 2 (220 μm)</td>
<td>3.8</td>
</tr>
<tr>
<td>Circle 3 (284 μm)</td>
<td>6.3</td>
</tr>
<tr>
<td>Circle 4 (470 μm)</td>
<td>17.3</td>
</tr>
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</table>
Figure 4-5. Three-dimensional plot comparing differentiation rate versus time versus geometry. There is a significant difference in the cell differentiation rate based on the geometry of the pattern and not the cell adhesive area. The geometry of the pattern influences cell differentiation.
We hypothesize that the cells on the periphery of the pattern are able to sense the edge or corners of the pattern and are able to influence the total differentiation rate of the cells within the pattern. This pattern edge phenomenon is well known to influence cell division, cell cytoskeleton dynamics, and migration from patterns but has not been previously observed for stem cell differentiation.\textsuperscript{22-25}

4.3 Conclusions

We have developed a general methodology to study the subtle changes in stem cell differentiation rate versus cell population and geometry. We used a simple microfabrication technique to pattern hMSCs on transparent surfaces and developed a new method to quantify adipogenic differentiation. We found that the pattern geometry and not the cell population influences adipogenic differentiation from hMSCs. Furthermore, the cells within the pattern behave more like a tissue than like individual cells because a certain critical cell density is required to induce differentiation. By employing high-throughput analysis, surface chemistry, pattern geometry, small-molecule screens, and genomic profiling, a systematic approach may be developed to investigate and control various aspects of hMSCs and other cell differentiation processes.

4.4 Materials and Methods

*Microscopy*

All micrographs were imaged using a Nikon inverted microscope (model TE2000-E). All images were captured and processed by MetaMorph.
Patterned Substrates

An elastomeric stamp made of polydimethylsiloxane (PDMS) was used to pattern hydrophobic alkanethiols onto a gold substrate and therefore control the cell adhesion area, population, and geometry. After inking with a PDMS stamp and a solution of 1-hexadecanethiol (C₁₆SH), the PDMS stamp transfers 1-hexadecanethiol onto a gold substrate to form a hydrophobic SAM. A solution of a tetra(ethylene glycol)-terminated alkanethiol (1mM in ethanol, EG₄C₁₁SH) was then used to backfill the remaining bare regions of gold to generate cell adhesive and inert regions on the surface.

Cell Culture

Human mesenchymal stem cells (hMSCs), growth medium, and differentiation medium were obtained from Lonza. hMSCs were cultured as instructed by the vendor. After cells were seeded onto the SAM substrates, they were incubated at 37 °C in a humidified atmosphere of 5% CO₂ overnight to form confluent cell patterns. Adipogenic differentiation was induced by an induction medium and kept by induction/maintenance cycles as described in the Lonza protocol.

Immunohistochemistry

The substrates were washed with PBS and fixed in 4% formaldehyde for 30 min, followed by washing with sterile water and 60% isopropanol for 2-5 min. Samples were then stained by Oil Red O for 5 min, followed by Harris Hematoxylin for 1 min.
**Quantification of Adipogenic Differentiation**

Quantification was carried out with a Matlab program based on determining the precise ratio of the red area to the entire cell area after staining with Oil Red O and Harris Hematoxylin. As differentiation occurs, more red lipid vacuoles are observed. The program is designed to scan each cell patterned area and to calculate the ratio of red pixels to the total number of pixels in the patterned area. (Red pixels represent lipid vacuoles stained with Oil Red O and serve as a marker for adipocytes.) Because the cells are fully differentiated after 16 days, the data for 16 days was normalized to 1.0. All other cell differentiation times were normalized to the 16 day differentiation data. This method allows for the quantification of differentiation.

**Gene RT-PCR Analysis**

Human mesenchymal stem cells (hMSCs) were induced to undergo adipogenic differentiation for various durations. Total RNA was extracted by RNA isolation kits (Qiagen). One microgram of total RNA was converted to cDNA using AMV reverse transcriptase and random hexamer primers (Promega). The resulting cDNA was used in PCR with the primer Lpl (sense 5′-GAGATTTCTCTGTATGGCACC-3, antisense 5′-CTGCAAATGAGACACTTTCTC-3′, β2 mg (sense 5′-ACCCCCACTGAAAAAGATGA- 3′, antisense 5′-GCATCTTCAACCTCCATGAT-3′) at annealing temperatures of 52 and 53 °C. Amplification reactions were carried out for 1 min through 30 cycles, and the reaction products were subjected to 1% agarose gel electrophoresis. The reaction products are 276 bp (Lpl) and 116 bp (β2mg), respectively.
4.5 REFERENCES


5.1 Introduction

The ability to characterize, reproducibly synthesize and tailor nanomaterials has the potential to revolutionize science and engineering. Generating nanomaterials and developing probes to further understand their physical properties is under intense investigation and has lead to many diverse applications ranging from molecular electronics, solar cells, imaging, biosensors, drug delivery and tissue engineering. A research area that nanomaterials may have a major impact is in cell biology and in particular studying how the dynamic nanoarchitecture of cells direct cell behavior. Because this research area is a relatively new frontier in nanoscience, many initial studies have been to investigate how the nanomaterial may be interfaced with cells. A few important recent studies have shown that a material with nanoscale structure can have interesting and unpredictable affects on cells. Until recently, there has been little attention given to how surface functionalization of nanomaterial surfaces influences cell behavior and the nature of the cell-nanomaterial interaction. In order to further probe how the nano-environment affects cell behavior, a model substrate that presents nano features would be extremely useful for examining the interplay between the nanomaterial and cells. The ability to precisely tailor the surface chemistry of these nanomaterials with a variety of molecules for generating defined surface properties would have a major impact in
bio-nanoscience and especially cell biology. These materials are crucial for probing and further understanding the role of the nanoarchitecture within cells that regulate diverse processes ranging from adhesion, proliferation, migration and differentiation.\textsuperscript{17,18}

Herein, we report a general strategy for creating electroactive gold nanorod surfaces that can be tailored to present a variety of ligands/molecules for biospecific studies of cell adhesion and stem cell differentiation. We use the chemoselective reaction of oxyamine tethered ligands to nanorod bound quinone molecules to generate covalently linked oxime ligands for cell behavior studies. In particular, by using scanning electron microscopy and phase contrast microscopy we show these tailored nanorods can be interfaced with cells for biospecific cell adhesion and stem cell differentiation studies. This methodology provides the ability to modulate the nanorods surface physical and chemical properties for a variety of material and biological applications (Figure 5-1).
Figure 5-1. Scheme of tailoring gold nanorods with an electroactive chemoselective immobilization strategy. A redox active hydroquinone alkanethiol was formed on the nanorods. The hydroquinone group can be electrochemically oxidized to the quinone, which can chemoselectively react with oxyamine tethered molecules (R-ONH$_2$). This strategy allows for the ability to tailor the nanorods with a variety of molecules for material and biological applications.
5.2 Results and Discussion

To generate the gold nanorods we thermally evaporated 800 nm of silver to one side of an anodic aluminum oxide (AAO) template with a pore size of 50 nm. The AAO/Ag substrate was then placed silver-side down on top of a layer of conductive AG epoxy that had been deposited onto a glass slide. The AAO/Ag/Ag epoxy/glass substrate was then cured at 80 °C for 3 hours. The substrate was then used as the working electrode in a three-electrode cell to electrochemically deposit gold into the pores of the AAO template. Electrical contact was made by connecting an alligator clamp to an exposed strip of Ag epoxy. Gold nanorods with a length of ~ 500 nm were deposited by applying -1 Volt versus Ag/AgCl for 5 minutes in a gold electroplating solution. The alumina template was then dissolved with 2 M NaOH (aq) for a period of 30 minutes. Figure 3A and 3B show representative scanning electron micrographs of the gold nanorods.
Figure 5-2. (Top) Scheme of the oxidation-reduction reaction of the electroactive H$_2$Q SAM and the oxidation-reduction reaction of the electroactive oxime product after immobilization of oxyamine tethered ligands. (Bottom left) Cyclic Voltammograms (CV) for characterizing the oxidation-reduction reaction of H$_2$QC$_{11}$SH SAM and the immobilization reaction on flat gold. (Bottom right) Cyclic Voltammograms (CV) for characterizing the oxidation-reduction reaction of H$_2$QC$_{11}$SH SAM and the immobilization reaction on gold nanorods. The black cyclic voltammograms correspond to the redox peaks of H$_2$Q SAMs and the red lines correspond to the redox peaks of the oxime product on the flat gold and nanorod gold respectively. The ligand used was RGD-ONH$_2$ (1 mM for 4 hrs in 1M HClO$_4$). The reaction went to completion on flat gold surfaces evidenced by no redox CV signal for the H$_2$Q, but only went to approximately 40% completion on the nanorods (determined by integrating the peak area for the oxime product compared to the peak area of the starting H$_2$Q surface on the nanorod surfaces).
Figure 5-3. Scanning electron micrograph images of cells on gold nanorods. (A) Gold nanorods of approximately 50 nm in diameter and length of 500 nm. (B) Higher resolution image of the gold nanorods. (C) Mesenchymal stem cells adhere on electroactive gold nanorods containing the RGD peptide ligand. (D) High resolution image of mesenchymal stem cell lamellapodia on gold nanorods.
Figure 5-4. Comparison of phase contrast images of mesenchymal stem cells on flat gold and nanorods surfaces. (A) A undifferentiated stem cell on a flat gold surface. (B) A differentiated stem cell on flat gold surface showing red lipid vacuoles. (C) Undifferentiated stem cells on nanorods are transparent and are not seen by reflectance microscopy. (D) However, differentiated stem cells on nanorods surfaces are visible due to the red lipid vacuoles. All samples were stained by Oil Red O. Micrographs of cells on flat gold were obtained in transparent mode and micrographs of cells on nanorods were taken by reflectance mode microscopy.
In order to make the nanorods accessible for various surface modifications, a redox active molecule $\text{H}_2\text{QClSH}$ was synthesized and formed on the gold nanorods (1mM in ethanol for 12 hours). We have shown on flat gold surfaces, the hydroquinone can be oxidized to the quinone, which can then react chemoselectively with soluble oxyamine tethered molecules to generate covalently bound oxime linked ligands on the surface.\textsuperscript{19-24} The substrate is used as a working electrode where cyclic voltammetry can track the course of the reaction between quinone and hydroxylamine because the redox cycle for hydroquinone-quinone is distinct from that of the product oxime redox cycle (Figure 5-2). This important feature allows for the determination and potential quantification of the immobilization of a variety of functionalized oxyamines onto the nanorods.

To demonstrate biospecific cell adhesion and differentiation on the nanorod substrates, we immobilized two different molecules: a cell adhesive peptide (RGD-ONH\textsubscript{2}) and an inert molecule (tetra(ethylene)glycol-oxyamine). The RGD peptide is found in the extracellular matrix protein fibronectin and is known to facilitate adhesion to cells via their cell-surface integrin receptors.\textsuperscript{25} The ethylene(glycol) group, when presented on many different types of materials, has been shown to prevent non-specific protein adsorption and cell attachment.\textsuperscript{26} Figure 5-3C and 5-3D show representative cells attached to nanorods that were functionalized with the RGD peptide. Interestingly, cells had many more fillopodia extensions from their cell body on the nanorods than on flat gold surfaces presenting the same RGD peptides (Supplementary). The surface topology is clearly influencing the cell cytoskeleton and therefore how the cell samples its environment for subsequent growth, migration or differentiation. As a control, nanorods presenting ethylene (glycol) groups had significantly fewer cells attached and almost all adopted a rounded morphology.
We also interfaced the tailored nanorods with mesenchymal stem cells to examine differentiation. Mesenchymal stem cells were seeded onto the RGD-presenting nanorods and differentiated to adipocyte cells (Supplementary). We found most cells (~80%) completely differentiated to adipocytes in approximately 8 days compared to tissue culture plate differentiation (~10 days). To determine differentiation the cells were stained with Oil Red O, a marker dye that specifically visualizes lipid vacuoles in adipocyte cells. Figure 5-4 shows mesenchymal stem cells that have clearly differentiated into adipocytes on flat gold surfaces and nanorods respectively. For both surfaces the micrographs were obtained using phase contrast microscopy. As controls the non-differentiated cells showed no red lipid vacuoles on either surface (Supplementary).

5.3 Conclusions

We report a strategy for the fabrication of tailored electroactive nanorod substrates for biospecific studies of cell adhesion and stem cell differentiation. To control the interfacial properties of the nanorods we formed self-assembled monolayers of an electroactive hydroquinone group that is able to chemoselectively immobilize oxyamine tethered ligands. We use these substrates to demonstrate for the first time biospecific cell adhesion and mesenchymal stem cell differentiation on gold nanorods. SEM and phase contrast microscopy were used to examine cell morphology and differentiation status on the nanorods. The methodology presented provides the ability to molecularly control the surface chemistry of nanostructures and for creating tailored nanomaterials with specific surface properties for a variety of material and biological applications. We believe, by combining this strategy with photolithographic methods, patterned and tailored electroactive nanorods can be generated to
further probe the dynamic nanoarchitecture within cells that regulate cell behavior ranging from growth, migration to differentiation. The electroactive strategy on nanorods may also be used to selectively release ligands or nucleic acids into adhered cells and by integrating high resolution fluorescence microscopy may be used to probe in real-time various mechano-transduction cell behavior.\textsuperscript{27-30} For future engineering applications the nanorods can be functionalized with chemistries that interact with interdigitated conducting polymers for a range of solar cell and molecular electronic applications. The conclusions section should come at the end of article.

5.4 Materials and Methods

Cell Differentiation Staining

Oil Red O stock solution was prepared by weighing out 300 mg of Oil Red O powder and added to 100 ml of 99% isopropanol. The working solution is made by mixing 3 parts of Oil Red O stock solution with 2 parts deionized water and filtered before use. Fresh working solution was made each time and used within two hours. Cell samples were washed in PBS and fixed by 10\% formalin for 30 minutes. After discarding the formalin, samples were washed with water, followed by 60\% isopropanol for 2-5 minutes, and then Oil Red O working solution for 5 minutes. The sample was then gently rinsed with water to wash away the Oil Red O solution.

Scanning Electron Microscopy of Cells

Cell samples were washed with PBS and fixed by 10\% formalin for 30 minutes. After discarding the formalin solution and washing with water, samples were dehydrated stepwise
in 30%, 50%, 70%, 90% and 100% ethanol for 30 minutes. After using a critical point drying technique and sputtering 2 nm gold, the samples are ready for SEM imaging.

**Microscopy**

A phase contrast microscope was used to take cells pictures after staining with Oil Red O. All cell micrographs on flat gold surfaces were taken in transparent mode. The cell micrographs on gold nanorods substrates were taken in reflectance mode, due to the non-transparency of the substrate.

**Nanorod Fabrication**

Anodic aluminum oxide (AAO) templates (pore size = 50 nm) were purchased from Puyuan Nano, Ltd. (China) 800 nm of Ag (Kurt Lesker, Inc., 99.99%) was thermally evaporated onto one side of the template. The AAO/Ag substrate was then placed silver-side down on top of a layer of conductive Ag epoxy (Part No. H2OE, Epoxy Technology) that had been deposited onto a glass slide. The AAO/Ag/Ag epoxy/glass substrate was then cured at 80 °C for 3 hours. This substrate was then used as the working electrode in a three-electrode cell to electrochemically deposit Au into the pores of the AAO template. Electrical contact was made by connecting an alligator clamp to an exposed strip of Ag epoxy. The reference electrode was a Ag/AgCl (3 M NaCl) electrode from Bioanalytical Systems while the counter electrode was a platinum gauze electrode. The electrolyte was a gold electroplating solution purchased from Technic, Inc. (Orotemp 24, diluted by ½ with deionized water). Au nanorods with a length of ~ 500 nm were deposited by applying -1 V vs. Ag/AgCl for 5
minutes. The alumina template was then dissolved with 2 M NaOH (aq) for a period of 30 minutes.

_Electrochemical Measurements_

All electrochemical experiments were performed using a Bioanalytical Systems CV–100W potentiostat. Electrochemistry on SAMs was performed in 1M HClO₄ or Phosphate Buffered Saline (PBS) pH 7.2 using a platinum wire as the counter electrode, Ag/AgCl as reference, and the gold/SAM or gold nanorod/SAM substrate as the working electrode. All cyclic voltammograms were scanned at 50 mV/s.
5.5 REFERENCES


CHAPTER 6

Transferring Surface Chemistry Strategy to Carbon Nanotubes (CNTs) for Cell Adhesion and Migration Studies

6.1 Introduction

The ability to characterize, reproducibly synthesize and tailor nanomaterials has the potential to revolutionize science and engineering.1,2

Proper cell adhesion and cell migration is essential for many fundamental biological processes ranging from wound healing, development to inflammation.1,2 Devastating diseases and cancer may result when errors occur in these highly regulated and complex processes. Many of the signals that cells receive to initiate adhesion and migration come from the dynamic extracellular matrix. The extracellular matrix is composed of highly fibrous and insoluble large proteins that include fibrinogen, fibronectin, laminin and collagen and is considered a complex adhesive nanomaterial. To further understand cell adhesion and migration, many model substrates have been developed ranging from soft elastic materials to patterned self-assembled monolayers on gold. However, to our knowledge, there has been a lack of reports that integrate tailored nanomaterials and patterned surfaces to study cellular behavior.3-7

Since its discovery by Iijima,8 carbon nanotubes (CNTs) have been the source of intense investigations from both theoretical and experimental scientists.9,10 Due to its carbon composition, high aspect ratio, electrical and physical properties, CNTs have been widely
studied for potential applications in nanoelectronics,\textsuperscript{11-17} optoelectronics,\textsuperscript{18} biosensors,\textsuperscript{19-25} field-effect-transistors,\textsuperscript{26-29} and molecular transporters.\textsuperscript{30-35} An important sub-class of carbon nanotubes (CNTs), single wall carbon nanotubes (SWCNTs), which are usually generated via arc-discharge,\textsuperscript{36} laser ablation,\textsuperscript{37} or chemical vapor deposition (CVD),\textsuperscript{38} are normally considered as a one-dimensional material and have also been used for a range of applications.

In recent years, there has been growing interest in using these CNTs for biomedical and tissue engineering applications such as cell tracking and labeling, sensing cellular behavior, augmenting cellular behavior, and enhancing tissue matrices.\textsuperscript{39} Therefore, of particular importance is the development of strategies to modify or tailor the surface chemistry of CNTs. The ability to routinely integrate biomolecules onto the surface of CNTs, thereby modifying its chemical or physical properties, is crucial for use in fundamental cell behavior studies leading to potential future biomedical applications. Although, there have been few reports of functionalization strategies for CNTs, including hydrophobic wrapping, $\pi-\pi$ stacking, electrostatic attraction, and carboxylic coupling,\textsuperscript{10,40-47} there is no general electroactive and quantitative immobilization method to tailor CNTs with a variety of ligands.

Herein, we report a combined chemoselective bioconjugation strategy to tailor carbon nanotubes with cell adhesive ligands and a microfluidic strategy to pattern these CNTs on a surface for multiplex cell adhesion and migration studies. A new electroactive hydroquinone terminated pyrene molecule is generated that is able to assemble via $\pi-\pi$ stacking to the side walls of CNTs. These tailored CNTs can be oxidized and made to chemoselectively react with oxyamine tethered ligands to generate stable and covalent oxime linkages. The interfacial reaction is characterized by cyclic voltammetry and scanning electron microscopy. A cell adhesive peptide (Arg-Gly-Asp) RGD is immobilized to the CNTs and a new
microfluidic patterning method is employed to generate multiplex patterned surfaces for biospecific cell adhesion and migration studies. Fluorescence microscopy is used to examine cell behavior on the various tailored CNTs to compare cell spreading, stress fiber formation, focal adhesion size and structure and cell migration rates. This work demonstrates the integration of a new functionalization strategy to immobilize a variety of ligands to CNTs for a range of potential drug delivery, tissue imaging and cellular behavior studies and a microfluidic patterning strategy for generating complex high-throughput surfaces for biotechnological and cell based assay applications.

6.2 Results and Discussion

We first synthesized a new hydroquinone tethered pyrene molecule (Py-H\textsubscript{2}Q) that is able to assemble on CNTs via π-π stacking to create electroactive CNTs (H\textsubscript{2}Q-CNTs) (Scheme 6-1). The H\textsubscript{2}Q-CNTs can be electrochemically oxidized to the corresponding quinone (Q-CNT), which can then react chemoselectively with oxyamine tethered ligands (R-ONH\textsubscript{2}) to generate a covalent oxime linkage (Qox-CNT) (Figure 6-1).\textsuperscript{48,49} Due to the ease of introducing the oxyamine moiety into molecules through routine synthesis, the H\textsubscript{2}Q-CNTs can be tailored with a variety of ligands. Therefore, the chemical and physical properties can be modulated for a variety of biological and material applications.
Scheme 6-1. Synthesis of Py-H₂Q (1)
Figure 6-1. Strategy to chemoselectively tailor single wall carbon nanotubes (CNT) by combining $\pi-\pi$ stacking and electroactive immobilization. A Hydroquinone-terminated pyrene (Py-$\text{H}_2\text{Q}$) irreversibly adsorbs onto the sidewall of a CNT via $\pi-\pi$ stacking. The hydroquinone-CNT ($\text{H}_2\text{Q}$-CNT) is then electrochemically oxidized to the corresponding quinone (Q-CNT), which can react with various oxyamine-tethered ligands (R-$\text{ONH}_2$) to form stable oxime linkages (Q$_\text{ox}$-CNT). The oxime product is also redox active and provides a diagnostic electrochemical signal to characterize ligand immobilization.
We have shown previously that a hydroquinone terminated self-assembled monolayers on gold can be oxidized and made to react efficiently with oxyamine terminated ligands, where the kinetics and yield of reaction can be quantitatively monitored and controlled by electrochemistry. In this report, we transfer this immobilization chemistry to generate electroactive carbon nanotubes. In order to characterize oxime formation on carbon nanotubes, we used a conductive indium tin oxide (ITO) surface as the working electrode.
Figure 6-2. Electrochemical characterization of ligand immobilization to the electroactive hydroquinone carbon nanotube (H$_2$Q-CNT). The cell adhesive peptide (RGD-ONH$_2$) ligand is reacted with the oxidized form of the H$_2$Q-CNT. Cyclic voltammetry shows a shift in the peak potentials after peptide immobilization. Cyclic voltammetry (CV) was performed in 1M HClO$_4$ using a platinum wire as the counter electrode, Ag/AgCl as the reference electrode, and ITO substrate as the working electrode. All CVs were scanned at 100 mV/s.
Figure 6-2 shows the distinct oxidation and reduction peaks of the H₂Q-CNT and Q-CNT pair. After reaction with an oxyamine ligand, a shift in the cyclic voltammogram peaks shows formation of the redox active oxime linkage (Qox-CNT). By integrating the CV peaks, the amount of charge thus the amount of immobilized ligand can be precisely determined. Based on this electroactive immobilization strategy, quantitative installation of ligands onto CNTs can be accomplished for a variety of applications.

To prepare patterned surfaces, we developed a microfluidic strategy to directly pattern surfaces with tailored carbon nanotubes. This strategy allows for surfaces to be patterned with various chemistries or nanomaterials to simultaneously study a series of cell behaviors. We have used this microfluidic strategy previously as a lithography method to pattern alkanethiols in gradients on gold surfaces to study directional cell migration. The microfluidic delivery of CNTs has also been shown to align CNTs by laminar flow on SiO₂/Si substrates for potential electronic applications.

For the cell adhesion and migration studies, an oxyamine tethered cell adhesive peptide RGD-ONH₂ (Scheme 6-2) was chosen for tailoring CNTs to generate RGD-CNTs. It has been shown that the RGD peptide is the minimum cell adhesive peptide found in the extracellular matrix protein fibronectin that facilitates biospecific adhesion with integrin receptors on cells.
Scheme 6-2. Structures of surface molecules used in this study.
As shown in Figure 6-3, a PDMS microfluidic cassette was placed in direct contact with a gold substrate. A hexadecanethiol solution (C_{16}SH), CNT suspension and a RGD-CNT suspension were flowed through the patterned channels to generate patterned C_{16}SH SAM, CNTs and RGD-CNTs in specific regions on the surface. After rinsing the channels with ethanol and carefully removing the PDMS cassette, the entire substrate was washed thoroughly with ethanol and then immersed in a solution of a tetra (ethylene glycol) terminated alkanethiol (EG_{4}C_{11}SH, 1mM in ethanol) for 12 h. The ethylene (glycol) group is known to prevent non-specific protein adsorption and cell attachment to substrates.63 After seeding Swiss 3T3 fibroblasts to the entire substrate, the cells only adhered to the patterned regions presenting C_{16}SH monolayer, CNTs and RGD-CNTs. To these cell patterned substrates, scanning electron microscopy (SEM) and fluorescence microscopy were used to investigate cell adhesion by analyzing cell spreading, stress fiber formation and focal adhesion size and structure (Figure 6-4).

To these cell patterned substrates, scanning electron microscopy (SEM) and fluorescence microscopy were used to investigate cell adhesion by analyzing cell spreading, stress fiber formation and focal adhesion size and structure (Figure 6-5).
Figure 6-3. A microfluidic strategy to pattern hexadecanethiol (C\textsubscript{16}SH), CNTs and RGD-CNTs on gold surfaces for spatially controlled biospecific studies of cell adhesion and cell migration. (a) A PDMS microfluidic cassette is placed in direct contact with a bare gold substrate and a solution of C\textsubscript{16}SH (red), a suspension of CNTs (black) and RGD-CNTs (blue) are flowed through the channel. (b) After rinsing with ethanol and removal of the microfluidic cassette, a surface pattern of deposited CNT, RGD-CNT and C\textsubscript{16}SH were formed. (c) The substrate was then backfilled with a solution of tetra (ethylene glycol)-terminated alkanethiol (EG\textsubscript{4}C\textsubscript{11}SH), generating a SAM inert to nonspecific cell adhesion on the non-patterned regions. (d) After cell seeding, a spatially controlled cell array is formed on the C\textsubscript{16}SH, CNTs and RGD-CNTs regions. Comparative cell adhesion and cell migration behavior can be studied simultaneously on one chip containing different zones of surface chemistries and carbon nanotubes.
Figure 6-4. Scanning electron micrographs (SEM) of patterned CNTs and fibroblast cells. (a) C$_{16}$SH and CNTs were patterned on the gold surface by a microfluidic lithography strategy. (b) Spatially controlled cell patterns were formed on the regions presenting C$_{16}$SH SAMs, CNTs and RGD-CNTs. (c) and (d) SEM of cells on the regions presenting RGD-CNTs. (e) and (f) SEM images of the cell-CNT interface.
Focal adhesions are large, dynamic protein complexes through which the cell cytoskeleton is connected to the ECM. The assembly and morphology of focal adhesions play a crucial role in signal transduction, cell adhesion, and cell migration. Since paxillin is an important protein found within focal adhesions, an antipaxillin antibody was used to visualize and analyze focal adhesion structures. Cells were seeded at a low density (5000/mL) in order to facilitate cell spreading and focal adhesion development. Fluorescence micrographs were taken on the three types of surfaces with 40X oil lens objective. Cells exhibited characteristic behavior on the various surfaces but were observed to have more stress fibers and focal adhesions on the RGD-CNT than on the CNT and C16SH patterns. The representative cell images on C16SH, CNTs and RGD-CNTs are shown in figure 6-5a, 6-5c and 6-5e respectively. The larger paxillin (green) spots within cells shown in 6-5e (RGD-CNT surface) are characteristic of well-spread and tightly adhered cells with well-formed focal adhesions. The chemical schemes in 5b, 5d and 5f represent the corresponding surfaces the fibroblasts are attached to in 6-5a, 6-5c and 6-5e respectively. Interestingly, the vast majority of cells had large needle like focal adhesions on the RGD-CNT surfaces compared to control surfaces that presented mixed monolayers of 50% RGD and 50% ethylene(glycol) alkanethiol (EG4C11SH). The combination of high ligand density and topology on the CNTs may cause the cells to adhere more strongly than the flat SAM gold surface. These results show the combination of tailored electroactive CNTs and microfluidic patterning will allow for the generation of various tailored and patterned nanomaterials for many future studies of biospecific ligand mediated cell behavior.
Figure 6-5. Representative fluorescent micrographs of 3T3 Swiss Albino mouse fibroblasts adhered to C_{16}SH SAM, CNTs and RGD-CNTs. Cells were stained for nuclei (blue), actin (red), and paxillin (green). Images were taken by fluorescence microscopy with a 40X oil lens objective. (a), (b) and (c) Micrographs of cell on C_{16}SH, CNTs, and RGD-CNTs respectively. (d), (e) and (f) Schemes representing the corresponding surfaces of (a), (b) and (c): C_{16}SH, CNT, and RGD-CNT. The cells have striking differences in cell spreading, stress fiber formation and focal adhesion size and structure on the various materials.
For cell migration studies, we examined cell migration rates on the modified carbon nanotube surfaces. Time-lapse microscopy of cell movements on C<sub>16</sub>SH SAM, CNTs, H<sub>2</sub>Q-CNTs and RGD-CNTs were recorded and analyzed using metamorph software. Approximately 40 movies were recorded of cell movements on the microfluidic patterned surfaces. From the comparative analysis, it was found that cells moved much slower on bare CNTs than on a C<sub>16</sub>SH SAM. Cells were observed to migrate the slowest on RGD-CNTs, while they had similar velocities on CNTs and H<sub>2</sub>Q-CNTs. These results imply that the more cell adhesive RGD-CNT surfaces cause cells to migrate more slowly due to the polyvalent nature of the RGD peptide ligand interaction with the integrin receptors of the cell. Interestingly, on control gold SAM surfaces presenting 50% RGD and 50% EG<sub>4</sub>C<sub>11</sub>SH, the cells migrated much faster. Therefore the ligand density, topology or presentation of the RGD on the non-flat CNT surface has a significant effect on the ability of the cell to form focal adhesions, stress fibers, and for lamelapodia and filopodia protrusions necessary for cell migration.
Figure 6-6. Comparison of cell migration rate on the tailored surfaces. Cell migration rates were obtained by time lapse recording of cell movement by phase contrast microscope and processing by metamorph software. For each surface, approximately 40 movies were produced and analyzed to obtain average cell migration rates (24 hours). The columns with different colors represent the migration rate on varying surfaces, including $C_{16}$, CNT, $H_2O$-CNT and RGD-CNT. Cells had the slowest migration rate on the RGD-CNT due to the high adhesivity of the peptide ligands for integrin receptors on the cells.
6.3 Conclusions

In summary, we report a new methodology for chemoselectively tailoring electroactive CNTs with a variety of ligands and applied these CNTs to study biospecific cell adhesion and cell migration. The synthetic tailoring methodology combines π-π stacking and a quantitative electroactive immobilization strategy. By using this method, CNTs can be easily functionalized with a range of ligands, thus allowing powerful control over various chemical and physical properties of CNTs. We also incorporate a new microfluidic technique to directly pattern CNTs onto gold surfaces to create a multiplexed and spatially controlled surface for parallel cell adhesion and migration studies. We characterized the tailored CNTs by cyclic voltammetry, transmission electron microscopy and scanning electron microscopy.

We also investigated cell behavior on the tailored CNTs by examining, with fluorescence microscopy, cell spreading, stress fiber formation, focal adhesion size and structure and cell migration rates. As an added feature, the electroactive immobilization strategy can also selectively release ligands in a suitable reducing environment. This important feature will allow for the potential tailoring of the CNTs and upon delivery to a cell, where the internal cytosolic environment is reducing, release the bound ligand. The ability to routinely functionalize CNTs has wide ranging implications for drug delivery, tissue imaging, tissue scaffolds and as nanomaterials for further cell behavior studies (cell growth, differentiation, adhesion migration, apoptosis). Future experiments will incorporate other peptide ligands and small molecules for cell polarization and stem cell differentiation studies. Finally, the microfluidic patterning technique allows for a powerful method to generate multiplex and high-throughput surfaces for a range of cell based
assays and as a biotechnology platform for patterning tailored CNTs and for subsequent delivery of reagents to cells adhered to the CNTs to study cell behavior.

6.4 Materials and Methods

All chemicals were obtained from Sigma-Aldrich. CNTs were obtained from Carbon Solutions, Inc.

_Synthesis of Pyrene-Terminated Hydroquinone (1) (Py-H$_2$Q)_

1,4-bis(tetrahydro-2H-pyran-2-yl)benzene (3). To a stirred solution of hydroquinone (2) (3.3 g, 30 mmol) and 3,4-dihydro-2H-pyran (DHP, 6.5 g, 77 mM) in dichloromethane (30 ml) was added HCl (0.15 mmol) drop wise and the solution was stirred at room temperature for 2 h. The solution was evaporated under vacuum and the product was extracted with dichloromethane and water. The organic phase was washed with water (3 x 50 ml), dried by Na$_2$SO$_4$, filtered, concentrated and recrystallized in hexanes to yield THP-protected hydroquinone 3 (6.7 g, 81%). $^1$H-NMR (CDCl$_3$): δ 6.96 (d, 4 H), 5.28 (d, 2 H), 3.93 (m, 2 H), 3.58 (m, 2 H), 1.55-1.99 (m, 12 H).

2,2'-(2-(6-bromohexyl)-1,4-phenylene)bis(oxy)bis(tetrahydro-2H-pyran) (4). To a stirred solution of 3 (1.9 g, 6.8 mmol) in 50 ml anhydrous THF was added 1.7 M t-BuLi (5 ml, 8.5 mmol). The solution was kept at 0 ºC for 1 h and warmed to room temperature for 3 h. Then 1,6-dibromohexane (3 ml, 19.5 mmol) was quickly added and the reaction was stirred at room temperature overnight. The solution was evaporated under vacuum. The crude product was then washed with saturated NH$_4$Cl (1 x 50 ml), water (3 x 50 ml), brine (1 x 50 ml), dried by Na$_2$SO$_4$, filtered and concentrated in vacuo and separated by column
chromatography with 10:1 hexane/ethyl acetate to afford 2.5 g (83%) of colorless oil 4. $^1$H-NMR (CDCl$_3$): $\delta$ 6.95 (m, 1H), 6.81 (m, 2H), 5.27 (m, 2H), 3.89 (m, 2H), 3.56 (m, 2H), 3.37 (t, 2H), 2.57 (t, 2H), 1.35-1.99 (m, 20H).

2-(6-(2,5-bis(tetrahydro-2H-pyran-2-yloxy)phenyl)hexyl)isoindoline-1,3-dione (5). To a stirred solution of potassium phthalimide (1.89 g, 10.2 mmol) in 15 ml DMF at 80 ºC was added a solution of 4 (1.5 g, 3.4 mmol) in 5 ml DMF. The resulting mixture was maintained at this temperature overnight. The reaction mixture was evaporated under high vacuum to get rid of DMF and then dissolved in 30 ml CH$_2$Cl$_2$. The crude product was washed with saturated NH$_4$Cl (1 x 30 ml), water (2 x 30 ml), brine (1 x 30 ml), dried by Na$_2$SO$_4$, filtered and concentrated in vacuo, and separated by column chromatography with 10:1 hexane/ethyl acetate to yield 1.5 g (87%) of yellow oil 5. $^1$H-NMR (CDCl$_3$): $\delta$ 7.83 (m, 2 H), 7.72 (m, 2 H), 6.95 (m, 1H), 6.81 (m, 2H), 5.27 (m, 2H), 3.89 (m, 2H), 3.68 (t, 2 H), 3.56 (m, 2H), 2.57 (t, 2H), 1.25-1.99 (m, 20H).

6-(2,5-bis(tetrahydro-2H-pyran-2-yloxy)phenyl)hexan-1-amine (6). To a stirred solution of 5 (0.6 g, 1.2 mmol) in 10 ml CH$_2$Cl$_2$ was added a solution of H$_2$N-NH$_2$ (1 M in THF, 5 ml). The reaction mixture was stirred at room temperature for 2 h. The resulting residue was filtered and evaporated by vacuo to afford 0.41 g (92%) of yellow oil 6. $^1$H-NMR (CDCl$_3$): $\delta$ 6.87 (m, 1H), 6.70 (m, 2H), 5.15 (m, 2H), 3.80 (m, 2H), 3.48 (m, 2 H), 3.56 (m, 2H), 2.56 (t, 2H), 2.45 (t, 2H), 1.25-1.87 (m, 20H).

2,5-dioxopyrrolidin-1-yl-4-(pyren-1-yl)butanoate (8). To a stirred solution of 1-pyrenebutanoic acid (7) (0.5 g, 1.7 mmol) and N-hydroxysuccinimide (0.58 g, 5.0 mmol) in DMF (20 ml) at 0 ºC was added dicyclohexylcarbodiimide (1.3 g). The reaction mixture was stirred for 3 h. The resulting residue was filtered, concentrated by vacuo and separated by
column chromatography with 10:1 hexane/ethyl acetate to yield 0.52 g (78%) of 8. $^1$H-NMR (CDCl$_3$): $\delta$ 7.88-8.28 (m, 9 H), 3.48 (t, 2 H), 2.88 (m, 4 H), 2.73 (t, 2 H), 2.32 (m, 2 H).

N-(6-(2,5-bis(tetrahydro-2H-pyran-2-yloxy)phenyl)hexyl)-4-(pyren-1-yl)butanamide (6).

Molecule 8 (0.18 g, 0.47 mmol) and 6 (0.2 g, 0.53 mmol) were dissolved in CH$_2$Cl$_2$ (10 ml) and stirred at room temperature overnight. The reaction mixture was evaporated under vacuum and separated by column chromatography with 10:1 hexane/ethyl acetate to yield 0.25 g (83%) of brown oil 9. $^1$H-NMR (CDCl$_3$): $\delta$ 7.83-8.28 (m, 9 H), 6.95 (m, 1H), 6.80 (m, 2H), 5.25 (m, 2 H), 3.89 (m, 2H), 3.56 (m, 2H), 3.39 (t, 2H), 3.20 (q, 2 H), 2.59 (t, 2H), 2.16-2.25 (m, 4 H), 1.25-2.01 (m, 20H).

N-(6-(2,5-dihydroxyphenyl)hexyl)-4-(pyren-1-yl)butanamide (1).

Molecule 9 (0.2 g, 0.31 mmol) was dissolved in a mixed solution (30 ml) of acetic acid, THF and H$_2$O (3:1:1). The reaction mixture was stirred at room temperature for 3 h. The resulting mixture was evaporated under vacuum, extracted with ethyl acetate/H$_2$O and separated by column chromatography with 9:1 hexane/ethyl acetate to yield 0.13 g (88%) of dark brown oil 1. $^1$H-NMR (C$_3$D$_6$O): $\delta$ 7.88-8.39 (m, 9 H), 6.48-6.67 (m, 3 H), 3.36 (t, 2H), 3.21 (q, 2H), 2.53 (t, 2 H), 2.31 (t, 2 H), 2.12 (m, 2 H), 1.25-1.62 (m, 8 H).

Solid-Phase Peptide Synthesis of GRGDS-oxyamine (12) (RGD-ONH$_2$).

Oxyamine functionalized GRGDS peptide (RGD-ONH$_2$) was synthesized using an automated peptide synthesizer (CS Bio) as previously described.$^{48-55}$ Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acid was used with Fmoc-Ser(tBu)-Rink Amide-MBHA resin. The synthesized peptide was cleaved from the resin by agitating in a solution of trifluoroacetic acid (TFA):water:triisopropylsilane (95:2.5:2.5) for 3 h. Excess
TFA was evaporated and the cleaved peptide was precipitated in cold diethyl ether. The water-soluble peptide was extracted with water and lyophilized. MS (ESI) (m/z): [M+H⁺] calculated for linear RGD-ONH₂ (C₂₅H₄₅N₁₁O₁₁), 676.69; found, 676.5.

Carbon nanotube (CNT) Functionalization

A mixture of a 1 mM methanol solution of Py-H₂Q and 1 mg/mL methanol suspension of CNTs (Carbon Solutions, Inc.) was sonicated for 1 h, and then stirred overnight. The resulting dark suspension was centrifuged to remove aggregates. The supernatant was collected and the excess Py-H₂Q was removed by repeated filtration through a centrifugal filter device (10k Da MWCO, Millipore Amicon) and extensive washing with methanol.

Microfluidic Lithography

Specially designed PDMS microfluidic cassette was fabricated by soft lithography as previously described.⁶⁹,⁷⁰ After placing the PDMS cassette in direct contact with a bare gold substrate, an 1 mM ethanol solution of C₁₀SH or 0.5 mg/mL methanol suspension of CNTs/RGD-CNTs were flowed through the channels. After drying for a few seconds, the microfluidic channels were rinsed with ethanol and then the PDMS cassette was removed.

Electrochemistry

All electrochemical experiments were performed using a Bioanalytical Systems CV–100W potentiostat. Electrochemistry on SAMs was performed in 1M HClO₄ using a platinum wire as the counter electrode, Ag/AgCl as reference electrode, and ITO substrate as the working electrode. All cyclic voltammograms were scanned at 100 mV/s.
**Cell Culture and Microscopy**

3T3 Swiss Albino mouse fibroblasts were seeded on the patterned substrate, incubated overnight in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) with 10% bovine calf serum and 1% penicillin/streptomycin. After washing with Dulbecco’s PBS buffer (Sigma, St. Louis, MO), the patterned cells were fixed with 3.2% formaldehyde for 15 minutes and then permeated with 0.1% Triton X–100 for 10 minutes. A combination of fluorescent dyes were used to visualize the fibroblasts and show the focal adhesion: DAPI (4’,6-diamidino-2-phenylindole dihydrochloride, Sigma, St. Louis, MO), for the nucleus, phalloidin-tetramethylrhodamine B isothiocyanate (Sigma, St. Louis, MO) for the F-actin cytoskeleton, anti-paxillin, (BD Biosciences, San Jose, CA) with a fluorescent tagged secondary antibody (Cy-2 conjugated goat anti-mouse IgG, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for focal adhesion position. Fluorescence images were taken using a Nikon Eclipse TE2000-E inverted microscope (Nikon USA, Inc., Melville, NY).

**Scanning Electron Microscopy of Cells**

Cell samples were washed with PBS and fixed with 3.2% formaldehyde for 30 min. After washing with water, samples were dehydrated stepwise in 30%, 50%, 70%, 90% and 100% ethanol for 30 min respectively. The samples were then dried by the critical point drying technique and sputtered with 2 nm gold, before taking SEM image.
6.5 REFERENCES


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CHAPTER 7

Transferring Surface Chemistry Strategy to Indium Tin Oxide (ITO) for Cell Studies

7.1 Introduction

Strategies to tailor materials have proven to be important for a variety of research fields ranging from heterogeneous catalysis, high throughput microarrays, molecular electronics to designing new tissue engineering platforms.\(^1\)\(^5\) The most common materials for biointerfacial studies are based on either glass (siloxane) or gold (conductive) surfaces.\(^6\)\(^7\) Both of these surfaces have revolutionized the use of materials for small molecule microarrays, cell based assays as well as a variety of diagnostic biosensor technologies.\(^6\)\(^\)\(^-\)\(^1\)\(^1\) Glass surfaces are robust and commonly used as a material for cell biological studies due to its biocompatibility, surface chemistry modification, patterning and optical transparency.\(^1\)\(^2\) The major limitations are the difficult synthetic strategies to tailor the surface and few in-situ surface characterization techniques to study dynamic interfacial associations that are only available for conductive surfaces. Due to the flexibility of surface chemistry and conductivity, gold has been widely used for biointerfacial studies and as a platform for many biotechnologies.\(^7\) However, due to golds efficient quenching of fluorescence, limited optical transparency and lack of long term stability, it has found limited use in practical cell biological and biosensor applications.\(^1\)\(^3\)\(^,\)\(^1\)\(^4\)
We believe, a material that combines the advantages of both glass and gold for biointerfacial studies that has been relatively unexplored and undervalued is indium tin oxide (ITO). ITO substrates have been widely used in optoelectronic applications that require both high transparency and good conductivity, such as liquid crystal displays, organic light-emitting diodes and solar cells. Although, there are distinct advantages of using ITO, the surface chemistry to tailor the material is difficult and relies on weak carboxylic acid linkages or siloxane or phosphonate linkages with varying stability in different pH ranges. There have been few studies to take advantage of the inherent ITO surface properties for biosensor or cell biological studies.

Herein, we install an electroactive chemoselective self-assembled monolayer strategy on ITO surfaces to generate 1. Robust renewable surfaces and 2. Biospecific surfaces for live cell high resolution fluorescence microscopy of cell culture. We also compare the surface chemistry properties on both ITO and gold surfaces for applications in fluorescent biosensing and cell biological studies. We have previously shown the utility of a quantitative electroactive interfacial oxime reaction to immobilize ligands, proteins and cells on gold surfaces in patterns and gradients. We also used this strategy to generate dynamic surfaces, molecularly controlled gradient surfaces and nanopatterned surfaces to study cell adhesion, cell polarity and cell migration. In combination with microfluidic technology, we have generated patterned SAM and partially etched patterned SAM surfaces for co-culture and directed cell polarity studies with a new polarity sensing cell line.
Figure 7-1. Scheme of renewable tailored electroactive Indium Tin Oxide (ITO) surface. The ITO surface is coated with a redox active hydroquinone that can be reversibly oxidized to the quinone form for a subsequent interfacial oxime reaction with soluble oxyamine tethered ligands (RONH₂). The oxime is also redox active and can undergo a reversible redox process at low pH 1M HClO₄ (pH = 0). However, electrochemical reduction [Red] of the oxime in PBS buffer at pH 7 spontaneously reverts the oxime to the original hydroquinone via release of the immobilized ligand.
7.2 Results and Discussion

The scheme in Figure 7-1 describes the re-usable immobilization and release strategy on a conductive and transparent ITO surface. A hydroquinone-phosphonate SAM is generated on the ITO surface and reversibly oxidized to the quinone. The quinone form can react chemoselectively with soluble oxyamine-tethered ligands to generate a stable covalent interfacial oxime linkage. The bound ligand can also be released by application of a mild reductive potential to re-generate the hydroquinone. Based on this strategy, multiple rounds of ligand immobilization and release can be achieved on ITO surfaces.

To control and characterize the immobilization and release of ligands from the electroactive ITO surface we used electrochemistry where the ITO is the working electrode. Figure 7-2 shows the structures of the redox active hydroquinone/quinone couple and subsequent redox active oxime conjugate with the corresponding cyclic voltammograms. By using cyclic voltammetry each step of the immobilization and release can be quantitatively monitored and controlled in real time in-situ. For these studies, we used the model ligand aminooxy acetic acid. After oxidation of the hydroquinone to the quinone (Figure 7-2A) and reaction with aminooxy acetic acid, a stable oxime product is formed. The oxime conjugate is also redox active but with distinct diagnostic peaks to distinguish from the hydroquinone/quinone redox pair (Figure 7-2B). By application of a redox potential at pH 7.0 in PBS, we noticed the spontaneous breakdown of the oxime conjugate where the ligand is released with re-generation of the hydroquinone (Figure 7-2C). This reversion to the hydroquinone surface allows for subsequent rounds of immobilization and release (Figure 7-2D). This strategy, in combination with microfabrication techniques, may be used as an
Figure 7-2. Electrochemical characterization and control of the interfacial immobilization and release of ligands on ITO surfaces by cyclic voltammetry (CV). The chemical schemes (left panel) correspond to the CV characterization (right panel). (A) The redox active hydroquinone monolayer on ITO undergoes reversible oxidation-reduction between hydroquinone and corresponding quinone. (Ox 330 mV, Red -185 mV) (B) By introducing a soluble oxyamine tethered ligand (aminooxy acetic acid) to the monolayer presenting quinone, a stable redox active interfacial oxime conjugate product is formed with new diagnostic peaks in the CV (Ox 130 mV, Red 15 mV). (C) To release the ligand from the surface and regenerate the hydroquinone, the pH was increased to 7.0 and a reductive potential was applied. The oxime conjugate spontaneously decayed and released the ligand ($k = 0.011 \text{ s}^{-1}$). (D) The regenerated hydroquinone can perform several more rounds of immobilization/release with a variety of oxyamine-tethered ligands.
optically transparent renewable surface for multiple rounds of immobilization and release of
different ligands for a variety of cell biological or biosensor applications. This method may
also be used as a new platform for renewable small molecule, carbohydrate, peptide or
DNA/RNA microarrays.
Figure 7-3. Comparison of ITO and gold surfaces robustness to generate renewable and reusable surfaces for multiple rounds of ligand immobilization and release. Y axis represents percentage of immobilized ligand and X axis represents number of immobilization and release cycles. HQC_{11}SH monolayer coated gold and HQ-PA monolayer coated ITO surfaces were both electrochemically oxidized and reacted with 1M aminooxy acetic acid for one hour to near completion. The oxime conjugate ligand was released by application of a reducing potential at -450 mV for 1 minute in PBS (pH 7.0). The surfaces were then cycled repeatedly and the amount of immobilization and release was determined by cyclic voltammetry. The gold monolayers were able to cycle approximately 3 times to produce functional monolayers and then rapidly deteriorated. The ITO monolayers were much more robust and were able to cycle more than 10 times to generate functional monolayers. Note: functional monolayers refers to the ability of the surface to present ligands without deterioration of the monolayer.
To validate the use of ITO surfaces as a platform for bio-immobilization we compared the robustness of the immobilization/release strategy with gold SAM surfaces. Hydroquinone SAMs were installed on both Gold and ITO surfaces and multiple rounds of immobilization and release of oxyamine acetic acid was performed. Each surface was reacted with oxyamine acetic acid for 1 hr to generate the oxime conjugate. The rate constants for the gold and ITO surfaces were approximately the same (k = 0.05 min\(^{-1}\)). The rounds of immobilization and release were performed on both surfaces with the same solution conditions (oxyamine acetic acid 1 mM for 1 hr) and electrochemical potentials (−400 mV to 800 mV) and scan rate (100 mV/s). The data in Figure 3 shows that ITO surfaces are much more robust and can perform at least 10 rounds before degradation while gold SAMs degrade after only 3 rounds. It should also be noted that the ITO SAMs can be left in ambient conditions for weeks with little degradation whereas gold SAMs degraded rapidly within 1 week due to the weaker gold-sulfur interaction and spontaneous gold oxidation. Furthermore the ITO SAM surfaces have a much greater electrochemical redox potential range than gold SAM surfaces. We were able to use a +2.2 V to −1.0 V window for the ITO surfaces without loss of SAM function whereas there was significant loss or damage to the gold SAM surfaces beyond +1.1 V or -850 mV. These direct comparisons clearly show ITO surfaces are superior in terms of durability and assay robustness than gold surfaces as a renewable material.

In order to use this ITO surface modification system for biointerfacial studies and potential use as a biosensor, we tailored the surfaces for biospecific cell adhesion studies. A major research tool to study internal cell signaling and cell motility behavior is the use of live-cell high resolution fluorescence microscopy. These powerful fluorescent microscopy
techniques are difficult to implement on gold SAM surfaces due to thin films of gold not being completely optically transparent and the very efficient gold quenching of fluorophores. Due to these major limitations and the fact that ITO is optically transparent and therefore superior to gold SAM surfaces for cell biological behavior studies requiring optical microscopy techniques such as fluorescence, TIRF and FRET we interfaced the tailored ITO surfaces with a stably transfected fluorescent cell line. Our first study was to make the ITO surfaces inert to non-specific protein adsorption or cell attachment, a crucial requirement for biospecific cell adhesion, cell migration studies and biosensor applications. We synthesized an oxyamine-ethyleneglycol and immobilized it to an ITO surface (200 mM, 9 hrs) presenting a full monolayer of quinone groups (supporting information). Addition of cells (50,000 cells/ml) showed almost no cell attachment, indicating the surface had been functionalized with the ethyleneglycol group and rendered inert.

To show biospecific cell adhesion we immobilized a RGD-oxyamine ligand to the electroactive ITO surfaces. The RGD motif is the minimum ligand known to facilitate cell adhesion by interaction with cell surface integrin receptors. Figure 4 shows a comparison of the ITO SAM and Gold SAM surfaces for biospecific cell adhesion characterized by fluorescence microscopy. A stable rat2 cell line containing GFP-actin is visible in the fluorescence mode of the microscope on ITO surfaces where on gold the cells are invisible. As controls, immobilization of a scrambled RDG peptide or no peptide showed no cell attachment to the surfaces. Furthermore, cells could be detached from the RGD presenting surfaces by addition of soluble RGD (1 mM, 30 min).
Figure 7-4. Comparison of fluorescent micrographs of live-cell GFP-actin transfected Rat 2 fibroblasts on ITO and gold surfaces presenting the biospecific RGD peptide. (Left) Brightfield image and corresponding fluorescence image of the same cells on ITO surface. (Right) Bright field image and corresponding fluorescence image of the same cells on gold surface. ITO is optically transparent and amenable to live-cell high resolution fluorescence microscopy whereas gold surfaces are much more efficient at quenching fluorescence and not ideal surfaces for live-cell high resolution fluorescence microscopy monitoring of cell behavior.
7.3 Conclusion

In conclusion, we have successfully transferred a sophisticated electroactive immobilization and release strategy to ITO surfaces. We demonstrated the ITO surfaces are superior to gold as a renewable surface, robustness (durability) and as an optically transparent material for live-cell high resolution fluorescence microscopy. These advantages will make ITO surfaces a desired platform for numerous biosensor, microarray and model surfaces for cell biological studies. Until now, ITO surface chemistry manipulation was difficult and more complex than glass or gold surface systems due to the limited synthetic chemistry strategies to generate phosphonate linked molecules and slow kinetics to generate monolayers. Ongoing studies will aim to generate microcontact printed features, gradients of ligands and model surfaces for studies of cell behavior. ITO surfaces may also be combined with other surface technologies such as MALDI mass spectrometry and high throughput fluorescence detection microarray strategies to generate novel hybrid devices for a variety of applications ranging from molecular electronics to tissue engineering.

7.4 Materials and Methods

All chemicals were obtained from Sigma-Aldrich. Indium tin oxide-coated glass slides (1"x 3"x 1.1 mm, 10 Ohm/sq) were obtained from NANOCs.
Scheme 7-1. Synthesis of HQ-PA

\[ \text{Scheme 7-1. Synthesis of HQ-PA} \]
**1,4-bis(tetrahydro-2H-pyran-2-yloxy)benzene (6)** To a stirred solution of hydroquinone (3.3 g, 30 mM) and 3,4-dihydro-2H-pyran (DHP, 6.5 g, 77 mM) in dichloromethane (30 ml) was added HCl (0.15 mM) drop wise and the solution was stirred under room temperature for 2 hours. The solution was evaporated under vacuum and the product was separated between dichloromethane and water. The organic phase was washed by water (3 x 50 ml), dried by Na₂SO₄, filtered, concentrated and recrystallized in Hexane to yield THP protected hydroquinone 6 (6.7 g, 81%). ¹H-NMR (CDCl₃): δ 6.95 (d, 2H), 6.81 (d, 2H), 3.89 (m, 2H), 3.56 (m, 2H), 1.55-1.99 (m, 12H).

**2,2′-(2-(6-bromohexyl)-1,4-phenylene)bis(oxy)bis(tetrahydro-2H-pyran) (7)** To a stirred solution of 6 (1.9 g, 6.8 mmol) in 50 ml anhydrous THF was added 1.7 M t-BuLi (5 ml, 8.5 mmol). The solution was kept at 0 ºC for the first hour and left to room temperature for 3 hours. Then 1,6-dibromohexane (3 ml, 19.5 mmol) was quickly added and the reaction was kept under room temperature overnight. The solution was evaporated under vacuum. The crude product was then washed by saturated NH₄Cl (1 x 50 ml), water (3 x 50 ml), brine (1 x 50 ml), dried by Na₂SO₄, filtered and concentrated in vacuo and separated by column chromatography with 10:1 hexane/ethyl acetate to afford 2.5 g (83%) of colorless oil. ¹H-NMR (CDCl₃): δ 6.95 (m, 1H), 6.81 (m, 2H), 5.27 (m, 2H), 3.89 (m, 2H), 3.56 (m, 2H), 3.37 (t, 2H), 2.57 (t, 2H), 1.35-1.99 (m, 20H).

**Diethyl 6-(2,5-dihydroxyphenyl)hexylphosphonate (8)** A solution of compound 7 (1 g, 2.27 mM) in 15 ml of triethyl phosphate (87.5 mM) was refluxed overnight under nitrogen. Excess triethyl phosphate was removed in vacuo and the crude product was purified by
column chromatography with 1:1 hexane/ethyl acetate, yielding a thick colorless oil (0.5 g, 67%). \(^1\)H-NMR (CDCl\(_3\)): \(\delta\) 6.59-6.68 (m, 3H), 4.03-4.09 (m, 4H), 2.57 (t, 2H), 1.27-1.81 (m, 10H).

**2-(1,4-dihydroxybenzene)-hexyl phosphonic acid (HQ-PA) (1)** To a stirred solution of 8 (0.2 g, 0.6 mmol) in 10 ml dry dichloromethane was added SiMe\(_3\)Br (0.8 ml, 6.0 mmol) drop wise under nitrogen. The reaction was kept under room temperature for 6 hours. Then unreacted SiMe\(_3\)Br and solvent were evaporated under vacuum to yield a yellowish oil. The oil was dissolved in 10 ml methanol and stirred for 2 hours followed by removing the methanol under vacuum. The crude product was dissolved in methanol and recrystallized from diethylether to yield 13.5 mg (82 %). \(^1\)H-NMR (CDCl\(_3\)): \(\delta\) 6.59-6.68 (m, 3H), 2.57 (t, 2H), 1.21-1.92 (m, 10H).

**Solid-Phase Peptide Synthesis of GRGDS-oxyamine (2).** Oxyamine functionalized GRGDS peptide was synthesized using a peptide synthesizer (CS Bio) as described before.\(^1\) Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids were used on Fmoc-Ser(tBu)-Rink Amide-MBHA resin. Synthesized peptide was cleaved from the resin by agitating in a solution of trifluoroacetic acid (TFA):water:triisopropylsilane (95:2.5:2.5) for 3 hours. TFA was evaporated and the cleaved peptide was precipitated in cold diethyl ether. The water-soluble peptide was extracted with water and lyophilized. Mass spectral data confirmed the peptide product. MS (ESI) (m/z): [M+H\(^+\)] calculated for linear RGD-oxyamine (C\(_{25}\)H\(_{45}\)N\(_{11}\)O\(_{11}\)), 676.69; found, 676.5.

134
Scheme 7-2. Synthesis of $C_{11}ONH_2$
2-(undec-10-enyloxy)isoindoline-1,3-dione (9) To a stirred solution of N-hydroxyphthalimide (3.15 g, 19.3 mmol) in 20 ml DMF was added NaHCO₃ (1.62 g, 19.3 mmol). The mixture was kept at 80 °C for around half an hour until it turns to dark brown. Then 11-bromoundecene (3.0 g, 12.9 mmol) was added drop wise and the reaction was kept under 80 °C overnight. The mixture was filtered and evaporated under vacuum. The crude product was dissolved in ethyl acetate and washed by saturated aqueous NH₄Cl, water, brine, dried by Na₂SO₄ and concentrated under vacuum. Then the product was purified by column chromatography with 1:4 ethyl acetate / hexane, yielding a colorless oil (3.2 g, 79%). ¹H NMR (CDCl₃): δ 7.82 (m, 2H), 7.72 (m, 2H), 5.80 (m, 1H), 4.95 (m, 2 H), 4.17 (t, 2 H), 2.03 (m, 2H), 1.77 (m, 2 H), 1.32 (m, 2 H), 1.23-1.28 (br s., 10 H).

O-(undec-10-enyl)hydroxylamine (4) To a stirred solution of 9 (2.6 g, 8.2 mmol) in DCM (20 ml) was added a solution of 1M hydrazine in THF (25 ml, 25 mmol) and the mixture was stirred at room temperature for 3 hours. The reaction mixture was filtered and concentrated under vacuum. The product was purified by column chromatography with 1:2 ethyl acetate / hexane, yielding a colorless oil (1.26 g, 82%). ¹H NMR (CDCl₃): δ 5.80 (m, 1H), 4.95 (m, 2 H), 3.65 (t, 2H), 2.03 (m, 2H), 1.55 (m, 2H), 1.32 (m, 2 H), 1.23-1.28 (br s, 10 H).
Scheme 7-3. Synthesis of TEGONH₂
2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (10) To a stirred solution of tetra(ethylene glycol) (25.5 g, 131 mmol) in 60 ml THF was added triethylamine (18.3 ml, 131 mmol). Then a solution of tosyl chloride (10 g, 52.5 mmol) in 20 ml THF was added to the reaction mixture drop wise and the reaction was kept at room temperature over night. The reaction mixture was filtered and concentrated under vacuum. The crude product was washed by saturated aqueous NH₄Cl, water, brine, dried by Na₂SO₄, and purified by column chromatography with 1:3 hexane / ethyl acetate, yielding a colorless oil (13.9 g, 76%). ¹H NMR (CDCl₃): δ7.77 (d, 2H), 7.32 (d, 2H), 4.13 (t, 2H), 3.58-3.70 (m, 14H), 2.42 (s, 3H), 2.22 (br s, 1H).

2-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethoxy)isoindoline-1,3-dione (11) To a stirred solution of N-hydroxyphthalimide (9.0 g, 55.1 mmol) in 30 ml DMF was added NaHCO₃ (4.6 g, 55.1 mmol). The mixture was kept at 80 ºC for around half an hour until it turns to dark brown. Then a solution of 10 (9.6 g, 27.6 mmol) in 10 ml dichloromethane was added drop wise and the reaction was kept under 80 ºC overnight. The mixture was filtered and DMF was removed under vacuum. The crude product was dissolved in dichloromethane and washed by saturated NH₄Cl, water, brine, dried by Na₂SO₄ and concentrated under vacuum. Then the product was purified by column chromatography with 1:1 ethyl acetate / hexane, yielding a colorless oil (6.7 g, 72%). ¹H NMR (CDCl₃): δ7.99 (br s, 1H), 7.81 (m, 2H), 7.72 (m, 2H), 4.35 (t, 2H), 3.83 (t, 2H), 3.55-3.70 (m, 12H).

2-(2-(2-(2-(aminooxy)ethoxy)ethoxy)ethoxy)ethanol (5) To a stirred solution of 11 (3.6 g, 10.6 mmol) in DCM (20 ml) was added a solution of 1M hydrazine in THF (30 ml, 30 mmol)
and the mixture was stirred at room temperature for 3 hours. The reaction mixture was filtered and concentrated under vacuum. The product was purified by column chromatography with 3:1 ethyl acetate / hexane, yielding a yellowish oil (1.93 g, 87%). $^1$H NMR (CDCl$_3$): $\delta$3.79 (t, 2H), 3.52-3.76 (m, 14H). ESI mass H2O calcd 209.1, found 209.1.

**SAM formation on ITO**

ITO slides were boiled for 15 minutes each in dichloromethane, acetone and ethanol sequentially. Then the freshly cleaned ITO slides were placed in an aqueous solution of HQ-PA (0.3 mM) at RT, for 16 hours.

**Electrochemical activation and characterization on ITO**

All electrochemical experiments were performed using a Bioanalytical Systems CV–100W potentiostat. Activation and characterization of the monolayer on ITO were performed in 1M HClO$_4$ or Phosphate Buffered Saline (PBS) pH 7.0 using a platinum wire as counter electrode, Ag/AgCl as reference electrode and ITO/SAM as working electrode. All cyclic voltammograms were scanned at 100 mV/s.

**Contact Angle Measurement**

Contact angle measurement was performed on functionalized ITO substrates to study the hydrophobicity of the surface after modification by certain ligand immobilization. The contact angle measurements were repeated 8 times from different water droplets, and the reported angles are the average values.
Cell Culture

GFP-cells (Rat 2 Actin Grean Fluorescent Protein) were maintained in Dulbecco’s Modified Eagle Medium supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO2 atmosphere. The cells were detached by treating with a solution of 0.05% trypsin/0.53 mM EDTA for 3-5 minutes in the incubator (37 °C in a humidified 5% CO2 atmosphere). Then serum containing media was added and cells were precipitated by centrifugation at 800 rpm for 5 minutes. The cell pellet was resuspended in serum-free medium and diluted to ~10000 cells/mL) and added onto the substrates for 2 h before transferred to serum-containing media.

Scanning Electron Microscopy of Cells

Cell samples were washed with PBS and fixed by 10% formalin for 30 minutes. After discarding the formalin solution and washing with water, samples were dehydrated stepwise in 30%, 50%, 70%, 90% and 100% ethanol for 30 minutes. After using a critical point drying technique and sputtering 2 nm gold, the samples are ready for SEM imaging.
7.5 REFERENCES


8.1 General Conclusion

The research described in this dissertation is focused on developing surface chemistry strategy and building surface system to investigate cell-surface interactions and surface effects on cellular behaviors, including cell adhesion, cell migration and stem cell differentiation. The surface system is based on self-assembled monolayers (SAMs) on different materials including planar gold, gold nanorods, indium tin oxide (ITO), and carbon nanotubes (CNTs). On the specially fabricated surfaces, cell adhesion, migration and stem cell differentiation have been studied. In chapter 2 and chapter 3, electroactive and quantitative microarrays have been developed and applied to study stem cell differentiation. By making a microarray of various alkanethiols SAMs on gold substrate, high-throughput study of surface chemistry effects on cellular behaviors can be performed. In chapter 4, microcontact printing strategy was employed to fabricate surfaces with spatial and geometric control in order to study the effects of cell population and surface geometry on stem cell differentiation. In chapter 5, we transferred our electroactive and chemoselective conjugation method onto gold nanorods to produce tailored electroactive nanorods for studying the combined effects of surface chemistry and surface topography on cell behaviors. In chapter 6
and chapter 7, our sophisticated chemoselective conjugation method was applied to ITO and CNTs to study cell adhesion and migration on tailored materials.

8.2 Future Directions

Future studies are aimed at two directions. First, based on the electroactive and quantitative microarray strategy, a large library of biomolecules can be installed onto one surface in microarray format to study surface effects on various cell behaviors, such as cell adhesion, migration, proliferation, apoptosis, and stem cell differentiation. In this work, only adipogenic differentiation from hMSCs has been studied. In the future, other differentiation lineages of hMSCs, as well as other stem cells, will be investigated by the microarray strategy. Furthermore, the microarray strategy also provides a direct writing tool to conveniently pattern SAMs and cells in any precisely controlled locations. By patterning different surfaces, selective cell adhesion and co-culture study can be accomplished. Second, transferring the electroactive and chemoselective immobilization strategy onto different types of materials, including gold nanorods, gold colloid, indium tin oxide (ITO), titanium oxide, carbon nanotubes (CNTs), graphene, and polymers, will lead to a series of tailored materials platforms that can be used in studying cell-materials interactions as well as electronics studies.