ABSTRACT

HASLAUER, CARLA MARIA. Design and Validation of Three-Dimensional Scaffolds for Bone Tissue Engineering Applications Using Human Adipose-Derived Adult Stem Cells. (Under the direction of Elizabeth G. Loboa).

Functional bone tissue engineering has arisen to address the need for implantable bone tissue in cases of trauma or disease. Some challenges to generating functional bone tissue have included the need to identify biocompatible substrates with appropriate mechanical properties, as well as a suitable cell source. Subsequently, human adipose-derived adult stem cells (hASCs) have been used in an assortment of tissue engineering applications in recent years. They are attractive cells for use in clinical applications due to their multi-lineage potential, relative abundance, and ease of harvest relative to other cell lines. The pluripotency of hASCs isolated from adipose tissue and used in the various studies described here has been tested by inducing both osteogenic and adipogenic differentiation pathways. These hASCs were then seeded on three different types of three-dimensional scaffolds. In the first study, hASC viability and proliferation were compared between custom-designed titanium implants and commercially available implants of the same composition. In the second study, viability and proliferation of hASCs were assessed for cell-seeded collagen-PCL sheath-core electrospun scaffolds. Osteogenic differentiation of hASCs on these scaffolds was also quantified. In the final study, islands-in-the-sea nanoporous fibers were designed.
and fabricated for bone tissue engineering applications. Initial cell viability was examined over a 4 week time period for hASCs seeded on the novel scaffolds.

Titanium implants are commonly used in medical procedures such as hip replacements. There is a need however, to design patient specific titanium scaffolds. An important factor in scaffold acceptance by the body is osseointegration, or bone ingrowth. Micromotion between the implant and surrounding tissue can hinder osseointegration, and ultimately lead to implant failure. Using computed tomography (CT) data, micromotion can be minimized by designing patient-specific implants. Implants from these designs can then be fabricated by electron beam melting (EBM) using titanium powder, built layer by layer to the appropriate specifications. In this study, hASCs were used to validate the effects of EBM processing when compared to commercially available medical-grade titanium implants. Results suggest EBM fabricated porous scaffolds promote hASC proliferation and do not adversely affect biocompatibility.

Natural polymers have also been investigated for bone tissue engineering applications. This study describes the design of electrospun fibers, which are on a similar size scale to the native collagen fibrils found in human bone tissue. Polycaprolactone (PCL) electrospun fibers were coated with type I collagen, a natural component of bone, during a co-axial electrospinning process, generating a sheath-core fiber morphology. Human ASCs seeded on the scaffolds were differentiated down the osteogenic lineage, and calcium deposition was compared to
that of hASCs seeded on uncoated PCL scaffolds. The results indicated the collagen coating increased cell spreading and osteogenic differentiation of hASCs seeded on the sheath-core electrospun scaffolds.

Nanoporous fibers are also desirable for bone tissue applications, as the micropores should enhance nutrient delivery and waste removal. In the final study, melt spun islands-in-the-sea fibers were extruded with a polylactic acid (PLA) sea matrix and islands comprised of EastONE, a water-dispersible sulfopolyester. The EastONE polymer was also added to the sea matrix in various concentrations, yielding micropores within the PLA sea after washing. These fibers were then knitted into three-dimensional fabrics, and the physical properties characterized. Microscopic observations revealed the presence of micropores throughout the fiber structure. Removal of the EastONE additive was also confirmed by weight loss measurements obtained before and after washing. Human ASCs were cultured on these three-dimensional fabrics for four weeks in vitro, and examined for cell viability. Confocal images confirmed the presence of viable hASCs throughout the four week culture period on both solid and porous scaffolds.
Design and Validation of Three-Dimensional Scaffolds for Bone Tissue Engineering Applications Using Human Adipose-Derived Adult Stem Cells

by
Carla Maria Haslauer

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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APPROVED BY:

Dr. Elizabeth G. Loboa
Committee Chair

Dr. Behnam Pourdeyhimi

Dr. Nancy Monteiro-Riviere

Dr. Ola Harrysson

Dr. Russell Gorga
DEDICATION

To my parents, for the sacrifices they made to give me the best education possible and their unwavering support of my academic pursuits.
BIOGRAPHY

Carla Maria Haslauer was born on September 27, 1983 in New Orleans, Louisiana to David John Haslauer and Maria Viviano Haslauer. Carla is the eldest of four girls, whose younger sisters Monica Alyssa, Alexandra Marisa, and Erica Arianna Haslauer. Carla attended St. Christopher the Martyr Elementary School in Metairie, Louisiana and in May 2001 graduated from St. Mary’s Dominican High School, New Orleans. She attended Louisiana State University, Baton Rouge where she received a Bachelor of Science degree in Biological and Agricultural Engineering in May 2005. While at LSU, she performed undergraduate research under the direction of Dr. William Todd Monroe and Dr. David D. Pollock. Carla moved to North Carolina in June 2005 to begin doctoral studies in the Joint Department of Biomedical Engineering at the University of North Carolina Chapel Hill and North Carolina State University. She was awarded a fellowship through the Whitaker Foundation for her first year of graduate studies. She was later awarded a second fellowship through the National Science Foundation Graduate Research Fellowship program for the following three years of study and research. Carla was also recognized twice by the Nonwoven Cooperative Research Center for their Best Paper Award (November 2006 and May 2009).
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I would like to thank my family for their unconditional support throughout this journey. This would not have been possible without my mom lending a listening ear or my dad going through the effort of boiling crawfish for my friends and me every time I returned home.

I would like to thank LSU for providing me with a strong undergraduate education upon which I could build my graduate studies. I greatly appreciate the guidance of Dr. Todd Monroe and Dr. Marybeth Lima during my undergraduate tenure, and their continued help and support throughout my graduate career. I am also indebted to Dr. Julianne Forman Audiffred for mentoring me as an undergraduate, then becoming a great resource as a colleague while we pursued our doctoral degrees.

These studies would not have been possible without the advice and guidance of my committee members. Thank you to Dr. Loboa, for serving as my mentor and for challenging me to expand my technical expertise. I would also like to thank Dr. Monteiro-Riviere and Dr. Harrysson for their guidance as well as the opportunity to pursue collaborative research projects with their groups. I would also like to thank Dr. Gorga for his contributions to my final study. Special thanks to Dr. Pourdeyhimi for his support and guidance throughout the porous fiber study.
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I am grateful for the funding provided by the Nonwovens Cooperative Research Center (NCRC), and the aid and support of Sue Pegram, Steve Sharp, and Wendy Cox. I would like to thank Hills Inc. and Eastman Chemical for generously donating supplies and machine time, as well as valuable advice. I would like to thank Chuck Mooney, Ka C. Wong, Stan Long, and Birgit Anderson for their technical assistance. I would also like to thank Jeremy Immer for his technical assistance with the BET analysis.
I would also like to thank all of my other friends and colleagues for their encouragement along this journey and for helping me understand the reason James Taylor sang “you must forgive me if I’m up and gone to Carolina in my mind.”
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>Au-Pd</td>
<td>Gold-Palladium</td>
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<tr>
<td>BET</td>
<td>Brunauer, Emmett and Teller</td>
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<tr>
<td>CCS</td>
<td>Cleaning Cross Section</td>
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<td>CT</td>
<td>Computed Tomography</td>
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<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
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<td>EBM</td>
<td>Electron Beam Melting</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>FBS</td>
<td>Fetal bovine Serum</td>
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<tr>
<td>FIB</td>
<td>Focused Ion Beam</td>
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<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectrophotometer</td>
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<tr>
<td>HFIP</td>
<td>1,1,1,2,2,2-hexafluoroisopropanol</td>
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<tr>
<td>hASCs</td>
<td>Human adipose-derived adult stem cells</td>
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<tr>
<td>hMSCs</td>
<td>Human Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>ISE</td>
<td>Induced Secondary Electron</td>
</tr>
<tr>
<td>MCT</td>
<td>Mercury Cadmium Telluride</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>PCL</td>
<td>Poly-(\varepsilon)-caprolactone</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
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<tr>
<td>3D</td>
<td>Three-dimensional</td>
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PREFACE

The work presented in this dissertation involved collaborative research projects with many investigators. The research presented in Chapter 2 has been accepted for publication in *Medical Engineering and Physics* with co-first author Jessica Springer and co-authors Ola Harrysson, Elizabeth Lobo, Nancy Monteiro-Riviere, and Denis Marcellin-Little. This work was funded by two grants from the North Carolina State University College of Veterinary Medicine (Marcellin-Little and Lobo, 1/2008 - 3/2009). Titanium scaffolds were fabricated by Jessica Springer. The work presented in Chapter 3 has been submitted for publication to *Journal of Biomaterials Science: Polymer Edition* with co-first author Ajit K. Moghe and co-authors Buhpender Gupta, Jason Osborne, and Elizabeth Lobo. This work was funded by a grant from the National Textile Center (Electrospun Core-Sheath Fibers for Soft Tissue Engineering- Gupta 5/2005-3/2009). Electrospun scaffolds were fabricated by Ajit Moghe. Some work presented in Chapter 4 has been submitted for publication to the *Journal of Applied Polymer Science* with co-authors Ruwan Sumanasinghe, Behnam Pourdeyhimi, and Elizabeth Lobo. Focused ion beam cross-sectioning and imaging presented in Chapter 5 has been published in *Microscopy and Microanalysis* with co-authors Ka C. Wong, N. Anantharamaiah, Behnam Pourdeyhimi, A. Dale Batchelor, and D Griffis. Imaging was performed by
Ka Wong. The study presented in Chapter 5 when prepared for publication will include co-authors Behnam Pourdeyhimi, Matthew Avery, and Elizabeth Loboa.
1. Introduction

1.1 Background

1.1.1 Three-dimensional Bone Tissue Engineering Scaffolds

Nearly 800,000 surgical procedures involving bone occur annually in the United States alone, making it the most commonly replaced tissue of the body [1]. Replacement bone tissue is typically obtained from the patient, with an autograft, or from a donor source, termed allograft. Problems with autografts, which are typically taken from the iliac crest, include donor site morbidity, limited quantity, and increased operative time [1, 2]. Allografts on the other hand, have the potential for contamination or other complications including infections, fracture, or a host immune response [1]. Tissue engineering techniques which incorporate autologous cells are believed to provide a better grafting solution by easing the problems of supply limitation/ donor scarcity of bone, as well as immune rejection or transfer of pathogens [3]. Tissue engineering scaffolds should be biocompatible and biodegradable, as well as possess proper mechanical properties and encourage tissue development at the graft site while maintaining cell viability [3].

Maintenance of cell viability in a three-dimensional construct is an important design consideration for tissue graft substitutes. One common problem encountered with tissue engineering scaffolds is the formation of a necrotic core as cells rapidly deposit extracellular matrix (ECM) proteins on the outer edge of the scaffold, blocking mass transport to the central region of cell-seeded scaffolds [4]. To
improve nutrient delivery to, and waste removal from, cells in the center of the three-dimensional (3D) structure, it is desirable to incorporate a macroarchitecture into the scaffold. This could be achieved through the addition of channels and pores, both random and controlled. Improvements in nutrient delivery and waste removal are expected to reduce the likelihood of developing a necrotic core, as cells which have migrated into the scaffold center will still have access to vital nutrients.

In addition to nutrient delivery, three-dimensional bone tissue engineering scaffolds must be designed to maintain their structural integrity once implanted, while allowing cells to proliferate and secrete extracellular matrix proteins [5]. As the scaffolds gradually degrade, space for new tissue growth and matrix deposition is created until the tissue has been fully replaced [5]. Previous studies have indicated that for bone tissue engineering applications, osteoblasts prefer pores 200-400µm in diameter [6]. Osteoblasts can then migrate into the porous structure and excrete extracellular proteins to replace the degrading scaffold. The combination of cellular proliferation and extracellular matrix accumulation should fill any voids left by the degrading scaffold material.

A variety of biocompatible, biodegradable polymers have been assessed for tissue engineering applications. Polymers such as poly(lactic) acid (PLA) have been shown to be nontoxic, and its degradation products are natural metabolites which can be eliminated from the body in the form of carbon dioxide and water [3]. One benefit of using synthetic polymers is that researchers can take advantage of the
differing degradation kinetics of synthetic polymers as needed for a particular application. The rate of degradation can be tailored for specific applications by altering crystallinity and molecular weight, or through the addition of other polymers [3]. For example, it has been shown that polycaprolactone (PCL), another nontoxic biodegradable polymer, can take several years to degrade in vivo [7, 8]. PLA, with its hydrophobic methyl groups, can take more than 5 years to degrade when comprised of L-lactic repeating units or as little as 1 year for amorphous PLA [5, 8]. PCL degrades similarly to PLA, with hydrolytic cleavage of ester bonds as well as weight loss of oligometric species diffusing from the bulk. PCL degradation is considered relatively slow, especially in comparison to some natural polymers, with complete removal from the body requiring three years [9]. Composite scaffolds of various synthetic and/or natural polymers have been considered for tissue engineering applications to tailor scaffold degradation rates. Beyond controlling degradation rates, composite scaffolds also have the additional advantage of combining properties to suit the mechanical and physiological demands of the surrounding host tissue [5]. The combination of naturally occurring polymers, such as collagen, with synthetic polymers has the potential to provide both cellular attachment sites as well as mechanical strength.

Collagen is a naturally occurring fibrous protein, and is the primary organic constituent of the extracellular matrix of bone [3]. Mizuno et al. have indicated that a type I collagen matrix aids the induction of osteoblastic differentiation, but this is not
seen by cells seeded on other types of collagen matrices (type II, III, or V collagen) [10]. The surface chemistry of most synthetic polymers is not familiar to cells, whose typical extracellular matrix consist of collagen, laminin, elastin, and fibronectin, among other proteins [11]. Since type I collagen is a major protein constituent of the ECM, it is easily recognized by cells and has been used as a scaffolding material, with other synthetic polymers, for bone tissue engineering applications.

1.1.2 Use and Healing Potential of Human Adipose-Derived Adult Stem Cells

Adipose tissue develops both pre- and postnatally from the mesodermal layer of the embryo [12, 13]. Human adipose-derived adult stem cells (hASCs) typically have a cell doubling time of 2-4 days which is dependent on factors such as culture medium and passage number [14, 15]. Stem cells, including ASCs, are characterized by their ability to self-renew as well as differentiate along multiple lineage pathways. In order to be used in regenerative medicine applications, stem cells should be found in abundant quantities, involve a minimally invasive procedure for harvesting, be capable of differentiation along multiple pathways, and safely and effectively transplanted to either an autologous or allogenic host [16].

How could human adipose-derived adult stem cells be used in tissue engineering applications? Approximately 400,000 liposuction surgeries are performed in the US each year [17]. Human ASCs isolated from these surgeries could be injected into a site of injury or disease. The hASCs may then secrete
cytokines and growth factors to stimulate recovery in a paracrine manner. The hASCs may also recruit endogenous stem cells to the site and promote their lineage appropriate differentiation. Autologous hASCs offer a variety of advantages, from histocompatibility and infectious perspectives to providing regulatory aid. While autologous cells are preferable when feasible, it is not always possible for a patient to provide his own therapeutic cell product. Initial studies indicate that passaged ASCs exhibit a reduced expression of histocompatibility antigens and no longer exhibit a lymphocyte reaction, and have been shown to actually suppress immunoreactions [18-20]. These results suggest that ASCSs implanted in vivo may not elicit a cytotoxic T-cell response, and subsequently suggest transplanted ASCs would not elicit a robust immune response [16]. However, in order to improve the feasibility of culturing hASCs in vitro prior to implantation, there also exists a need for serum free culture medium to avoid exposure to bovine spongiform encephalopathy or other xenogenic infections [16].

One reason stem cells are often chosen for research and therapeutic applications is because of their self-renewal capabilities. This means that when a stem cell divides, it does so asymmetrically, producing one cell like the mother cell, and another that is functionally different from the mother cell. Stem cells thereby give rise to progenitor cells, which, for example, are ultimately necessary to give rise to osteoblasts. Stem cells, however, also maintain the capability for self-renewal, unlike the progenitor cells which can only proliferate and expand in number [21]. Stem
cells are also categorized by their potency. In many bone tissue engineering applications, multipotent adult stem cells are induced down an osteogenic lineage through the addition of soluble factors to the medium, electrical stimulation, fluid shear stress, or applied tension. While osteoblasts only have a life span of approximately forty days \textit{in vivo}, they are important in that they give rise to the matrix of new bone tissue as well as the osteocytes and bone lining cells that comprise bone tissue [21]. The ability to differentiate into bone forming cells, combined with decreased disease transmission risks and increased immune compatibility, is an important consideration when designing bone-graft substitutes.

1.1.3 Bone-graft Substitutes

Traditional treatment methods used to address skeletal disorders, including bone trauma and disease, rely on autografts or allografts. Autografts, the current gold standard for bone-graft procedures, are desirable because of the decreased risk of disease transmission or of graft rejection by the immune system. However, they require additional painful and expensive surgeries to extract the patient’s own tissue from a secondary site, from which there is often a limited supply of tissue which can be safely removed [22]. Additionally, the associated risk of major and minor complications occurring from autologous bone harvesting is 8.6% and 20.6% respectively [23]. Allografts in comparison rely on cadaver tissue, eliminating the secondary surgery and providing a larger tissue source in a variety of shapes and
sizes [22, 24]. Although the tissue is carefully screened and matched for immune purposes, there is always the risk that the tissue has been infected or will be rejected by the host patient. To further address patient needs, tissue engineering has emerged as a method of gaining insight into tissue development as well as a resource for replacement surgeries.

Tissue engineering interests in recent years have focused on the development of viable, implantable tissue constructs, including bone-graft substitutes. For several years now, adult stem cells have been harvested and isolated from patients and coaxed into a differentiated state by various chemical and mechanical processes [25-30]. Human adipose-derived adult stem cells have been shown to differentiate and proliferate in both monolayer culture conditions and on various polymer scaffolds [29, 31-33]. The relative abundance of adipose tissue, combined with a significantly reduced risk of infection or immune rejection, makes hASCs a great potential cell source for bone-graft substitutes.

When designing graft substitutes, it must also be remembered that bone has a complex architecture, and its properties vary within individuals. Properties such as porosity, pore size and mechanical properties have all been noted to vary based on the patient’s age, nutrition, and activity levels [34]. These considerations must be examined prior to implantation to ensure a proper match with the physical and mechanical nature of the native tissue [22]. As others have suggested, it is also important to remember that the validation of a bone-graft substitute in one clinical
location may not exhibit the same performance in a different location [24]. Therefore, the site-specific effectiveness, as well as the efficacy of the graft, can only be clearly determined in human trials. The work here describes the initial in vitro validation of potential bone-graft substitutes. Additional in vivo studies will be required to assess properties such as capillary formation and examine the interface between the grafts and native tissue, as well as note any immune response.

1.2 Project Overview and Objectives

The various projects described here were designed to address multiple issues in functional bone tissue engineering. The objectives of this research include: 1) the evaluation of hASC response to a custom titanium implant fabrication method; 2) the examination of hASC proliferation, viability, and osteogenic differentiation on three-dimensional collagen-PCL sheath-core bicomponent electrospun scaffolds in comparison to monocomponent PCL scaffolds; 3) the design of a porous fiber with interconnected micropores for bone tissue engineering applications; 4) evaluation and characterization of hASC response to islands-in-the-sea fibers designed for bone tissue engineering applications.
2. Evaluation of Human Adipose-Derived Adult Stem Cell Response to Custom Titanium Implant Fabrication Methods

In the previous chapter, the need to design appropriate scaffolds for bone tissue engineering applications was discussed. In weight bearing applications, it is sometimes necessary to rely on other implanted materials for strength. Titanium is one such biocompatible material capable of supporting a patient’s weight. Patient specific titanium implants can be generated from computed tomography (CT) scans using electron beam melting (EBM). However, the response of human cells to EBM processing has not previously been examined. In this chapter, the response of human adipose-derived adult stem cells (hASCs) to EBM processed discs was compared to commercially available titanium discs. Human ASC proliferation, viability, and cytokine production were analyzed over a one week period.
2.1 Introduction

The most prominent role of bone is to provide skeletal integrity and allow locomotion [35]. However, it may sometimes be necessary to replace bone, such as in cases of disease or trauma. Titanium is one such replacement option, and is commonly used because it is not degradable, thereby maintaining its material properties over time [35]. Titanium is also a popular implant choice because it is biocompatible and possesses high strength and favorable fatigue behavior, and can resist corrosion from body fluids.

Integration at the bone-implant interface, termed osseointegration, improves stability and proper load transfer of implants [36-39]. A variety of factors can influence osseointegration including implant characteristics, mechanical loading, and bone quality [40, 41]. Osseointegration requires close proximity between the bone and implant [37, 39, 42]. Also, the motion between implant/bone interface should be < 150 µm to allow bone ingrowth. Greater motion at the implant/tissue interface lead to fibrous tissue ingrowth [43]. Fibrous ingrowth impedes future bone ingrowth and causes inefficient stress transfer from implant to bone, typically resulting in implant failure [41, 44]. Acrylic bone cement has been used as an anchoring device for implants to help reduce interfacial motion, however it has been suggested that the cement may actually lead to aseptic loosening of the implant. Aseptic loosening can be caused by a multitude of factors, including bone remodeling, cement failure, and interfacial failure [45]. It has been suggested that bone cement can lead to chemical...
necrosis of surrounding tissue due to release of unreacted methyl methacrylate monomer [45]. It can also result in the formation of cement particles, which evoke an inflammatory response from the surrounding tissue, further increasing destruction of the bone around the implant [45]. To avoid the necessity of bone cement, while still reducing interfacial micromotion, custom-designed, patient-specific implants have been developed.

The fabrication of a custom implants using conventional fabrication methods (machining, casting) has major limitations because of the unique and complex geometry of bones. Additionally, fabrication of both solid and porous portions on the implant surface is often a time and labor intensive process, increasing the cost of production [46]. Other possible fabrication methods for titanium implants include laser sintering by an EOSINT M-270 system (EOS, Krailling, Germany) or using Laser Engineered Net Shaping technology developed by Sandia National Laboratories. The EBM fabrication method described here similarly reduces production costs of multi-piece assembly by allowing the structure to be generated in one step, with the added advantage of being more energy efficient than the laser based models.

Long-term success and mechanical stability of an implant depends on osseointegration [42]. The implants are intended to be secured to the surrounding bone, forming a mechanical interlock termed “functional osseointegration” [42]. To enhance osseointegration, implants have been designed with microscopic surface
textures imparted by processes such as mechanical grit-blasting or chemical etching [42]. Researchers have compared a variety of other titanium surfaces treatments as well, such as sintered porous-surfaced and plasma-sprayed implants. The results indicated greater matrix mineralization and tissue integration occurred on porous-surfaced implants, and these implants subsequently exhibited greater interfacial stiffness and attachment strength [42]. These results suggested that porous surfaces could improve early implant stability, thereby accelerating the body’s healing response [42]. Research has similarly suggested that surface texture has a significant impact on direct bone fixation [47]. It was found that roughened surfaces exhibited greater bone apposition, as opposed to fibrous tissue encasement for smooth implant surfaces, leading to greater interface stiffness and shear strength [47]. It has also been shown that bone ingrowth into these porous metal surfaces is dependent on the micromotion between the bone and implant, surface porosity, and the presence of gaps between the native bone and the implant surface [48]. Custom-designed implants are uniquely poised to reduce gaps and possess appropriate porosity, thereby reducing the chances of implant failure.

Another design consideration for implants is the reduction of stress-shielding effects. It is known that bone regeneration is aided by mechanical loads, however there is a worry that titanium implant will carry a disproportionate quantity of the biological load as its properties are much stiffer [49]. By increasing the porosity of the titanium implant though, Thelen et al. have suggested that its stiffness similarly
decreases [49]. The incorporation of pores will also affect the strength and fatigue resistance, thereby reducing the stress shielding effects [49]. Their results suggested a porous titanium material would possess a lower material modulus, and that the stiffness could be oriented in specific directions by designing elongated pores [49]. Porous titanium structures have also been shown to facilitate bone tissue ingrowth, improving the anchorage at the bone/implant interface [50]. Animal studies of rapid prototyped porous titanium implants revealed bone inside the pores and between the struts of the implants [50]. This ingrowth provided biological fixation of the implant to the skeleton [50]. These results suggest that implants could be improved by incorporating the patient's own bone marrow cells in a rapid prototyped, porous titanium implant [50].

In this study, commercially produced solid, polished Ti6Al4V discs (control group) were compared to 3 types of EBM-produced Ti6Al4V discs (experimental groups) with different surface morphologies. Human adipose-derived adult stem cell (hASC) viability, proliferation, and release of proinflammatory cytokines IL-6 and IL-8 were compared between the experimental EBM samples and the commercial control. We hypothesized that the increased surface area of the unpolished EBM samples would increase hASC proliferation without increasing IL-6 and IL-8 cytokine release. It was further hypothesized that the porous structure of the EBM samples would allow for the greatest hASC proliferation, compared to the solid commercially
available control discs, because cells would be able to migrate into the three-dimensional porous structure.
2.2 Materials and Methods

2.2.1 Scaffolds Preparation

Four groups of titanium scaffolds (Figure 2.1), 10mm diameter and 6mm height, were produced: A) polished commercially-produced (control), B) polished EBM-produced, C) unpolished EBM-produced, and D) unpolished, porous EBM-produced. Triplicate scaffolds (n=3) of each group were analyzed for hASC proliferation, viability, and cytokine production.

![Figure 2.1](image)

Figure 2.1. Photographs of titanium discs. A) polished commercially-produced (control), B) polished EBM-produced, C) unpolished EBM-produced, and D) unpolished, porous EBM-produced
Commercially-produced, high strength titanium (Grade 5) was purchased from McMaster-Carr Supply Company (Santa Fe Springs, CA) and machined to the final dimensions (10mm diameter, 6 mm height) for 6 discs (Group A) using a computer numerically controlled (CNC) lathe (MAG Cincinnati, Hebron, KY). For the solid, polished experimental samples (Group B), a solid rod of the same diameter (5/16”) was fabricated via EBM using Ti-6Al-4V powder, and further machined in a manner identical to the control scaffolds. Two additional groups of six discs were fabricated via EBM: solid, unpolished discs (Group C) and porous discs (Group D).

Silicon carbide paper was used to abrade the two groups of polished scaffolds (Groups A and B) successively, in grades from 240 to 2000 grit. These scaffolds were subsequently polished with 6µm diamond, 1µm Al₂O₃, and 0.3µm Al₂O₃. The unpolished scaffolds, both porous and solid (Groups C and D), were blasted with titanium powder to remove any excess powder using titanium from the scaffold build as the blasting medium. All four scaffold groups were ultrasonically cleaned for 10 mins with isopropanol alcohol, rinsed with distilled water for 10 mins then treated with Prepzyme® X.F. eXtreme Foam (Ruhof Corporation, Mineola, NY). Scaffolds were subsequently ultrasonically washed and rinsed with distilled water, dried, and steam sterilized.
2.2.2 Human Adipose-Derived Adult Stem Cell (hASC) Isolation

Excess human adipose tissue was obtained from an elective abdominoplasty surgery with donor consent from a Caucasian female (50-years-old) in accordance with protocols approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (04-1622). Briefly, the hASCs were isolated from the adipose tissue using a density and differential adhesion based assay modified from Zuk et al. and as reported previously by the Cell Mechanics Laboratory [32, 51-54]. The tissue was rinsed with an equal volume of phosphate buffered saline (PBS) and 100 units/ml Penicillin/ 100 µg/ml Streptomycin (Mediatech, Inc., Herndon, VA) to remove excess blood. The tissue was then minced with a scalpel and combined with 0.075% Collagenase I (Worthington Biochemical Corp., Lakewood, NJ), 10,000 I.U. Penicillin / 10,000 µg/mL Streptomycin and alpha-modified minimal essential medium (α-MEM with L-glutamine, Invitrogen), and incubated on a rotator for 30 minutes at 37°C. After incubation, the collagenase was neutralized by adding an equal volume of growth medium (α-MEM with L-glutamine, 10% fetal bovine serum (Premium Select, Atlanta Biologicals, Lawrenceville, GA), 10,000 I.U. Penicillin / 10,000 µg/mL Streptomycin, 200 mM L-glutamine (Mediatech, Inc.)) to the digest, and the suspension was centrifuged at 12,000g for 10 minutes. The pellet was resuspended in 160mM NH₄Cl to lyse the red blood cells. The suspension was again centrifuged at 12,000g for 10 minutes and then resuspended in growth
medium and seeded into tissue culture flasks (one 75 cm$^2$ flask per 5 grams initial tissue) in 25 mL growth medium. The culture flasks were washed with PBS to remove non-adherent cells after 24 hours, and fresh growth medium was added. Isolated hASCs were then characterized by their ability to differentiate down osteogenic and adipogenic pathways. Human ASC cultures were passaged and cryopreserved at 70-80% confluency, then thawed and re-seeded at a density of 100,000 cells per 75 cm$^2$ tissue culture flask. Cells used in this study were third passage cells following isolation.

**Figure 2.2.** Images of hASCs after induction down the adipogenic (left) and osteogenic (right) pathways. Red stain indicates presence of oil droplets (left) and calcium deposits (right).

**2.2.3 Cell Seeding on Scaffolds**

Three of each of the four scaffold types were placed in individual wells of a 24-well, non-tissue culture treated plate (Sarstedt, Inc., Newton, NC). A seeding
density of 20,000 hASCs/20 µL was used for each scaffold, and cells were allowed to adhere for 30 mins before 2mL growth medium was added to each well.

2.2.4 Cell Proliferation

Human ASC viability and proliferation was determined with an alamarBlue assay (AbD Serotec, Raleigh, NC) at 0, 24, 48, 72 h, and 1 week post-seeding. AlamarBlue was added to each well five hours before the sampling time point at a volume of 10% of the culture medium. After incubation of the scaffolds in the alamarBlue solution, 200µL samples were taken in triplicate and the absorbance read at 570 and 600 nm using a Tecan GENios microplate reader (Tecan, Switzerland). Triplicate scaffolds (n=3) for each of the four groups were assayed. The percent reduction of alamarBlue for cells growing on the EBM porous, EBM polished and EBM unpolished scaffolds was compared to reduction by cells on the commercial samples. Greater alamarBlue reduction was indicative of greater hASC proliferation. Statistical analysis was performed with a repeated measures design, using PROC MIXED in SAS (SAS 9.1 for Windows; SAS Institute, Cary, NC). A value of p<0.05 was considered significant.

2.2.5 Cell Viability

Cell viability was assessed 8 days after seeding using the Live/Dead® Viability/ Cytotoxicity Kit (Molecular Probes, Eugene, OR). Each scaffold was
washed twice in PBS and incubated in a calcein AM / ethidium homodimer-1 solution for 15 min in the dark. A fluorescence microscope (Leica Microsystems Inc., Bannockburn, IL) was used to image the cells on the scaffolds with a 10x objective. SimplePCI image analysis software (Compix Inc. Imaging systems, Cranberry Township, PA) was used to acquire the images.

2.2.6 Cytokine Analysis

Culture media was extracted at days 0, 1, 2, 3, and 7 post-seeding to analyze cytokine production. Extracted media was frozen at -20°C so all samples could be analyzed simultaneously. Interleukin-6 and -8 (IL-6, IL-8) from the media were simultaneously quantitated utilizing the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA) and 50µL of culture medium from each well. Beads (5.6µm) conjugated to a capture antibody specific to IL-6 and IL-8 were incubated in a 96-well filter plate with each sample (3 replicates per sample). The beads were then incubated with a fluorescent-labeled reporter molecule that specifically binds the analyte. The contents of each well were analyzed in the Bio-Plex array reader (Luminex xMAP™ Technology, Luminex Corporation, Austin, TX), with IL-6 and IL-8 quantitated relative to the standard curve specific to that cytokine.

Cell viability data, as determined by alamarBlue, was incorporated in the cytokine analyses to determine cytokine concentration normalized to cell viability data and expressed as pg/mL. Mean values for cytokine concentration for each
scaffold were also calculated and the significant differences (p<0.05) between scaffolds determined using the least significant difference in the PROC ANOVA (SAS 9.1 for Windows; SAS Institute, Cary, NC). Multiple comparisons among different scaffolds were conducted using the Student's t-test at p<0.05 level of significance.
2.3 Results

2.3.1 Cell Proliferation

Significantly greater alamarBlue reduction was noted for the porous samples on days 3 and 7 in comparison to the commercial samples, indicating greater cell proliferation on the porous EBM samples (Group D) relative to the commercially produced polished samples (Group A) (Figure 2.2). There was also significantly greater alamarBlue reduction on the EBM unpolished samples than the commercial samples on day 7 (Figure 2.2).

Figure 2.2. Percent reduction of alamarBlue by hASCs on four titanium discs. p<.05 is considered statistically significant.
2.3.2 Cell Viability

Cell viability after 8 days in culture was observed using Live/Dead staining and fluorescence microscopy (Figure 2.3, live=green, red=dead). Control polished, commercial scaffolds (Group A) and the EBM polished scaffolds (Group B) both appeared to have no, or very limited, negative impact on hASC viability (Figure 2.3 A, B). Though changes in surface topography sometimes obscured the view of hASCs in the two-dimensional image, similar numbers of live cells were observed on the unpolished (Group C) and porous (Group D) scaffolds (Figure 2.3 C, D). From images obtained at varying depths, live cells appeared attached and spread along the struts of the porous scaffolds (Figure 2.3D, not all data shown).
**Figure 2.3.** Representative fluorescence microscopy (10X objective, scale bar=100µm) Live/Dead images of hASCs seeded on titanium discs after 8 days of culture (live= green, red= dead). A) polished commercially-produced (control), B) polished EBM-produced, C) unpolished EBM-produced, and D) unpolished, porous EBM-produced.

**2.3.3 Release of Cytokines IL-6 and IL-8**

Human ASC cytokine production (IL-6 and IL-8) was quantified on each scaffold type at days 0, 1, 2, 3, and 7 and mean values reported (Figures 2.4 and 2.5). The EBM polished (Group B) scaffold elicited a significant (p<0.05) increase in
IL-6 release (10,210 pg/mL) after day 7, followed by the commercial (Group A), EBM unpolished (Group C) and then the EBM porous (Group D) scaffolds (Figure 2.4). The limit of detection for IL-6 was 1.1pg/mL.

The greatest release of IL-8 occurred between day 0 to 1 and between days 3 to 7, with the commercial (Group A) and EBM polished (Group B) scaffolds eliciting a significantly higher response than the EBM porous (Group D) (p<0.05) at days 1, 3, and 7 (Figure 2.4). By day 7, the IL-8 concentration in the commercial (Group A) and EBM polished (Group D) scaffolds had a significant increase in IL-8 release (above 1100 pg/ml) followed by the EBM unpolished (Group C) and EBM porous (Group D) scaffolds. The limit of detection for IL-8 was 0.5pg/mL.
Figure 2.4. Mean IL-6 release (± SEM), normalized to proliferation data, by hASCs on titanium implants. Histogram with different letters (A-C) denote mean values that are statistically different at p<.05. If no letters are present, then no significant difference was noted.
Figure 2.5. Mean IL-8 release (± SEM), normalized to proliferation data, by hASCs on titanium implants. Histogram with different letters (A and B) denote mean values that are statistically different at p<.05. If no letters are present, then no significant difference was noted.
2.4 Discussion

Custom-designed, patient-specific implant fabrication is possible with EBM, however, the biological response of human adult-derived stem cells to EBM-processed titanium structures has not previously been reported. Titanium implants have been fabricated using a variety of other methods, including rapid prototyping, and porous coatings have been applied by a variety of means such as plasma-spraying, machining, shot-blasting and acid etching [34, 50]. This was a preliminary study to explore the biocompatibility of implants fabricated via EBM, enabling patient-specific implants when designed using CT images. EBM fabrication offers advantages over other fabrication methods because it may be used to build patient-specific implants with solid and porous portions directly from CAD files. Little was known, however, about the biological response to EBM-processed titanium structures [55]. In this study, proliferation, cytokine expression, and viability of hASCs were examined. Results indicated that cell proliferation occurred on EBM scaffold types, and cells remained viable throughout the eight day culture period, suggesting these scaffolds are capable of supporting ingrowth of native tissue and cells. Increased cell proliferation of hASCs on the EBM porous implants, combined with Live/Dead images revealing viable cells on multiple layers of the scaffold, suggest that this design may improve the mechanical interlock between the implant and the surrounding native tissue.
Pore size has previously been shown to be an important factor in bone ingrowth. Researchers have noted that higher porosity and pore size result in greater bone ingrowth in the *in vivo* environment [34]. Others have designed porous titanium implants comprised of meshes with 60% porosity and pore sizes of 800 and 1200 µm [50]. Results indicated bone ingrowth occurred on scaffolds of both pore sizes, with no significant difference noted between the two pore sizes [50]. In this study, the porous discs were comprised of 76% porosity and pores approximately 640 µm in diameter. Results from this study similarly indicated that increased porosity aided cell viability and proliferation, suggesting EBM processed titanium would be a good candidate for orthopedic applications as the porous structure yielded greater hASC proliferation at earlier time points.

Cell viability and proliferation was assessed using the alamarBlue assay. Significant increases in alamarBlue reduction were noted on days 3 and 7 for the EBM porous scaffolds compared to the commercial samples. Reduction of the alamarBlue dye is performed by living cells, indicating a significantly greater cell number on the porous scaffolds at these two time points. The significant increase in cell number at these time points may have resulted from the increased surface area in EBM porous discs. The porous design allowed for cell growth into the sample along the struts, and the presence of large pores likely allowed for better nutrient delivery and waste removal for cells which had migrated into the scaffold. Increased cell proliferation was additionally noted on day 7 for the EBM unpolished samples.
This difference was likely due to an increased surface area of the unpolished samples compared to the polished samples. These results were supported by previous observations which noted that the attachment and proliferation of anchorage-dependent bone forming cells are enhanced by surface roughness [56].

The Live/Dead staining images obtained in this study confirmed the results of the proliferation study, indicating that viable cells were present on all scaffold types throughout the experimental period and that few dead cells were present. Images obtained from the EBM porous scaffold also showed viable cells on various layers of the scaffold (only one layer was reported here). Similarly, the three-dimensional nature of the EBM unpolished sample resulted in dark areas correlating to Ti6Al4V surface peaks. Viable hASCs were therefore observed on all implant types, with few dead cells. This suggests that cells were capable of adhering and proliferating along the EBM implants, and should thereby improve tissue adherence.

The release of proinflammatory cytokines IL-6 and IL-8 indicate a cellular response to stimulation. IL-6 is a pro-inflammatory cytokine which can be produced by almost all human cells, including adipocytes, in response to stimulation [57-59]. For example, Sumanasinghe et al. has previously shown that cyclic tensile strain induces IL-6 and IL-8 expression in human mesenchymal stem cells (hMSCs) [29]. The primary activity of IL-6 lies in the acute phase response, and is often correlated with an elevated body temperature [59]. It has been concluded previously that early high serum levels of IL-6 may be an indication of transplant-related complications.
Therefore, it has been suggested that IL-6 plays the most significant role during the initial inflammatory response by triggering cytokine release [59]. Additionally, since IL-6 is produced by almost all cells, it is not uncommon to note elevated levels in pathological conditions [59]. Both IL-6 and IL-8 are believed to be pro-inflammatory, and are involved in both systemic and local inflammatory responses [59]. IL-8 is described as a chemokine, and is now also known as CXCL8 [60]. Chemokines act on leukocytes rolling along the blood vessel wall causing the leukocyte to bind, travel through the vessel wall, and migrate to an infection site following the concentration gradient of the chemokine [60]. At each time point, the quantity of IL-6 and IL-8 released by hASCs on EBM unpolished and EBM porous scaffolds was equal to, or significantly lower than, the quantity released by cells on the commercial samples. These results suggest EBM processing does not adversely affect the properties of the titanium, as it does not elicit an increased immunogenic response in comparison to the commercially available titanium implants.

Findings from this in vitro study indicate that EBM structures can successfully host living cells. Furthermore we have shown that EBM-fabricated porous titanium structures generally result in reduced cytokine production and greater cell proliferation than commercially-produced titanium scaffolds, suggesting that it would be a good candidate for orthopedic applications and potentially promote cell ongrowth and ingrowth.
2.5 Summary

Custom designed, patient-specific implants may reduce micromotion at the implant-bone interface by improving fit, leading to improved ingrowth and overall success of the implant. Patient-specific implants are difficult to manufacture with conventional techniques due to complex bone contours however, novel free-form fabrication methods, such as electron beam melting (EBM), have enabled the direct fabrication of custom implants using titanium and other biocompatible metals. EBM was used to produce titanium structures of various surface topographies (polished, unpolished, and porous), which were compared to commercially available titanium discs. Viability, proliferation, and cytokine production of hASCs on these EBM titanium discs was assessed in vitro. The results of these studies indicated that a porous EBM structure supported increased hASC proliferation and did not exhibit an increase in production of cytokines IL-6 and IL-8 in comparison to the commercially available titanium implants. These results suggest EBM processed porous structures may enhance implant stability by promoting cell ingrowth.
3. Three-dimensional Collagen-PCL Sheath-Core Bicomponent Electrospun Scaffolds Increase Osteogenic Differentiation and Calcium Deposition of Human Adipose-Derived Adult Stem Cells

In the previous chapter, four surface coatings for titanium implants were assessed for cell viability and proliferation. Results from that study showed a porous mesh design created using EBM supported significantly increased cell proliferation in comparison to commercial-grade titanium discs. These EBM porous scaffolds did not show an increase in cytokine production compared to commercially available scaffolds, suggesting similar biocompatibility. These scaffolds were designed to facilitate bone ingrowth and reduce micromotion for weight-bearing implants. In non-weight-bearing injuries where less tissue is required, it may be preferable to incorporate a biodegradable implant. In this chapter, sheath-core electrospun scaffolds were designed for such bone tissue engineering applications.

Polycaprolactone (PCL) monocomponent control scaffolds were compared to novel sheath-core electrospun scaffolds possessing a type I collagen sheath and PCL core. Human ASCs were qualitatively and quantitatively examined for viability and proliferation on both scaffold types. Calcium deposition was also measured after two weeks of culture in osteogenic medium.
3.1 Introduction

Tissue engineering scaffolds need to mimic the structure and function of the native extracellular matrix (ECM) by providing both mechanical support and sites for cell interaction [61]. Various strategies have been developed to improve cell-substrate interactions on polymer scaffolds [62-65]. The ideal scaffold requires a porous network which is biocompatible and biodegradable with controlled degradation kinetics, while providing appropriate mechanical strength.

The natural ECM of bone tissue is comprised of type I collagen fibers on the nanoscale range. Collagen I fibril diameters generally vary between 260 and 410 nm, and through their orientation create a three-dimensional, porous, and multi-fibril network [66]. These nanoscale fibrils often form a rope-like structure, such as that observed in Figure 3.1. Through the implementation of electrospinning techniques, it is possible to create a three-dimensional, porous network of fibers with diameters on a similar size scale to these collagen fibrils [67].
The natural ECM of bone is comprised of many nanoscale proteins and features. For this reason, electrospun nanofibrous structures are being used, with increasing interest, as scaffolds for tissue engineering [67-70]. The structures have unique characteristics, in that they provide extraordinarily high specific surface area combined with high porosity and a three-dimensional interconnected pore network conducive to cell growth and proliferation [71-73]. In the conventional electrospinning technique, a charged polymer solution flows out of a capillary and is accelerated toward a grounded collector plate in a strong electrostatic field [74, 75]. The electric
field causes the droplet emerging from the capillary end to undergo deformation into a conical shape, commonly called the ‘Taylor cone’ [74]. This shape is assumed as the surface tension of the solution, the electric charges developed in the solution, and the electrostatic force acting on the droplet, interact. As the strength of the applied potential is increased to the point at which the electrostatic force has overcome the surface tension of the solution, a fine jet of the solution emerges. The jet, after an initial straight path, undergoes bending instability and the characteristic whipping motion due to the charge-charge repulsion that occurs between the excess charges present in the jet. During this phase, the jet is drawn by at least two orders of magnitude, the solvent evaporates, and the dry nanofibers deposit onto the collector. These fibers, in the form of a fibrous mat such as the one seen in Figure 3.2, can then be removed from the collector plate and used as a tissue engineering scaffold.
Various biodegradable polymers of both synthetic and natural origin have been used to prepare nanofibrous scaffolds via the electrospinning technique [61, 67, 76]. The primary reason for using natural polymers has been their inherent biocompatibility established with the presence of specific cell recognition sites that are capable of binding cells [67, 76]. Alternatively, wide interest in synthetic polymers remains high due to their favorable mechanical properties, controlled degradation rates, and low cost [67, 77, 78]. Regenerated natural polymers, although greatly biocompatible, are weak and degrade rapidly and uncontrollably. However, synthetic polymers, although mechanically more stable, do not possess
cell recognition sites. Accordingly, when used alone, neither material provides an ideal base for long-term development of tissues. Hybridization of synthetic and natural polymers could, therefore, provide an effective approach to utilize the unique properties of the materials while overcoming the drawbacks. Although the blending of materials can improve cell growth on the scaffold, a more appropriate approach could be a differentially biodegradable, bicomponent, nanofiber with a natural polymer sheath and synthetic polymer core [79]. The efficacy of using the polymers in this way has been demonstrated with human dermal fibroblasts by Zhang et al [62]. They created sheath-core nanofibers using collagen Type I from calf skin (sheath) and polycaprolactone (PCL) (core). They showed that the density of human dermal fibroblast increased linearly from 19.5% on day 2 to 31.8% by day 6 as compared to the initial cell density [62]. This increase was shown to be significantly higher than that on the pure PCL nanofiber scaffolds. However, no longer term behavior of the fibroblast cells was examined.

Researchers have also examined the use of blended PCL/collagen nanofibers with various compositional ratios (PCL/collagen = 1/3, 1, and 3 by weight). Lee et al. examined pre-osteoblastic MC3T3-E1 cell viability on collagen/PCL (50 wt% collagen) and pure PCL scaffolds and reported significantly increased cell viability on the collagen/PCL scaffolds at 3h, 3 and 7 days relative to pure PCL. Gene expression of bone-associated genes collagen type I, osteopontin, and alkaline phosphatase was also analyzed at 1, 3, and 5 days using real-time RT-PCR. They
reported a significant upregulation of these genes on the collagen/PCL scaffolds, relative to pure PCL, with upregulation most increased on days 1 and 3 [65].

These prior studies examined cells cultured on similar PCL and collagen I polymers at early time points, but none have looked at the effects of a collagen sheath on human cell osteodifferentiation over a longer, e.g. 14 day, period. Further, no previous studies have examined the effects of such sheath-core configurations on hASCs. Therefore, in the present study we investigated the potential of bicomponent, sheath-core, collagen-PCL fibers for bone tissue engineering using hASCs. After seeding, the hASCs were allowed to proliferate on two biocompatible scaffolds, and then were induced down the osteogenic lineage through the addition of soluble osteogenic inductive factors (dexamethasone, β-glycerolphosphate, and ascorbic acid) to the complete growth medium. The combined effects of scaffold architecture and surface chemistry, imparted by both the electrospinning process and a collagen I coating, on PCL nanofibers were analyzed for their effects on hASC viability, proliferation, and osteogenic differentiation. We examined cell viability and proliferation over 14 days (analysis at time points 1, 2, 3, 7, and 14 days), and osteogenic differentiation after 14 days of culture in osteogenic medium. The goal of this study was to determine the ability of this three-dimensional scaffold to increase osteogenic differentiation of hASCs, thus verifying its potential to serve as a bone tissue engineering scaffold. We hypothesized that the 3D bicomponent collagen-PCL sheath-core scaffold would
accelerate hASC osteodifferentiation and increase calcium deposition onto the scaffold.
3.2 Materials and Methods

3.2.1 Materials

Polycaprolactone (PCL) (MW 80 KDa) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Collagen (Type I, Lyophilized) extracted from calf skin was obtained from MP Biomedical (Solon, OH, USA). To prepare pure PCL scaffolds, a mixture of glacial acetic acid (ACS Reagent grade, Fisher Scientific, Pittsburgh, PA, USA), and pyridine (ACS Reagent grade, Sigma-aldrich, St. Louis, MO, USA), was used as a solvent for the polymer. 1,1,1,2,2,2-hexafluoroisopropanol (HFIP) obtained from Alfa-Aesar (Ward Hill, MA, USA) was used as a solvent for both collagen I and PCL to produce sheath-core bicomponent nanofiber scaffolds.

3.2.2. Fabrication of PCL Scaffolds

The PCL solution was prepared by dissolving 1.25 grams of PCL in 10 ml glacial acetic acid. A small amount of pyridine (2.75 %, vol/vol) was added to increase the solution conductivity in order to achieve uniform and small diameter (~300 nm) PCL fibers. The polymer-solvent mixture was stirred gently (200 rpm) overnight (10 hrs) at 35°C. A conventional electrospinning device was set up to prepare PCL scaffolds [74]. The solution was kept in a 5 mL plastic syringe (Becton-Dickinson, Franklin Lakes, NJ, USA) with a metal capillary needle (27 Gauge, 0.2 mm ID, 0.4 mm OD, 50 mm length, McMaster-Carr, Atlanta, GA). The flow rate of the solution was maintained constant at 0.3 mL/hr using a syringe pump (NE- 500,
New Era Pump Systems, Inc., Farmingdale, NY, USA). An aluminum plate of 15 cm diameter was used as a collector. The distance between the end of the capillary and the point of collection was kept constant at 15 cm. A high voltage power supply (ES-30P, Gamma High Voltage Research, Ormond Beach, FL, USA) was connected between the metal capillary and the collector plate and the voltage was maintained at 11 kV.

3.2.3. Fabrication of Sheath-Core Nanofibrous Scaffolds

To create the 4% (wt/vol) collagen solution, 40 mg collagen was dissolved in 1 mL HFIP. Similarly, 12% (wt/vol) PCL solution was prepared by dissolving 1.2 grams of PCL in 10 mL of HFIP. The polymer-solvent mixtures were stirred gently (200 rpm) for four hours in order to obtain homogeneous solutions.

A co-axial electrospinning approach was used to produce the hybrid collagen/PCL sheath-core structures. A compound spinneret to hold solutions for the sheath (collagen) and the core (PCL) was constructed using two plastic syringes (5 mL, Becton-Dickinson) (Figure 3.3). A longer capillary (27 Gauge, McMaster Carr) was connected to the back syringe and a shorter (20 Gauge, McMaster Carr) capillary was connected to the front syringe to create the ‘co-axial’ configuration at the tip. The front syringe contained the sheath polymer (collagen) solution, while the back held the core (PCL) solution. The syringes were independently driven by syringe pumps (NE-500, New Era Pump Systems, Inc.) to control the flow rates of
the two materials. A high voltage supply (Gamma High Voltage Research) was connected between the co-axial capillary and the aluminum collector plate. The distance between the end of the capillary and the point of collection was kept constant at 15 cm. The applied voltage and the flow rates of the solutions were adjusted as needed to achieve the maximum stability of the compound Taylor cone. The voltage was maintained at 9.5 kV and the flow rates for the collagen sheath and PCL core solutions were maintained at 0.3 mL/hr and 0.5 mL/hr, respectively.

Figure 3.3. Schematic of the co-axial electrospinning system. (Adapted from: [80])

3.2.4. Characterization of Fiber Morphology

All samples were sputter coated with gold-palladium (Au-Pd) using Anatech Hummer 6.2 sputter coater. The samples were examined using scanning electron...
microscopy (SEM) (JEOL JSM- 5900 LV) to analyze fiber morphology. Fiber diameter and distribution were assessed using NIH Image J software. The image pixels were calibrated using the pixel length of a magnification bar in nanometers generated on the SEM image. To measure the fiber diameter, a line was drawn on the fiber perpendicular to its longitudinal axis. The length of the line was automatically converted into nanometers by the software, producing a fiber diameter value at that location. One hundred readings were taken from each image to calculate the average value of the fiber diameter for each electrospun scaffold and to determine the standard deviation.

Sheath-core morphology within the fibers was confirmed by a freeze fracturing technique [79]. The fiber mats were immersed in liquid nitrogen for approximately 10 seconds. While still in the liquid nitrogen bath, the scaffold fibers were fractured at various locations using sharp tweezers to induce differential fibrillation between the collagen sheath and the PCL core. The fractured fibers were then viewed using SEM (FESEM- JEOL 6400F).

3.2.5 hASC Isolation and Expansion

Excess human adipose tissue from elective plastic surgery procedures were obtained with donor consent from three Caucasian females (one 36 and two 50-year-old donors) in accordance with a protocol approved by the Institutional Review Boards of the University of North Carolina at Chapel Hill (04-1622). The hASCs
were isolated from the tissue using a differential adhesion and density based assay modified from Zuk et al. and as reported previously by the Cell Mechanics Laboratory [32, 51-54]. Following the steps described previously in Chapter 2 Section 2.2, adipose derived adult stem cells were isolated, characterized, and cryopreserved. Human ASCs were then thawed and re-seeded at a density of 100,000 cells per 75 cm² flask prior to experimental use. Cells used in this study were first passage cells following isolation.

3.2.6 Cell Seeding

Each 1cm² electrospun scaffold was sterilized by ultraviolet irradiation overnight prior to cell seeding, and subsequently pre-wetted with PBS. The PBS was then aspirated, and 20µL of a cell suspension with a concentration of 1,000 cells/µL media was added to each scaffold and allowed to adhere for 5 minutes before flooding the well with growth medium.

3.2.7 hASC Proliferation

Cellular proliferation was assessed using alamarBlue (AbD Serotec, Raleigh, NC) at days 1, 2, 3, 7, and 14 after seeding. Reduction of the alamarBlue dye occurs as a result of cellular proliferation, resulting in a change in reagent color from an oxidized blue to the reduced pink color. Four hours before each time point, the scaffolds were rinsed with PBS, and a solution consisting of growth medium with a
10% volume of alamarBlue was added to each well. Following the 4h incubation period, the medium was sampled in triplicate and the absorbance read at 570 and 600 nm using a Tecan GENios microplate reader (Tecan, Switzerland). Proliferation was determined by calculating the reduction of alamarBlue dye per the manufacturer’s protocol for the treated (collagen-coated PCL bicomponent) and control (monocomponent PCL) hASC-seeded scaffolds in the wells. Greater alamarBlue reduction was indicative of greater cell proliferation and number. Three scaffolds of each type, for all three cell donors, were analyzed at each time point (18 replicate scaffolds).

3.2.8 Cell Viability

Cell viability was assessed at days 1, 2, 3, 7, and 14 after seeding using the Live/Dead® Viability/ Cytotoxicity Kit (Molecular Probes, Eugene, OR). Each scaffold was washed twice in PBS and incubated in a calcein AM / ethidium homodimer-1 solution for 15 min in the dark. An Olympus IX71 microscope (Center Valley, PA) was used to image day 1, 2, and 3 time points with a 10X objective. IPLab image analysis software (Rockville, MD) was used to acquire the images. A confocal microscope (Leica TCS NT, Bannockburn, IL) with a 10X objective was used to image the day 7 and 14 time points.
3.2.9 Calcium Quantification

Human ASCs were seeded on the scaffolds and grown in complete growth medium for one week. Cells were then cultured in osteogenic medium (growth medium supplemented with 0.1uM dexamethasone, 50uM ascorbic acid, and 10mM β-glycerol phosphate) and calcium was quantified after 1 and 2 wks. Extent of osteogenic differentiation was determined by quantifying calcium deposition per scaffold. Calcium deposits were quantified using the Calcium LiquiColor assay (Stanbio, Boerne, TX). Three scaffolds of each type (PCL alone and collagen/PCL sheath-core), for all three cell donors, were analyzed in triplicate at each time point (18 replicate scaffolds).

To perform the Calcium LiquiColor assay, the medium was aspirated from each well, and scaffolds were carefully rinsed two times in PBS. Next, 0.5N HCl (Fisher, Fair Lawn, NJ) was added to each scaffold, then the scaffolds were vortexed and frozen at -20°C. All samples were later thawed and placed on an orbital shaker at 4°C overnight to extract the calcium. Per the manufacturer’s protocol, the working solution was added to each sample, from which the absorbance was measured and compared to a standard curve of known calcium concentrations.
3.2.10 Statistical Analysis

To investigate the possible effect of scaffold type and time on hASC proliferation, a mixed effects model appropriate to the repeated measured design was used. In the design, 18 replicate scaffolds were used, three for each combination of the three cell donors and two scaffold types, and repeated measurements were made at five time points on each scaffold. This model involved fixed effects for scaffold type and time and their interaction, and random effects for scaffold, nested within the combination of cell donor and scaffold type. To investigate the effects of scaffold type on calcium deposition by hASCs, a simpler mixed effects model was considered with fixed effects for scaffold type and random effects for donor and type-by-donor interaction. Standard error bars reflect variability in the estimated mean response (cell count or calcium deposition) and tests were conducted at significance level .05.
3.3 Results

3.3.1. Scaffold Morphology

PCL monocomponent scaffolds possessed a uniform fiber structure with narrow fiber diameter distribution (Figure 3.4). The average fiber diameter was 280 ± 51 nm and the average thickness of the PCL monocomponent scaffold was approximately 200 μm (Figure 3.4). The collagen-PCL sheath-core scaffolds also exhibited uniform fiber diameters (Figure 3.5) with an average diameter of 442 ± 45 nm and scaffold thickness of approximately 180 μm (Figure 3.5). The freeze fracture method induced differential fibrillation in the collagen sheath and the PCL core and provided evidence of the sheath-core arrangement of the two components (Figure 3.6). It was observed that the fiber diameter was reduced during this technique which could be attributed to some loss of collagen from the sheath during the freezing-thawing action.
Figure 3.4. SEM image of the PCL scaffold structure. Average fiber diameter: 280 ± 51 nm. (Adapted from: [80])
Figure 3.5. SEM image of the collagen-PCL sheath-core scaffold structure. Average fiber diameter: 442 ± 45nm. (Adapted from: [80])

Figure 3.6. SEM images of freeze-fractured nanofibers (Sheath-Collagen; Core-PCL). (Adapted from: [80])
3.3.2 Cellular Interactions

Proliferation of hASCs on both the bicomponent collagen/PCL sheath-core and monocomponent PCL scaffolds was assessed at days 1, 2, 3, 7, and 14 by calculating the reduction of alamarBlue (Figure 3.7). Data indicated a trend of increased cell number on collagen coated PCL scaffolds compared to PCL controls for all time points, although the differences were not significant. When considering all three donor cell lines together, there is no evidence of a scaffold type effect, regardless of whether the time point was considered as a fixed effect or an average effect.
Figure 3.7. Relative absorbance units of alamarBlue dye reduced by hASCs seeded on the bicomponent and monocomponent scaffolds. Standard error bars reflect variability in the estimate of the mean. No statistical significance was noted between groups at any time. (Adapted from:[80])

Similarly, live/dead images revealed viable cells on both scaffold types (Figure 3.8 and 9). At 24 hrs, however, hASCs appeared well spread on collagen coated PCL scaffolds (Figure 3.8A), and, in comparison, appeared to have a clumped morphology on PCL alone (Figure 3.8B). By 1wk this distinction disappeared, and viable cells covered all scaffold surfaces. Further examination with confocal microscopy revealed viable cells throughout and within the scaffold
structures at 1 and 2 wks (Figure 3.9A-D), indicating continued cell proliferation and migration. Few dead cells were present on either scaffold type.
Figure 3.8. Representative images (10X) of cells seeded on collagen-coated and uncoated PCL scaffolds showing live (green) and dead (red) cells. (A) collagen-coated after day 1; (B) uncoated PCL after day 1; (C) collagen-coated after day 2; (D) uncoated PCL after day 2; (E) collagen-coated after day 3; (F) uncoated PCL after day 3 (scale bar = 100µm). (Adapted from: [80])
Figure 3.9. Representative confocal microscopy images (10X) of cells seeded on collagen-coated and uncoated PCL scaffolds with calcein AM and ethidium homodimer-1 showing live (green) and dead (red) cells. (A) collagen-coated after day 7; (B) uncoated PCL after day 7; (C) collagen-coated after day 14; (D) uncoated PCL after day 14. Scale bar = 100µm. (Adapted from: [80])
Calcium content from each of the three hASC lines was determined on three scaffolds of each type after 14 days of culture in osteogenic medium (Figure 3.10). Significantly higher calcium deposition was found on collagen-PCL bicomponent scaffolds as compared to pure PCL controls. On average, a four to five-fold higher calcium deposition was observed for each hASC line on the bicomponent collagen-PCL scaffolds.

**Figure 3.10.** Calcium deposition (µg/scaffold) is quantified after 14 days for three different age (36-50), gender (female), and ethnicity (Caucasian) matched hASC lines growing on bicomponent and monocomponent scaffolds. Standard error bars reflect variability in the estimate of the mean. Scaffold type is significant (*) at a level of p=.03 when calcium deposition is averaged over all three cell lines. (Adapted from: [80])
3.4 Discussion

One of the greatest challenges in tissue engineering is the design and development of biomimetic scaffolds. Several factors contribute to the overall feasibility of a particular structure serving as a cell scaffold, including its porosity, fiber diameter, and the presence of cell adhesion molecules. Nanofibrous structures produced by electrospinning techniques are capable of addressing each of these important areas. The results from this study are significant in that they show that further optimization of the nanofibrous structures for bone tissue engineering can be achieved through the addition of a collagen coating.

The data presented represents an original report of the differentiation of hASCs on a sheath-core collagen-PCL bicomponent nanofibrous structure. This novel scaffold showed significant advantages beyond those noted using the individual components. Pure collagen nanofibers are not mechanically stable and tend to lose the fiber form unless cross-linked. Cross-linking, on the other hand, reduces the availability of the functional groups necessary for cell attachment. PCL nanofibers are mechanically more stable, however, lack the cell interaction sites. Therefore, a hybrid structure with a collagen sheath for effective cell attachment and a PCL core to provide essential mechanical stability has significant potential for tissue engineering applications.
3.4.1 Scaffold Fabrication

PCL scaffolds were fabricated using a conventional single-fluid electrospinning technique. A novel solvent system consisting of acetic acid and pyridine was used to dissolve PCL as we have previously described [81]. Briefly, the solvent system involved an acid-base reaction to produce weak salt complexes that served to increase the conductivity of the polymer solution in order to form small diameter fibers with narrow fiber diameter distribution.

To produce sheath-core nanofiber scaffolds, the co-axial electrospinning technique was used. The process of co-axial electrospinning is conceptually similar to that of the single jet electrospinning approach [82, 83]. When the polymer solutions are charged using high voltage, the charge accumulation occurs predominantly on the surface of the sheath liquid coming out of the outer co-axial capillary [83]. The pendant droplet of the sheath solution elongates and stretches due to the charge-charge repulsion to form a conical shape. Once the charge accumulation reaches a certain threshold value due to the increased applied potential, a fine jet extends from the cone. The stresses generated in the sheath solution cause shearing of the core solution via “viscous dragging” and “contact friction” [84]. This causes the core liquid to also deform into the conical shape and a compound co-axial jet develops at the tip of the cones (Figure 3.11). On the way to the collector, the jet undergoes bending instability, follows a back and forth whipping
trajectory (see Figure 3.3), and thins out greatly [82, 85]. The two solvents evaporate, and nanofibers consisting of a sheath-core morphology are collected.

Figure 3.11. Schematic illustration of compound Taylor cone formation. A) Surface charges on the sheath solution, B) viscous drag exerted on the core by the deformed sheath droplet, C) Sheath-core compound Taylor cone formed due to continuous viscous drag (Adapted from: [80])

It is expected that as long as the compound cone is stable, the core will be uniformly incorporated into the sheath and the sheath-core morphology will develop. Many factors govern the stability of the compound cone, which include the interfacial tension between the sheath and the core solutions, and the applied voltage [79]. In order to minimize the interfacial tension between the collagen and the PCL solutions, a common solvent (HFIP) was used for the two polymers. Further, by maintaining the voltage relatively constant (~ 9.5 kV), a stable compound cone was obtained that led to highly uniform fibers (Figure 3.5).

The 3D scaffolds obtained using two electrospinning approaches (traditional and co-axial) led to somewhat different fiber diameters, with the bicomponent having a greater diameter than the monocomponent. The effect of fiber diameter was not examined in this study. Moghe had previously examined the in vitro degradation
behavior of collagen-PCL sheath-core nanofibers in PBS, and found that for a structure similar to that used in the current study, the diameter of the bicomponent fiber decreases by approximately 13% within the first hour of PBS treatment due to the degradation of collagen from the sheath [86]. The rate of collagen mass degradation decreases logarithmically with time, resulting in a similar trend for the reduction in diameter. We would expect that the bicomponent fiber in this study would exhibit similar behavior. Therefore, it was also expected that the fiber diameters for the two types of scaffolds in this study would be comparable after seeding with hASCs and the results obtained were not due to differences in fiber diameters of the two scaffolds.

3.4.2 Cellular Interactions

One significant advantage of the electrospinning process is that nanoscale fibers of a constant diameter and a controlled porosity can be produced. Since the collagen fibrils which comprise the majority of the ECM of bone tissue are also randomly oriented and possess diameters on the nanometer-scale, the electrospun fibers used in this study are similar in size to what cells may experience in native tissue [87]. The nature of the electrospinning process additionally allows for the development of a highly porous structure. This high porosity enhances nutrient delivery and the removal of metabolic wastes, while simultaneously providing space for cell penetration and migration into the structure [87].
From the results of the alamarBlue assay (Figure 3.7), no significant difference in cell proliferation was observed on either scaffold at any of the five time points when averaging over all three hASC lines. There appeared to be an initial increase in cell proliferation at days 1, 2, and 3 for hASCs on each scaffold type. By day 7, hASCs were confluent (as confirmed by live/dead images) and experienced a reduced proliferation rate. Results from day 7 and 14 therefore reflect a decrease in metabolic activity as compared to day 3, but not a decrease in overall cell number. Since both scaffolds were fabricated with biocompatible polymers, and were on the size scale similar to that of collagen fibrils found in native tissue, it was expected that viable cells would proliferate on, and within, the two biocompatible polymer scaffolds.

The addition of a collagen sheath on the PCL core resulted in greatly increased calcium deposition for all three hASC lines after two weeks of culture in osteogenic medium. Previous work has shown that human mesenchymal stem cells plated on two-dimensional surfaces coated with collagen I exhibit the greatest osteogenic differentiation in comparison to cell growth on an uncoated substrate [88]. Though it is thought that a variety of ECM proteins may support cell attachment to the matrix, the most significant role in osteogenic differentiation appears to involve the interaction of the COL I receptor α1β1 integrin [88]. It has been shown that although interactions with the ECM alone are capable of inducing osteogenic
differentiation, the collagen matrix also enhances cell sensitivity to osteogenic
differentiation medium [88].

A similar effect was observed in this study for hASCs seeded on collagen-
PCL sheath-core nanofibers arranged as a three-dimensional bone tissue scaffold. Less than one microgram calcium was measured on either scaffold type after one week of culture in osteogenic medium (data not shown). However, after two weeks in culture there was a significant increase in calcium deposition on the collagen coated scaffolds (four to five-fold), indicating greater hASC differentiation down the osteogenic lineage. Since alamarBlue data reflected a similar number of cells present on each scaffold type, it appears that the increased calcium deposition was an effect of increased osteogenic differentiation and not due to the presence of a greater quantity of hASCs.

Results from this work are promising as all results noted were consistent across hASCs from multiple donors. These results might indicate the use of a bicomponent sheath-core collagen-PCL electrospun scaffold as a potential patient specific solution to repairing bone defects using a patient’s own hASCs. Early cell-spreading and early osteogenic differentiation could allow for quicker tissue growth to fill a critical defect.
3.5 Summary

The objective of this study was to determine if the addition of a collagen type I sheath to the surface of poly(ε-caprolactone) (PCL) nanofibers enhances the viability and osteogenic differentiation of human adipose-derived adult stem cells (hASCs). This is the first study to examine the differentiation behavior of hASCs on collagen-PCL sheath-core bicomponent nanofiber scaffolds. Scaffolds were developed using a co-axial electrospinning technique. Use of sheath-core configuration ensured a uniform coating of collagen on the surface of the PCL nanofibers throughout the three-dimensional scaffolds. PCL nanofiber scaffolds were prepared using a conventional electrospinning technique and served as controls. Human ASCs were seeded at a density of 20,000 cells/cm² on 1 cm² electrospun nanofiber (PCL controls or collagen-PCL sheath-core) sheets. Fluorescent confocal images and cell proliferation data confirmed the presence of viable cells after two weeks in culture on all scaffolds, indicating cell attachment and spreading occurred on both matrices. Initial observations, however, revealed greater cell spreading on the bicomponent collagen-PCL scaffolds at earlier time points. The hASCs were induced to differentiate along the osteogenic lineage through the addition of soluble osteogenic inductive factors. Results from calcium quantification analyses showed that calcium deposition was approximately five times higher on bicomponent collagen-PCL sheath-core scaffolds than on monocomponent PCL controls, indicating that the collagen-PCL bicomponent scaffolds promoted greater hASC osteogenic
differentiation after two weeks of culture in osteogenic medium. This is the first study to look at collagen coated sheath-core composite fibers on hASC osteogenic differentiation. While the collagen I coating did not enhance proliferation, it significantly increased calcium deposition by hASCs, indicating that collagen-PCL sheath-core bicomponent structures have potential for bone tissue engineering applications with hASCs.
4. Initial Design Iterations of Porous Melt-spun Fibers

In the previous chapter, effects of a type I collagen coating on a PCL electrospun fiber were described. The surface coating appeared to enhance cell sensitivity to osteogenic supplements. In addition to osteogenic growth factors, nutrient delivery and waste removal also play important roles in cell viability and differentiation. This chapter describes the initial design phases of a nanoporous fiber. The goal of these fibers was to aid the development of a three-dimensional scaffold with enhanced fluid flow properties. These enhanced diffusion properties were expected to increase the delivery of osteogenic supplements to hASCs throughout the construct. In this chapter, the polymers, geometry, and physical properties were analyzed and modified to generate a biocompatible fiber with interconnected micropores. This chapter reflects the chronological progression of the nanoporous fiber design, which will be further discussed in Chapter 5 for its capabilities of serving as a bone tissue engineering scaffold.
4.1 Introduction

Nanoporous fibers are desirable for a variety of applications, including surgical drapes, microfiltration, water desalination, gas separation, and tissue engineering scaffolds. Nanoporous fabrics have been generated using a variety of techniques including gas foaming, solvent casting, particulate leaching and thermally induced phase separation. Other methods of producing porous structures, such as those described previously in Chapters 2 and 3, include electrospinning and rapid prototyping. Emulsion freeze-drying and three-dimensional (3D) printing techniques have also been used to generate porous structures [1]. Porous fabrics are particularly interesting for bone tissue engineering applications, as they possess increased diffusional properties which aid the delivery of nutrients to cells throughout the structure.

Of the 6.2 million fractures which occur annually in the United States, approximately 5-10% of them experience impaired or delayed healing, or result in a non-union [89]. Critical sized defects, described as the “smallest size intraosseous wound that will not heal spontaneously during the lifetime of the animal” require medical intervention to achieve proper healing [90]. One such intervention method includes pre-seeding a biodegradable scaffold with adult stem cells, inducing osteogenic differentiation, and then implanting this construct in the defective site. During normal fracture healing, many regulatory factors such as hormones, cytokines, and ECM are recruited to interact with a variety of cell types, such as
bone forming cells or even mesenchymal stem cells from surrounding muscle tissue [89]. A biodegradable scaffold possessing a porous network should encourage cell migration from the surrounding tissue, as well as deliver nutrients, excreted hormones and cytokines to the cells found within the construct.

Biodegradable materials have been used in many medical applications, such as in cases of cartilage, meniscal, or bone repair, as well as fracture fixation and drug delivery [91, 92]. Polymer degradation in vivo is influenced by a variety of factors, including the site and size of the implant, molecular weight, presence of impurities, degradation mechanism (e.g. hydrolysis or enzymatic), applied stress to the implant and the patient’s age [91]. Poly(lactic) acid (PLA) is a commonly used biodegradable polymer, whose degradation results in acidic by-products, which can further accelerate the degradation process due to the relatively slow diffusion of by-products from the implant center. This slow rate of diffusion creates an acidic environment, autocatalyzing the reaction, leading to increased resorption of the central material [91]. This can cause the mechanical strength of the implant to decline well before fracture union or tissue healing has occurred [91]. Therefore, the degradation kinetics of the PLA scaffold should be modified based upon factors such as implant size and location to achieve an optimal tissue ingrowth/ implant degradation relationship. Porous scaffolds are also preferable, as they allow for the products of degradation to spread throughout the medium, preventing the autocatalysis seen in non-porous scaffolds [5]. Biodegradable implants should thus
incorporate a porous design to aid removal not only of cell waste, but also degradation by-products to assist in the uniform degradation of the scaffold.

The degradation kinetics of nanoporous fibers can be controlled through a variety of means, such as the polymer processing history, crystallinity, environmental conditions, additives, and overall hydrophilicity [5]. As mentioned, the degradation products of PLA include lactic acid and water, as well as small debris particles. Studies have shown that macrophages are capable of phagocytizing PLA debris particles formed during the degradation process [93]. In this study, PLA, a known biocompatible polymer described and used in Chapter 5, is examined as a potential polymer for fabricating porous biodegradable scaffolds for bone tissue engineering. Several design iterations were attempted and characterized, culminating in a fiber comprised of PLA combined with EastONE polymer, a water dispersible sulfopolyester, to fabricate biodegradable scaffolds with improved diffusion properties.
4.2 Experimental Approach

4.2.1 Polymer Characterization/Production of Filaments

Preliminary studies were performed to determine potential polymers for this nanoporous fiber application and their optimal spinning conditions. These studies tested the feasibility of removing an additive from poly(lactic acid) (PLA) polymer. Three different microfilm samples were created: a control sample of PLA, an experimental sample containing sodium chloride (NaCl) and a second sample containing sucrose. The microfilms were then imaged both before and after washing with water (Figure 4.1). These results indicated that by including additives that could be removed with water, it was possible to create pores in the surrounding polymer. However, the NaCl and sucrose did not afford much control over the pore size and distribution, as these additives were relatively large. To gain better control over the pore size and distribution, we decided to include a second polymer that could similarly be removed with water. The polymer chosen for this application was the water-dispersible sulfopolyester, EastONE (Eastman Chemical Company, Kingsport, TN).
Figure 4.1. Microscopic images of PLA microfilms before (top) and after (bottom) washing.

Thermal characterization of the chosen polymers, PLA and EastONE, was performed using differential scanning calorimetry (DSC) to determine the melting points and glass transition temperatures. The glass transition temperature of PLA was determined to be 64°C and the melting point 165°C. The glass transition temperature of the water dispersible sulfopolyester (EastONE) was determined to be approximately 50-55°C. No melting was observed with EastONE, indicating that this polymer is completely amorphous. Ground PLA was then physically blended with a water dispersible polyester (EastONE) at concentrations (by weight) of 100%PLA, 100%EastONE, 99%PLA/1%EastONE, 97%PLA/3%EastONE, 95%PLA/5%
EastONE, and 90% PLA/10% EastONE in a Thermo Haake Minicompounder (Figure 4.2) at 175°C and subsequently spun into fibers. The spinning was followed by hot drawing at a constant draw ratio. For all subsequent polymer concentrations, it will be reported as 90/10 PLA/EastONE to indicate a concentration of 90% PLA and 10% EastONE, for example.

![Thermo Haake Minicompounder](image)

**Figure 4.2.** Thermo Haake Minicompounder used to extrude solid monofilaments of varying polymer concentrations.

4.2.2 Removal of Secondary Polymer

The resultant fibers were then washed for two hours in a shaker bath (Boekel Grant) at 140 rpm and subsequently for two hours in an ultrasonicator at 65°C. Fibers were then dried in a vacuum oven for 24 hrs. Both washed and unwashed fibers were analyzed using a scanning electron microscope (SEM) (Jeol JSM 5900).
Figure 4.3. SEM images of unwashed (A, C, E, G, and I) and washed (B, D, F, H, and J) composite fibers acquired at a magnification of 2500X. (Adapted from: [94])
As seen in Figure 4.3, SEM studies revealed the presence of random pores on the 99/1 PLA/EastONE, 97/3 PLA/EastONE, 95/5 PLA/EastONE, and 90/10 PLA/EastONE fiber surfaces after the sulfopolyester had been washed. Pores on the latter fibers were clearly visible at 200X and their areas ranged from 0.08 to 16.0 µm². From the SEM images, we determined that the water dispersible polymer was successfully washed from the surface and from Figure 4.4 it was observed that the pores extended into the fiber. The result was well distributed, random pores on the fiber surface. It was noted that as the concentration of EastONE increased, the frequency and size of the pores on the surface also increased.

Figure 4.4. SEM image of washed 10%EastONE/ 90% PLA fiber showing micropores that continue deep into the fiber (magnification: 5000X, scale bar 1µm). (Adapted from: [94])
4.2.3 Examination of Fiber Cross-Section

Further experiments were performed to characterize the distribution of EastONE throughout the PLA. Washed fiber samples were embedded within resin using the PELCO Eponate 12T embedding kit (Ted Pella Inc. Redding, CA), and then cut into 40 µm sections using a microtome (Reichert Microscope Services, Depew, NY). These samples were then examined using SEM (Figure 4.5).

**Figure 4.5** SEM images of unwashed (A,B) and washed (C,D) composite fibers. A,B) 99/1 PLA/EastONE C,D) 90/10 PLA/EastONE (Adapted from: [94])

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Unwashed fiber samples were similarly embedded within the epoxy resin, and microtomed into 20 µm sections for Fourier Transform Infrared spectroscopy (FTIR) analysis. Overlaid spectra of PLA and EastONE (Figure 4.6) show a peak that is unique to EastONE at 1717.7 cm\(^{-1}\). Microtomed cross-sections were then analyzed at that wavelength to identify locations of EastONE polymer. FTIR contour maps from five sites (Figure 4.7) across the fiber cross-section reveal EastONE locations/intensity. FTIR mapping of the composite fibers show a random dispersion of EastONE throughout the PLA matrix. This random arrangement was more prominent in composite fibers consisting of 10% EastONE.

![FTIR overlaid spectra for 100%EastONE and 100%PLA polymers.](image)

**Figure 4.6.** FTIR overlaid spectra for 100%EastONE and 100%PLA polymers. (Adapted from: [94])
Figure 4.7. Contour maps for 90/10 PLA/EastONE showing the variation in peak height of 1717.7 cm\(^{-1}\) band indicative of localization of EastONE polymer in regions of an unwashed composite fiber cross-section. A) Top edge, B) Right edge, C) Bottom edge, D) Right edge, E) Center of the fiber, and F) Arrangement of maps from fiber cross-section. (Adapted from: [94])

Additional FTIR contour maps confirmed the results that EastONE is dispersed across the fiber cross-section. **Figure 4.8A** shows the digital image of the cross-section, with the area to be mapped outlined in red. This area spans the diameter of the fiber, and results indicate the presence of EastONE in several locations, with the red areas showing the greatest intensity, throughout the center of this fiber (**Figure 4.8B**). These results further validate the previous conclusions drawn from SEM images and FTIR mapping that the localization of the EastONE additive in the PLA matrix is fully dispersed throughout the fiber diameter.
Figure 4.8. Variation in peak height of 1717.7 cm$^{-1}$ band (localization of EastONE) across the diameter of an unwashed 90/10 PLA/EastONE composite fiber. A) Digital image of mapped area, B) Contour map with intensity gradients (Adapted from: [94])

4.2.4 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was used to examine thermal properties of the two polymers and the composite blends. After melt extruding the fibers, glass transition temperatures of both PLA and EastONE decreased. This change indicates a microstructural change in both of the polymers resulting from the extrusion process. Analysis of the DSC data also showed that the melt extrusion
process resulted in completely amorphous 100% PLA and composite fibers. The presence of a single glass transition temperature for the composite fibers also indicates that the polymers formed a miscible blend.

4.2.5 Viscosity Average Molecular Weight

The viscosity average molecular weight of PLA was determined by dissolving the PLA in chloroform. The reduced specific viscosity of various PLA solutions (Equation 4.1) were then measured using an Ubbelohde viscometer at 25°C and plotted against concentration of PLA solutions. From this plot, it was determined (Equation 4.2) that the intrinsic viscosity of PLA was 1.88 dL/g. This value was then used in the Mark-Houwink equation (Equation 4.3) to calculate the viscosity average molecular weight (M) of PLA which was found to be approximately 70,000.

\[
\text{Reduced specific viscosity: } (\frac{\eta}{\eta_0} - 1)/C \quad \text{(Equation 4.1)}
\]

\[
\eta = \text{Solution viscosity}
\]

\[
\eta_0 = \text{Solvent viscosity}
\]

\[
C = \text{Concentration of solution (dL/g)}
\]

\[
\eta = \text{Intrinsic viscosity: } [\eta] = \lim_{C \to 0} \frac{\ln (\eta/\eta_0)}{C} \quad \text{(Equation 4.2)}
\]

\[
\eta = KM^\alpha \quad \text{(Equation 4.3)}
\]

For PLA in chloroform at 25°C: \( K = 5.45 \times 10^{-4} \)

\( \alpha = 0.73 \)
4.2.6 Extrusion of Solid Monofilaments

Solid monofilaments were created using the Fuji Filter Melt Spinning Tester MST-CII seen in Figure 4.9. A variety of polymer blends, ranging from 5, 10, 15, and 20% EastONE were created at 230°C. The fibers were subsequently washed in a shaker bath (Boekel Scientific, Feasterville, PA) for two hours, followed by two hours in an ultrasonicator (Cole-Parmer Instrument Company, Chicago, IL). These fibers were then examined using SEM. Figure 4.10 shows a representative image of the fiber surface. It was observed that the pores were only visible in streaks on the fiber surface. This was likely due to the fact that there was no mixing of the polymer prior to extrusion. It was hypothesized that this problem would be resolved if the mixture was compounded.
Figure 4.9. Fuji Filter Melt Spinning Tester MST-CII.

Figure 4.10. SEM image of washed 20%EastONE/80% PLA fiber prior to compounding.
To test this hypothesis, PLA polymer was shipped to Eastman Chemical (Kingsport, TN), where it was compounded with EastONE in an 85/15 PLA/EastONE ratio. The compounded polymer was then extruded as a solid monofilament at 231°C. As the polymer stream was initially not consistent, it was extruded directly into a cold water bath with an approximate 2 inch air gap. Figure 4.11 shows the surface of these solid fibers comprised of compounded polymer on the left, and 100% PLA on the right. Several dispersed pores were observed on the surface of the compounded fiber, ranging from approximately 0.522 to 4.198 µm in diameter. The dark spots seen on the 100% PLA fiber are a function of the SEM beam, and were not present when observing the surface prior to image capture. Though a more consistent distribution of pores was noted on the entire fiber surface, these pores did not appear to be fully interconnected nor span the entire fiber diameter.

Figure 4.11. SEM image of washed 15%EastONE/85% PLA fiber (left) and 100% PLA fiber (right) at 5000X.
4.2.7 Viscosity

After successfully extruding various combinations of hollow monofilaments, it was desired to scale up the experiments to a multi-filament extruder. Prior to spinning the spools of fibers, the compounded polymer (80/20 PLA/EastONE) was analyzed to determine the viscosity of the material at 210°C. The viscosity at a shear rate of approximately zero was found to be 238 ± 15 Pa-s.

![Viscosity vs. Shear Rate Graph](image)

**Figure 4.12.** Plot of viscosity vs. shear rate.

4.2.8 Melt-Spinning Fibers

Six spools of fibers were obtained using the melt spinning line at Hills Inc. (W. Melbourne, FL). Fiber composition and spinning speeds are noted in **Table 4.1**. All
of the homocomponent fibers were obtained using a standard 250 mesh. All fiber spools were comprised of 72 filaments and were extruded at 210°C.

**Table 4.1.** Polymer composition and spinning speeds of each fiber spool.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Composition (PLA/EastONE)</th>
<th>Spinning Speed (mpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>80/20</td>
<td>50</td>
</tr>
<tr>
<td>Run 2</td>
<td>80/20</td>
<td>100</td>
</tr>
<tr>
<td>Run 3</td>
<td>90/10</td>
<td>100</td>
</tr>
<tr>
<td>Run 4</td>
<td>90/10</td>
<td>200</td>
</tr>
<tr>
<td>Run 5</td>
<td>90/10</td>
<td>250</td>
</tr>
<tr>
<td>Run 6</td>
<td>90/10</td>
<td>200</td>
</tr>
</tbody>
</table>

**4.2.9 SEM Images**

For each polymer combination, SEM was used to examine the surface morphology before and after the washing procedure. As in the previous washing steps, the fibers were placed in deionized water and then on a shaker table for 2 hours at 70°C, and then in an ultrasonicator at the same temperature for an additional 2 hours. All images were taken at 8000X (**Figure 4.13**).
Figure 4.13. SEM images at 8000X of washed (left – A, C, E, G, I, K) vs. unwashed (right- B, D, F, H, J, L) samples. Fiber composition: A-D) 80/20 PLA/EastONE; E-L) 90/10 PLA/EastONE. Spinning speeds: A,B) 50mpm; C-F) 100mpm; G,H,K,L) 200 mpm; I,J) 250mpm. scale bar = 2 µm)

4.2.10 Pore Size Measurement of Multifilament Fibers

For each sample the pore sizes were measured using SEM images obtained at 8000X. The diameters were measured using the Revolution image analysis software. The average size plus standard deviation are shown in Table 4.2. The far right column shows the range of pores found in the images, which vary in size from 8 to 600nm. From this table, we observed that the two samples with the largest pore sizes were Runs 1 and 2. These samples were the only two fiber spools spun with
the 80/20 PLA/EastONE composition, suggesting increased EastONE polymer concentration yielded a larger average pore size after washing.

**Table 4.2.** Polymer composition and spinning speeds of each fiber spool.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Avg Pore Size (nm)</th>
<th>Range of Pore Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1 (80/20 P/E)</td>
<td>312±122</td>
<td>57- 571</td>
</tr>
<tr>
<td>Run 2 (80/20 P/E)</td>
<td>319±157</td>
<td>146- 600</td>
</tr>
<tr>
<td>Run 3 (90/10 P/E)</td>
<td>78±31</td>
<td>31- 151</td>
</tr>
<tr>
<td>Run 4 (90/10 P/E)</td>
<td>107±48</td>
<td>62- 249</td>
</tr>
<tr>
<td>Run 5 (90/10 P/E)</td>
<td>127±50</td>
<td>73- 191</td>
</tr>
<tr>
<td>Run 6 (90/10 P/E)</td>
<td>18±8</td>
<td>8- 29</td>
</tr>
</tbody>
</table>

4.2.11 *Creation of Hollow Monofilaments*

Though prior compounding of the polymer appeared to improve the dispersion of the EastONE polymer in the fiber, we were not able to observe pores that spanned the fiber diameter. Therefore, the fiber geometry was changed from a solid filament to a hollow one. The hollow geometry was first tested on the monofilament extruder. To obtain hollow fibers, the spinneret was changed and pressure on the monofilament extruder was set to 500 psi, and varied by approximately 25% throughout the run. The 85/15 PLA/EastONE compounded
polymer was extruded at approximately 200°C and a take-up speed of 32 m/min. Samples were then washed in a shaker bath at 70°C for 2 hours, before placing in the ultrasonicator for an additional 2 hours. Figure 4.14 shows these hollow monofilaments, after (left) and prior (right) to the washing process. From the image on the left, evenly dispersed micropores can be seen on the surface of the monofilament. These micropores ranged in size from approximately 0.074 to 0.451 µm. The image on the right shows the fiber prior to washing, and lacking the presence of surface pores. A seam was present on these fibers, where the polymer streams did not fully meld together after exiting the spinneret.

Figure 4.14. SEM image of washed 15%EastONE/85% PLA hollow fiber at 5000X (left) and unwashed fiber at 100X (right).
4.2.12 Extrusion of Hollow Fibers

As the micropores present in the solid filaments did not appear to be large enough or interconnected to such a degree as to span the entire fiber diameter, it was decided that a new iteration of fibers should be extruded with a hollow channel in the center. Though single hollow fibers had been extruded previously, the next step involved the combination of the hollow fiber monofilament and the scaled up extrusion process. Using the meltspinning line at North Carolina State University, 69 hollow filaments were extruded with three different polymer combinations: 80/20 PLA/ EastONE, 90/10 PLA/ EastONE, and 100% PLA. Figure 4.15 shows the fiber cross-sections before and after washing. Micropores were observed on both the 80/20 and 90/10 polymer combinations, with a greater number apparent on the 80/20 sample. However, since the fiber wall surrounding the hollow channel is approximately 20 microns, it still seemed unlikely that there would be sufficient interconnectivity of the micropores. This observation led to the final design geometry, islands-in-the-sea.
Figure 4.15. Unwashed (left) and washed (right) images of 90/10 PLA/EastONE (A,B) and 80/20 PLA/ EastONE (C,D) hollow fibers at 1500X (A, B, C) and 1200X (D). (scale bar = 10µm)
4.3 Summary

The design and characterization process for fabricating a highly porous, biocompatible fiber has been described. Initial proof of concept experiments involved the use of additives contained within a polymer film. Microscopic observations indicated that after washing with water, the additives had dissolved leaving pores in the film. However, the size and distribution of these pores were difficult to manipulate, which led to the inclusion of a secondary polymer in place of the sugar or salt additive. The polymer chosen for this application was EastONE, a water-dispersible sulfopolyester. Monofilaments were extruded with varying ratios of the EastONE polymer, and were examined with SEM after washing. These images revealed the presence of pores on the fiber surface, though their distribution was not homogeneous. Therefore, the polymer combinations were compounded prior to extrusion, which improved pore distribution and size in subsequent filaments. Examination fibers from a large scale production (72 filaments) line showed that while the micropores were distributed throughout the fiber and its cross-section, these micropores were not fully interconnected. This resulted in a change in fiber geometry, resulting in production of hollow fiber monofilaments.

Initial images of hollow fiber monofilaments continued to reveal the presence of micropores in the cross-section, leading to the simultaneous extrusion of 69 hollow filaments. Washed hollow fibers were freeze fractured, and examined with SEM. These images showed the best results— a large hollow center would allow for
fluid flow through the center of the fiber, and widely distributed micropores would allow fluid flow peripherally. The problem remained, however, that the fiber wall was quite thick (approximately 20 µm) and would likely hinder fluid flow. A decision was therefore made to change the fiber geometry one last time, to that of an islands-in-the-sea design.

This islands-in-the-sea configuration will be discussed in the following experimental chapter, as it was extensively characterized. Three-dimensional knitted fabrics were created with these fibers, and these fabrics served as a scaffold for preliminary bone tissue engineering experiments using hASCs.
5. Characterization of Porous Islands-In-The-Sea Biodegradable Scaffolds for Bone Tissue Engineering Applications

In the previous chapter, the design of a nanoporous fiber for bone tissue engineering applications was described. Through several design iterations, an islands-in-the-sea geometry was chosen. The sea components of these fibers were comprised of PLA, and the islands of EastONE (a water-dispersible sulfopolyester). In this chapter, the islands-in-the-sea fibers were analyzed for their potential to serve as a scaffold for bone tissue engineering using hASCs. Prior to cell seeding, the islands-in-the-sea fibers were further examined, including the weight loss of each fabric sample after washing. Focused Ion Beam (FIB) techniques were used to section and image the fiber cross-sections. The Brunauer, Emmett and Teller (BET) method was also used to approximate the surface area of the various fiber samples. After the fabrics had been fully characterized, they were assessed for their ability to serve as a bone tissue engineering scaffold. Human ASCs were seeded on the knitted fabrics and hASC seeded scaffolds were examined for cell viability over a 28 day culture period.
5.1 Introduction

Previous research has indicated that cells cannot migrate more than 500µm from the surface of a scaffold or implant, a depth which is governed by the lack of nutrients and oxygen supply [95]. This lack of nutrients and method of waste removal means that cells cannot migrate into the center of the scaffold, resulting in continued cell proliferation at the periphery. These cells then act as a barrier to nutrient and oxygen diffusion to the scaffold center, which consequently results in cell viability only at the scaffold surface [95]. To combat this problem, the human body uses blood vessels to supply nutrients and oxygen to cells throughout the entire tissue. In this study, the incorporation of hollow channels within the fibers might act as an artificial vascular system, increasing mass transport within the scaffold.

As described in previous chapters, porous biodegradable scaffolds are currently being examined for use as bone graft substitutes. Biodegradable tissue engineered scaffolds are attractive due to the fact that there is no removal operation required once the tissue has healed. Consequently, biodegradable fracture fixation devices and scaffolding constructs are often more appealing than metal ones, such as the titanium scaffolds described in Chapter 2. It is imperative the scaffold degradation rate match the rate of bone regeneration as closely as possible, however, to both provide mechanical stability and an appropriate environment for new tissue growth [3]. Poly(lactic) acid (PLA) is a commonly used biodegradable
polymer for tissue engineering applications, as the degradation rates can be altered depending on the particular application. For example, resorption times for PLA have ranged from 40 weeks to over 5.7 years depending on the implantation site and other material properties.

The chemical properties of PLA allow it to undergo hydrolytic degradation through de-esterification, producing lactic acid as a by-product [5, 95]. The degradation products of PLA are present in the human body and can be removed by naturally occurring metabolic pathways, such as the tricarboxylic acid cycle [95]. As the PLA polymer degrades, lactic acid is released, which reduces the pH of the surrounding environment. This reduction in pH can result in autocatalysis of the polymer, further accelerating the degradation rate. Without proper diffusion of the by-products, autocatalysis can lead to increased degradation at the scaffold core, jeopardizing the mechanical integrity of the construct. One way to combat the accumulation of degradation products and reduce the effects of autocatalysis is to incorporate a network of pores within the scaffold, capable of removing the lactic acid molecules.

The incorporation of an inherent porous network in the scaffold geometry could potentially slow the rate of autocatalysis of the scaffold, with the added benefit of removing the acidic by-products of degradation from the cell environment. Removal of the acidic by-products is also desirable as research suggests that a highly acidic environment resulting from polymer degradation adversely affects
cellular function [96]. It has also been shown that small particles released during degradation can elicit an inflammatory response and induce bone resorption [95]. However, through the incorporation of a porous network these problems can be averted by transporting the acidic by-products away from the scaffold center and allowing cells in neighboring tissue to metabolize the lactic acid molecules. Thus, PLA has been approved by the US Food and Drug Administration for use in a variety of medical products and devices, such as degradable sutures [5]. PLA is a popular synthetic polymer for biomedical applications, as it can be easily processed and has degradation rates and material properties which can be adjusted through the incorporation of other polymers or by changing the molecular weight. The degradation kinetics can also be controlled through the processing history, chain orientation, crystallinity, environmental conditions, additives, and the overall hydrophilicity [5]. In the previous chapter, a water-dispersible polymer was incorporated into a PLA matrix, yielding micropores after washing. Further manipulation of this polymer system could generate scaffolds suitable for a wide variety of tissue engineering applications.

The purpose of this study was to generate a nanoporous scaffold. An islands-in-the-sea fiber geometry was used to incorporate EastONE (water dispersible) islands within a PLA sea matrix. It was hypothesized that after washing in water, removal of these polymer islands would yield 12 channels within each fiber, which are expected to enhance nutrient delivery and waste removal. It was further
hypothesized that through the incorporation of the EastONE polymer in the sea matrix, it would be possible to obtain interconnected micropores in addition to the micron sized channels. These fibers were knitted into three-dimensional fabrics, characterized through a variety of techniques, and seeded with human adipose-derived adult stem cells (hASCs) to examine initial cell viability.
5.2 Materials and Methods

5.2.1 Fiber Extrusion and Scaffold Fabrication

Four fiber combinations were generated with an islands-in-the-sea geometry. The pressure for both the islands and sea extruders was set to 750psi. The temperature of the melt was set to 235ºC. The draw ratio used during fiber uptake was 2:1, and the denier of extruded fibers was 870. The compositions of the four fiber types fabricated are described in Table 5.1, and subsequently will be referred to using the abbreviation. Each islands-in-the-sea fiber combination was knitted into individual fabrics, using a 1 by 1 knit, 7 cut structure on a Brevets Dubied knitting machine.

Table 5.1. Table reflecting the polymer compositions for the islands and sea matrices for the four fiber types. The abbreviation for these fibers is also listed in the far right column.

<table>
<thead>
<tr>
<th>Sea</th>
<th>Islands</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA</td>
<td>PLA</td>
<td>P/P</td>
</tr>
<tr>
<td>PLA</td>
<td>EastONE</td>
<td>P/E</td>
</tr>
<tr>
<td>80% PLA / 20% EastONE</td>
<td>EastONE</td>
<td>80/20</td>
</tr>
<tr>
<td>90% PLA / 10% EastONE</td>
<td>EastONE</td>
<td>90/10</td>
</tr>
</tbody>
</table>
5.2.2 Fabric Washing

Fabrics were submerged in deionized water and were then placed in the benchtop shaker table (SteadyShake 757 GYROMAX Benchtop Incubator Shaker, Amerex Instruments Inc., Lafayette, CA) for 2 hrs, at 70ºC and 100 rpm. The fabrics were then transferred to an ultrasonicator (Cole-Parmer Instrument Company, Chicago, IL) and sonicated for 2 hrs at 69ºC.

5.2.3 Weight Loss

Fabrics were cut into 1 X 1.25 in rectangles, and placed into individual 50mL conical tubes. Fabrics were dried for 3 days, then weighed on a balance (AB 104-S, Mettler Toledo, Columbus, OH). Fabrics were then individually washed in 40mL deionized water per the protocol described in Chapter 5 Section 2.2. Washed fabrics were then dried overnight in normal atmosphere, before being placed in a dessicator for 1wk. Fabrics were then transferred to an oven (Precision, Winchester, VA) set at 70ºC, for two days. Fabrics were then weighed again, and the percent weight loss calculated. A one-way analysis of variance (ANOVA) model was specified in Proc GLIMMIX (SAS version 9.2, SAS Institute, Cary, NC) with scaffold type being the only variable. Pair-wise differences were calculated using a Tukey adjustment, and significance determined at p<.05.
5.2.4 Focused Ion Beam Imaging

A FEI Quanta 200 3D Dual Beam FIB system (FEI Company, USA) was used for focused ion beam (FIB) cross-sectioning and imaging. All FIB nanomachining and imaging was done using 30kV Ga\(^+\). Prior to FIB nanomachining, all fibers were coated with approximately 20 nm of gold-palladium (AuPd) using a Denton Desk II (Denton Vacuum, USA) sputter coater. The sputtered coating was to protect the surface of the fiber from ion beam damage and erosion during the subsequent platinum (Pt) deposition. After deposition of the AuPd protective coating, a Pt layer consisting of a 3µm thick platinum strip was deposited over the surface of regions to be cross sectioned. This platinum strip serves as a protective cap to further preserve the fiber surface and protect the cross-section face from any damage from the ion beam tail during the nanomachining process. Following deposition of the Pt coating, a 20nA Ga\(^+\) ion beam rastered in a rectangular pattern of approximately 50µm by 150µm was used to rapidly remove most of the polymer material and expose the fiber cross-section. Following this large mass removal step, the ion beam was employed in a stepped line by line fashion termed a cleaning cross section (CCS) pattern. Using the CCS scan pattern, 5nA, 1nA, 500pA and 100pA Ga\(^+\) currents were used in that order to provide increasingly fine polishing of the cross-section face. In general, the nanomachined area dimension along the fiber axis length (longitudinal cross-section) was three times the nanomachined area.
dimension perpendicular to the fiber axis width (radial cross-section). An overview of the nanomachining procedure is illustrated in Figure 5.1.

Figure 5.1. Illustration of the fiber cross-sectioning process. After a protective AuPd coating has been applied and a site of interest is chosen (a), a 3µm wide platinum cap layer is then deposited with a 500pA Ga⁺ beam (b). Mass removal with a 20nA Ga⁺ ion beam (c) and subsequent polishing with 5nA, 1nA, 500pA and 100pA Ga⁺ beams give rise to the final cross-section (d). (Adapted from: [97])

After the cross sectioning process, a 30kV Ga⁺ ion beam was used to image the cross-sections. Imaging was performed using a 10pA Ga⁺ ion beam at a working distance of approximately 30mm and at an incident angle of ~52°. Ga⁺ FIB induced secondary electron (ISE) micrographs are acquired with dwell times of 55µs per pixel and 1024x768 pixel resolution. Micrographs were acquired until contrast was optimized. The brightness and contrast (only) of selected micrographs were adjusted using Adobe Photoshop CS2 Edition.

5.2.5 BET Surface Area Analysis

To compare the available surface area of the various fabric samples, the BET method was used. The BET method was first described by Brunauer, Emmett and
Teller in 1938, and is used to calculate the surface area of solids by physical adsorption of gas molecules [98]. Washed fabric samples were cut into small sections, approximately 1mm in length. These sections were then collected and 15-25mg of each sample (n=1) was placed in the U-tube of the Micromeritics FlowSorb II 2300 (Micromeritics, Norcross, GA) using 30.0% N₂ (balance He) gas (National Welders Supply Co., Charlotte, NC). Each sample set was dried by repeated N₂ adsorption/desorption to remove excess water prior to analysis. From this, the surface area of each sample was measured 3-4 times then divided by the measured mass of the sample, resulting in a m²/g determination for each sample.

5.2.6 Transverse Wicking

Each fabric was cut into 1x1.25 in sections, washed, and dried using the method described in Chapter 5 Sections 2.2 and 2.5. A modified version of the Woolmark Test Method: Transverse Wicking of Fabric was used to analyze each fabric type (Australian Wool Innovation Limited, Sydney, Australia). Green food dye (McCormick and Co., Inc., Hunt Valley, MD) was diluted 1:1 with dionized water. This dye solution was then added to each fabric by ejecting 10µL just above the fiber surface. After 30 seconds, fabrics were imaged using a Sony Cybershot digital camera at a constant zoom and distance. The Image J image analysis software, developed by the NIH, was used to measure the diameter of the stained area in the vertical, horizontal, and diagonal directions. The image pixels were calibrated using
the pixel length of a line drawn on the image of a ruler at the same magnification. The length of the line was converted into centimeters, then used to measure the diameter of the stained area.

5.2.7 Sterilization and Cell Seeding

Washed PLA/PLA and PLA/EastONE fabrics were dried in a dessicator for two days, then cut into individual circular scaffolds 1.25cm in diameter using a punch (McMaster-Carr, Aurora, OH). Scaffolds were then soaked in 70% ethanol for 15 minutes. The ethanol was aspirated and the remainder evaporated over 16 hrs. Scaffolds were then soaked in complete growth medium for 3hrs prior to cell seeding, and placed in 24 well non-tissue culture treated plates (Sarstedt, Inc., Newton, NC). Human ASCs were isolated from a 50 yr old Caucasian female per the protocol described previously in Chapter 2, Section 2.2. Human ASCs were seeded on the circular scaffolds, at a final density of 60,000 cells/scaffold, in three phases. In the first phase, 20,000 cells in 20 µL medium were added to each scaffold. Scaffolds were then centrifuged at 500g for 2 minutes. Seeded scaffolds were then incubated for 15 minutes. In the second phase, another 20,000 cells in 20µL medium were added to each scaffold then placed in the incubator for 15mins to allow cell attachment. All scaffolds were then turned over, and seeded with an additional 20,000 cells in 20 µL medium. Scaffolds were placed in the incubator for 15 mins, then each well was flooded with 1mL medium.
Human ASCs were cultured in complete growth medium for 1 wk, and then cultured in osteogenic medium (growth medium supplemented with 0.1 μM dexamethasone, 50 μM ascorbic acid, and 10 mM β-glycerolphosphate) for the remaining 3 weeks.

5.2.8 Human ASC Viability

Effects of the scaffolds on cell viability was assessed via the Live/Dead assay (Live/Dead® Viability/ Cytotoxicity Kit, Molecular Probes, Eugene, OR) at days 1, 21, and 28. At each time point, one scaffold of each type was rinsed twice with PBS. Scaffolds were then submerged in 1 mL of the calcein AM and ethidium homodimer-1 stain and kept in the dark. Scaffolds were then individually imaged using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, New York) with a 10X objective.
5.3 Results

5.3.1 Weight Loss

The percent weight loss for each of the four fabric types was quantified (n=5) and reported in Figure 5.2. Significance was determined at p values < .05, and is represented by differing letters. The percent weight loss for each fabric type is significant, though the percent difference between the 80/20 and 90/10 is relatively small.

Figure 5.2. Percent weight loss of the four fabric samples. Bars reflect standard deviation of the measured mean for each sample. Different letters denote significance < .05.
5.3.2 FIB Imaging

Focused ion beam imaging was used to section and image both washed and unwashed islands-in-the-sea fibers. **Figure 5.3** shows the longitudinal and radial view of an unwashed 80/20 fiber. Smooth areas represent EastONE islands, and the surrounding polymer corresponds to the sea matrix. **Figure 5.4** reveals the cross section of a fiber of the same composition as seen in **Figure 5.3**, after washing. **Figure 5.4a** shows the removed EastONE islands and as the image is zoomed to that shown in **Figure 5.4c**, pores are revealed within the sea matrix.

**Figure 5.3.** Micrographs of a biaxial cross-section of an unwashed 80/20 PLA/EastONE fiber showing the distribution of the EastONE islands within the sea matrix: a) longitudinal view, b) biaxial view, and c) radial view. The smooth regions correspond to the EastONE islands and the rough regions correspond to the EastONE/PLA sea matrix. (Adapted from: [97])
Figure 5.4. Micrographs of a biaxial cross section of an 80/20 PLA/EastONE fiber after washing. A biaxial cross section of the washed fiber is displayed in (a). A portion of the fiber is further magnified (region denoted by the dotted line) in the subsequent micrographs (b and c). (Adapted from: [97])

5.3.3 BET Surface Area Analysis

The surface area of each washed fabric type was analyzed using the BET method. The results of the analysis are reported in Figure 5.5. Statistical significance could not be determined, as the analysis involved multiple measurements of only one sample. However, a trend of increasing surface area was observed as the concentration of EastONE polymer increased.
Figure 5.5. Fiber surface area (m$^2$/g) for each of the four samples measured using the BET method. Error bars reflect standard deviation.

5.3.4 Transverse Wicking

Fabrics were stained with 10 µL dye, and imaged as shown in Figure 5.6. The extent of transverse wicking was measured three times for each sample, and reported in Figure 5.7.
Figure 5.6. Photographs of fabrics following washing and drying, and the addition of 10 µL of dye. Fabrics were imaged after 30 seconds. A) PLA/PLA; B) PLA/EastONE; C) 80/20; D) 90/10
Figure 5.7. Average transverse wicking diameter for each fabric type. Error bars reflect standard deviation from the mean.

5.3.5 Viability

Human ASC viability was assessed after 1, 7, 14, 21, and 28 days using a Live/Dead stain. Viable cells appear green, and dead cells appear red. In general, cell attachment, spreading and proliferation increased over time for hASCs seeded on PLA/PLA and PLA/EastONE scaffolds.
**Figure 5.8.** Live/Dead images after 1 day in culture of hASCs seeded on:
A) PLA/PLA; B) PLA/EastONE (scale bar = 100µm)

**Figure 5.9.** Live/Dead images after 21 days in culture of hASCs seeded on:
A) PLA/PLA; B) PLA/EastONE; (scale bar = 100µm)
Figure 5.10. Live/Dead images after 28 days in culture of hASCs seeded on: A) PLA/PLA; B) PLA/EastONE; (scale bar = 100µm)
5.4 Discussion

One large problem with current three-dimensional scaffolds remains the inability of nutrients to permeate throughout the scaffold and provide adequate sustenance to cells in the center, while carrying away metabolic wastes. To address this challenge of nutrient delivery/waste removal, a three-dimensional porous scaffold with 12 hollow channels was developed. The incorporation of micropores and channels into the fiber structure should enhance fluid flow and allow for increased cell growth in the center of the scaffold. These fluid flow properties are thus expected to promote cell viability over long term culture \textit{in vitro}, as well as yield an implantable bone-graft substitute capable of supporting cell viability \textit{in vivo} until adequate capillary formation is achieved.

An islands-in-the-sea geometry was used to generate fibers with increased fluid flow properties. It was expected that the incorporation of a water-dispersible polymer would produce voids in the PLA matrix after washing. Removal of the EastONE polymer was confirmed by weighing fabric samples before and after washing. The results, summarized in Figure 5.2, reflect the mean percent weight loss of each fabric type. Statistical analysis indicated a significant ($p<.05$) increase in percent weight loss as the concentration of EastONE polymer increased in each fiber type. Very little weight loss was noted for the PLA/PLA fabrics (<2%), however there was approximately 53% weight loss for PLA/EastONE fibers. This percent increased for the fibers containing EastONE in the sea matrix, to approximately 68%
and 69% for the 90/10 and 80/20 PLA/EastONE sea combinations respectively. The difference between these last two samples was considered statistically significant, but may not be considered practically relevant as the difference in percent weight loss was relatively small. These results confirm the successful removal of the EastONE polymer from the islands-in-the-sea fibers after washing, as a significant increase in weight loss was observed as the concentration of EastONE polymer in the unwashed fiber increased.

Previous attempts to characterize the micro-porosity in PLA monocomponent fibers were performed using microtomy as shown in Figure 4.5. Briefly, fibers were embedded within an epoxy resin and cross-sectioned using a microtome at room temperature. Scanning electron micrographs revealed the presence of some micropores, but these micropores were not well-defined as a result of microtome induced artifacts. Freeze-fracturing followed by SEM observation was also used to investigate PLA/EastONE hollow fibers after washing (Figure 4.15). Although micropores were evident in the matrix of the fiber cross-section and inside the channels, it was suspected that the blade used to fracture the material during the freeze-fracture process may have occluded the smallest of the pores and that preferential fracture paths may also have distorted the cross-sectional image. This may have limited the ability to accurately determine the extent of the porosity [99, 100].
Therefore, to better image the internal structure of the fiber cross-sections, FIB cross sectioning followed by imaging was employed. Micrographs of biaxial FIB nanomachined cross sections of 80/20 PLA/EastONE islands-in-the-sea fibers are shown in Figure 5.3 (before EastONE removal with water) and Figure 5.4 (after EastONE removal). In addition to the ability to clearly distinguish the islands and the sea in Figure 5.3, porosity can clearly be seen in the 80/20 PLA/EastONE sea even though this fiber has not yet been washed to remove the water dispersible EastONE polymer. This porosity is believed to be induced by the extrusion process, as similar porosity was also observed in 100% PLA fibers extruded under the same conditions. This porosity can be seen in the micrographs of both the radial and longitudinal cross sections of the unwashed EastONE/PLA islands-in-the-sea fiber (Figure 5.3).

Micrographs of the washed EastONE/PLA islands-in-the-sea fiber in Figure 5.4 also show that even though the EastONE islands have been removed, the fiber retained its structural integrity during the FIB nanomachining process. It is interesting to note that the porosity of the cross section of the sea in this fiber is significantly greater than would have been predicted from its 80/20 PLA/EastONE composition. This unexpected void content was observed in FIB prepared and imaged cross-sections of other fibers of the same composition and extruded using the same process, and is believed to be a result of the extrusion process combined with the removal of the EastONE polymer. The biaxial cross section in Figure 5.4c additionally suggests that the porosity must be relatively uniform and contiguous, as
water induced dissolution of the EastONE would not have occurred otherwise. Results from FIB imaging therefore revealed 12 channels within the fiber cross-section in washed samples possessing EastONE islands. Additional micropores were noted for fiber samples also possessing EastONE polymer within the PLA sea matrix. These images provided visual confirmation that both channels and micropores could be fabricated within melt-spun fibers. However, this analysis was largely qualitative.

The quantitative determination of the percent weight loss after washing, in combination with the qualitative analysis provided by the FIB images, confirmed the removal of EastONE polymer after washing, yielding micropores in the sea matrix as well as 12 channels in the fiber cross-sections. Further characterization of the fibers was performed using a BET surface area analysis technique. The surface area of each washed fabric type was analyzed several times, however as repeated measurements were taken from only one sample of each type, statistical significance could not be determined. The mean values reported in Figure 5.5 do reveal a trend of increasing surface area, correlating to the increased concentration of EastONE polymer in the fibers. This trend is similar to the one observed for percent weight loss in Figure 5.2, confirming the removal of EastONE polymer after washing, forming hollow channels within each fiber.

The transverse wicking properties of each fabric type was of interest, as the cell suspension is typically added to each scaffold in this manner. From the images
in Figure 5.6, it was observed that the droplet diameter appears to vary according to scaffold type. Figure 5.7 is a histogram of the mean dye diameters observed on each fabric type. It appears that the PLA/PLA fabric sample possess the largest transverse wicking diameter. The apparent significant decrease in wicking diameter observed for the fibers with EastONE channels may have resulted from dye entering the fiber channels and micropores. The dye may have remained trapped in these voids, instead of spreading along the fiber surface. The results of this examination suggested that unequal cell seeding may occur if the hASC suspension was applied in a similar manner. Additionally, the use of centrifugal seeding has previously been shown to increase the seeding efficiency of porous, biodegradable scaffolds [101]. Therefore, a combination of centrifugal and static cell seeding was chosen to improve cell dispersion for this application.

Human ASCs were seeded on the PLA/PLA and PLA/EastONE scaffolds to examine cell viability over 4 weeks. Initial cell viability was assessed at days 1, 21, and 28. Confocal images after one day of culture (Figure 5.9) revealed viable cells, many of which had already attached to and spread along the fiber surface. By day 21 (Figure 5.10), hASCs had increased in cell number and appeared to cover the surface of the fabrics. Further cell proliferation appears to have occurred by day 28 (Figure 5.11). In this assay, live cells appear green and dead cells appear red. However, some staining/autofluorescence of the fibers occurred, resulting in the red appearance seen most clearly in Figure 5.9. The results from the Live/Dead assay
indicate that cell viability can be maintained on both solid and porous fiber scaffolds. Furthermore, these results suggest that residual EastONE polymer does not affect cell viability over long term culture. Though there appears to be a slightly greater number of cells present on the solid PLA/PLA fibers at later time points, this may be due to greater initial hASC attachment seen on day 1. After day 7 the culture medium was changed to osteogenic differentiation medium, which other studies have suggested results in a decrease in cell proliferation [35]. Higher initial hASC seeding densities or longer culture times in complete growth medium may be used in future studies to address this difference.

The design and characterization of a biodegradable scaffold with interconnected micropores and channels has been described. Characterization of the knitted fabrics indicates the use of a water-dispersible additive may produce fibers possessing a controlled porous geometry. The use of the additive in the surrounding sea matrix appears to further increase the porosity of the fabric after washing. Initial cell studies suggest human adipose-derived adult stem cells will remain viable on these three-dimensional constructs over a 28 day period.
5.5 Summary

There exists a need for three-dimensional, biocompatible and biodegradable scaffolds for bone tissue engineering applications. The incorporation of pores and channels inherent to the fiber structure should aid nutrient delivery as well as removal of cell waste and by-products of scaffold degradation. Porous fibers were fabricated with an islands-in-the-sea geometry using PLA and a water-dispersible additive. These fibers were then characterized revealing significant mass loss after washing, as well as the presence of hollow channels and pores within the fibers. Initial hASC viability images revealed viable cells on porous scaffolds after 28 days of culture. These results indicate the porous scaffolds could serve as bone-graft substitutes, providing nutrients to cells throughout the construct through the porous network.
6. Conclusions and Recommendations for Future Research

6.1 Conclusions

This work describes the design and validation of various three-dimensional scaffolds designed for bone tissue engineering applications. In the first study, human adipose-derived adult stem cells were seeded on EBM fabricated titanium implants to examine effects on cell viability. In the second study, fibers on a similar size scale to those observed *in vivo* were electrospun. Osteogenic differentiation was compared between hASCs seeded on control scaffolds and scaffolds possessing a type I collagen sheath. In the final study, nanoporous fibers with micron sized channels were fabricated to enhance diffusion of nutrients/waste. Initial viability experiments revealed viable cells after 4 weeks of culture.

Human ASC proliferation was assessed *in vitro* on EBM fabricated titanium implants and reported in Chapter 2. All EBM scaffolds were compared to a commercially available, polished titanium scaffold. Results from the alamarBlue assay indicated a significant increase in hASC proliferation at both day 3 and 7 for the porous EBM scaffolds. An increase in proliferation was also noted on the unpolished EBM scaffold at day 7. A significant decrease in IL-6 release was noted on day 1 for all EBM processed implants, and day 7 for the unpolished and porous EBM scaffolds. Similarly, a decrease in IL-8 release was noted for the unpolished and porous EBM scaffolds at days 1, 3, and 7. The results from this study suggest that through EBM processing, patient-specific implants with a porous surface could
be generated to improve cell proliferation, biocompatibility, and eventually lead to better implant fixation.

In Chapter 3, bone formation from hASCs seeded on electrospun scaffolds in the presence of osteogenic supplements was assessed *in vitro*. Novel collagen-PCL sheath-core bicomponent fibers obtained using co-axial electrospinning served as a scaffolding material that supported hASC osteogenic differentiation and increased their calcium deposition. This bicomponent scaffold combines the beneficial properties of each material, namely the natural ECM ligands provided by the collagen and the material properties of PCL. The results indicate that the coating did not affect hASC proliferation on the scaffold, but did enhance early cell spreading. The addition of the collagen coating significantly increased calcium deposition compared to non-coated PCL scaffolds, signifying an increase in the number of osteodifferentiated hASCs. This supports the feasibility of using bicomponent nanofibers as a scaffolding material to promote bone formation. Findings from this study indicated that collagen-coated PCL nanofibers lead to increased osteogenic differentiation of hASCs, indicating that cell ligand attachment may promote calcium deposition when compared to uncoated nanofibrous PCL structures. This is the first study of its kind to describe osteogenic differentiation of hASCs on a three-dimensional collagen-PCL sheath-core scaffold, the results of which indicate a collagen coating is advantageous for bone tissue engineering applications using hASCs.
Chapter 4 describes several designs which were generated, with various polymers and additives, in an attempt to fabricate nanoporous melt-spun fibers. In all fibers tested, successful removal of the additive was observed after washing. In order to achieve the desired pore interconnectivity, the overall geometry of the fibers was changed from a solid filament, to a hollow one, and finally an islands-in-the-sea cross-sectional geometry. These islands-in-the-sea fibers were further characterized in Chapter 5, by measuring the weight loss achieved by washing and using focused ion beam imaging to observe the porous structure. Initial cell viability experiments suggest the EastONE polymer does not adversely affect hASC viability on washed islands-in-the-sea fabrics.

6.2 Future Research

Future experiments should now examine the EBM processed scaffolds in an in vivo environment. The design of a site-specific implant and examination of its interaction with the surrounding tissue, along with the host’s immune system, would further validate the EBM scaffold’s potential as an implantable scaffolding material.

The sheath-core collagen-PCL scaffolds indicated improved osteogenic differentiation of hASCs. However, these scaffolds were created as very thin, flat sheets. Further attempts should be made to design a collector that would enable the spinning of three-dimensional scaffolds of different sizes and shapes. Other extracellular matrix proteins, such as fibronectin and elastin, could also be
incorporated into the collagen sheath. The effects of this protein blend should then be examined for their effects on cell viability and differentiation and potential use in vivo.

The design of the nanoporous fibers included the creation of fibers of various geometries until the final islands-in-the-sea design was chosen. The properties of these fibers could be tailored by choosing different polymers with their own mechanical properties and degradation kinetics. Processing parameters could also be adjusted to change the crystallinity and molecular orientation of the fibers. Other additives could also be included in the polymer fibers, which would be released upon polymer degradation. These additives could provide cells with nutrients or possess antimicrobial properties which would aid scaffold acceptance. These three-dimensional fabrics could also be seeded with cells, and then implanted into an animal model. After several weeks of in vivo growth, the implants could then be analyzed for blood vessel ingrowth, cell viability at the scaffold center, and implant fixation with the surrounding tissue.
7. References


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