EFFECT OF SURFACE FUNCTIONAL GROUPS ON CHONDROCYTE BEHAVIOR

USING MOLECULAR GRADIENTS

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EFFECT OF SURFACE FUNCTIONAL GROUPS ON CHONDROCYTE BEHAVIOR

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Recently, efforts addressing osteoarthritis (OA) have focused on preserving the host joint by addressing the underlying mechanical changes in cartilage during OA progression. There are the limitations to using OA chondrocytes including low numbers and the metabolic imbalance between expressions of catabolic matrix cytokines. For progression to be successful, it is necessary for implants to develop optimal surface conditions to facilitate expansion of cell number and bioactivity while preserving the narrow cellular phenotype. Initial studies have shown that competitive surface-engineering approaches such as soluble protein signaling factors and surface chemistry system have a profound effect on lineage commitment and cellular function. Collectively, functionalized amine and hydroxyl surfaces have been studied and found to promote stem cell viability, cell adhesion and migration. Unfortunately, chemical concentrations have not been widely examined even though the cell behavior has been shown strongly influenced by the concentration of chemical functionality. Herein we examined the effects of surface functional group concentrations, examining chondrocyte proliferation and phenotype maintenance within continuously variable one-dimensional methyl/amine/hydroxyl concentration gradients. The gradients were fabricated using the “vacuum away” confined channel vapor deposition method which is highly tunable and
versatile. Cell number and phenotype maintenance were found to vary along the chemical gradients. Thus spatial presentation of surface chemistry shows a significant impact in modulating human osteoarthritic chondrocyte behavior.
DEDICATION

This master thesis is dedicated to my parents, my advisor, Dr. Becker, my group members and all my friends who helped me tremendously.
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# TABLE OF CONTENTS

LIST OF FIGURES................................................................................................................. ix

CHAPTER

I. INTRODUCTION ................................................................................................................ 1

1.1 Interaction Between Cells And Surface Chemistry ...................................................... 2

1.2 Surface Modification of Biomaterials .......................................................................... 5

1.3 Gradient Fabrication ................................................................................................. 8

1.4 Surface Analytical Techniques .................................................................................... 15

1.5 Osteoarthritis (OA) ................................................................................................. 17

II. EXPERIMENTAL SECTION .......................................................................................... 21

2.1 Materials .................................................................................................................. 21

2.1.2 Chemical Reagent ............................................................................................... 21

2.1.2 Cell Culture Supplements ..................................................................................... 22

2.1.3 Cell Fixation, Fluorescence Staining Agents ....................................................... 22

2.2 Contact Angle Measurement ..................................................................................... 23

2.3 X-ray Photoelectron Spectroscopy (XPS) ................................................................. 23

2.4 Fluorescence Microscopy ......................................................................................... 24

III. FABRICATION OF ONE-DIMENSIONAL CONCENTRATION GRADIENTS BY VAPOR DEPOSITION ...................................................................................................................... 25

3.1 Experimental Section ............................................................................................... 25

3.1.1 Methyl-terminated Gradient Fabrication ............................................................. 25
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1: Light micrographs of endothelial cells attached to the surface of hydrogels without RGDS (A), and hydrogels with 5.0 mM Acr-PEG-RGDS (B). Reprinted from Langer et al.\textsuperscript{14} with permission from the American Chemical Society. Copyright (2004)</td>
<td>4</td>
</tr>
<tr>
<td>1.2: Schematic illustration of UV-ozone treatment to generate gradient immobilization and further “click” reaction. DMAP: 4-methylaminopyridine; DIC: diisopropylcarbodiimide; DMSO: dimethylsulfoxide. Reprinted from Becker et al.\textsuperscript{32} with permission from the WILEY-VCH. Copyright (2007)</td>
<td>10</td>
</tr>
<tr>
<td>1.3: Device schematic. (a) Reservoirs are loaded in one side of Teflon insert. (b) Insert is loaded into the deposition chamber. (c) The cross-section of reservoir and substrate (d) dynamic vacuum is pulled from one side of the insert. Reprinted from Epps et al.\textsuperscript{8} with permission from the American Chemical Society. Copyright (2009)</td>
<td>12</td>
</tr>
<tr>
<td>1.4: (a) The fabrication scheme for functional self-assembled monolayer concentration gradients using a confined channel diffusion method. (b) A two-step vapor deposition strategy to fabricate orthogonal concentration gradient, enabling a “dual-click” reaction. Reprinted from Becker et al.\textsuperscript{34} with permission from the American Chemical Society. Copyright (2013)</td>
<td>14</td>
</tr>
<tr>
<td>1.5: Differences in the physiologic environment, metabolic rate, and cellular make-up of bone and cartilage have profound effects on the potential to engineer these tissues\textsuperscript{41}. Reprinted from Athanasiou et al with permission from the American Association for the Advancement of Science (2012)</td>
<td></td>
</tr>
<tr>
<td>3.1: Scheme of vapor deposition device for concentration gradient using confined channels.</td>
<td>26</td>
</tr>
<tr>
<td>3.2: Reaction scheme of hydroxyl and amine functionalized surface. (a) The amine end group was connected to the surface via phosphanimine hydrolization. (b) The hydroxyl end group was connected onto the surface via SN\textsubscript{2} substitution of the chlorine with azide and alkyne-azide cycloaddition. DMF: N, N-Dimethylformamide; RT: room temperature; PPh\textsubscript{3}: Triphenylphosphine</td>
<td>30</td>
</tr>
</tbody>
</table>
3.3: Reaction scheme for coupling YIGSR peptide to vinyl-terminated gradient substrates via thiol-ene “click” reaction..........................................................................................................................32

3.4: Reaction scheme of PEG-like Fmoc peptide synthesis. PEG-like Fmoc peptide was prepared for modification of YIGSR peptide.........................................................................................................................33

3.5: Reaction scheme of standard peptide synthesis, achieved using solid phase synthesis of Fmoc-amino acid-Wang resin and Fmoc-amino acid. .........................................................................................................35

3.6: Structure of YIGSR peptide which was synthesized by standard solid phase peptide synthesis and modified with PEG like amino acid. ........................................................................................................38

3.7: $^1$H-NMR spectrum of PEG-like Fmoc peptide. Peak between 7.23 and 7.37 ppm is assigned to the chloroform-$d$ solution.........................................................................................................................39

3.8: Mass spectrom of YIGSR showing the isotopic distribution. Peak at 1002.5 m/z is assigned as the main peak of [YIGSR+NH$_3$] and all these four peaks are assigned as isotopic distribution....................................................................................................................................................41

3.9: A scheme of contact angle and XPS sample points distribution. The data of static contact angle and XPS was collected from five equidistant points on the surface at 5 mm intervals................................................................................................................................................................................43

3.10: The methyl end group gradient profiles were measured by static water contact angle. The static contact angle changed gradually across the surface, reflecting the organosilane concentration changing. Deionized water static contact angle (mean ± S.D., $n = 3$) ........44

3.11: The chlorine profiles were measured by static water contact angle. The static contact angle change gradually across the surface, reflecting the changing organosilane concentration. Deionized water static contact angle (mean ± S.D., $n = 3$).................................46

3.12: The amine gradient profiles were measured by static water contact angle, achieved by phosphanimine hydrolization. Compared with the chlorine gradients, the contact angle changes indicate the amine groups were successfully functionalized onto the surface. Deionized water static contact angle (mean ± S.D., $n = 3$) .................................................47

3.13: The hydroxyl gradient profiles were measured by static water contact angle, achieved by alkyne-azide cycloaddition. Compared with the chlorine gradients, the contact angle changes indicate the successful “click” reaction. Deionized water static contact angle (mean ± S.D., $n = 3$) .................................................48
3.14: The vinyl end group gradient profiles were measured by static water contact angle. The static contact angle changed gradually across the surface, reflecting the changing organosilane concentration. Deionized water static contact angle (mean ± S.D., n = 3).

3.15: The XPS high resolution of C$_{1s}$ spectra for each gradient position on n-octyldimethilchlorosilane (-CH$_3$) covered surface. C$_{1s}$ curves of XPS spectra were fitted based on Gauss-Lorentz function.

3.16: Peak fitting of XPS C$_{1s}$ spectra for each gradient position on YIGSR covered surface. C$_{1s}$ curves of XPS spectra were fitted based on Gauss-Lorentz function.

3.17: Surface concentration and surface coverage fraction varied as a function of position across the methyl-terminated surface. Surface concentration and surface coverage fraction were determined by XPS.

3.18: Surface concentration and surface coverage fraction varied as a function of position across the YIGSR-terminated surface. Surface concentration and surface coverage fraction were determined by XPS.

3.19: Survey spectra (0-700 eV) from XPS for chlorine surface gradient. Peak at 201 eV was assigned as Cl$_2p$ element which verified the chlorine has been successfully tethered onto the surface.

3.20: Survey spectra (0-700 eV) from XPS for amine surface gradient. The disappearing Cl$_2p$ peak confirmed the quantitative conversion of chlorine to amine.

3.21: Survey spectra (0-700 eV) from XPS for hydroxyl group surface gradient. The disappearing Cl$_2p$ peak and appearing N$_{1s}$ peak confirmed the quantitative conversion of chlorine to hydroxyl group.

3.22: Reaction verification by high resolution Cl$_{2s}$ (a) and N$_{1s}$ (b) from XPS after SN2 substitution, alkyne-azide cycloaddition and phosphanimine hydrolization. Figure shows the loss of the Cl$_{2s}$ signal (a) and acquisition of the N$_{1s}$ signal following the azide reaction, alkyne-azide cycloaddition and phosphanimine hydrolization.

4.1: Immunofluorescent staining of actin (red), vinculin (green) and nuclear (blue) after (A) 1 day, (B) 3 days and (C) 7 days of culture. Images were taken every 5 mm down the length of the gradient. Scale bar=20 μm.
4.2: Human primary chondrocytes density response as a function of methyl concentration on methyl surface gradient. After 1, 3, 7 days of culture, cell number was collected at each sample position (mean ± S.D., n = 3) ..........................................................................................................................70

4.3: Immunofluorescent staining of CD 14 (red), CD 90 (green) and nuclear (blue) after (A) 3 day, (B) 7 days and (C) 14 days of culture. Images were taken every 5 mm down the length of the gradient. Scale bar=20 μm. .................................................................................................72

4.4: Chondrocyte survival of each gradient position based on phenotype maintenance using CD14/CD90 ratios response as a function of methyl concentration on methyl surface gradient for days 3, 7, and 14. The CD14/CD90 ratio was obtained by dividing the fraction of cells expressing CD14 by the fraction of cells in expressing CD90 (mean ± S.D., n = 3) ........................................................................................................................................73
Osteoarthritis, the most common form of joint disease, affects the joints in the hands, knees, and hips as well as other widely used joints in the body. It occurs when the protective cartilage at the end of bones wears down and is accompanied by pain and discomfort. Nearly 27 million people among U.S. ages 25–74 years were affected by clinically defined OA of some joint in 2008 (up from the estimate of 21 million for 1995)\(^1\). Although osteoarthritis becomes more common with age, it can be caused by acute trauma, normal daily activities and genetic disposition\(^2\). Unlike bones, cartilage has a limited capacity to repair itself inhibiting the recovery process\(^3\). Its prevalence and the frequent disability has economic backlash for both the individual and society which costs 0.25%-0.50% of a country’s Gross Domestic Product (GDP) on osteoarthritis\(^4\). Recently, efforts to osteoarthritis (OA) have focused on preserving the host joint by addressing the underlying mechanical changes in cartilage during OA progression\(^5\). However, rational development of OA treatment is still faced some challenges. Current limitations for using OA chondrocytes including low numbers and the metabolic imbalance between
expressions of catabolic matrix cytokines\textsuperscript{6}. For progression to be successful, it is necessary for implants to develop optimal surface conditions to facilitate expansion of cell number and bioactivity while preserving the narrow cellular phenotype. Initial studies have shown that competitive surface-engineering approaches such as soluble protein signaling factors and surface chemistry system have a profound effect on lineage commitment and cellular function\textsuperscript{7}. Collectively, functionalized amine and hydroxyl surfaces have been studied and found to promote stem cell viability, cell adhesion and migration\textsuperscript{8}. Unfortunately, chemical concentrations have not been widely examined even though the cell behavior has been shown strongly influenced by the concentration of chemical functionality. Herein we examined the effects of surface functional group concentrations, examining chondrocyte proliferation and phenotype maintenance within continuously variable one-dimensional methyl concentration gradients.

1.1. Interaction between Cells and Surface Chemistry

When in contact with a biological environment, the surface chemistry, surface conditioning growth factors, such as peptides, and topography of the material will have direct consequences on the protein adsorption, cell interaction, and ultimately the host response\textsuperscript{9}. For example, cell attachment is directed by the presence of specific binding proteins and cell proliferation, maintenance and differentiation require specific cytokines and appropriate growth factors\textsuperscript{10}. 

2
Xia et al coated polycaprolactone (PCL) nanofibers with laminin, an ECM glycoprotein, and showed that neurite outgrowth was significantly increased on aligned nanofibers with immobilized laminin relative to untreated samples. It showed that the alignment and laminin directed axon growth directs the axons of maturing neurons to innervate targets through a combination of contact-mediated.

The short amino acid sequence of the signaling domains of the whole of ECM proteins has been proven to be as effective as the whole protein and primarily interact with cell membrane receptors.

Erkki Ruoslahti and Michael D. Pierschbacher reviewed that Arg-Gly-Asp (RGD) tripeptide was found to be crucial for its interaction with its cell surface receptor among 2,500 amino acids in the fibronectin polypeptide. Synthetic peptides containing the RGD sequence inhibited cell attachment to fibronectin as well as number of other proteins, including vitronectin, which indicated that synthetic RGD played the same role as whole proteins.

Langer et al developed a method of fabricating photo-cross-linked hydrogels with gradients with an immobilized RGD sequence. The study used low cell numbers of Human Umbilical Vein Endothelial cells (HUVECs) to observe cell spreading and found that the hydrogels without the RGD motif showed no spreading (as shown in Figure 2A), whereas hydrogel functionalized with RGD showed significant spreading across the entire hydrogel microstructure (as shown in Figure 2B).
Figure 1.1: Light micrographs of endothelial cells attached to the surface of hydrogels without RGDS (A), and hydrogels with 5.0 mM Acr-PEG-RGDS (B). Reprinted from Langer et al.\textsuperscript{14} with permission from the American Chemical Society. Copyright (2004)
1.2 Surface Modification of Biomaterials

In the last few decades, the rapidly evolving field of biomedical devices holds significant promise for cell-based therapies for bone, cartilage, nerve, and cardiovascular tissue engineering. Biomaterials that have been investigated for implantation include silicone, lipid-based material, natural polymers and synthetic polymers. Most of them have excellent bulk properties such as mechanical stability, elasticity, strength and desired stability towards degradation and low toxicity. For example, medical grade silicone is widely used to manufacture cerebrospinal fluid (CSF) shunt devices such as catheters, access chambers, suture clamps and external valve housings showing to its chemical stability, minimal biological reactivity, low toxicity and noncarcinogenicity\(^9\). Consequently, artificial materials overcome the major limitations associated with autografting technique including donor site morbidity and lack of suitable graft material\(^{15}\).

Although incorporation of biomaterials is effective in treating diseases, complications such as inflammation, infections, obstruction, aseptic loosening, local tissue waste and mechanical failure are common problems which can be contributed to inadequate interaction between synthetic materials and cells\(^{16-17}\).

To reduce the inflammatory tissue response around implanted materials, surface-engineering approaches\(^{18}\) with bioactive factors and surface chemistry have been executed, increasing the interfacial interaction between the materials and cells\(^{19}\).
For cell-binding growth factors, the approaches involve incorporation into biomaterials can be separated into chemical modification or physical modification. The long chains of ECM proteins such as fibronectin (FN), vitronectin (VN), and laminin (LN) have been used in early work for promoting cell adhesion and proliferation. Surface modification can be modified by direct coating of with these proteins via physical adsorption including hydrophobic interactions and electrostatic interaction20-21. The mechanism involved with physical adsorption is that the energetics of adsorption is dominated by an entropic gain in releasing the otherwise low-entropic water that is near the relatively hydrophobic material interface22. The protein thus acts like a surfactant, and its adsorption reduces the free energy of the system.

Involving the chemical modification and the complicated physical adsorption, the use of a short peptide chain or other surface chemical groups for modification is advantageous over the long chain of native ECM proteins which tends to be randomly folded upon adsorption to surface and the receptor binding domains are not always sterically available20. In the period of 1960s to 1990s, the most common chemical surface modification technique was radiation graft copolymerization with selected monomer compositions23. This could be followed by bonding of functional biomolecules onto the active groups introduced in the graft copolymer. Plasma gas discharge (or radio frequency glow discharge, RFGD) has more recently emerged as another interesting and unique way to modify biomaterial surfaces. For example, the reactive parts on the model surface, usually NH2, are chemically reacted with certain functional groups, usually COOH, that
are present within the bioactive peptide\textsuperscript{24}. A bi-functional crosslinker that has a long spacer arm can be used for the immobilization of the peptide to the surface, which can enable the immobilized peptide to move with flexibility in the biological environment\textsuperscript{25}.

With the rapid development of technologies and science, the chemical modification get to a new level and some novel methods are introduced, such as self-assembled monolayer (SAM) formation, graft copolymerization, Layer-by-layer assembly and genetically engineered surface\textsuperscript{26, 27, 28, 29}. Consequently, creating tethered peptides or proteins allows the concentration of those peptides and proteins to be more closely controlled and characterized. This allows us to overcome the problems of other gels or materials with free floating proteins that will diffuse and may cause adverse effects on other tissues.
1.3 Gradient Fabrication

To study surface chemistry concentration-dependent cell behavior, gradients provide a fast, efficient and reliable strategy by incorporating a series of concentration values in one single substrate. The first generation of palladium gradient on cellulose acetate with the vapor deposition technique was investigated by Carter et al. in 1965\textsuperscript{30}, various kinds of gradient fabrication methods have been developed depending on tissue target or specific cell responses. These techniques can be generally subdivided into several groups simply on the basis of mechanisms: (1) Reaction time-dependent gradients, (2) Diffusion control gradients, (3) Temperature-dependent gradients, (4) Pump density gradient gradients, (5) Electrical potential control gradients, (6) Plasma gradient deposition gradients, (7) Microfluidic system gradients\textsuperscript{31}, (8) All other gradient methods.

In terms of time-dependent gradients, it is generated by the gradually rising reaction time which leads to increasing amounts of conversion of reactive chemical sources. The time controlling can be achieved by changing ultraviolet-ozone (UVO) or plasma, and taking advantages of certain shields, masks, templates or pumps. The most commonly used technique in biomedical gradients is the ultraviolet-ozone (UVO) based oxidation gradient. For example, Becker et al.\textsuperscript{32} developed a system where exposure time of the organic self-assembled monolayer to UV light is changed upon fabrication of the linear concentration gradient profiles shown in Figure 1.2. Reactive atomic oxygen species are generated and react oxidatively with the C-terminal methyl group on the SAMs to
produce carboxylate, carbonyl and other oxygenated functional groups. The increase in UV exposure time led to rising amounts of ozone-derived oxidation of n-octyldimethyldichlorosilane SAM and generated gradients across the slide, which can be further reacted for peptide concentration gradients with a high efficient click reaction.
Figure 1.2: Schematic illustration of UV-ozone treatment to generate gradient immobilization and further “click” reaction. DMAP: 4-methylaminopyridine; DIC: diisopropylcarbodiimide; DMSO: dimethylsulfoxide\textsuperscript{32}. Reprinted from Becker \textit{et al.}\textsuperscript{32} with permission from the WILEY-VCH. Copyright (2007)
For diffusion controlled gradients, including vapor and liquid, are based on Fick’s diffusion laws.

\[
J = -D \frac{\partial c(x,t)}{\partial x} \quad \text{Equation 1.1}
\]

\[
\frac{\partial c(x,t)}{\partial t} = -D \frac{\partial c^2(x,t)}{\partial^2 x} \quad \text{Equation 1.2}
\]

For specific boundary cases

\[
x = 0 \quad \frac{\partial c(0)}{\partial t} = 0
\]

\[
x = \pm \infty \quad c(\pm \infty) = 0
\]

when \( x \ll \sqrt{Dt} \)

\[
c(x,t) = c(0)[1 - 2 \frac{x}{2\sqrt{Dt}^3}] \quad \text{Equation 1.3}
\]

Where \( D \) is the diffusion coefficient; \( J \) is the diffusion flux; \( c \) is the concentration per unit volume; \( x \) is the position. For relatively short distances, \( x \ll 2(Dt)^{1/2} \), the solution for equation 2 can be approximately simplified to equation 3. Therefore, the concentration almost has a linear relationship with distance.

Under this mechanism, diffusion time varies along the diffusion direction and the concentration gradient forms\(^8,33\). Introduced by Genzer et al, the vapor diffusion method is utilized prepared for many different variations of chlorosilane molecular gradients on silicon wafer with various geometries. However, the wide usage of this method is limited by imprecise control of the slope and functional properties, low batch to batch reproducibility, and the poor versatility in chemical groups on the surface. The complexity of devices and singularity of surface functionality reactions restrict use from further development. To overcome these limitations, Epps et al focused on a confined
vapor deposition device which can generate a linear gradient of chlorosilane on a silicon substrate, employing dynamic vacuum (as shown in Figure 1.3) \(^8\).

Figure 1.3: Device schematic. (a) Reservoirs are loaded in one side of Teflon insert. (b) Insert is loaded into the deposition chamber. (c) The cross-section of reservoir and substrate (d) dynamic vacuum is pulled from one side of the insert. Reprinted from Epps et al. \(^8\) with permission from the American Chemical Society. Copyright (2009)
To achieve a multifunctional surface, a new gradient strategy is raised. It is best suited for the fabrication of diverse functions with gradual changes across surface, with simple devices and easy chemistry. The chemical source (organosilane) and substrate (silicon or glass) is loaded into a confined insert in a sealed chamber. Using vapor deposition method, the silane solution is first vaporized by vacuum from the opposite direction and then the vapor diffused freely along the channel to graft onto the surface. The diffusion is quenched by methanol. The reaction between the chlorine atom of silane and the hydroxyl groups on the surface are involved in the grafting process. The simple and easily controllable process only takes about half minute to complete and overcome many of the aforementioned difficulties. In addition, two-dimensional (2D) orthogonal chemical concentration gradients are achieved by using a sequential deposition protocol that enables the orthogonal engineering of peptide functionalized SAMs in our previous study, which means it has successfully overcome the functional singularity of other gradient strategies.
Figure 1.4: (a) The fabrication scheme for functional self-assembled monolayer concentration gradients using a confined channel diffusion method. (b) A two-step vapor deposition strategy to fabricate orthogonal concentration gradient, enabling a “dual-click” reaction. Reprinted from Becker et al. 34 with permission from the American Chemical Society. Copyright (2013)
1.4 Surface Analytical Techniques

Well-defined surface characterizations play an important role in the surface gradient fabrication and applications. This is especially true to meet the stringent need for standardized and quantifiable surfaces for biological studies. Several factors, such as composition, surface energy, thickness, stability, morphology or wettability are all needed to be taken into consideration choosing an appropriate analysis technique for surface gradients.

X-ray photoelectron spectroscopy (XPS), near edge X-ray absorption fine structure (NEXAFS), Fourier transform infrared spectroscopy (FTIR) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) techniques afford elemental and molecular analysis of the topmost atomic layers of solid surfaces and precise quantitative concentration information. Atom force microscopy (AFM) and scanning electron microscopy (SEM) are widely used to investigate surface morphology. Contact angle measurement, the mostly common used analysis method in surface analysis, can provide general data of surface energy as well as surface composition. However, the resolution of contact angle is limited. Ellipsometry is a popular optical technique for studying the thickness, roughness and composition on the surface, playing an important role in self-assembled monolayer area. Fluorescence imaging and surface charger analysis (SCA) are also qualified candidates for quantitate characterization.
Contact angle measurement and XPS are discussed in detail since they are the most common used tool in this gradient study.

Surface wettability is the most common surface property for biomaterials and it can be characterized by contact angle methods simply and conveniently. Moreover, Contact angles are more sensitive than optical ellipsometry or X-ray photoelectron spectroscopy to certain small structural changes in monolayers since it obtains the information for the surface within only a few atomic layers depth without bulk effect\textsuperscript{36}. For example, contact angles are sensitive to the composition so long as the wettability of the two components having the same chain length but different tail groups while XPS and ellipsometry reduce the ability to discriminate between two monolayers that differ slightly in composition.

XPS measurements are performed on surface energy measurements to verify the respective functional group concentration and “click” reaction. XPS is used to measure the elemental concentration at each sample position on the gradient, providing more precise information on peptide concentration than contact angle.
Osteoarthritis (OA)

Osteoarthritis, also known as degenerative joint disease, is the most common form of arthritis and a major cause of disability. It is characterized by joint pain, swelling, stiffness, dysfunction, even joint contractures, muscle atrophy and limb deformity in end-stage of osteoarthritis\(^37\) (as shown in Figure 1.5). Thus, osteoarthritis accounts for more trouble with daily activities such as walking and any other disease, even lead to disability\(^37\).

Although the pathogenesis of osteoarthritis is multifactorial, heterogeneous and not fully understood, the major independent risk factor of osteoarthritis can be contributed to chronological age of patients\(^2\). However, aging and osteoarthritis are inter-related but not inter-dependent in conjunction with other factors both for intrinsic (e.g., alignment, overloading) and extrinsic (e.g., genetics) to the joint\(^38\). In young patients, the etiology of osteoarthritis is predominantly affected by an unfavorable biomechanical environment at the joint\(^37-38\). For instance, misalignments of bones caused by congenital injury; excess body weight, mechanical injury and loss of strength in the muscles supporting a joint can also lead to osteoarthritis. In detail, it occurs when the protective cartilage on the ends of bones, the connective tissue, breaks and wears down over time. This progressive decline may lead to degenerative joint disease with earlier onset of osteoarthritis\(^39\). In the long term, the mechanical disruption of joint motion, body abnormalities formation, mechanical wear in the involved compartment may result in more widespread joint
degeneration and osteophytes growing on the edges of the joint. Consequently, when bones rub together in the absence of a synovial joint, it can cause more pain and damage especially when bits of bone break off and remain inside joint.

Furthermore, the molecular mechanism of osteoarthritis directly related to chondrocyte senescence and contributes to the risk of the losing proteoglycan, decreasing mitotic and synthetic activity and decreasing responsiveness to anabolic growth factors\(^{40}\),\(^{41}\) (as shown in Figure 1.6). During which a water content increase will disorganize the collagen network. All of these factors decrease in the ability of chondrocytes to maintain and repair the tissue, resulting in an accelerated damage to the joint surface and end-stage osteoarthritis\(^{42}\). Unlike bone, the cartilage undertakes the large articulating motion and continuously bears weight which can damage newly developed tissues that are unable to possess lubrication, compressive and tensile properties. However, cartilage integration is precluded by its hyaline and nonadhesive nature\(^{41}\) and poor nutrient supply from its surroundings, which indicates the difficulties in regenerative therapies. Thus, the limited capacity to repair itself makes the recovery process complicated\(^3\) and more robust exogenous approaches are needed.

In the United States, nearly 27 million people ages 25–74 years were affected by clinically defined osteoarthritis of some joint in 2008 (up from the estimate of 21 million for 1995)\(^1\). It is growing economic burden for both the individual and for society which costs 0.25% - 0.50% of a country’s Gross Domestic Product (GDP) for social cost of osteoarthritis \(^4\). Such high prevalence, distinctive structure and function of hyaline
cartilage and its inherent low healing potential are believed to be the challenge in management of cartilage disease.
Figure 1.5: Differences in the physiologic environment, metabolic rate, and cellular make-up of bone and cartilage have profound effects on the potential to engineer these tissues\textsuperscript{41}. Reprinted from Athanasiou \textit{et al} with permission from the American Association for the Advancement of Science (2012)
CHAPTER II

EXPERIMENTAL SECTION

2.1 Materials

Specific commercial equipment, instruments and materials are identified in this thesis in order to specify the experimental procedure adequately. All other chemicals and solvents were purchased from commercially available sources without further purification unless specified.

2.1.1 Chemical reagents

4-Chlorobutyldimethylchlorosilane and n-octyldimethylchlorosilane were purchased from Gelest (Morrisville, PA). Toluene (99.5%), methanol (99.8%), 3-butyln-1-ol (97%), copper(II) sulfate(99%), (+)-Sodium L-ascorbate (98%) and sodium azide (99.0%) were purchased from Sigma-Aldrich (St. Louis, MO). Triphenylphosphine (PPh₃) and propargyl alcohol were purchased from ACROS (Hampton, NH). Tetrahydrofuran (THF, certified) and EDTA (Ethylenediaminetetraacetic acid, 0.5M, pH 8.0) were purchased from Fisher Scientific (Waltham, MA).
Microscope glass slide (75 × 25 mm, Fisher Scientific) were used for general gradient fabrication and surface tension evaluation; and silicon wafers (Si [100], one side polished, Silicon Quest Int.) were cut down to 25 × 25 mm for X-ray photoelectron spectroscopy (XPS) analysis.

2.1.2 Cell Culture Supplements

In cell culture, the Reduced Serum Eagle's Minimum Essential Media (Opti-MEM® I Reduced Serum Media (1x), Gibco) was purchased from Invitrogen (Grand Island, NY, USA), supplemented with 0.1 mg/mL primosin and 0.05 mg/mL ascorbate. The Phosphate-Buffered Saline (PBS) buffer was bought from Fisher Scientific. Collagenase type II (230 u/mg) was purchased from Worthington Biochemical Corporation (Lakewood, NJ).

2.1.3 Cell Fixation, Fluorescence Staining Agents

Donkey serum, secondary antibodies anti-rabbit Alexa Fluor 546 and anti-goat Alexa Fluor 488 were purchased from Invitrogen (Grand Island, NY, USA). Cytoskeleton stabilization (CS) buffer was made with 0.1 M PIPES, 1 mM EGTA, and 4% (w/v) 8000 MW polyethylene, pH=6.9. The Phosphate-Buffered Saline (PBS) buffer was bought from Fisher Scientific (Waltham, MA). Formaldehyde, sodium borohydride, and sodium azide, sodium citrate and TritonX-100 were purchased from Acros Organics (Hampton,
Primary antibodies, CD14 (SC9150) and CD90 (SC6071) were purchased from Santa Cruz (Dallas, TX, USA).

2.2 Contact Angle Measurement

Surface tension was measured on an Advanced Goniometer (Ramé-Hart Instrument, Co., Model 500) at 25 °C using deionized water (2 μL) as the probe fluid. ImageJ and a drop analysis plugin (free download from the National Institute of Health, Bethesda, MD at http://rsb.info.nih.gov/ij/ and http://bigwww.epfl.ch/demo/dropanalysis/) were used to determine the contact angle of drops at each position.

The standard uncertainty of the contact angle measurement at each point along the gradient was calculated by the standard deviation between three independent measurements on three samples prepared under identical conditions.

2.3 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) measurements were performed on a Kratos AXIS Ultra DLD spectrometer with monochromated Al Ka source over a 300×700 μm sample area. The survey spectra were acquired at pass energy 160 eV while high-resolution C1s scans were acquired at 20 eV. Each spectrum was collected using silicon slides as substrates.
2.4 Fluorescence Microscopy

IX81 motorized inverted system microscope (Olympus) was used with appropriate filters and mercury bulb excitation. Mercury bulb excitation was provided by Olympus U-RFL-T Power Supply for High Pressure Mercury Burner.
3.1 Experimental Section

Fabrication of one-dimensional concentration gradients by vapor deposition was described in this chapter.

3.1.1 Methyl-terminated Gradient Fabrication

A solution (10%) of n-octyldimethchlorosilane (150 μL) in toluene was used as a chemical diffusion source. A 2.5 × 2.5 cm square silicon or glass slides were washed with methanol and toluene three times each and blown dry with N₂ gas. Slides were then pretreated by Ultraviolet Ozone Cleaner (Jelight Company Inc. Model No. 42A) for 1 hour to clean up the organic contaminants on the surface. The system for fabricating molecular concentration gradients using a confined channel diffusion method is shown in Figure 3.1.
After the whole vacuum line and devices were set up (as shown in Figure 3.1), the n-octylidimethylchlorosilane solution (150 μL) was injected into a Teflon reservoir (1.5 × 2.5 × 1.1 cm) and a pretreated slide was placed on a Teflon substrate support (7.5× 2.5 × 1.0 cm), inserted into the rectangular glass tubing (30 × 2.5 × 1.3 cm) both. The glass tubing was placed in a sealed metal chamber and a syringe port with methanol (40 mL) was connected to the camber from the opposite direction. A dynamic vacuum was executed from the side near to the Teflon substrate support for 10 s to create the diffusion concentration gradient, the valve of methanol container was turned on to quench the diffusion process with methanol flow. After the vapor deposition process, the glass slide was washed with methanol, toluene and methanol alternately to remove the non-covalently bonding silane and blown dry with N₂ gas. Samples are stored in vacuum desiccators at room temperature until used.
3.1.2 Amine-terminated Gradient Fabrication

A solution (10%) of 4-Chlorobutyl(dimethyl)chlorosilane (150 μL) in toluene was used here as chemical diffusion source and 150 μL solution was injected into the reservoir and a pretreated slide was placed on a Teflon substrate support, inserted into the rectangular glass tubing both. The glass tubing was loaded in a sealed metal chamber\textsuperscript{34} and a syringe port with methanol (40 mL) was connected to the camber from the opposite direction. A dynamic vacuum was executed from the side near to the Teflon substrate support for 10 s to create the diffusion concentration gradient, the valve of methanol container was turned on to quench the diffusion process with methanol flow. After the vapor deposition process, the glass slide was washed with methanol, toluene and methanol alternately to remove the non-covalently bonding silane and blown dry with N\textsubscript{2} gas. Next, two subsequent surface reactions were carried out immediately after chlorine-terminated gradient fabrication, including a surface SN\textsubscript{2} substitution of chlorine end group with –N\textsubscript{3} end group and reduction of azide with amine group. First, chlorine concentration gradients were incubated in NaN\textsubscript{3} solution (4 mg/mL in DMF) at 65 °C for 2 days and small amount of 18-crown-6 was also added as phase-transfer catalyst. After 48 hours, the slides were rinsed sequentially with methanol, toluene and methanol and blown dry with N\textsubscript{2}. Then these slides were immersed into PPh\textsubscript{3} (0.78 g, 3 mM)/ H\textsubscript{2}O (54 μL, 3 mM)/THF (30 mL) solution, stirring at room temperature for 12 hours. Next, samples were rinsed sequentially with methanol, toluene and methanol and blown dry with N\textsubscript{2}.
3.1.3 Hydroxyl-terminated Gradient Fabrication

A solution (10%) of 4-Chlorobutyldimethylchlorosilane (150 μL) in toluene was used here as chemical diffusion source and 150 μL solution was injected into the reservoir and a pretreated slide was placed on a Teflon substrate support, inserted into the rectangular glass tubing both. The glass tubing was loaded in a sealed metal chamber and a syringe port with methanol (40 mL) was connected to the camber from the opposite direction. A dynamic vacuum was executed from the side near to the Teflon substrate support for 10 s to create the diffusion concentration gradient, the valve of methanol container was turned on to quench the diffusion process with methanol flow. After the vapor deposition process, the glass slide was washed with methanol, toluene and methanol alternately to remove the non-covalently bonding silane and blown dry with N₂ gas. Next, two subsequent surface reactions were carried out immediately after chlorine-terminated gradient fabrication, including a surface SN2 substitution of chlorine end group with –N₃ end group and reduction of azide with amine group. First, chlorine concentration gradients were incubated in NaN₃ solution (4 mg/mL in DMF) at 65 ℃ for 2 days and small amount of 18-crown-6 was also added as phase-transfer catalyst. After 48 hours, the slides were rinsed sequentially with methanol, toluene and methanol and blown dry with N₂. Then these slides were immersed into 3-butyln-1-ol (60 μL, 0.79 mM)/CuSO₄ (60 mg, 0.37 mM)/ Sodium ascorbate (120 mg, 0.60 mM)/ THF (15 mL)/H₂O (15 mL)
solution, stirring at room temperature for 48 hours. Next, samples were washed with methanol, toluene and methanol alternately and blown dry with N₂ gas.
Figure 3.2: Reaction scheme of hydroxyl and amine functionalized surface. (a) The amine end group was connected to the surface via phosphanimine hydrolization. (b) The hydroxyl end group was connected onto the surface via SN$_2$ substitution of the chlorine with azide and alkyne-azide cycloaddition. DMF: N, N-Dimethylformamide; RT: room temperature; PPh$_3$: Triphenylphosphine.
3.1.4 YIGSR-terminated Gradient Fabrication

A solution (10%) of 5-hexenyldimethylchlorosilane (150 μL) in toluene was used as a chemical diffusion source and 150 μL solution was injected into the reservoir and a pretreated slide was placed on a Teflon substrate support, inserted into the rectangular glass tubing both. The glass tubing was loaded in a sealed metal chamber and a syringe port with methanol (40 mL) was connected to the camber from the opposite direction. A dynamic vacuum was executed from the side near to the Teflon substrate support for 10 s to create the diffusion concentration gradient, the valve of methanol container was turned on to quench the diffusion process with methanol flow. After the vapor deposition process, the glass slide was washed with methanol, toluene and methanol alternately to remove the non-covalently bonding silane and blown dry with N₂ gas.

The surface thiol–ene reaction in click chemistry was employed to graft a peptide onto the surface. Irgacure 2959 was used as a photoinitiator which was widely used in medical applications for its robust bonding, high efficiency, and orthogonal synthesis properties. YIGSR peptide (0.080 g, 0.0812 mmol) and Irgacure 2959 (0.040 g, 0.1783 mmol) were dissolved in a DMF/H₂O (20 mL, 9:1) solution. Glass slides with 5-hexenyldimethylchlorosilane silane SAMs on the surface were immersed into the solution, treated with UV light for 2 h.
Figure 3.3: Reaction scheme for coupling YIGSR peptide to vinyl-terminated gradient substrates via thiol-ene “click” reaction.
3.1.5 Synthesis of PEG-like Fmoc Peptide

Figure 3.4: Reaction scheme of PEG-like Fmoc peptide synthesis. PEG-like Fmoc peptide was prepared for modification of YIGSR peptide.
A solution of succinic anhydride (0.1858 g, 1.858 mM) in ACN (5 mL) was added to a solution of 2,2’- (ethylenedioxy)bis(ethylamine) (295 μL, 2.020 mM) in ACN (10 mL) dropwise for 30 min in a 100 mL three-neck round bottom boiling flask with a stir bar. The reactor was set up on a magnetic stirrer for 2.5 h until a waxy substance was formed.

The magnetic stirrer was turned off and the mixture was left standing for 1 h and suspended the organic solvent was removed. Then the waxy substance was dissolved in ACN/ H₂O (1:1) (20 mL). The reaction was merged into an ice-bath, cooled for 0.5 h and a Fmoc-Cl solution (0.673 g, 2.60 mM) in ACN (2 mL) was added dropwise. When the last addition was finished, the pH value of the solution was adjusted to 7-8 with 5% NaHCO₃/ H₂O solution. The reaction was stirred overnight at room temperature. It was then concentrated in vacuum and the product remaining was dissolved in 5% NaHCO₃/H₂O (20 mL) and the aqueous layer was washed with EtOAc (4×30 mL) for 4 times. A solution of 1M HCl/H₂O was added to acidify the aqueous layer to pH = 2 and washed 4 times with EtOAc (4×30 mL). The combined organic layer was washed with H₂O (2×30 mL), dried over MgSO₄, filtered and concentrated in vacuum. The final product was obtained as clear oil and was used directly (as shown in Figure 3.6).

Product: 0.599 g; 1.426 mM; 77% yield.
3.1.6 Solid Phase Peptide Synthesis

Figure 3.5: Reaction scheme of standard peptide synthesis, achieved using solid phase synthesis of Fmoc-amino acid-Wang resin and Fmoc-amino acid.
Fmoc-Arginine-Wang resin (0.300 g, 0.47 mmol, 1 equiv.) was dissolved in a sintered funnel-like reactor with DMF (10 mL) for 20 min and protected by N₂ gas. The solvent was pumped out and a deprotection reaction was carried out first. Piperidine (10 mL) and DMF (30 mL) were added into the system to remove the protective Fmoc-function group and mixed by N₂ flow (as shown in Figure 3.4). Thorough mixing was necessary for good reaction rates and high yields because this reaction takes place mainly on the boundary between liquid and solid resin surface. After one hour reaction, the solution was pumped out and the residual solid resin was washed by DMF, DCM and methanol three times respectively. Serine residue were coupled to the resin by adding Fmoc-serine amino acid (0.369 g, 2.35 mmol, 5 equiv.) into the reactor with HOBt (0.294 g, 4.7 mmol, 10 equiv.), DIC (300 μL, 4.7 mmol, 10 equiv.) and 40 mL DMF. HOBt and DIC lead to the formation of the corresponding ester which was able to react with the carboxylic group of serine amino acid. This reaction took three hours to go and product was washed by DMF, DCM and methanol for three times respectively. This method was repeated for the five remaining amino acid additions in an order of Glycine, Isoleucine, Tyrosine, Glycine and PEG-like Fmoc peptide. Particularly, the coupling reaction for PEG-like Fmoc peptide was extended to 6 hours.

After the last Fmoc-functional group was removed, a cleavage reaction was carried out in trifluoroacetic acid (19 mL), triisopropylsilane (0.5 mL) and deionized H₂O (0.5 mL) for 30 min.
The final product was obtained by concentrating the trilouroacetic acid and adding the concentrated solution into cold diethyl ether dropwise for precipitation. The precipitate was centrifuged and dialyzed in a dialysis bag (MWCO: 100-500D, Biotech CE Tubing, Spectrum Laboratories, Inc.) for two days and lyophilized for two days. Product was characterized with mass spectroscopy (MS) (as shown in Figure 3.7). Product: mol. wt. 985.4 g/mol.
Figure 3.6: Structure of YIGSR peptide which was synthesized by standard solid phase peptide synthesis and modified with PEG like amino acid.
3.2 Results and Discussion

All the results from Nuclear Magnetic Resonance (\(^1\text{H-NMR}\)), Mass Spectrometry (MS), Contact Angle Measurement and X-Ray Photoelectron Spectroscopy (XPS) were shown below and discussed.

3.2.1 Nuclear Magnetic Resonance (\(^1\text{H-NMR}\))

![Figure 3.7: \(^1\text{H-NMR}\) spectrum of PEG-like Fmoc peptide. Peak between 7.23 and 7.37 ppm is assigned to the chloroform-\(d\) solution.](image)
$^1$H NMR (500 MHz, Chloroform-$d$) ppm 2.42 - 2.58 (m, 2 H) 2.58 - 2.75 (m, 2 H) 3.20 - 3.40 (m, 1 H) 3.41 - 3.50 (m, 3 H) 3.50 - 3.59 (m, 2 H) 3.59 - 3.67 (m, 4 H) 4.19 - 4.35 (m, 1 H) 4.37 - 4.55 (m, 2 H) 7.14 (br. s., 1 H) 7.23 - 7.37 (m, 2 H) 7.41 (t, $J=$7.46 Hz, 2 H) 7.51 - 7.69 (m, 2 H) 7.78 (d, $J=$7.58 Hz, 2 H)

Figure 3.7 shows the $^1$H NMR spectrum for the PEG-like Fmoc peptide prior to solid phase YIGSR peptide synthesis, confirming that the reactions has been completed. The resonance peaks at around 1.25 ppm and 2.09 ppm are assigned to the protons of residual EtOAc solvent. And peaks at around 5.59 ppm, 6.37 ppm, and 7.14 ppm are supposed to be the shifting peaks of amino groups.
3.2.2 Mass Spectrometry (MS)

Figure 3.8: Mass spectrum of YIGSR showing the isotopic distribution. Peak at 1002.5 m/z is assigned as the main peak of [YIGSR+NH$_3$] and all these four peaks are assigned as isotopic distribution.

We were able to identify the exact mass of the expected product and to confirm the structure of the YIGSR peptide. Figure 3.8 shows the isotopic distribution and the theoretic isotope pattern of singly-charged cluster of YIGSR peptide, which may be in the form of [M+NH$_3$]. So the molecular weight here was 985.0 g/mol which was in
accordance of YIGSR structure. This result reveals the successful synthesis of YIGSR peptide.

3.2.3 Contact Angle Measurement

To verify the linear peptide concentration profiles, static contact angle measurement and X-ray photoelectron spectroscopy (XPS) were employed for quantitative analysis of surface energy, surface concentration and surface coverage ratio. The data of static contact angle and XPS was collected from five equidistant points on the surface at 5 mm intervals (as shown in Figure 3.9).
Figure 3.9: A scheme of contact angle and XPS sample points distribution. The data of static contact angle and XPS was collected from five equidistant points on the surface at 5 mm intervals.
Figure 3.10: The methyl end group gradient profiles were measured by static water contact angle. The static contact angle changed gradually across the surface, reflecting the organosilane concentration changing. Deionized water static contact angle (mean ± S.D., n = 3).
Due to the hydrophobicity of n-octyldimethlchlorosilane, the surface energy changed with the gradual changing of organosilane concentration. Therefore we were able to monitor gradient deposition by measuring the water contact angle.

Figure 3.10 shows the identification of the one direction gradient formation by the linear correlation between static contact angle and distance across the surface. The solid red line refers to the linear regression of the data and the slope of gradient is \(-15.2^\circ/\text{cm}\). This decrease in n-octyldimethlchlorosilane leads to decreased contact angle. The contact angle decreases from 65±5° to 27±4° down the length of the gradient. The standard uncertainty of contact angle measurements at each position was determined by the standard deviation between four independent measurements on three samples prepared under identical conditions.
Figure 3.11: The chlorine profiles were measured by static water contact angle. The static contact angle change gradually across the surface, reflecting the changing organosilane concentration. Deionized water static contact angle (mean ± S.D., n = 3).
Figure 3.12: The amine gradient profiles were measured by static water contact angle, achieved by phosphanimine hydrolization. Compared with the chlorine gradients, the contact angle changes indicate the amine groups were successfully functionalized onto the surface. Deionized water static contact angle (mean ± S.D., n = 3)
Figure 3.13: The hydroxyl gradient profiles were measured by static water contact angle, achieved by alkyne-azide cycloaddition. Compared with the chlorine gradients, the contact angle changes indicate the successful “click” reaction. Deionized water static contact angle (mean ± S.D., \( n = 3 \)).
Figure 3.12 and Figure 3.13 show the identification of the final one direction gradient by static contact angle comparison between original chlorine gradients and modified amine and hydroxyl gradients. Solid red lines refer to the linear regression of the data. Because chlorine is more hydrophobic than amine and hydroxyl group, average contact angle on chlorine surface is higher than latters. The $10^\circ$ decreasing contact angle in average after surface reaction show the amine and hydroxyl groups are successfully tethered onto the surface. The standard uncertainty of contact angle measurements at each position was determined by the standard deviation between four independent measurements on three samples prepared under identical conditions.
Figure 3.14: The vinyl end group gradient profiles were measured by static water contact angle. The static contact angle changed gradually across the surface, reflecting the changing organosilane concentration. Deionized water static contact angle (mean ± S.D., $n = 3$)
Figure 3.14 shows the identification of the one direction gradient formation by the linear correlation between static contact angle and distance across the surface. The solid red line refers to the linear regression of the data and the slope of gradient is -12.6 °/cm. The decrease in 5-hexenyldimethylchlorosilane concentration leads to decreased contact angle. The contact angle decreases from 65±5 ° to 40±4 ° down the length of the gradient. The standard uncertainty of contact angle measurements at each position was determined by the standard deviation between four independent measurements on three samples prepared under identical conditions.
3.2.4 X-Ray Photoelectron Spectroscopy (XPS)

For this project, the overall concentration of grafted reagent was what is what is being probed. The concentration profile and functionality were verified using high resolution C$_{1s}$ and survey spectra (0-700 eV). Core peaks were analyzed using a linear background and Figure 3.15 showed the peak fitting and overall peak envelope on methyl surface. The peak area was calculated by integration. The peak area decreased linearly with position along the substrate, matching the linear results obtained from contact angle measurement. According to the physical basis of XPS which measures the elemental composition, empirical formula, chemical and electronic state of the elements that exist within a material, quantitative XPS is used to determine the methyl concentration and surface coverage fraction. The surface coverage fraction was first calculated by the equation 3.1.
Figure 3.15: The XPS high resolution of C$_{1s}$ spectra for each gradient position on n-octyldimethylchlorosilane (-CH$_3$) covered surface. C$_{1s}$ curves of XPS spectra were fitted based on Gauss-Lorentz function.
Figure 3.15 shows the XPS high resolution of $C_{1s}$ spectra for each gradient position on n-octyldimethylchlorosilane (-CH$_3$) covered surface. $C_{1s}$ curves of XPS spectra were fitted based on Gauss-Lorentz function with Multipak software. The $C_{1s}$ consists of peaks assigned to C-C and C-H groups at 283.5eV and C-Si groups at 284.7-285.8 eV. Overall peak area was analyzed with Multipak software assuming a linear background.
Figure 3.16: Peak fitting of XPS C$_{1s}$ spectra for each gradient position on YIGSR covered surface. C$_{1s}$ curves of XPS spectra were fitted based on Gauss-Lorentz function.
Figure 3.16 shows the XPS high resolution of C\textsubscript{1s} spectra for each gradient position on YIGSR covered surface. C\textsubscript{1s} curves of XPS spectra were fitted based on Gauss-Lorentz function with Multipak software. The C\textsubscript{1s} consists of peaks assigned to C-C and C-H groups at 283.5 eV, C-N, C-O groups at 286.3 eV and N-C=O groups at 289.5 eV. Overall peak area was analyzed with Multipak software assuming a linear background.

\[
\text{Surface coverage fraction} = \frac{A_{\text{peak}} / \text{number of carbon atom of sample}}{A_{\text{peak,0}} / \text{number of carbon atom of standard}} \\
\text{Equation 3.1}
\]

The \(A_{\text{peak,0}}\) was obtained by testing a standard sample in a specific condition, which was the same as the condition our sample was tested. Then the overall area was divided by the number of carbon atom per molecule. Surface concentration was calculated with surface coverage fraction using the following equation 3.2.

\[
\text{Surface concentration} = \text{coverage fraction} \times \text{concentration of 100\% coverage} \\
\text{Equation 3.2}
\]

The surface concentration of 100\% surface coverage was 465 pmol/cm\textsuperscript{2} \textsuperscript{43}, obtained from literature research, representing the surface concentration with 100\% coverage fraction. Then the surface concentration and surface coverage fraction were plotted below in Figure 3.17 and Figure 3.18.
Figure 3.17: Surface concentration and surface coverage fraction varied as a function of position across the methyl-terminated gradient surface. Surface concentration and surface coverage fraction were determined by XPS.
Figure 3.18: Surface concentration and surface coverage fraction varied as a function of position across the YIGSR-terminated surface. Surface concentration and surface coverage fraction were determined by XPS.
Figure 3.19: Survey spectra (0-700 eV) from XPS for chlorine surface gradient. Peak at 201 eV was assigned as Cl$_2p$ element which verified the chlorine has been successfully tethered onto the surface.
Figure 3.20: Survey spectra (0-700 eV) from XPS for amine surface gradient. The disappearing Cl$_{2p}$ peak confirmed the quantitative conversion of chlorine to amine.
Figure 3.21: Survey spectra (0-700 eV) from XPS for hydroxyl group surface gradient.

The disappearing Cl$_2$p peak and appearing N$_{1s}$ peak confirmed the quantitative conversion of chlorine to hydroxyl group.
Figure 3.22: Reaction verification by high resolution Cl$_{2s}$ (a) and N$_{1s}$ (b) from XPS after SN2 substitution, alkyne-azide cycloaddition and phosphanimine hydrolization.
Figure shows the loss of the Cl$_2$s signal (a) and acquisition of the N$_{1s}$ signal following the azide reaction, alkyne-azide cycloaddition and phosphanimine hydrolization.

Two step azide-alkyne cycloaddition and phosphanimine hydrolization chemical reaction were used to achieve the amine-terminated and hydroxyl-terminated concentration gradients respectively. The loss of chlorine peak showed that chlorine group was efficiently displaced via S$_{N2}$ reaction by an azide salt and subsequently reduced to a primary amine (as shown in Figure 3.22).
CHAPTER IV

EFFECT OF SURFACE FUNCTIONAL GROUPS ON CHONDROCYTE BEHAVIOR
USING MOLECULAR GRADIENTS

4.1 Experimental Section

Effect of surface functional groups on chondrocyte behavior using molecular gradients was shown in this chapter.

4.1.1 Cell Isolation

Institutional Review Board (IRB) approval was obtained in Department of Orthopaedics, Summa Health System involved for use of human tissue. Chondrocytes cells were isolated from the tibial plateaus and femoral condyles of patients undergoing total knee arthroplasty (average age: 62 years, range: 61-63 years, total 2 knees (female and male)).

Isolated tissue was placed in 4 mg ml⁻¹ collagenase in Hank’s buffered salt solution for at least 4 h and washed twice with phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA). Cell suspension was then passed through a 22 mm diameter ~80 μm stainless steel syringe filter to remove cellular debris. The cells were precultured immediately after isolation in complete medium, Reduced Serum Eagle's Minimum
Essential Media (Opti-MEM® I Reduced Serum Media (1x), Gibco, BRL, Grand Island, NY, USA) supplemented with 0.1 mg/mL primosin and 0.05 mg/mL ascorbate. Cells are incubated in a humidified 5% CO₂-balanced air atmosphere at 37°C for 24 hours. The cells were plated in Corning® 75cm² rectangular canted neck cell culture flask (Corning, NY) and the medium was changed every other day.

4.1.2 Proliferation and Maintenance Assay

The precultured primary human chondrocytes were used in this study. The cells were seeded evenly onto each engineered substrates (molecular gradients) in Reduced Serum Eagle's Minimum Essential Media at a density of 3000 cells/cm² in 6-well plates. Cells were incubated in a humidified 5% CO₂-balanced air atmosphere at 37°C for 24 hours, 3 days, 7 days and 14 days and the medium was changed every other day. After each incubation period, supernatant was aspirated (media and unattached cells) from each well and different immunofluorescence were carried out according to different research interests of proliferation and chondrocyte phenotype maintenance assay. For cell proliferation assay, day 1, day 3 and day 7 time point were studied. Day 3, day 7 and day 14 time points were studied for chondrocyte phenotype maintenance assay.

4.1.3 Immunofluorescence

Human primary chondrocyte cells have two principal characteristics, proliferation and chondrocyte phenotype maintenance. Both of them must be monitored in order to ensure
safety and efficacy when chondrocytes are used as a cell source for cartilage treatment.

After each incubation period, supernatant was aspirated (media and unattached cells) from each well and all samples were fixed in warm (37°C) 3.7% formaldehyde solution at 37 °C on dry block for 5 minutes. Then the cells were permeabilized in 0.5% TritonX-100 in PBS at 37 °C for 10 minutes. Samples were rinsed for three times with cytoskeleton stabilization (CS) buffer in water, pH=6.9 at room temperature for 5 minutes each wash, and soaked in 0.05% fresh sodium borohydride in PBS in each well to quench activity and remove excess formaldehyde and incubated at room temperature for 10 minutes. 5% donkey serum in PBS was added in each well to block non-specific binding at 37°C for 20 minutes. Main wash was carried out with PBS three times at room temperature for 5 minutes each wash. Samples for proliferation assay were stained with rhodamine phalloidin (1:200 in PBS) at room temperature for 1 hour and then rinsed three times with PBS for 5 minutes each. Samples were then incubated in Alex Fluo 488 CS-maleimide (1:200) for 1 hour and washed three time and 5 minutes each. DAPI (1:1000 in PBS) was used to stain the cell nuclei for 20 minutes, followed by 5 minutes 3 times wash. Finally, a drop of mounting media was added onto a cover slip and the samples were flipped and faced down then, sealed with polish before use.

For chondrocyte phenotype maintenance assay, samples were fixed and blocked, following the previously mentioned protocol. After main wash was carried out with PBS three times at room temperature for 5 minutes each wash, whole mount samples for chondrocyte phenotype maintenance assay were incubated in 0.1% donkey serum with
0.01% sodium azide in PBS for 30 min at room temperature. Then samples were blocked by donkey serum (10%) for 1 hour and wash it with PBS once. After that, samples are incubated in primary antibodies (CD 14, 1:100 in PBS and CD 90, 1:100 in PBS) overnight. Then they are stained with appropriate Alexa Fluor secondary antibodies, anti-rabbit Alexa Fluor 546 and anti-goat Alexa Fluor 488, at 37°C for 1 hour, washed in PBS three times for five minutes each. Then cell nuclei are stained with DAPI (1:1000) for 20 minutes, washed and then viewed.

4.1.4 Statistics

Randomly picked images were taken for each concentration section along the gradients with 5 mm interval in between on each sample under 10×, 20× and 40× magnification. 3 gradients or control samples were prepared for statistical analysis and they were recorded in montage. The cell number and the CD14/CD90 ratio which was obtained by dividing the number of cells expressing CD14 by the number of cells expressing CD90 were calculated and averaged based on three samples. CellSens (bought from Olympus Corporation) was used to count cell number. The average number of cells per square micron in immunofluorescence was determined from nuclear staining.

4.2 Results and Discussion

The results from proliferation assay and chondrocyte chondrocyte phenotype maintenance assay were shown below and discussed.
4.2.1 Proliferation Assay

As such, actin, C5-Maleimide and nuclei organization were observed in our system over 7 days of culture. After one day of culture, chondrocytes in the gradient region with 243 pmol/cm\(^2\) methyl concentration were less spread than chondrocytes in regions with 172 pmol/cm\(^2\) methyl concentration. Then the same phenomenon was observed in the next 3 days and 7 days study (as shown in Figure 4.1). The statistic data revealed that relative high methyl hydrophobicity (contact angle of 65°), which correlated with increased concentration in our system (Figure 4.2), can inhibit cellular proliferation at first and led to a relative low cell density (27-55 cell/mm\(^2\)). When methyl concentration decreased across the gradient (from 243 pmol/cm\(^2\) to 172 pmol/cm\(^2\)), the cell density reached a maximum (54 cell/mm\(^2\)) at 0.75 cm (202 pmol/cm\(^2\)) and goes down gradually (to 24 cell/mm\(^2\)) in day 1 study. The cell density at day 3 and day 7 followed the same tendency at day 1, reaching the maximum (56 cell/mm\(^2\) and 41 cell/mm\(^2\)) at 1.25 cm (190 pmol/cm\(^2\)) for both and goes down gradually (to 41 cell/mm\(^2\) and 23 cell/mm\(^2\))
Figure 4.1: Immunofluorescent staining of actin (red), vinculin (green) and nuclear (blue) after (A) 1 day, (B) 3 days and (C) 7 days of culture. Images were taken every 5 mm down the length of the gradient. Scale bar=20 µm.
Figure 4.2: Human primary chondrocytes density response as a function of methyl concentration on methyl surface gradient. After 1, 3, 7 days of culture, cell number was collected at each sample position (mean ± S.D., n = 3).
4.2.2 Chondrocyte Phenotype Maintenance Assay

In this project, Surface marker CD 14 and CD 90 were used to test these two properties. Surface marker CD14 is a lipopolysaccharide receptor found on freshly isolated chondrocytes, which can be used to quantify chondrocyte phenotype maintenance while surface marker CD90, a glycosylphosphatidylinositol-anchored glycoprotein, is associated with cellular proliferation. The CD14/CD90 ratio was obtained by dividing the fraction of cells expressing CD14 by the faction of cells in expressing CD90. The CD14/CD90 ratio increased rapidly with time from 1.9±0.3 to 5.1±0.3 at the first 7 days and fall down to 1.0±0.1 in the end. However, the ratio along the methyl concentration gradient didn’t vary significantly which means the phenotype of human primary cells maintained almost the same throughout the methyl concentration gradients (from 243 pmol/cm$^2$ to 172 pmol/cm$^2$) (shown in Figure 4.3). Since red dye was bind to CD 14 and green dye was bind to CD90, the expression of CD 14 was much intensive than the CD 90 at the first 7 days in Figure 4.3 which matches the result from statistic data in Figure 4.4.
Figure 4.3: Immunofluorescent staining of CD 14 (red), CD 90 (green) and nuclear (blue) after (A) 3 day, (B) 7 days and (C) 14 days of culture. Images were taken every 5 mm down the length of the gradient. Scale bar=20 μm.
Figure 4.4: Chondrocyte survival of each gradient position based on phenotype maintenance using CD14/CD90 ratios response as a function of methyl concentration on methyl surface gradient for days 3, 7, and 14. The CD14/CD90 ratio was obtained by dividing the fraction of cells expressing CD14 by the fraction of cells in expressing CD90 (mean ± S.D., n = 3).
CHAPTER IV

SUMMARY

This thesis outlined the urgent clinical demand of implant material for cartilage medicine applications with high added values. The strategic fabrication of surface chemical gradients they were used to measure the optimal modification concentrations of bioactive growth factors and surface chemistry. The substrates provide a testing platform to investigate the optimal concentrations in an efficient manner. Gradient substrates were fabricated using the “vacuum away” confined channel vapor deposition method which is highly tunable and versatile. Methyl, amine and hydroxyl end groups, as well as YIGSR peptide, were investigated here as a target chemistries. Herein we examined the effects of methyl surface functional group concentration on chondrocyte proliferation and phenotype maintenance on continuously variable one-dimensional methyl concentration gradients. Cell numbers were found to vary along the chemistry concentration gradients and changed with time, reaching a maximum cell density (41-56 cell/mm²) at 190-202 pmol/cm² methyl concentration and chondrocyte phenotype was maintained throughout
the gradients. Spatial presentation of surface chemistry has a significant impact in modulating human osteoarthritic chondrocyte behavior.
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