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Techniques For Type I Collagen Organization

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Techniques for Type I Collagen Organization

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Biographical Sketch

LaTecia Diamond Anderson-Jackson was born on August 2, 1988, in Cheverly, Maryland. She received her Bachelor of Science degree in Chemical Engineering from North Carolina Agricultural and Technical State University in 2010. As an undergraduate LaTecia was a part of many research programs. In 2007, she was considered as a Solar Energy Trainee for the Department of Energy (DOE). She became a research intern for the National Renewable Energy Laboratory (NREL) in Golden, CO in 2008. LaTecia also became a research assistant working under Dr. Shamsuddin Ilias for the National Oceanic and Atmospheric Administration (NOAA) in 2009. While attending North Carolina Agricultural and Technical State University as undergraduate she featured in two publications. One of those two publications was submitted and published by the Materials Research Society (MRS), titled Light Induced passivation of Si by Iodine Ethanol solution in 2008. The other publication Analysis of the Sol-Gel synthesis of Tungsten-Based nanomaterials in a multiphase semi-batch reactor was presented at the American Institute of Chemical Engineers (AIChE) annual meeting in 2008. She is a candidate for the Master of Science degree in Nanoengineering.

Dedication

This book is dedicated to my biggest supporters my mother and stepfather, Deirdrea Hill & Charles Hill Jr, and father and stepmother, Wilbert Jackson Jr & Tecia Jackson. I would also like to dedicate this book to my siblings Monique Khan, Dwayne Khan, Samantha Hill, Laila Jackson, and Mikaila Jackson. Thank you for all the support each of you has given me in reaching my goals over the years. Love you all.

Acknowledgements

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List of Abbreviations

Abbreviations	Meaning
HIM	Helium Ion Microscope
AFM	Atomic Force Microscope
HRSA	Human Resource of Service Administration
ECM	Extracellular Matrix
nm	nanometer
PDMS	Polydimethylsiloxane
THF	Tetrahydrofuran
DC	Direct Current
PAN	Polyacrylonitrate
PA	Peptide-Amphiphile
PVA	poly(vinyl alcohol)
PVP	poly(vinyl pyrroldine)
UV	Ultraviolet
DI/dH_2O	Deionized Water
PBS	Phosphate Buffer Saline
PVD	Physical Vapor Deposition

Abstract

Tissue Engineering is a process in which cells, engineering, and material methods are used in amalgamation to improve biological functions. The purpose of tissue engineering is to develop alternative solutions to treat or cure tissues and organs that have been severely altered or damaged by diseases, congenital defects, trauma, or cancer. One of the most common and most promising biological materials for tissue engineering to develop scaffolds is Type I collagen. A major challenge in biomedical research is aligning Type I collagen to mimic biological structures, such as ligaments, tendons, bones, and other hierarchal aligned structures within the human body. The intent of this research is to examine possible techniques for organizing Type I collagen and to assess which of the techniques is effective for potential biological applications. The techniques used in this research to organize collagen are soft lithography with solution-assisted sonication embossing, directional freezing, and direct poling. The final concentration used for both soft lithography with solution-assisted sonication embossing and direct poling was 1 mg/ml, whereas for directional freezing the final concentration varied between 4mg/ml, 2mg/ml, and 1 mg/ml. These techniques were characterized using the Atomic Force Microscope (AFM) and Helium Ion Microscope (HIM). In this study, we have found that out of the three techniques, the soft lithography and directional freezing techniques have been successful in organizing collagen in a particular pattern, but not alignment. We concluded alignment may be dependent on the pH of collagen and the amount of acetic acid used in collagen solution. However, experiments are still being conducted to optimize all three techniques to align collagen in a unidirectional arrangement.

CHAPTER 1

Overview

1.1 Motivation

The purpose of tissue engineering is to develop alternative solutions to treat or cure tissues and organs severely altered by diseases, congenital defects, trauma, or lost by cancer. In order for artificial tissue or organs to be utilized they must be developed using biomaterials and cells. However, it is not required but is recommended for the artificial tissue or organ's to be conformed to that specific tissue or organ native state. Collagen is a popular material used in biomedical research because of its natural composition and compatibility within the human body. Because 90% of the body make-up is collagen it is an important component in the human body make-up, from bone structure to skin composition. Because of this factor, a fundamental question in tissue engineering is: How can collagen contribute to successful biological applications? Scientist and engineers have been seeking answers to this question for over a decade. Applying collagen is an important biological process to understand in order to make it applicable to various applications, such as, biomedical research (regenerative medicine), biomaterials research, and studies involving cell adhesion and growth.

The intent of this research is to explore organization of Type I collagen fibrils for potential biological applications. There are three consistent objectives within this study: (1) observing collagen fiberization in its native state without interference of techniques, (2) characterizing organization of Type I collagen using soft lithography, directional freezing, and electric poling, and (3) determining the technique that is most effective. This thesis will further explain each techniques advantages and disadvantages. Another question to be answered in this research is what technique is classified to be time efficient and inexpensive to implement.

1.2 Thesis Outline

In this thesis, I present a comprehensive study of tissue engineering, including basic knowledge of specific features in my research. I begin in Chapter 2, with an introduction to tissue engineering, consisting of tissue engineering history, statistical data in regards to the United States, and treatments that have been used prior to tissue engineering. I follow with introducing the background and theory of tissue engineering and how this process is used for biological applications. I then present information to provide a fundamental understanding of the materials and techniques utilized in my research. Chapter 3 will contain a literature review of research that has been conducted to date in regards to general alignment, collagen alignment, electric poling alignment, and directional freezing alignment. Therefore, this chapter will also include research background on collagen alignment specifically. Chapter 4 introduces the methodology and procedures of my research. This chapter will consist of materials and methods used for each alignment technique. In Chapter 5, results are presented for soft lithography, direct current (electrical poling), and directional freezing by characterization. Chapter 5 will introduce the two characterization methods used in this research, entailing the use of Helium Ion Microscope (HIM) and Atomic Force Microscope (AFM) along with a discussion. In Chapter 6, I conclude with answering my thesis questions and providing possible future research.

CHAPTER 2

Introduction

The first successful organ transplantation was in 1954 between two identical twins in Boston's Bringham and Women's Hospital and established organ transplantation as the best solution for saving lives after tissue and organ damage (U.S Department of Health and Human Services, 2013). According to Health Resources and Services Administration (HRSA) under the U.S Department of Health and Human Services, in 1988, the national data collection of organ transplantation began and more than 560,000 transplants have been successfully performed in the United States (HRSA, 2013). Due to organ transplantation the success rates of recipients post-transplant have increased in the number of years. However, organ transplantation is not available to all patients. Often, the number of patients in need of transplants outnumbers the amount of donors. While the number of donors increases over the years, it still has not kept pace with the demand of organs needed. On July 9, 2013 data was analyzed by HRSA quantifying living and deceased donor donations. As of 2012 an estimate of 14,013 of people donated organs, whereas, the number of people in need of transplantations estimated to be 117,040 (HRSA, 2013)

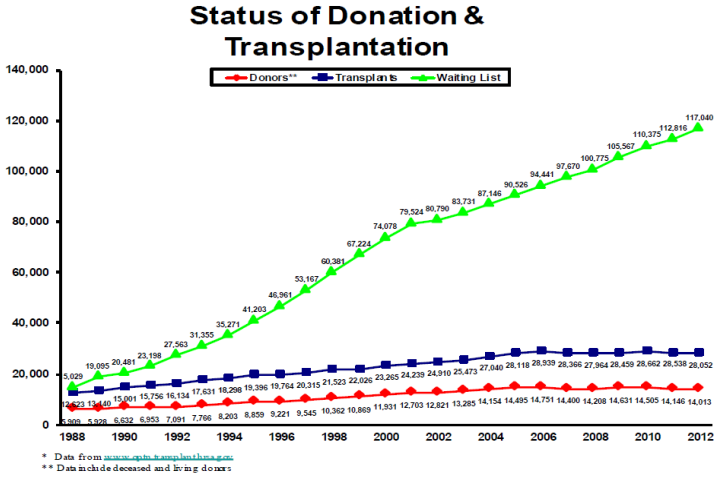

Figure 2.1 A graph indicating the large gap of donors to recipients every two years (HRSA, 2013).

One of the major contributors to the nation's health care costs is patients who suffer from organ failure and tissue loss. Approximately 8 million surgical procedures are performed annually in the US to treat these disorders (Langer & Vacanti, 1993). Over the past 50 years, current treatment of organ failure and tissue loss consists of reconstructive surgical techniques, transplantations of various tissues, and replacements with mechanical devices (Fuchs, Nasseri, & Vacanti, 2001). Each therapy has transformed medical practices and shown significant improvement in patient outcomes, however, they have limitations. As stated before transplantations of various tissues or organs are restricted by the donor shortages that increase a substantial amount annually in comparison to the transplants that are needed. As a consequence of donor shortages many patients die while awaiting an organ or tissue to become available for transplantation. Another issue with transplantation is immune rejection post-transplantation. Mechanical devices only provide a temporary solution or benefit to the body because it cannot perform all the functions of a single organ. Also, patients would have to go through some type of therapy due to the body becoming dependent on the mechanical device performing the function needed once it has been removed. Reconstructive surgery may or may not be a permanent solution depending on reoccurrence or mistakes possibly made by the physician. A possible solution for the shortcomings of the current treatments of organ failure and tissue loss is the use of tissue engineering, which is an area of which the scientific, engineering, and medical communities are undertaking as the next best alternative treatment.

2.1 Background and Theory

In the beginning tissue engineering was based on cell and tissue culture approaches. In the early 1970s a scientist by the name of W.T. Green embark on many experiments to generate

cartilage using a chondrocyte culture technique in combination with a bone scaffold (Meyer, 2009). Although, Green was unsuccessful in developing a method to generate new cartilage, he established the theoretical and practical concept to incorporate cells with scaffolds. In the mid-1980s the term 'tissue engineering' was loosely utilized in literature in regards to surgical manipulation of tissues and organs. The term was also applied in a broader sense when prosthetic devices or biomaterials were used (Fox, 1988). In 1987, tissue engineering was introduced to medicine and a new definition of the term was established. Meyer et al (2009) stated in his documentation the definition of tissue engineering was define as "the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure function relationships in normal and pathologic mammalian tissue and the development of biological substitutes to restore, maintain, or improve function". Approximately 30 years ago the term tissue engineering was formed to signify a new perception that focuses on regeneration of tissues from cells with the support of biomaterials and growth factors (Ikada, 2006). The purpose of tissue engineering research is to establish new clinical technology that can contribute to potential medical treatments for diseases that have been too difficult to be treated or cured by conventional methods. However, the primary goal of the various approaches of tissue engineering is the repairing of function through the delivery of living components which become integrated into the patient (Vacanti & Langer, 1999). Although, some techniques of tissue engineering are steered towards only matrices and other approaches are directed to use cells only, most researchers are trying to achieve new tissue formation by incorporating both factors together. To regenerate new tissues or organs, biomedical engineering uses three basic tools; cells, scaffold, and growth factors (Ikada, 2006). Cells can be tissue specified and the matrix may be a natural or synthetic scaffold. The objective of the scaffold is to resemble the extracellular

matrix (ECM) that is naturally a part of the body make-up, therefore, it could be fibrous (collagen or gelatin), a foam, a hydrogel, or capsules.

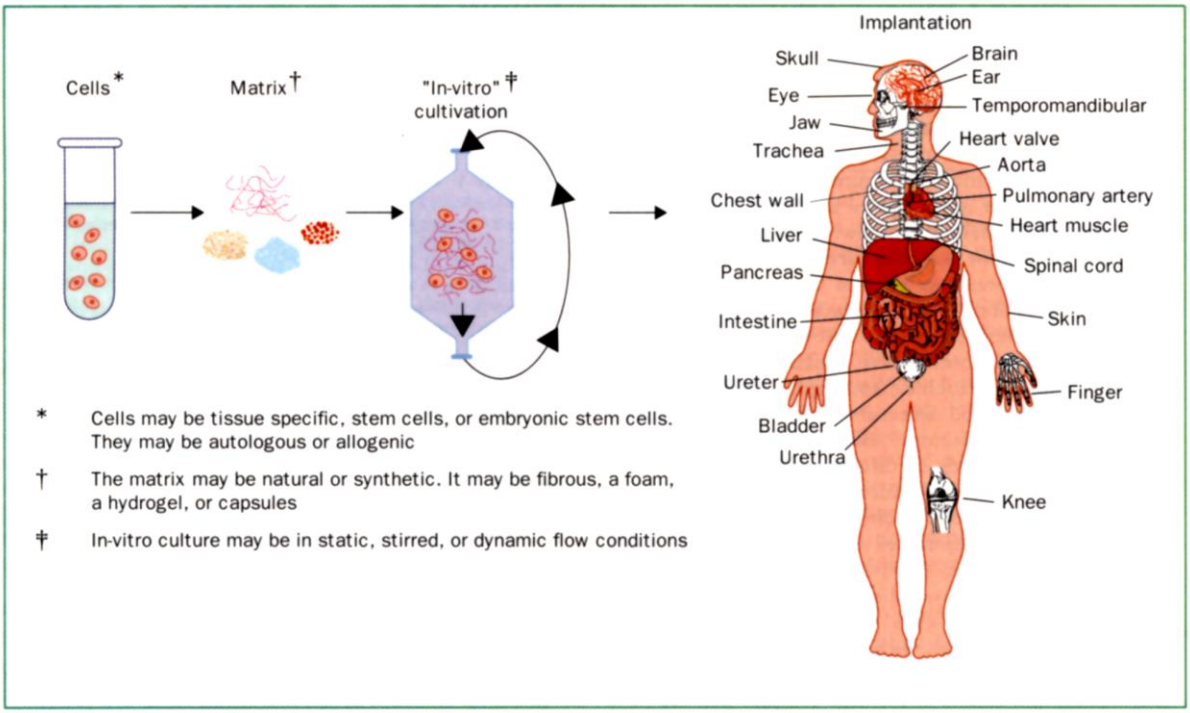

Figure 2.2 Schematic diagram of tissue engineering process (Vacanti & Langer, 1999).

When tissues or organs have been severely diseased, damaged by cancer, congenital abnormality, or trauma that conventional pharmaceutical treatments are no longer applicable, artificial organs or tissues is one of the first alternative options to reconstruct the damaged tissue or organ (Ikada, 2006). A unique characteristic of tissue engineering is the capability of regenerating patient's own tissues and organs that are completely free of poor biocompatibility, low bio-functionality, and low severe immune rejection. Tissue engineering is considered to be the ideal medical treatment due to its exceptional advantages.

2.1.1 Basic Collagen Structure

The main function of collagen is to hold cells and tissues together along with protecting structures (i.e. skin) by inhibiting absorption and spreading pathogenic substances, micro-organisms, environmental toxins, and cancer cells in the body (Mandal, 2012). It is also vital for strengthening blood vessels and providing elasticity and tensile strength to skin. Collagen makes up approximately 25% to 35% of the proteins within the body and is considered to be the most abundant protein in mammals because it is the main component in connective tissue (Sikorski, 2001). Collagen is the main insoluble fibrous protein in the extracellular matrix and connective tissue in the human body. It is made up of specific amino acids, glycine, proline, hydroxyproline, and arginine; which are built up from hydrogen, oxygen, and carbon (Di Lullo, Sweeney, Körkkö, Ala-Kokko, & San Antonio, 2002). These strong and tough structures can be found in bones, tendons, ligaments, blood vessels, and skin (Brandt, 2007).

There are over 16 types of collagen, however, approximately 80 to 90 percent of collagen in the human body consist of collagen types I, II, and III. Collagen types I, II, and III are collagen molecules that are pack together to form long thin fibrils that average around 300 nm long (Lodish, 2000). Along these fibrils are quartered staggered packing array of molecules that produces a characteristic of banding patterns (Orgel & Irving, 2006). The structural unit of the fibril-forming collagen is called tropocollagen. The subunits of tropocollagen produce the self-assembled fibrils whose molecules staggered by each other approximately sixty-seven nanometers (nm) a part in collagen type I (Orgel & Irving, 2006). The structure of the fibrous proteins is the main source of strength in connective tissue. The staggered arrangement collagen strands within the fibril is shown in Figure 2.3 (c). In natural bones, this space is filled with a calcium phosphate, and hydroxyapatite, and known to be of vital importance to bone formation.

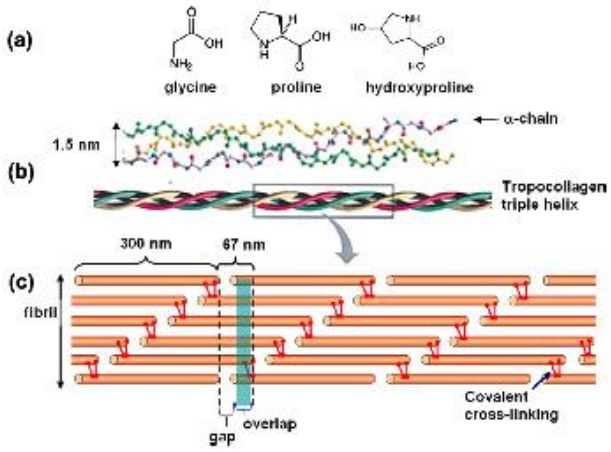

Figure 2.3 (a) major amino acid residues found in collagen; (b) triple helical structure of tropocollagen formed by three collagens strands; (c) schematic of assembled tropocollogen strands into collagen fibrils (Chen, P. Y. et al., 2012).

The three most common residues found in collagen, are composed of Glycine, Proline, and Hydroxyproline. A unique feature of collagen is the systematic arrangement of the amino acids in each of the three chains, self-assemble fibrils, of the collagen subunits (JPK Instruments, 2009). The sequence usually follows the pattern Glycine-Proline-X, whereas, X may be any of the various other amino acid deposits (Szpak, 2011). The triple helical structure can be represented by repeating the Glycine-Proline-X, sequence in the axial direction of the molecule. Rotation of the polypeptide chains are limited by the amino acids located at the X_{AA} and Y_{AA} positions of the $(Gly\text{-}X_{AA}\text{-}Y_{AA})_n$ repeat unit. The location of the amino acids side-chains, in the X_{AA} and Y_{AA} positions, are based on the conformation of the triple helix. These chains are located on the surface of the molecule, allowing them to interact with other molecules or neighboring chains. The structure of the bi-functional interchain cross-link is the relatively complex condensation product of a reaction involving lysine and hydroxylysine residues; this reaction continues as the organism matures, thereby causing the collagen of older animals more difficult to extract from tissues (Prockop & Kivirikko, 1995).

Type	Class	Composition	Distribution	Pathology
I	Fibrillar	$\alpha1[I]_2\alpha2[I]$	Abundant and widespread: Dermis, bone, tendon, ligament	Osteogenesis imperfecta, Ehler-Danlos syndrome, Osteoporosis
II	Fibrillar	$\alpha1[II]_3$	Cartilage, Vitreous	Osteoarthrosis, Chondrodysplasia
III	Fibrillar	$\alpha1[III]_3$	Skin, Blood Vessels, Intestine	Ehler-Danlos syndrome, Arterial aneurysms
IV	Network	$\alpha1[IV]_2\alpha2[IV]$ $\alpha3[IV]\alpha4[IV]\alpha5[IV]$ $\alpha5[IV]_2\alpha6[IV]$	Basement membranes	Alport syndrome
V	Fibrillar	$\alpha1[V]_3$ $\alpha1[V]_2\alpha2[V]$ $\alpha1[V]\alpha2[V]\alpha3[V]$	Widespread: Bone, dermis, cornea, placenta	Ehler-Danlos syndrome
VI	Network	$\alpha1[VI]\alpha2[VI]$ $\alpha3[VI]^a$ $\alpha1[VI]\alpha2[VI]\alpha4[VI]$	Widespread: Bone, dermis, cornea, cartilage	Bethlem myopathy
VII	Anchoring fibrils	$\alpha1[VII]_2\alpha2[VII]$	Dermis, Bladder	Epidermolysis bullosa acquisita
VIII	Network	$\alpha1[VIII]_3$ $\alpha2[VIII]_3$ $\alpha1[VIII]_2\alpha2[VIII]$	Widespread: Dermis, brain, heart, kidney	Fuchs endothelia corneal dystrophy
IX	FACIT	$\alpha1[IX]\alpha2[IX]\alpha3[IX]$	Cartilage, Cornea, Vitreous	Osteoarthrosis, Multiple epiphyseal dysplasia
X	Network	$\alpha1[X]_3$	Cartilage	Chondrodysplasia
XI	Fibrillar	$\alpha1[XI]\alpha2[XI]\alpha3[XI]$	Cartilage, Intervertebral disc	Chondrodysplasia, Osteoarthrosis
XII	FACIT	$\alpha1[XII]_3$	Dermis, Tendon	-
XIII	MACIT	-	Endothelial cells, Dermis, Eye, Heart	-
XIV	FACIT	$\alpha1[XIV]_3$	Widespread: Bone, dermis, cartilage	-
XV	MULTIPLEXIN	-	Capillaries, Testis, Kidney, Heart	-
XVI	FACIT	-	Dermis, Kidney	-
XVII	MACIT	$\alpha1[XVII]_3$	Hemidesmosomes in epithelia	Generalised atrophic epidermolysis bullosa
XVIII	MULTIPLEXIN	-	Basement membrane, Liver	Knobloch syndrome
XIX	FACIT	-	Basement membrane	-
XX	FACIT	-	Cornea (chick)	-
XXI	FACIT	-	Stomach, Kidney	-
XXII	FACIT	-	Tissue junctions	-
XXIII	MACIT	-	Heart, Retina	-
XXIV	Fibrillar	-	Bone, Cornea	-
XXV	MACIT	-	Brain, Heart, Testis	Amyloid formation
XXVI	FACIT	-	Testis, ovary	-
XXVII	Fibrillar	-	Cartilage	-
XXVIII	-	-	Dermis, Sciatic nerve	Neurodegenerative disease

Figure 2.4 Table of collagen types, class, composition, distribution, and pathology (Jain, Kaur, Pandav, Dewan, & Saxena, 2014)

Research on collagen containing biological systems, has been shown to exhibit carefully arranged multilayered hierarchal systems. There are multiple steps involved in the biological assembly of a collagen fiber from amino acids to the staggered fibril arrangement. Initially, the collagen molecule is synthesized from its precursor in the fibroblast cell known as procollagen. Each subunit of the procollagen precursor contains extension peptides located at their amino and carboxyl termini. Next, the procollagen chains self-assemble into left handed aggregates forming a right handed triple helical bundles. The extension peptides are necessary in order for the chains to obtain proper alignment with respect to one another, which are then removed after procollagen bundles are secreted into the extracellular matrix (ECM). Subsequent collagen microfibrils form when collagen molecules aggregate in the ECM near the surface of the fibroblasts. Finally, these collagen molecules pack into a quasi-hexagonal lattice at an interchain

distance of about 1.3 nm, which is a quarter of its length from its neighboring molecules in the bundle, so that there is complete overlapping only at intervals of five molecules. In the microfibril aggregates, a distance between the C-terminus and N- terminus of consecutive collagen molecule is about thirty-two nm.

Each type of collagen is subject to different parts of the body and due to the nature of this research, collagen type I would be the main focus. Collagen type I representative tissues are skin, tendon, bone, ligaments, dentin, and interstitial tissues. The COL1A1 gene produces a component of type I collagen called the pro-alpha1 chain, by which combines with another pro-alpha1 chain and a pro-alpha2 chain created by the COL1A2 gene to make a molecule of type I pro-collagen. These pro-molecules have to be processed by enzymes outside of the cell and after being processed they arrange themselves into long thin fibrils that cross-link with one another in spaces around the cell. The cross-link between these long thin fibrils forms a formation of very strong type collagen I fibers (Sikorski, 2001). Since collagen is an abundance of tendon-rich tissue, for experimentation purposes rat tail collagen type I is used because it is easy to isolate and earlier research has shown rat tail type I collagen was the first to be characterized (Lodish, 2000). Type I collagen can self-assemble into structures called fibrils, which are estimated to be tens to hundreds of nanometers in diameter.

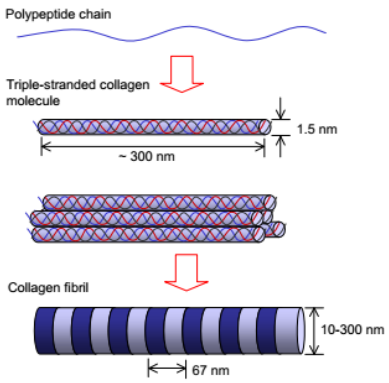

Figure 2.5 A schematic diagram of Type I collagen fibril structure (JPK Instruments,2009).

Side chain interactions between pairs of residues, together with backbone features and solvation, determine the conformation and stability of such proteins. Figure 2.4 shows the stages of fiber formation from an initial tropocollagen molecule. A unit referred to as the 'D' band changes slightly along the length of the fibril depending upon the hydration state of the aggregate and also repeats itself in the micro-fibril. Collagen fibrils are arranged in diverse combinations and concentrations in various tissues to provide changeable tissue properties. For example, in bone, the whole collagen triple helices lie in parallel, staggered array, approximately forty nanometer gaps between the ends of the tropcollagen subunits that serves as a nucleate site for deposition of hydroxyapatite, mineral component composed of fine crystals (Ross & Pawlina, 2011). Whereas, the arrangement in skin is a network of fibers and fiber bundles that are arranged in various directions, constructing a densely interwoven fiber pattern. The interwoven fiber arrangement gives skin and bone the ability to withstand mechanical stresses of tension and provide tensile strength (Meyer, Neurand, & Radke 1981). Although, collagen is known and used in research for its mechanical properties, it also has electrical properties that are sometimes used in biological research. Electrical properties of solid-state proteins allow classification of proteins as conducting semiconducting or dielectric materials (Gauza, 2009).

2.1.2 Soft Lithography

Soft lithography is a fabrication technique that is used to replicate structures measured on the micrometer to nanoscale. The name “soft” comes from the usage of elastomeric materials to make stamps, molds, and photomasks. Soft lithography can also be considered as rapid phototyping, suited for replicating a pattern with nonphotolithographic methods (McDonald, 2000). Polydimethylsiloxane (PDMS) is most commonly used in this particular technique because it is inexpensive, easy fabrication technique, flexibility and its optical transparency (Zhang, Dung, Korman, Li, & Zaghlool, 2013). PDMS belongs to a polymeric organosilicon (carbon-silicon bonds) compound group that is referred as silicones (Joint Assessment of Commodity Chemicals, 2011). The physical appearance of PDMS is optically clear with characteristics of being inert, non-toxic, and non-flammable. PDMS exhibits viscoelastic mechanical behavior, meaning it has both viscous and elastic properties when undergoing deformation. At long flow times and/or high temperatures PDMS is a viscous liquid. However, at short time flow times or low temperatures it is an elastic solid, similar to rubber. Therefore, if PDMS is left on a surface for an extended period of time it will flow to cover the surface and mold to any surface imperfections, which is used in soft lithography.

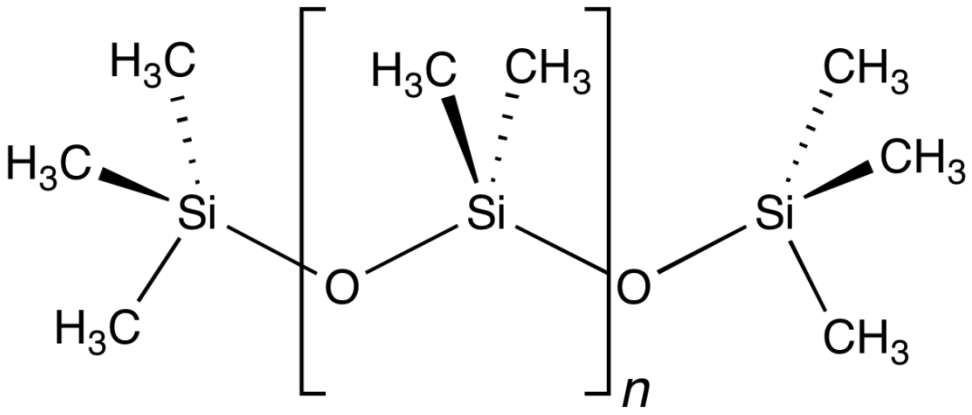

Figure 2.6 PDMS chemical structure (American Physical Society, 2014)

PDMS contains repeating units of $–OSi(CH_3)_2–$ groups, causing its surface to be hydrophobic in nature. Exposure of the surface to oxygen, or air plasma, will change the surface to one which is hydrophilic. Such exposure to plasma introduces Si-OH ("silanol") groups, and also destroys $Si-CH_3$ ("methyl") groups. Such a modification to standard PDMS creates what is known as "Plasma-oxidized PDMS" and can be wetted by aqueous, polar solvents. If the oxidized PDMS is allowed to stand for prolonged times, the hydrophilic surface reverts back to a hydrophobic one. The mechanism behind such is due to surface reconstructions of the non-crosslinked components. Therefore, it is possible to keep a plasma treated PDMS surface hydrophilic indefinitely by keeping the surfaces in contact with water or polar organic solvents (Tang & Whitesides, 2009).

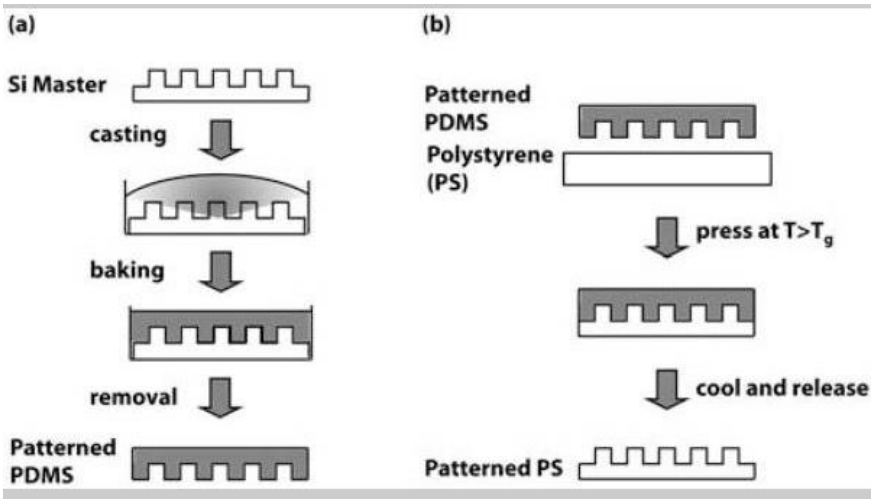

Figure 2.7 Depiction of Soft Lithography technique process (a) PDMS molded from Si master after baking and removal. (b) PDMS mold used for photo imprinting onto polymeric substrate (Chalut,Kulangara, Giacomelli, Wax, & Leong, 2010)

As a result of PDMS chemical make-up most organic solvents, such as, chloroform, ether, and tetrahydrofuran (THF) will diffuse into the material and cause the PDMS to swell, making them incompatible with PDMS devices. However, PDMS can be used in combination with water and alcohol solvents without material deformation. Some organic solvents produce

small swelling that can be used with PDMS, such as, acetone, 1-propanol, and pyridine. In order to reduce the absorption of small molecules and essentially swelling by nonpolar organic solvents, PDMS can be modified with silica particles or the surface can be coated with a glass-like layer using sol-gel chemistry. The primary usage of PDMS in soft lithography is to transfer patterns from the master substrate, usually only a few nanometers in size, onto glass, silicon, or polymeric surfaces. This technique is most useful for biomedical research and optic telecommunications.

2.1.3 Direct Current (Electrical Poling)

Direct Current (DC) is the uninterrupted unidirectional flow of electric charge, meaning the current flows in only one direction. In context of some applications, DC is also considered as the continuous flow of charged particles. In clinical applications the flow of charged particles must be continuous with no interruptions for at least one second to be considered as direct current (Robinson, 2008). DC is produced by a fixed-magnitude voltage applied to a conductor with a fixed resistance that develops an electrical circuit. The electric current is created by a source, such as, batteries, solar cells, thermocouples, and electrical machines incorporated with some type of electrical switch. DC is commonly used to flow through conductors, such as wires, but DC can also be used to flow through insulators, semiconductors, or a vacuum (ion or electron beams).

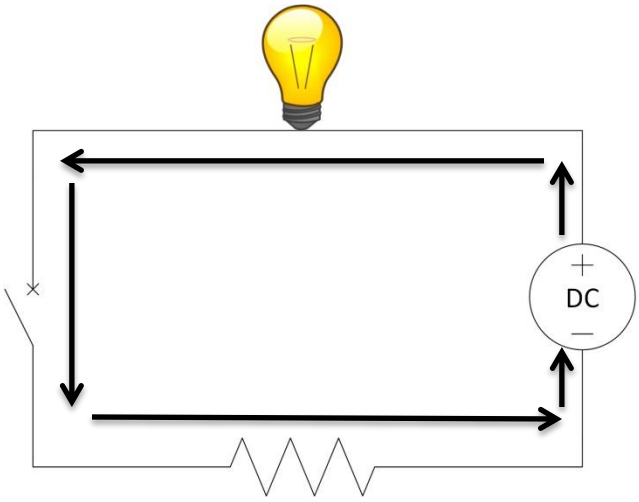

*Figure 2.*8 Schematic diagram of Direct Current (DC) circuit depicting direction of the current flowing in the circuit.

The fixed electromotive force that is used as a source develops a chemical reaction. This chemical reaction produces an abundance of electrons on one pole (cathode) and a shortage in electrons on the opposite pole (anode). Cathode is considered as the negatively charged electrode, attracting cations or positive charges. It is the source of electrons or referred to as the electron donor. Whereas, anode is the positively charged electrode, attracting electrons or anions, therefore, being a source of positive charge or an electron acceptor. The opposition of both the cathode and anode within the circuit represents as a resistor (Robinson, 2008). Typically, electrons flow from an area of high concentration (cathode) to an area of low concentration (anode) when the switch in the circuit is closed. The flow of the electrons, which are prevented from the resistance of the wire, will continue to flow until the charge difference between terminals is eradicated. This elimination occurs when the chemical reactions within the source can no longer provide free electrons to the negative terminal. Although, DC consist of charged particles moving from negative to positive terminals, the current, moves in the opposite direction from positive to negative terminals. Some systems of DC barely have no variations in voltage, but may have variations in output power and current. Electrical currents, such as, DC, has been

applied to biological applications. For example, DC current was applied to nerves to enhance regeneration(Shen and Zhu 1995).

2.1.4 Directional Freezing

Freeze drying (lyophilization) is the process of which solutions are frozen in a freezer, cool bath, or liquid nitrogen and then placed under a vacuum to remove frozen solvent through sublimation. In recent years, lyophilization has been recognized as a unique method to produce novel porous materials for tissue engineering and biological applications (Qian & Zhang, 2010). The freeze drying method is suitable for creating porous materials because the process inhibits impurities into samples and it is possible to produce a generous amount of pore morphologies and nanostructures. Porous structures area developed by voids left by the removal of the solvent in an aqueous solution (Qian & Zhang, 2010). Directional freezing is a technique used in freeze drying to control the direction of ice formation during the freezing process. It enables the growth of ice crystals to be orientated in one direction. This technique is accomplished by incorporating a high temperature gradient across or within the sample to allow ice crystals to form, growing from low to high temperature. During the freezing process the particles or polymeric molecules from solution are omitted from the ice and accumulates between the growing crystals in an orientated formation (Zhang & Cooper, 2007) .The drying process allows the removal of the orientated ice crystals and results in a material with unidirectional pores.

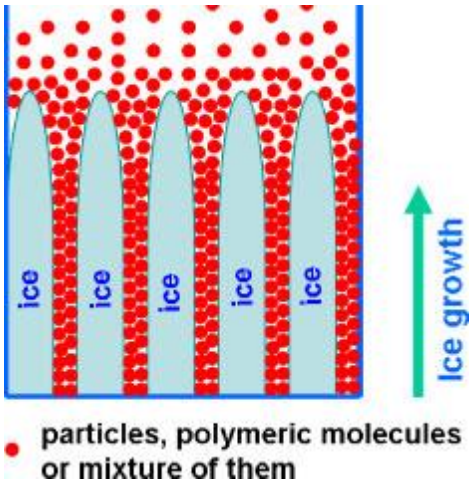

Figure 2.9 Particles or polymeric molecules are omitted from the solution and accumulates in between the growth of the ice crystals in a unidirectional formation (Zhang & Cooper, 2007).

CHAPTER 3

Literature Review

3.1 Fiber Alignment

Aligned methods have previously been used to produce orientated nanofibers for biological applications, electronics, composite materials, and other applications. This technique involves an assembly process, by which pre-existing elements that form an organized structure, which contains an interaction amongst the elements themselves without any outside directions. Some assembly of fibers consists of molecular self-assembly, which involves the usage of spontaneous molecules under equilibrium conditions into stable organized aggregates joined by non-covalent bonds (Whitesides, Mathias, & Seto, 1991). For example, Zhang et al studied amphiphilic molecules when elevating temperatures that supplied alignment of supramolecular fibrils that is visible to the naked eye (2010). In addition Feng et al aligned polyacrylonitrate (PAN) nanofibers by using templates with different pore diameters, while studying super-hydrophobic surface (2002). In recent years, researchers have been interested in developing alternative techniques to align nanofibers or using older techniques that could be applicable to aligning nanofibers. Soft lithography is a technique that has been used as a fabrication method to assist in creating nanostructures over a large scale. Hung et al uses soft lithography with incorporating a technique termed sonication-assisted solution embossing. Hung et al introduces this technique of alignment in peptide-amphiphile (PA) nanofibers by nanostructures self-assembling through solvent evaporation after applying a patterned elastomer stamp to PA solution while being agitated by ultra-sonication (2007). In this study it was concluded that alignment is achieved by spatial confinement and possible transitions of the lyotropic crystalline phase as the solvent evaporates (Hung & Stupp, 2007).

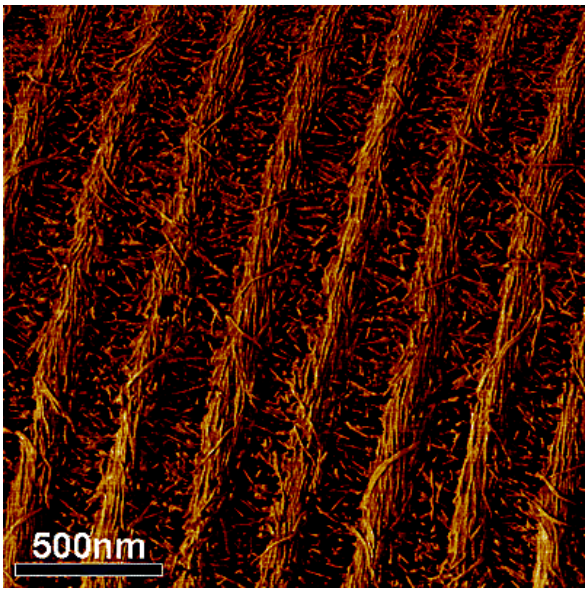

Figure 3.1 AFM phase imaging of Peptide-Amphiphile fiber alignment along channels produced by PDMS stamp (Hung & Stupp, 2007).

Another technique that is commonly used for organizing nanofibers is electrospinning. Electrospinning is a technique that has been recognized to be efficient in fabricating polymer nanofibers. Relatively, most nanofibers obtained in a non-woven form, which can be used for limited number of applications. This particular technique is challenging to achieve straight-lined nanofibers due to the "whipping" that occurs when electrospinning onto a target. However, Fong et al achieved an alignment formation from electrospinning nylon 6 and montmorillonite-nylon 6 composite nanofibers with diameters between 100 and 500 nm (2001). Another group succesful in aligning nonofibers was Luming et al who were able to electrospin poly (vinyl alcohol) (PVA) and poly (vinyl pyrroldine) (PVP) by using two oppositely metallic needles that are connected to positive and negative voltages. In this study the nanofibers were expelled from the two needles in a yarn format, which were combined and wounded by a cylinderical collector rotating at high speed (2006). In addition, Liu et al developed a unique technique entailing the effects of residual charges on nanofiber alignment using the gap method presented by Li et al that produced

electrospun nanofibers carrying residual charges after deposition across the gap of two electrodes (2008). Electrospinning is a known method used to apply an electric field, however, producing an elecric field without electrospinning has also been studied as its own application. Applying an electric field is effective method to align or fabricate nanostructures (Smith et al, 2000). Smith et al studied an electric-field assisted self-assembly technique to place single nanowires on a lithographical Silicon Oxide substrate after suspending the wires in a dielectric medium between two electrodes (2000). Therefore, aligning nanofibers of synthetic materials through various techniques empowers researchers to apply alignment techniques to natural biomaterials, such as, collagen.

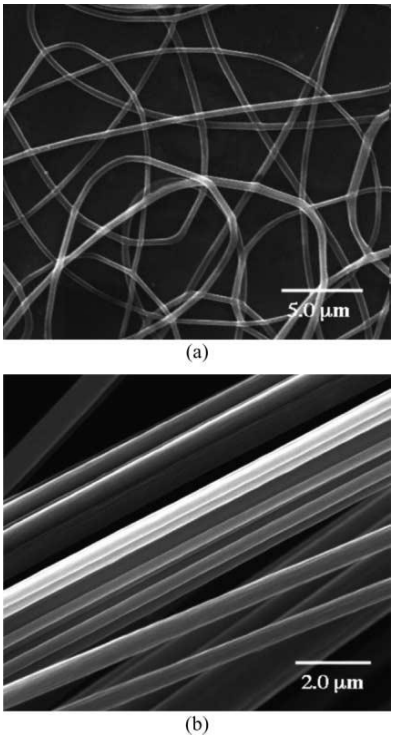

Figure 3.2 SEM images of electrospinning nylon-6 and montmorillonite-nylon 6 (a) non-woven and (b) aligned *yarn (Fong et al, 2001).*

3.2 Collagen Alignment

It is known that alignment affects the spatial and oriented organization of the collagen matrix, generally found in tendons, ligaments, and bone (Ziv, Wagner, & Weiner, 1996). The orientation of collagen fiber alignment plays an imperative role in cell signaling for numerous tissues in vivo; however, alignment has been difficult to accomplish in vitro (P. Lee, L. Lee, & Moon, 2006). Producing anisotropic collagen matrices that are unidirectional has been a challenge in tissue engineering for years. It has been realized that organizing collagen fibers isn't a straightforward process. Previous research states that collagen is usually found with the orientation of being parallel fiber bundles in bone, ligaments, and tendons (Riley, 2005). It is of importance to produce collagen patterns in the native state of tissues in order to study equivalent artificial tissues (Iannace, Sabatini, Ambrosio, & Nicolais, 1995). Another factor dependent on fiber alignment is cellular behavior, which consist of interactions, growth, and adhesiveness. For example, Wu et al reviewed the usage of organized collagen, an ECM protein, for controlling stem cell behavior by regulating stem cell adhesion, differentiation, and growth (2013). Provenzano et al used novel assays to organize collagen matrix, however, the study demonstrated reorganization of collagen matrix by tumor cells generating a mechanical force allowing contact guidance to mediate three-dimensional cell migration (2008). Meshel et al observed the reorganization of individual collagen fibers via fibroblast cells in real-time (2005). Elsdale et al was one of the earliest groups to experiment aligning collagen fibrils via film solution by predisposing a surface during polymerization, referred to as "drainage" method (1972).

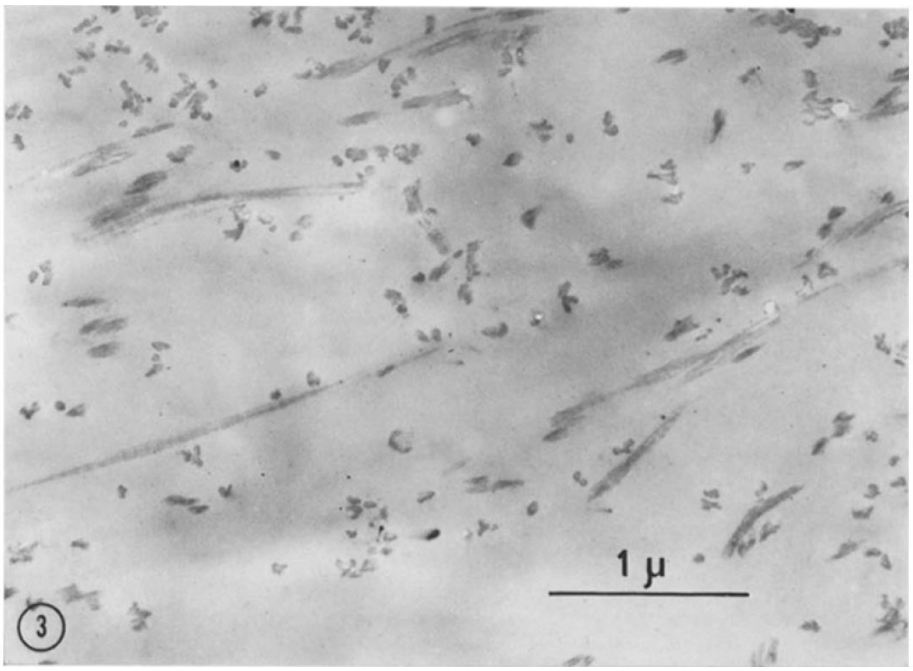

Figure 3.3 Electron Microscopy image of hydrated collagen lattice of collagen bundles dispersed within the watery environment (Elsdale et al, 1972).

Several research groups have utilized techniques to produce aligned and organized layer(s) of collagen fibrils, with the intention to develop scaffolds for guiding cell culture systems. Techniques implemented to impact collagen organization during self-assembly includes, electrospinning, magnetic fields, microfluidic channels, dip-pen nanolithography, and others. As mentioned previously, electrospinning is a fabrication process that uses an electric field to control deposition of polymeric nanofibers on a target substrate. Therefore, Phu et al electrospun aligned collagen fibrils that were small in diameter to mimic the microenvironment in the native cornea in order to study the effect of nanostructured scaffold and composition on corneal stromal cells (2011). Another approach for collagen alignment is the magnetic method, which utilizes magnetic beads and a magnet. Guo et al studied surface modified magnetic beads in a collagen gel solution that coupled together when attracted to magnet in an alignment formation (2007). In addition, microfluidic channels is another technique previous used for

aligning collagen fibrils. Lee et al studied microfluidic collagen fiber alignment by allowing the collagen solution to polymerize inside PDMS channels that ranged in width (2006). Lastly, two other techniques that can be used for collagen fiber alignment are electric poling and directional freezing. Electric poling also known as an electrochemical fabrication method is a process of which electrical currents are used to generate pH gradient. The second technique, directional freezing is a method that entails freezing collagen in one direction and drying to remove moisture that eventually leave voids that leaves collagen resembling a sponge. These two techniques along with the soft lithography technique will be the focused of this research due to other techniques mentioned being expansive and time consuming.

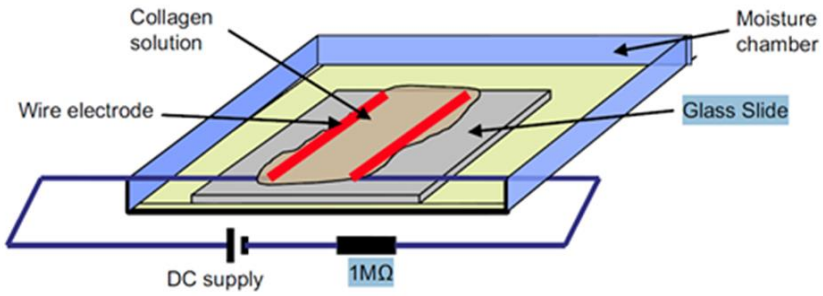

Figure 3.4 Schematic of electrochemical process of collagen alignment

3.3 Electric Poling Alignment

An alternative approach to produce highly orientated collagen fibers is to use a technique involving the manipulation of the electrochemical environment surrounding collagen molecules. Previous research focused on producing tightly packed aligned collagen threads to resemble the hierarchical and mechanical features of tendons by using an electrochemical process. Uquillas et al studied incubating aligned collagen threads after electrochemical manipulation in phosphate

buffer saline solution to stimulate fibrillogenesis (2011). Cheng et al controlled the self-assembly of the collagen molecules by applying electric currents to collagen solution which generated a pH gradient. In recent studies, the electrochemical process is shown to be the beginning stage to produce 3D scaffolds. Younesi et al utilized the electrochemical process to align collagen threads followed by incorporating a weaving process that resulted in producing a 3D scaffold (2014). Although, the use of this technique has been successful, it still remains a challenge to accomplish. Nevertheless, research is being conducted to optimize the electrical poling (electrochemical process) technique for future work.

3.4 Directional Freezing

A common approach to organize or align collagen fibers is the use of the freeze-alignment method. A variety of oriented nano-porous materials have been produced in future work on block copolymers, conducting polymers, and aluminum zinc alloys using the freeze-alignment method of crystals (Zhang & Cooper, 2007). Directional freezing is a most recent similar method to freeze-alignment used to control the direction of crystal growth. Directional freezing allows the orientation of the crystal growth to dictate the orientation of the porous material. For example, Schoof et al investigates unidirectional solidification application during the freezing process to produce a collagen porous structure (2001). Brien et al cross-linked collagen and glycosaminoglycan to form a stronger scaffold and utilize the directional freezing method to determine the effect of pore size on cell adhesion. Caliari et al developed a specialized freeze-drying technique that make use of unidirectional heat transfer to produce collagen-glycosaminoglycan scaffolds with aligned pores to mimic the native microstructure of tendon (2010). Most research incorporates a thermal gradient above or below the collagen sample to

achieve collagen organization. The novelty in our research is to incorporate a thermal gradient in the center of our sample to achieve organization radially.

CHAPTER 4

Methodology

The experimental methods for this research were developed to provide a more in depth understanding of using organized fibrils of collagen type I in contribution to biomedical research for future bio-applications. The literature review gave an overview analysis of most common methods used previously to organize collagen type I fibrils. The purpose of this research is to explore other various techniques to organize collagen and compare outcomes. Also, this research is to determine which method is best used for applications in future studies.

The first technique, soft lithography, is a technique utilized for its simple cleanroom free procedure and inexpensiveness. Also, using soft lithography allows patterning of non-covalent surfaces, prohibits degradation of the performance of patterned ultraviolet (UV) sensitive surfaces, and provides the capability of generating 3D structures. Present research has proven soft lithography as a technique to be useful in self-assembling, aligning, and patterning nanofibers in a solution. The soft lithography technique utilizes micro-molding for which a liquid is cast onto diffraction gratings with groove patterns that allows a patterned duplicate polymer (stamp). Soft lithography is demonstrated in conjunction with a method termed as embossing, by which the stamp is placed in conformal contact with the collagen film and sonicated while a uniform sufficient force is applied onto the stamp. This method eventually pushes the collagen fibers into the grooves of the stamp to form into the same or similar formation of the patterned surface of the stamp.

Electric poling is the second technique used to achieve collagen organization. It is presented as an electrochemical alignment technique to control the assembly of collagen type I molecules into vastly oriented and compact packed bundles of elongated fibers on a macro-scale.

A pH gradient is generated through this process by applying electric current to the collagen solution. Previous studies have observed the formation of collagen bands attributed to electrolysis, for which exhibited high pH values generated in the area of the cathode (Marino & Becker, 1969). The purpose of this technique is to create an electrochemical gradient that force collagen to align through the repulsive electrostatic forces aiding self-aggregation of molecules along the isoelectric point (pl).

Lastly, directional freezing is used to organize collagen with an alignment from a heat conductive material and ice crystals. Collagen gel is placed around a material (preferably a metal) and put into a freezer in between a material that can be used as an insulator. The expected outcome is to "freeze" the collagen gel in its current position, and when the material it is surrounding is removed the collagen maintains its structure. In previous research collagen-glycosaminoglycan scaffolds were manufactured by freeze-drying the suspension of collagen and glycosaminoglycan creating an interpenetrating network of ice crystals surrounded by collagen-glycosaminoglycan (Caliari, 2010). This study was used to develop a technique to produce aligned pores that mimic the native microstructure of tendon. The purpose of this technique is to promote alignment from an object or material (inward) to outward by freeze-drying.

4.1 Materials and Procedure

4.1.1 Soft Lithography

4.1.1.1 Substrate Cleaning

Plain microscope pre-cleaned glass slides (Fisher Scientific, USA) were cut into 1 x 1 squares and washed in Nano-strip (Cyantek, USA) to strip and clean organic and inorganic contamination. Nano-strip also contributed to rendering the surface of the slides hydrophilic. The substrates were soaked in nano-strip at room temperature in a glass beaker and sonicated for 1 hr.

The substrates were also immersed and sonicated in deionized (DI) water from the cleanroom twice for 10 min each, and finally stored in a 100 mL glass beaker of DI water until utilized. Before substrates are used for experimentation they are dried by a nitrogen tube.

4.1.1.2 PDMS Stamps

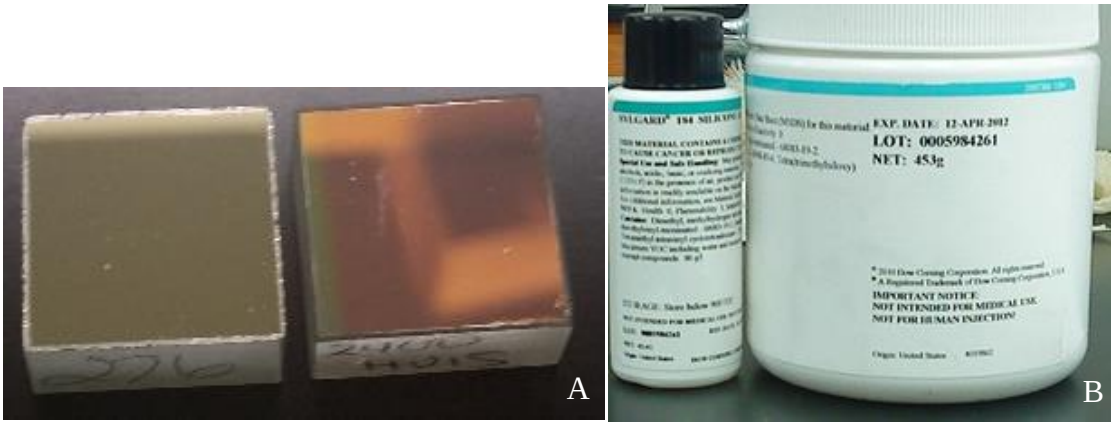

Figure 4.1 (a) Picture of optical diffraction gratings with groove densities of 1200 lines/mm (left) and 2400 lines/mm (right). (b) PDMS kit used to duplicate pattern from optical diffraction grating and produce elastomer stamp.

Optical diffraction gratings (Edmund Optics Inc.) with groove densities of 1200 and 2400 lines/mm with 833 nm and 416 spacing were used as master substrates to cast PDMS onto in order to produce patterned PDMS stamp molds. Sylgard 184 PDMS (Dow Corning) was prepared by following the procedure recommended by the manufacturer consisting of mixing the pre-polymer with a curing agent in a 10:1 ratio by weight. Next, the mixture was cast over the diffraction grating substrate in a heavy layer to form a thick stamp. The sample was placed on a workbench to begin curing at room temperature for 12 hrs. to produce even curing across master substrate. After pre-curing, the sample was placed in an oven at 60°C for at least a 24 hr. period, before removal of the polymer from the mold. Finally, edges of stamp were trimmed to create an even stamp and to keep tract of which direction of the grooves.

4.1.1.3 Stamp Cleaning

Prior to use, stamps went through a solvent wash to remove impurities, dirt and dust, from the surface that could possibly interfere with imaging quality. Stamp was sonicated in acetone, isopropanol, and then DI water (dH_2O) for 5 min each. As mentioned in Chapter 2 the two solvents used caused the PDMS stamp to swell, therefore, after solvent wash the stamp was placed in a desiccator (vacuum) for a 24 hr. period to minimize swelling and allowing stamp to dry.

4.1.1.4 Collagen Film Solution

Collagen film solution was prepared by following the recommended protocol provided by the manufacturer. 26 µL of Rattail type I collagen (BD Biosciences, USA) solubilized in 0.02 N acetic acid with a concentration of 3.83 mg/ml was pipetted and placed into an 1.5 ml eppendorf tube. BD protocol suggest adding additional diluted acetic acid to collagen to form a film solution, however, due to acetic acid prohibiting fibrillization (non-assembly), this step was disregarded for some samples, but used in others. Samples' containing additional acetic acid varied between 5 to 10 µL and calculations of the amount of dH2O was altered to balance the total amount of solution. To produce a final concentration of 1 mg/ml with a total volume of 100 µL, 72 µL of DI water was added to the collagen in the eppendorf tube. The stock solution was placed on a vortex machine for an estimate of 30 sec. Dilution formula, $M_1V_1=M_2V_2$, was used for calculations.

4.1.1.5 Solution Embossing via Sonication

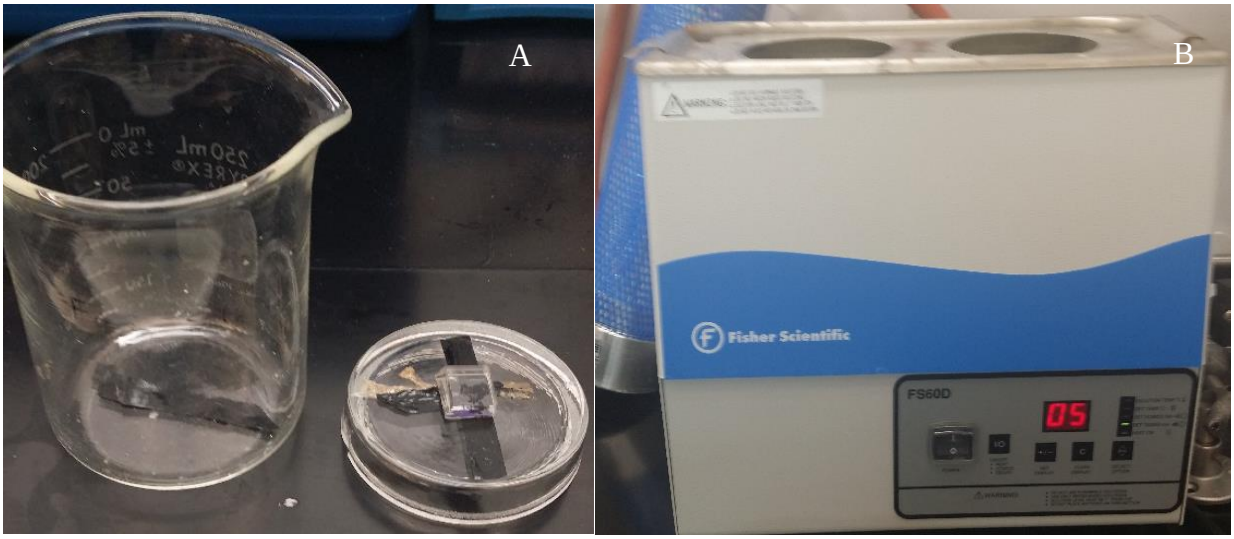

Figure 4.2 (a) Experimental setup for soft lithography technique with empty beaker (left) and sufficient weight object with PDMS stamp applied (right). (b) Sonicator used for embossing technique.

Double-sided tape was used to tape down a 1 x 1 inch cleaned glass slide substrate to the bottom of an empty 250 mL glass beaker. The stamp was placed patterned side up onto a circular glass weight, for which will apply a load or force on the stamp. 5 µL of 1 mg/ml solution was pipetted onto glass substrate, and the weighted PDMS stamp was placed on top of the collagen solution. The circular weight was chosen to be used as the object to apply load onto the stamp due to its tight fit within the glass beaker which is needed for successful embossing. The glass beaker was then immersed in a sonicator bath and sonicated for 90 min in a Fisher Scientific 60D sonicator. After sonication, the beaker was placed under a vacuum hood without any agitation for at least 8 hrs before the stamp was removed from substrate. Solution embossing is considered successful if a holographic imprint can be seen on the glass substrate after stamp removal. Solution embossing without the aid of a patterned stamp is performed by pipetting a 5 µL drop of

collagen solution onto a clean glass substrate. An unpattern stamp was placed onto the circular glass weight, and the weighted PDMS stamp was placed on top of the collagen solution. The same process is repeated as the patterned stamp and the solution is allowed to dry for a minimum of 8 hours before stamp removal.

4.1.2 Electric Poling

4.1.2.1 Substrate Cleaning and Preparation

Figure 4.3 (a) Substrates with gaps in between gold coated area. (b) Physical Vapor Deposition machine used to coat substrates in conductive metal.

Plain pre-cleaned microscope glass slides (Fisher Scientific, USA) were solvent washed with acetone, isopropanol, and DI water for 10 min before each use and dried with a nitrogen tube. Then electrical tape was used to design parallel gaps on the slides, with one gap being larger than the other. Gaps are developed by cutting a single strip of tape (varying from 3mm to 8mm in width) and applying the strip on to the slide. The glass slides are then place into a Physical Vapor Deposition (PVD) machine, which is used to coat samples with metal. Because the glass substrates need to be conductive for this technique, the metal of choice used was gold

and substrates were coated in PVD 75 (Kurt J. Lester Company, USA) for 20 to 30 min. After coating, the strips of take are removed from the glass substrates and the gap is revealed in between the gold coating. The substrates are placed into an oxygen plasma machine for 6 min at 21 mA to provide surface cleaning and rendering the surface super-hydrophilic.

4.1.2.2 Collagen Film Solution

As mentioned before in the previous method, collagen film solution was prepared by following the recommended protocol provided by the manufacturer. 13 µL of Rattail type I collagen (BD Biosciences, USA) solubilized in 0.02 N acetic acid with a concentration of 3.83 mg/mL was pipetted and placed into an 1.5 L eppendorf tube. 37 µL of 0.02 M acetic acid was added to collagen in eppendorf tube to produce a final concentration of 1 mg/ml with a total volume of 50 µL. Finally, the stock solution was placed on a vortex machine for an estimate of 30 sec. Dilution formula, $M_1V_1=M_2V_2$, was used for calculations.

4.1.2.3 Poling using Source Meter

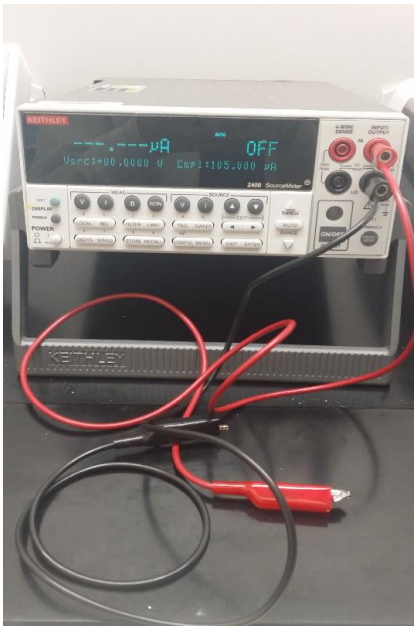

Figure 4.4 Source meter used to measure current and source voltage.

Series 2400 SourceMeter (Keithley Instruments, USA) was used according to the manual. Cables are connected to the low and high input/output connections and alligator clips are clipped to the coated glass samples to act as a circuit with an anode and cathode. For this procedure we are measuring the current (ammeter) and sourcing voltage to align the collagen. 2 µL was pipetted in positioned in the small gap and 4 µL was positioned in the big gap. Voltage applied ranged from 2 V and 4 V and was sourced for 20 to 45 min.

4.1.3 Directional Freezing

4.1.3.1 Collagen Gel Solution

Collagen gel solution was prepared by following the protocol given by the manufacturer. High concentrated (HC) rattail collagen type 1, 10 x PBS, DI water, and 2M Sodium Hydroxide (NaOH) was placed on ice before beginning mixture. The collagen gel mix consist of 467 µL of rattail type I collagen (BD Biosciences, USA) solubilized 0.02 N acetic acid with a concentration of 8.56 mg/ml, 50 µL of 10 X PBS, 5 µL 2M NaOH, and 478 µL of dH_2O to equal final volume of 1 mL of collagen mix. This mix is then used to create various concentrations for experimentations. Final concentrations consist of 4 mg/mL (collagen mix), 2 mg/ml (50 µL collagen mix + 50 µL dH_2O), and 1 mg/ml (25 µL of collagen mix + 75 µL dH_2O). Lastly, each stock solution was place on a vortex machine for an estimate of 30 sec. As stated previously, dilution formula was used for calculations.

4.1.3.2 Substrate Preparation

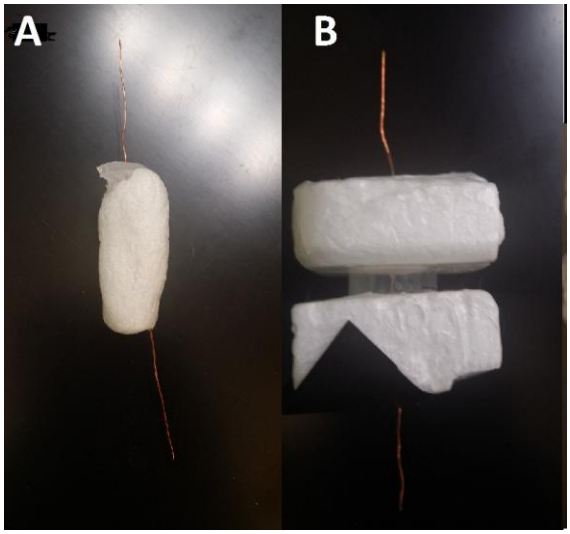

Figure 4.5 (a) Eppendorf tube substrate with copper wire going through middle of tube surrounded by Styrofoam. (b) Falcon plate substrate placed in between two squared Styrofoam portions with copper with going down the middle of substrate.

Two substrates were self-made to use for this procedure. The first substrate used was a 1.5 ml Eppendorf tube. A hole was drilled into the middle of the Eppendorf tube and a copper wire that is approximately 4” in length was inserted in the hole through the middle of the Eppendorf tube to act as a thermal gradient. 4 µL of collagen gel solution was pipetted around the bottom of the copper wire before placing Styrofoam around the wire to act as an insulator. The second substrate consists of a 5 ml falcon tube that was made into a small plate. A small cylindrical portion of the falcon tube was cut out and placed on Styrofoam that was cut into a square with parafilm placed side-up. A copper wire, approximately 4” in length was inserted in the middle of the self-made plate and through the Styrofoam to act as a thermal gradient as mentioned before in the Eppendorf tube substrate preparation. 4 µL of collagen gel solution was pipetted onto the parafilm around the copper wire, followed by placing another squared Styrofoam on top to create a sandwich.

4.1.3.3 Lyophilizing

Figure 4.6 Lyophilizer used to vacuum moisture from sample.

Substrates were placed into freezer at standard freezer temperature overnight until the solvent sublimates and ice crystals formed. After samples were completely frozen they were lyophilized to dry, by which, they go from a solid phase to a gas phase without turning into liquid form. Samples were lyophilized for 24 hrs., before being removed carefully from container, followed by removal of the copper wire from the middle of the samples.

CHAPTER 5

Results and Discussion

5.1 Objective 1

5.1.1 Observing collagen fibers in native state

Collagen gel solution was prepared by following BD Biosciences rattail type I gelation protocol. Figure 5.3 shows an overview of collagen gel solution with fibers and amorphous collagen surrounding the fibers, whereas, figure 5.4 show an up close view of collagen gel fibers with salt embedded in the collagen. The salt content was produced by the use of PBS in the collagen solution. Collagen film solution was prepared using BD Biosciences rattail collagen Type I protocol. As seen in both figures 5.1 and 5.2 collagen is naturally in a bundle arrangement.

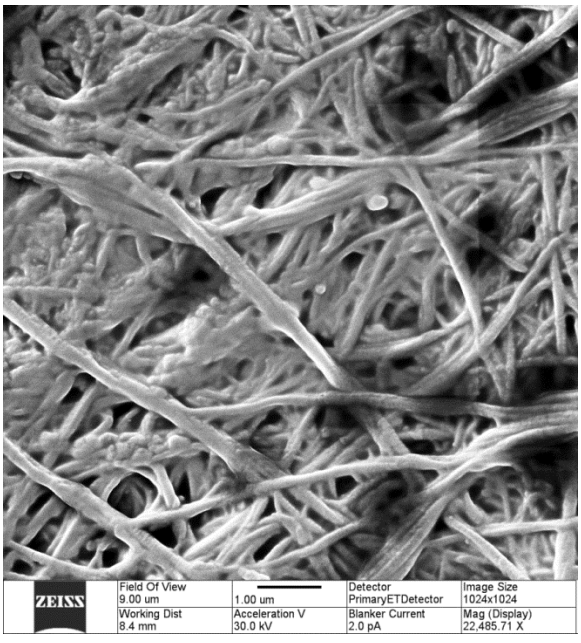

Figure *5.1* HIM image of collagen solution in native state.

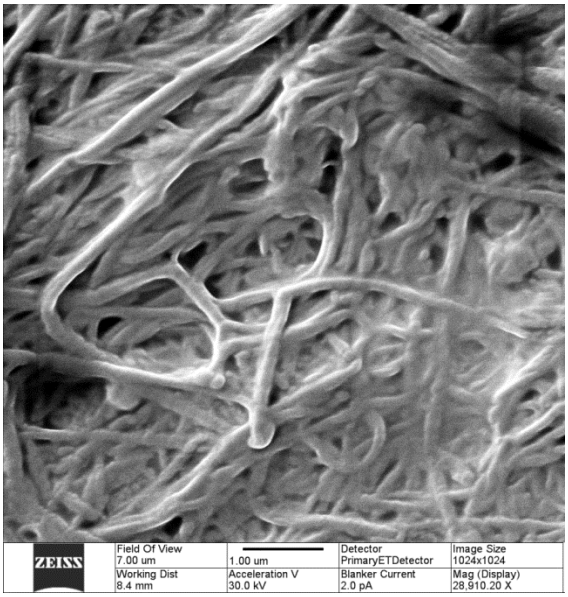

Figure 5.2 HIM of collagen fiber bundle in native state.

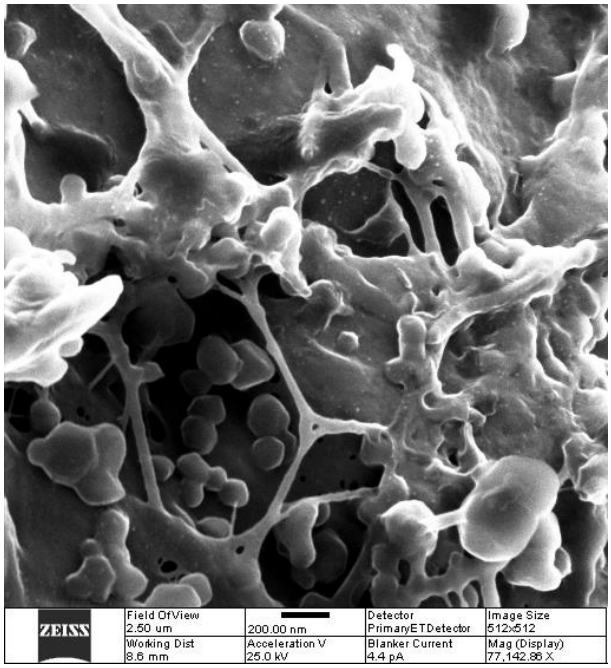

Figure 5.3 HIM image of collagen gel solution in native state with salts and amorphous collagen.

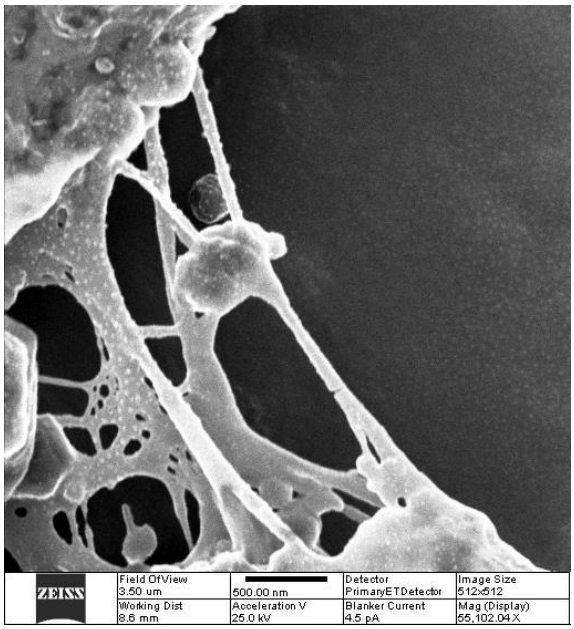

Figure 5.4 HIM image of collagen gel solution in native state with salt possibly interfering with collagen organization

5.2 Objective 2

5.2.1 Characterization of Collagen using Soft Lithography technique

Figures 5.5 and 5.6 is the result of using the soft lithography technique using grooves that have 1200 and 2400 line periods. After conducting much iteration, 1mg/ml concentration produced the best results within this study. Both figures show some fiberization , however, figure 5.5 show minimal fiberization due to amount of acetic acid in collagen solution, which consist of total concentration of 13.2 mM acetic acid. This was confirmed by testing the effect of acetic acid on the BD collagen stock solution. Figure 5.6 shows fiberization of fibers that agglomerated on one another with a total concentration of 9.2mM of acetic acid. In comparison to figure 5.5, figure 5.6 solution was prepared with minimum diluted acetic acid added into BD collagen stock solution.

Previous studies have indicated that acidification disassembles fibrillar structure. In theory, the soft lithography process with assisted sonication-solution embossing is supposed to allow collagen fibers to disassemble, rotate into the topography feature of the elastomer stamp that was duplicated from optical diffraction gratings, and finally reassemble together. However shown in *Figure 5.6* the collagen fibers disassembled, but instead of rotating into grooves the collagen fibers aggregated on top of one another across the groove in the orientation of the groove. A possible reason for fibers not aligning within the grooves is that the groove line period and width could be too small to allow spatial confinement of fibers in the groove. Studies have stated that collagen fibrillogenesis is commonly triggered by one of the following processes: (1) pH shift to neutral, (2) Increase in temperature to 30-37°C, and (3) diluting acidified collagen solution with sufficient amount of water (Kew et al, 2011). If one of these factors are violated it could stop fibrillogenesis from occurring. We observed that in *Figure 5.5* the solution use was possibly to acidic which allowed minimal fiberization from occurring.

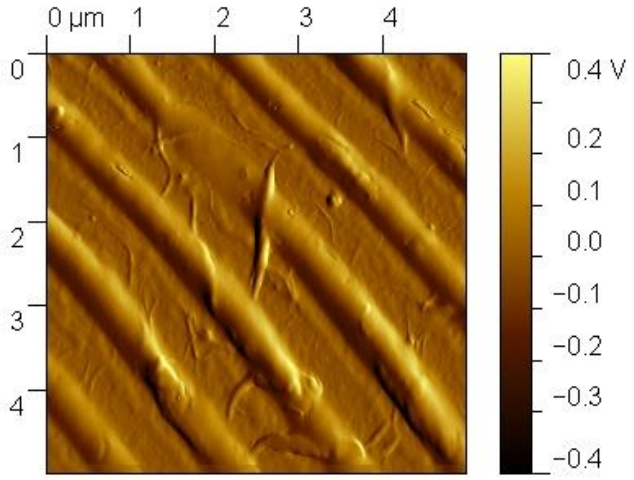

Figure 5.5 AFM image of soft lithography sample of 2400 grating with minimum fiberization

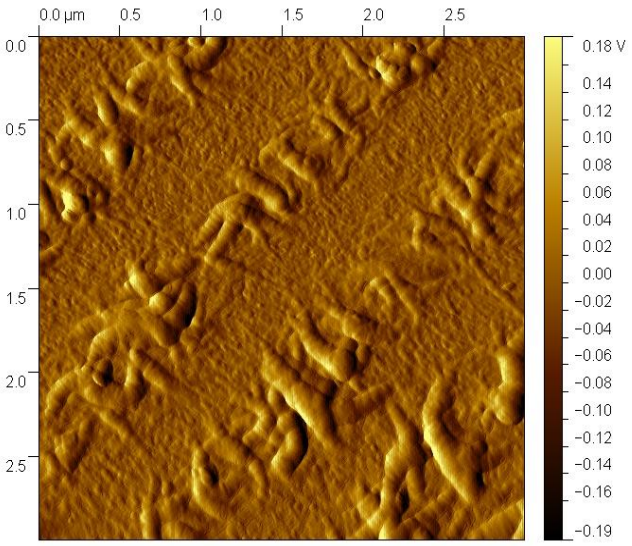

Figure 5.6 AFM image of soft lithography 1200 grating with fiberization organization on grooves

5.2.2 Characterization of Collagen of Direct Current technique

Electric poling samples were conducted multiple times using different voltages. Voltages applied to collagen solution varied from 2V to 10 V during testing using gap sizes of 2mm and 4mm, however, 2V and 4V were the two samples that provided a visual of fibers. Results show in both figures 5.7 and 5.8 a bundle of fibers but no fiber alignment within the gaps. Fibers moved from gap to gold cathode on sample during poling process, for which is shown in both figures.

In Figure 5.7 and Figure 5.8 show collagen fibers along the gold cathode of the sample. In electric poling, alignment is dependent on pH of solution and the size of the gaps. Within this study there two possible reasons for the collagen fibers not aligning are because thd gaps are too big and the pH of the solution was changed during poling because of salt content incorporated into the solution.

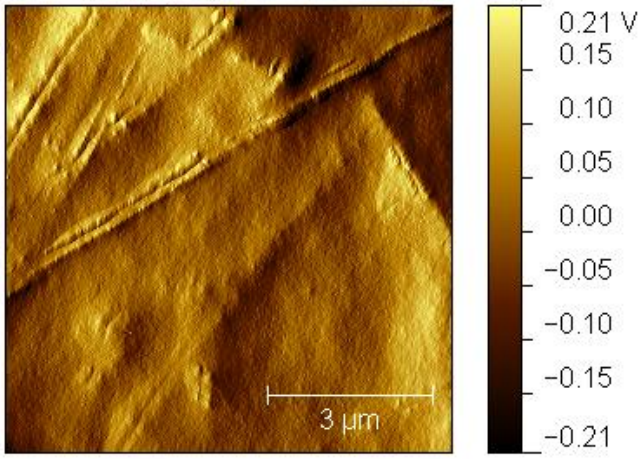

Figure 5.7 AFM phase image of 2V electric poling on gold cathode

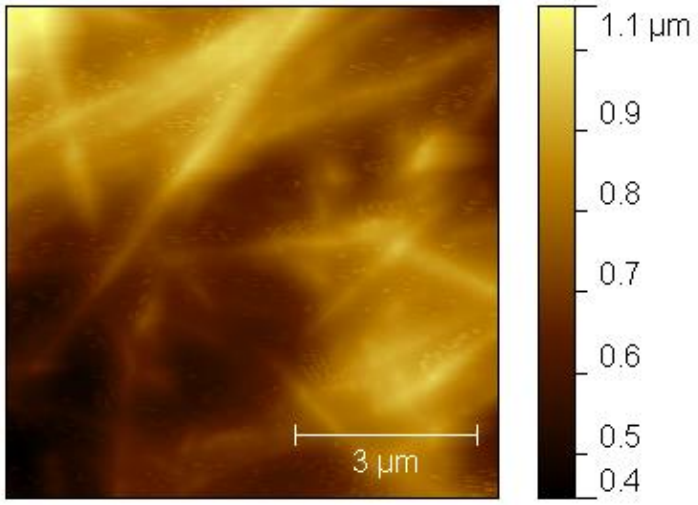

Figure 5.8 AFM topography image of 4V electric poling with fiber structure bundles.

5.2.3 Characterization of Collagen of Directional Freezing

Figures 5.9 and 5.10 depict the effects that the freeze-drying process has on the collagen gel. Results prove recent studies of ice crystal forming to eventually produce a porous structure

after drying process. Figure 5.9 was created using a concentration of 1 mg/ml collagen solution which provides a more dense porous structure, whereas figure 5.10 was prepared with a 2 mg/ml collagen solution which provided an agglomerated porous collagen structure.

We observe in Figure 5.9 a porous structure formed by solvent crystals. Former studies mentioned previously in literature review stated that porosity size is due to the rate of freezing, therefore, fast freezing rate produce small pores and slow freezing rate produce big pores. This figure confirms previous research observation by providing a visual of big pores because solution froze in freezer, which is a process that is consider being a slow freezing rate. Another observation is that directional freeze drying may be dependent on collagen solution because of agglomeration that occurs in the freezing process. Figure 5.10 displays collagen agglomeration in an outer area from thermal gradient. This lead us to believe that if there is too much collagen in the solution when ice crystals began forming, the crystals can push the excess collagen together in various outside areas in relation to the heating gradient.

Figures 5.11 and 5.12 are a representation of the collagen formation from where the thermal gradient (copper wire) was placed within the sample. The samples were prepared using a concentration of 1 mg/ml collagen solution, which resulted in figure 5.12 having collagen sheet alignment. Figure 5.11 is an overview of the radial formation of the collagen that was produced from the copper wire into a porous structure.

Also, Figure 5.11 and Figure 5.12 the radial formation is observed from where the wire was placed in sample. These figures do not depict individual fiber alignment, but we notice partial alignment of collagen sheets. We made the assumption that it is possible for collagen fibrils to align themselves to form a flat aligned sheet of collagen. However, future experimentation will have to be conducted to conclude this assumption.

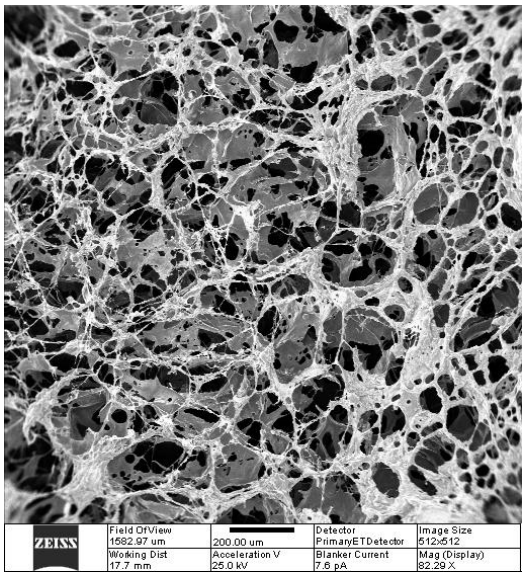

Figure 5.9 HIM image porous structure left by slow freezing collagen gel

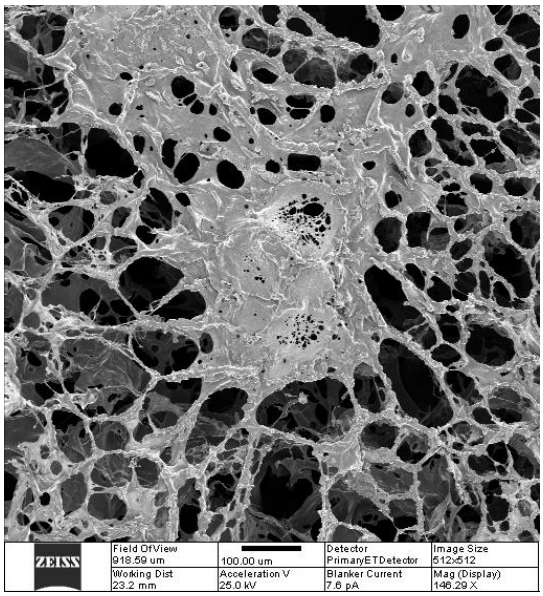

Figure 5.10 HIM image of collagen agglomerating from ice crystal formation

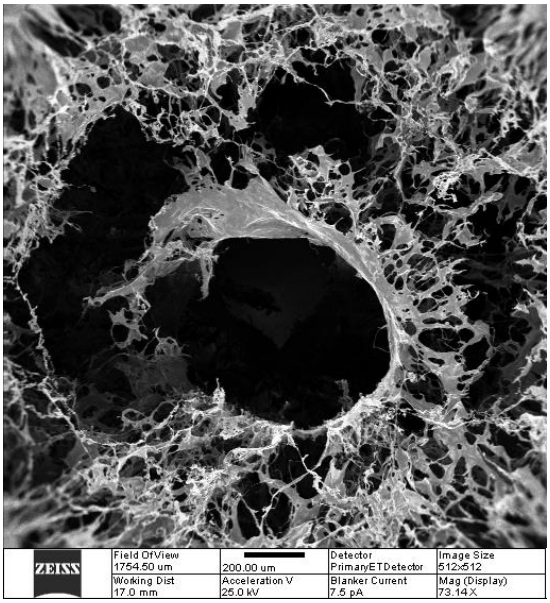

Figure 5.11 HIM image of collagen in a radial formation left by copper wire

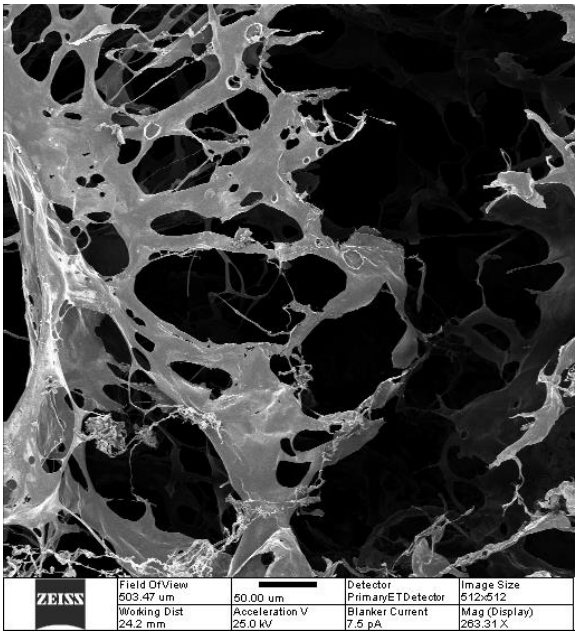

Figure 5.12 HIM image of collagen aligned collagen sheets

CHAPTER 6

Conclusion and Future Research

In conclusion, research is still being conducted to have more conclusive data. We found that soft lithography technique on collagen fiber alignment may be dependent on pH of collagen solution. Also, we found partial organization but no alignment in one direction, however, we could assume that alignment of fibrils within the grooves did not occur due to inconsistent parameters and groove width being too small. We found that in the directional freezing technique there may be partial alignment due to collagen sheets being aligned. Individual fibers cannot be seen; however, by sheets being flat and aligned it can be assumed that collagen fibrils are aligned and bunched together to create a sheet. We can also consider through observation that collagen agglomerates as it travels further from wire due to the ice formation pushing collagen outward. In the direct poling technique, collagen fibrils are extended, but are moved onto coated area of electrode. We can conclude that collagen organization did not occur because the gaps were too big in width.

Directional freezing is the most effective to use for collagen alignment because it can be used in actual biological applications as a result of being used as a 3D scaffold. Advantages of using the soft lithography technique are that it is a simple fabrication process, inexpensive, and repeatable. Disadvantages of this technique are that it is 2D and according to previous studies, it is not applicable for all fiber types. Advantages of using the directional technique are it is beneficial for cell migration and scaffolding; however, the main disadvantage is that this technique depends on time. Advantages of using the electric poling technique is if successful it aligns fibers in a unidirectional form, however, a disadvantage is that it depends on various parameters.

Future studies for soft lithography can incorporate adjusting pH of collagen solution to allow collagen fibers to dissemble to self-assemble back together within grooves, without utilizing too much acetic acid to permit collagen fibers from fiberizing. Another future study is to observe the effect of basic pH collagen solution on collagen organization. Lastly, future research is to explore collagen organization using soft lithography technique with heat-embossing method.

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Appendix

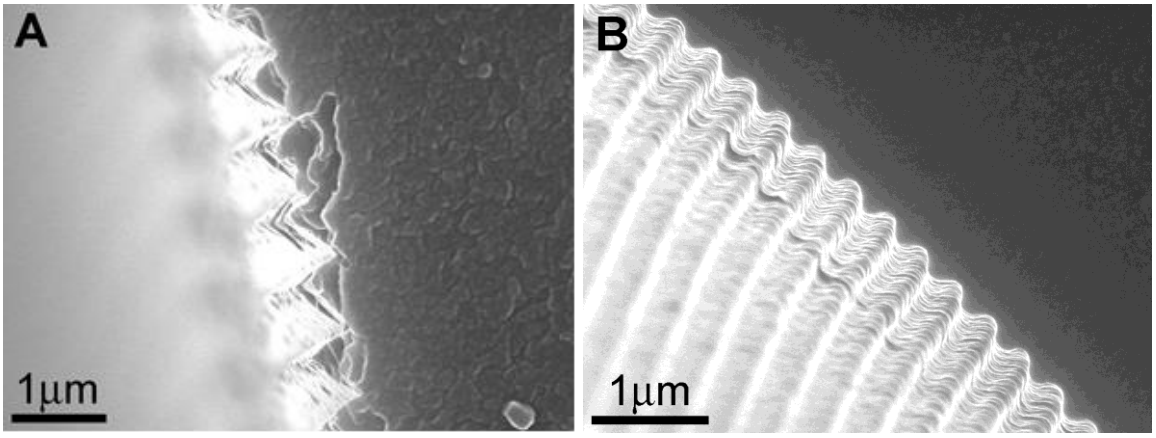

Figure A.1. SEM microraphs of PDMS stamps replicated from diffraction grating (A) 1200 lines/mm (B) 2400 lines/mm. (A. Hung et al., *Nano Lett.*, **2007**, *7* (5), pp 1165–1171)

Table A.1

Parameters of diffraction grating masters used for soft lithography method (Hung, 2007).

Geometric parameters of diffraction grating masters.

Line density (lines/mm)	Groove period (nm)	Groove Depth (nm)	Aspect ratio	Fabrication method
1200	833	626 ± 17	0.752	Mechanical blazing
2400	417	128 ± 4	0.307	UV interference
3600	278	63 ± 3	0.23	UV interference