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Development and Fabrication of Novel Woven Meshes as Bone Graft Substitutes for Critical Sized Defects

Jordan Anthony Stowe Gilmore
Clemson University

DEVELOPMENT AND FABRICATION OF NOVEL WOVEN MESHES AS BONE GRAFT SUBSTITUTES FOR CRITICAL SIZED DEFECTS

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In Partial Fulfillment
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by
Jordon Anthony Stowe Gilmore
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Accepted by:
Dr. Karen Burg, Committee Chair
Dr. Ken Webb
Dr. Timothy Burg
Dr. Kyle Jeray

ABSTRACT

With more than $2.5 billion spent per year, and over 2.2 million procedures conducted annually worldwide, bone grafting continues to be a large part of the treatment strategy for large non-healing bone defects (critical-sized defects, CSDs). But complication rates (>20%), donor shortage, and donor site morbidity, have led to bone tissue engineering as an important option in these cases. This work explores the creation of woven polymeric meshes as viable bone tissue engineering scaffolds and a bio-loom to fabricate the meshes.

Melt-spun poly-l-lactide (PL) and poly-l-lactide-co-ε-caprolactone (PLCL) fibers were studied to build variable mesh types to affect porosity, pore size, and cellular affinity. A custom bio-loom was designed and built based on dobby-loom textile technology for use with the resorbable polymer monofilaments. Fluid flow properties were characterized through the evaluation of permeability and wicking rate using a purpose-built permeameter.

Osteogenic viability was analyzed through studying cell adhesion and differentiation on the meshes. D1 murine bone marrow mesenchymal stem cells (MSCs) were used to characterize cell adhesion via integrin binding. Immunofluorescent analysis of Fibronectin (FN), Vitronectin (VTN), Type 1 Collagen (COL1), and Laminin-alpha 2 (LAMA2) adhesion was conducted. These proteins serve as ligands to osteogenic integrin subunits β1, α2, α5, and αV. Expression of integrin subunits was tested via real-time polymerase chain reaction (RT-PCR). Additionally, MSC osteogenic differentiation was evaluated by colorimetric Alkaline Phosphatase (ALP) expression, Alizarin Red stain for mineralization, and ALP and Osteocalcin (OC) gene expression via RT-PCR.

Results showed effective creation of meshes with variable properties and significant differences in cell metabolic activity and DNA concentration. Changes in mesh parameters significantly effected mesh permeability. ECM protein adhesion and integrin PCR results suggest

a means to control the early differentiation process by varying attachment and expression of VTN, β1, and αV. Early stage differentiation was verified by the consistent expression of ALP, shown through colorimetric and PCR experiments. Mature differentiation was shown through constant adhesion rates of FN, COL1, and LAMA2 with subunits β1, α2, and α5. Mineralization and OC gene expression results showed sparse mineralization and little expression of OC in the late stages of differentiation.

Additionally, work regarding the encouragement of Science, Technology, Engineering, and Math (STEM) career pursuit for underrepresented minority middle school students was conducted. Results showed that parental encouragement, external STEM environment, and extracurricular STEM exposure were closely related to a student's likelihood to express interest in a STEM career. Student interest in STEM careers significantly increased after participation in an interactive camp based on mesh-based modules. Further work explored the effect of early research experiences on the development of research identity for underrepresented minority science and engineering undergraduates. Results showed that students participating in this program significantly increased their research identity through increased self-recognition and competence in research activities.

DEDICATION

First, I dedicate this volume to my lovely wife Kimberly Gilmore who has been tremendously supportive and encouraging throughout the entire process of this work. I am happy, proud, and thankful to have shared this PhD journey with you through the good, the bad, and the ugly. Thank you for always understanding the late nights and trips to “the lab”. I pray that this experience has made us stronger as a couple and I look forward to building on this accomplishment as a part of our legacy together.

I would also like to dedicate this work to my wonderful parents, James and Betty Gilmore. You too have been extremely supportive and I thank you for encouraging me to pursue a dream, even when it was not always clear what the end goal would be. Thank you for providing me the tools, environment, and mindset to be successful in a variety of settings. Mom, all of those forced summer projects paid off. Thank you. Dad, thank you for teaching me about first and foremost about being a good citizen and a better man. Your lessons and methods, though sometimes rough in their delivery, have been more valuable to me than any book or class.

Lastly, I dedicate this work to the young people I have had the opportunity to influence, and those who I have yet to meet. I was once told that in order to be successful in this graduate journey I needed to sometimes become selfish, and focus on me. I disagree, and I thank you all for never letting me be comfortable with selfishness, and for keeping my motivations noble, and my intentions pure. I pray that my accomplishments have served and will serve as fuel for your accomplishments. Never stop marching onward and upward toward the light.

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PREFACE

There are an estimated two million bone grafting procedures per year worldwide, with a significant number of these being autologous bone grafting procedures. The next most common surgical approach is allograft bone grafting, requiring the donation of viable bone tissue. The need for viable donor tissue presents a number of concerns: 1) the need for donor tissue vastly outweighs the supply, and 2) a United States population that is generally increasing in age lends to an increased rate of fractures as the availability of healthy donor bone decreases. Therefore, there exists a significant fraction of the population for which bone grafting is not a viable treatment option, either due to unsuitable autologous tissue, lack of donor tissue, desire to avoid multiple surgical wounds, or existing comorbidities leading to compromised fracture healing (i.e. diabetes, obesity). For these patients, researchers and clinicians are investigating bone graft substitute materials and bone tissue engineering approaches to induce healing.

This work demonstrates development of a modular test system, i.e. a bone tissue engineering scaffold with variable properties, for the study of critical-sized defect healing. A system that is able to quickly modulate multiple properties, that has been shown to affect bone tissue differentiation and ingrowth, is needed in order that specific combinations of parameters can be applied to specific defect situations. This approach allows the design of scaffolds in a more patient-specific manner, and gives further understanding particular to the individual and collective contributions of each design parameter to overall fracture healing. The developed system was adapted from medical textile technology, specifically woven surgical mesh concepts. Weave configuration, fiber geometry, material type, and fiber spacing were manipulated to create a 2-D scaffolds capable of being arranged in 3-D configurations and affect the biologically significant factors of scaffold geometry, surface interaction/modification, biocompatibility, porosity/pore size, and mechanical strength/stability. These factors have been shown to affect cell

affinity, stem cell differentiation and phenotype, angiogenesis, nutrient/waste transport, and overall implant viability. The efficacy of this system was evaluated by characterizing the effect of parameter variation on fluid flow properties and the related cell response. Increased fluid transport properties have been shown to regulate nutrient/waste transport properties or levels shear stress on attached cells, thereby affecting cell metabolic activity or mechanical response, respectively. Scaffolds developed via the proposed system were finally cultured with D1 MSCs with the goal of stimulating cellular differentiation into osteoblasts. The effect of scaffold parameters on MSC adhesion and differentiation was evaluated to better understand the efficacy of specific parameter combinations as bone graft substitute materials. The ability to predict bone cell response based on material and configuration parameters *in vitro* will help direct future *in vivo* studies focused on repairing specific types of critical sized defects.

CHAPTER ONE
INTRODUCTION

Clinical Relevance

Bone trauma, more commonly referred to as a fracture, is one of the leading types of trauma leading to hospitalizations in the United States every year. It has been estimated that 3.5 million emergency room visits and 887,679 hospitalizations occur each year due to fracture.[1] More specifically, long bone fractures are one of the most prevalent types of bone trauma. Tibia and fibula fractures, alone, account for more than 7% of all fractures in 2010.[1] Recent market research reports that by 2050 the percentage of North Americans age 65 and older will reach 21.1%.[1] This rate in growth of the elderly population has led to the projection of a $10 billion global orthopedic biomaterial market, including allograft materials and bone graft substitutes.[6]

Generally, post-traumatic skeletal conditions such as delayed unions, nonunions, malunions, or other bone loss problems, are successfully addressed by the restoration of alignment and sufficient fixation of the bone during fracture healing. However, in some situations bone grafting has been employed by physicians to replace bone or to augment the natural bone healing process.[2] Bone grafting may be required in a variety of traumatic situations, during which the orthopedic surgeons may exercise their best judgment in the selection of materials with specific properties advantageous to specific bone healing needs. It was estimated in 2011 that more than 2 million bone grafting procedures were performed worldwide each year.[3] The gold standard for bone grafts is the autologous graft, usually taken from the patient's iliac crest; however, there are problems associated with bone graft procedures using this method. A study by Younger and Chapman reported a minor complication rate of 20.6%, and a major complication rate of 8.6% in bone graft surgeries.[4] Along with a high complication rate for autologous procedures, allograft procedures are hindered by the availability of donor bone material.[5] While

the graph in Figure 1.1 showed an upward trend in the number of bone graft donors from 1994-1999, this number was still significantly lower than the clinical need for these tissues at that time. This deficiency is further compounded today by the rapid increase in people age 65 or older, who statistically are more likely to need a bone graft after fracture. This population is also more likely to have osteoporotic bone unsuitable for donation.[6]

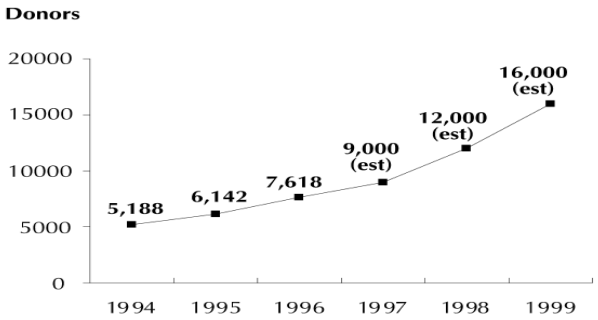

Figure 1.1: Estimated number of musculoskeletal tissue donors in the United States, 1994-1999.[5]

As a result of the high rate of complication and donor tissue shortage in these increasingly common procedures, clinicians and researchers have sought new biological and non-biological solutions. Non-biological developments classified as bone graft substitutes include mineralized composites, injectable cements, bioactive glasses, and polymers.[3] Materials are used independently or in combination with another treatment option to stimulate bone healing. Bone graft substitutes have become increasingly popular due to the future indications for bone fracture incidence and healing. Within the research regarding biological bone graft substitutes, bone tissue engineering applications have also been explored by clinicians and researchers. Tissue engineering may be considered a fairly immature field, having its roots in the early 1970s with attempts to develop new cartilage by seeding spicules of bone with chondrocytes [7] and attempts to build naturally derived dermal substitutes.[8] This early work, although rudimentary, provided the basic framework for the concept of tissue engineering. Figure 1.2 illustrates these basic concepts.

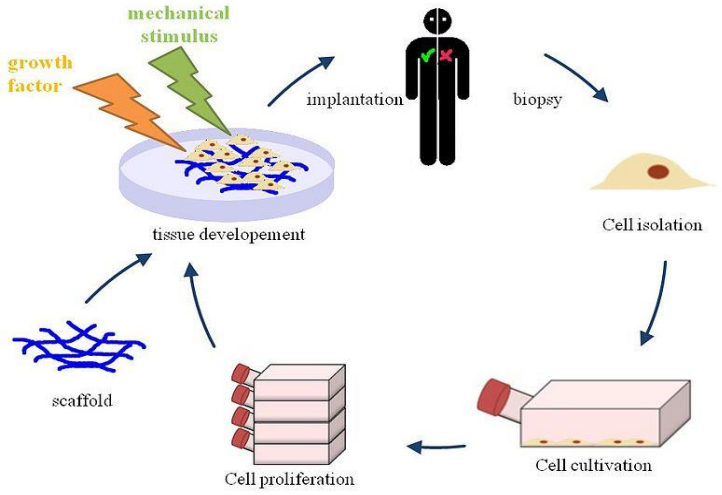

Figure 1.2: Classic tissue engineering schematic.[7]

In 1993, the work of Drs. Joseph Vacanti and Robert Langer propelled tissue engineering into the forefront of biomedical research with their work in designing appropriate scaffolding for cell delivery, in contrast to seeding cells on naturally occurring scaffolds.[7, 9] Tissue engineering approaches to bone graft substitutes may not only alleviate some clinical issues, but may also present a significant market opportunity for those addressing an increasingly active aging population.[10]. Greenwald and coworkers report that market was nearly $300 million in 1999.[5] Vacanti and others worked to develop functional tissue equivalents using synthetic biocompatible/biodegradable polymers configured as scaffolds and seeded with viable cells.[7] This line of work is the foundation for the research highlighted in this document.

Bone Structure and Fracture

Bone Structure

Bone tissue, a specialized form of connective tissue, is one of the more diverse tissue types in the human anatomy. On the macroscopic level, bones can be divided into four main types: long bones, short bones, flat bones, and irregular bones.[11]

Differences in the cortical and cancellous regions of long bones have motivated researchers to examine what possible physiological benefits may be derived from these structural variations. One such example of this work is research by Bayraktar and coworkers in which the elastic, tensile, and compressive yield properties were compared for cortical and cancellous bone.[12] Understanding the mechanical properties of these different bone types has become clinically relevant as researchers have attempted to design biomaterials capable of withstanding physiologic elastic, tensile, and compressive loads.

Understanding the mechanical properties of bone has become an increasingly critical aspect of orthopedic biomaterials research. Throughout the literature, observations have been made of the ability of cancellous bone to withstand substantial compressive loads, while cortical bone has been shown to significantly resist bending forces. However, researchers such as Rho and coworkers maintain that in order to better understand the true mechanical properties of bone it is necessary to examine bone tissue at the micro, and even nano, levels.[13]

Microscopic structure of bone is highly regular and its acellular components primarily comprise collagen fibers and calcium phosphate crystals.[14] The calcium phosphate crystals are termed hydroxyapatite, with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$. This formula has become increasingly important over the past 30 years. Researchers and physicians have sought to implement artificially made hydroxyapatite as an augmentation to bone defects, and as a coating for orthopedic implants. The material was heralded for its ability to chemically bond with the host bone, further stabilizing a fracture site or the implant – bone interface.[15] The collagen fibers in bone matrix are most often type I collagen, and are cross-linked to increase strength and insolubility.[14]

The cellular components of bone are generally broken down into three cell types: osteoblasts, osteocytes, and osteoclasts. Remodeling is carried out by osteoblasts, bone-forming

cells equipped to produce many proteins, and also by osteoclasts, bone-resorbing cells equipped to break down and phagocytize components of bone. Researchers have been highly interested in understanding the interaction between osteoblasts and osteoclasts in bone remodeling. For example, Lemaire and coworkers attempted to construct a model to understand and predict how the proportions of immature and mature osteoblasts affected osteoclast activity.[16]

Bone is formed through one of two different processes. Flat bones are formed through the process of intramembranous ossification, in which membranes of mesenchymal connective tissues are ossified.[14] The mesenchymal cells commit to either a cartilaginous pathway or a bony pathway. Researchers, such as Thompson and coworkers, believe that this pathway is determined primarily by the mechanical environment surrounding the tissue. This belief has been applied to fracture healing research exploring the benefits of external fixation.[17] The other process of bone formation, endochondral ossification, is responsible for the development of most bones in the skeleton, including long and short bones.[14] During endochondral ossification, chondrocytes progress through hypertrophy, and eventually die and become calcified. An influx of blood vessels and osteoblast precursor cells transitions this calcified tissue into woven bone, which may later be remodeled into cancellous or cortical bone.[18]

Along with the aforementioned functions, bone contains bone marrow. Bone marrow is either red marrow, which is active in blood cell formation, or yellow marrow, which is inactive.[19] The process of hematopoiesis results in the production of red blood cells (RBCs) rich in oxygenated hemoglobin, which has a characteristic red pigmentation. These RBCs, called erythrocytes, are housed in the red bone marrow. Yellow bone marrow may convert back to red marrow if the blood supply becomes deficient.[19]

Fracture Healing

There are several types of fracture, with the four most common types pictured below in Figure 1.3. Oblique, comminuted, spiral, and compound fractures all develop from one or a combination of the following forces: torsion, tension, or compression.[14] Fractures may also be pathologic in nature, when there is an underlying disease in the bone tissue. Some diseases increasing the probability of fractures include osteoporosis, Paget's disease, or osteopenia.

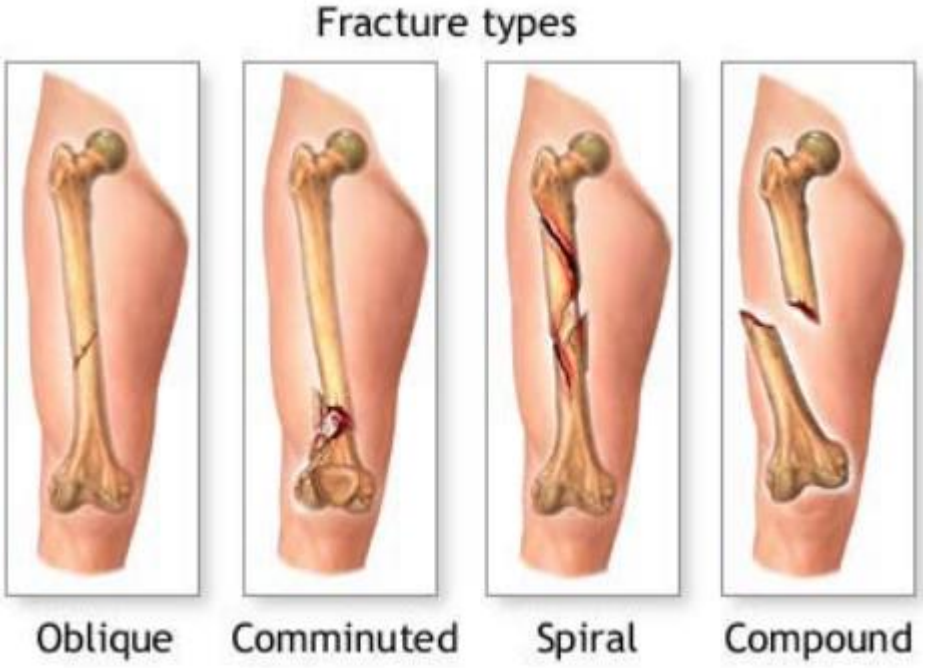

Figure 1.3: Fracture types. (http://www.nlm.nih.gov/medlineplus/ency/imagepages/1096.htm)

Clinically, fractures are addressed through reduction and fixation. The fixation method may be internal or external, but the goal is always to provide the necessary mechanical stresses on the bone to incite bone ingrowth and restored anatomical function at the injury site. Mechanical stresses, or biomechanical conditions, and vascularity at the injury site are considered to be the key factors determining effectiveness of fracture healing.[20]

At the micro level, bone healing occurs in four major steps. The four steps are hematoma formation/inflammation, soft callus formation, primary bone deposition, and bone remodeling.[21]

Fracture healing serves to repeat the process of initial bone development, resulting in bone tissue that is eventually consistent with surrounding unaffected tissue. Figure 1.4 displays the four major steps of fracture healing. The first step, hematoma formation and inflammation, begins immediately following the fracture. The disruption of vasculature, soft tissue, and marrow spaces incites the generic wound healing response.[22] This response begins with inflammation, characterized by redness, swelling, heat, and pain at the injury site.[19] The redness and swelling are caused by the initial vasodilation at the injury site. The increased blood flow to the area causes an influx of immune cells and molecular factors that carry on the healing cascade. Heat and pain at the injury site are a result of these molecular factors, which include interleukins such as IL-1 and IL-6, and tumor necrosis factor alpha (TNF-α).[23] The factors mentioned above, IL-1, IL-6, and TNF-α are considered pro-inflammatory cytokines, and are secreted by macrophages, monocytes, and leukocytes introduced to the area during vasodilation. These factors, along with others, have a chemotactic effect on other inflammatory cells which enhances extracellular matrix synthesis, stimulates angiogenesis, and recruits fibrogenic cells to the injury site.[24]

Inflammation during bone healing has been intensely studied as researchers have attempted to characterize the ideal conditions for bone remodeling. Claes and coworkers conducted work comparing fracture healing in a healthy environment versus one in which there was systemic inflammation, as would be seen in a trauma with multiple injury sites. In another study the same authors measured elevated levels of IL-6 in rat models with multiple injury sites. It was concluded that the increased inflammation levels resulted in inhibition of fracture healing.[20, 25]

Soft callus formation begins from the damage to surrounding tissue and the exposure of collagen, resulting in the influx of platelets activated by thrombin at the injury site.[26] The platelets release a series of growth factors, including platelet derived growth factor (PDGF) and

transforming growth factor beta (TGF-β) which induce mesenchymal stem cell (MSC) migration, activation and proliferation, angiogenesis, chemotaxis of other inflammatory cells, and further platelet aggregation.[27] Simultaneously, bone morphogenic proteins (BMPs) are released from bone matrix and are expressed by MSCs. Soft callus formation occurs as MSCs differentiate into chondrogenic or osteogenic cell types and new vasculature enters the injury site. The angiogenic process is a prerequisite for the continuation of the fracture healing process and is regulated by the expression of fibroblast growth factors (FGF) and vascular endothelial growth factors (VEGFs). The differentiation of MSCs to chondroblasts and the subsequent proliferation of the resulting chondrocytes yield a soft callus. Under proper mechanical stresses, the cartilaginous callus may be converted to bone tissue.[27]

The two methods of prenatal bone development are intramembranous ossification and endochondral ossification. Both of these phenomena occur during the fracture healing process. Together, these processes result in the primary deposition of bone that will eventually be remodeled. Instances of intramembranous ossification occur within 3-5 days of the fracture, generally close to the periosteal region and in the marrow where high cell density is present.[23] Cells from this region differentiate into osteoblast and osteoblast precursors and lay down woven bone with days of the fracture. Differentiation of cells to the osteoblast phenotype ceases at approximately 14 days, but osteoblast activity remains as woven bone is continuously deposited.[27]

Endochondral ossification results from the hypertrophy of chondrocytes in the soft callus region. TNF-α induces apoptosis of these chondrocytes and stimulates osteoclast activity, allowing the mineralized cartilage to be absorbed.[28] As osteoclasts resorb the calcified chondrocytes, osteoblasts migrate into the area to deposit woven bone. This woven bone is surrounded by marrow and vasculature as the angiogenic processes continue.[18, 27] As discussed

previously, the mechanical environment plays a key role in the development of bone. Claes and coworkers performed a study in which strain rate and hydrostatic pressure were used to influence bone development through intramembranous or endochondral ossification. It was shown that strain rates less than 15% and hydrostatic pressures greater than 0.15 MPa resulted in bone growth, with higher values yielding endochondral ossification. Mechanical environments outside this range resulted in connective tissue growth.[29]

The final phase of the fracture healing process involves the remodeling of the disorganized and mechanically weak woven bone. The return of bone tissue to physiologic levels of utility is highly dependent on the mechanical stress environment at the injury site.[30] Kenwright and coworkers report, in a review of tibial diaphyseal fractures, that interfragmentary stability and mechanical stresses are especially important in cases of comminuted fractures as pictured in Figure 1.3. This stage in healing is dominated by osteoclasts and osteoblasts engaged in the bone remodeling discussed in the previous section. Osteoclast release BMPs, which in turn regulate osteoblastic activity.[24] The balance of resorption and deposition of bone eventually results in a return to normal function at the injury site. Disruption of this process, mechanically or pathologically, may lead to a severely weakened area of bone, resulting in high probability of recurring fracture.[21]

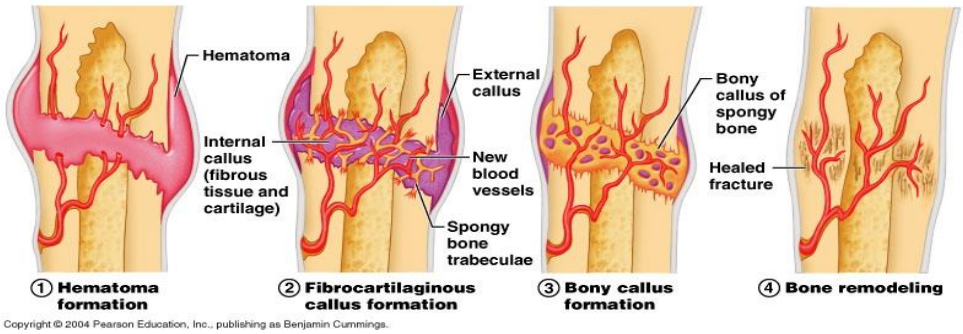

Figure 1.4: Fracture healing stages. (http://apbrwww5.apsu.edu/thompsonj.htm)

Causes of Critical-Sized Defects

The fracture healing process is highly refined; however, continually unhealed defects resulting from non-union fractures or tumor resection have proven to be problems clinically. Researchers and clinicians have come to call these types of defects critically-sized defects.[31] Critical-sized defects result from a bone defect large enough that normal fracture healing processes are not sufficient in restoring proper function.[32] Researchers have commonly accepted 5 mm round defects in mice and 6-8 mm round defect in rats as models for critical-sized defects in orthobiologic research.[33, 34] On a radiograph these defects will present as visible gaps, an absence of callus formation, or persistent fracture lines.[35] A visible gap can be seen in Figure 1.5 below.

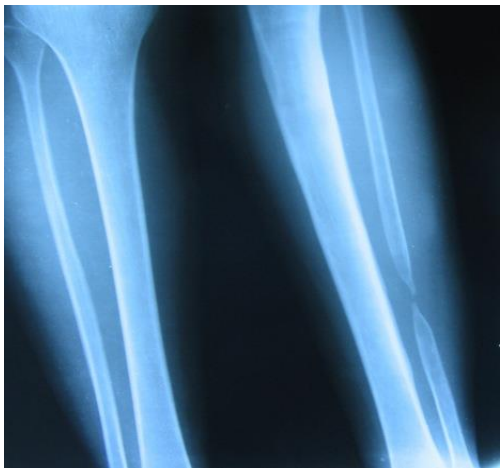

Figure 1.5: Non-union of a left fibular diaphyseal fracture.

(http://boneandspine.com/orthopaedic- images/xray-union-shaft-fibula/)

Generally, causes for non-unions can be placed in one of two categories. The first category includes biologic factors, which primarily involve damaged vasculature or the lack of quality bone from which remodeling can occur.[35] High energy fractures (generally forming compound or comminuted fractures as shown in Figure 1.3) are at particular risk for non-union due to the disruption of blood vasculature around the wound site. There is also an increased distance between bone fragments, resulting in frustration of healing supported by diffusion of

nutrients.[36] Harley and coworkers studied 242 tibial fractures to determine the effect of time-to-treatment on rate of non-union for patients suffering from open fractures. The mean time-to-treatment was 8 hours and 25 minutes with 16.5% of patients eventually experiencing non-union. Amongst patients who experienced non-union there was also a high occurrence of deep infection.[37]

The other category of factors contributing to critical-sized defects is mechanical. Fracture site instability is the primary mechanical factor leading to non-union. Failure to properly immobilize the fracture site, or failure to provide adequate internal or external fixation, will result in instability. This instability allows the movement of bone fragments which causes an initially high strain on the precursor cells attempting remodeling.[35] This high strain rate and subsequent movement at the fracture site will result in the development of connective tissue, as shown by the work of Claes and coworkers.[29] If movement is allowed to continue, pseudarthrosis, or a "false joint", will develop, making return to proper function more difficult.[27] In order to address the potential issues with critical-sized defects, specifically involving open or high energy fractures, physicians and researchers have developed a number of interventions.

Traditional Bone Grafts and Bone Graft Substitutes

The most common intervention to address posttraumatic critical-sized defects, such as delayed unions, nonunions, malunions, and significant bone loss, is a bone grafting procedure.[2] The earliest recorded bone grafting procedure was performed and recorded by Dutch surgeon Job van Meekren in 1668. Meekren reported successful implantation of a portion of dog skull into a patient with a cranial trauma.[38] The purpose of these bone grafts has traditionally been to stimulate the natural bone healing process. Bone graft has become the second most common transplantation tissue, with blood being by far the most common.[39] With advances in clinical research, orthopedic surgeons have a variety of bone graft options from which to choose. This

variety has positively affected the surgeon's ability to provide treatment with a high level of specificity for the condition, but has negatively affected the patient's potential outcomes due to a high degree of variability.[40] All bone graft materials are selected to address at least one of the following characteristics, considered essential to the stimulation of bone healing. Materials should be osteogenic, osteoinductive, and/or osteoconductive.[2, 3, 39, 40]

Osteogenesis

The osteogenic properties of a bone graft material are those characteristics which involve the presence and viability of surviving osteoblasts or osteoprogenitor cells, and the potential for proliferation and differentiation into osteoblasts, and eventually to osteocytes.[2, 3, 39] Osteogenesis has been described as a combination of factors, mainly consisting of osteoinduction, osteoconduction, and osseointegration.[3] Naturally, the most osteogenic material is autologous bone graft, due to the presence of surviving osteoblasts or osteoprogenitor cells.[2, 3, 39] However, researchers are attempting to develop solutions that may offer the same osteogenic properties without the negative aspects of harvesting autologous tissue.

Osteoinduction

The osteoinductive properties of a bone graft material include the ability of the material to stimulate and activate mesenchymal stem cells from the surrounding host tissue. These cells may differentiate into bone-forming osteoblasts. The stimulation and activation of these host cells is mediated by a cascade of biological signals, and by the activation of several extra- and intracellular receptors.[2, 3, 39] Several researchers and clinicians have attempted to manipulate the osteoinductive properties of some graft substitutes, mainly demineralized bone matrix (DBM). Among these researchers are Lee and colleagues, who conducted a study combining the osteoinductive qualities of DBM with the osteoconductive properties of hydroxyapatite (HA). The results of this study indicated that the addition of the osteoinductive material to the HA

served to enhance new bone formation in nude mice.[41] However, researchers are still attempting to pinpoint the mechanisms controlling bone cell induction, and also how these mechanisms may interact with other therapies. [41-44]

Osteoconduction

Osteoconductive properties of a bone graft material underly the material's ability to provide scaffolding for the ingrowth of new bone.[2, 3, 39, 40] In non-synthetic bone graft materials, the protein matrix and mineral phase of the bone provide this structure.[3] Osteoconduction also describes the facilitation and orientation of vasculature throughout the scaffold. [39] The osteoconductive properties of a graft material are not only critical for scaffolding and the influx of vasculature, but also for the modeling of bone structure, especially in cancellous applications. Both Al Ruhaimi and Bucholz point out, in their comparisons of synthetic osteoconductive graft material, the importance of anatomically consistent pore dimensions and mechanical properties.[45, 46] However, implementation of these types of architecture may present other complications that are detrimental to graft osteoconduction, such as brittle handling properties, variable rates of resorption, and poor performance in cortical applications.[46]

Other Considerations

In addition to osteogenesis, osteoinduction, and osteoconduction, structural support/strength and osseointegration are also key factors in the success of a bone graft material.[3, 39, 40] Being that one of the surgeon's principal indications for bone grafting is bone loss, adequate mechanical support and structural replacement are required for the proper healing of the bone. These characteristics may serve to provide the proper mechanical environment for fracture healing.[47] Osteointegration describes the surface bonding between the host bone and the grafting material.[39, 40, 48] Osseointegration is an increasingly important aspect in the application of bone graft substitutes, as biocompatible and biomechanical considerations must be included.[40] Hannink

and colleagues assessed bone graft substitutes, including an analysis of the effects of material osseointegration, osteoconduction, and osteoinduction on overall mechanical stability and biocompatibility.[49]

Naturally, an optimal replacement for a patient's bone tissue is bone tissue with similar physiology from that same patient. This logic is supported by the fact that autologous bone grafts are currently the gold standard for treatments requiring a graft.[2, 3, 38-40] In fact, Goulet and coworkers reported that there were approximately 200,000 autologous bone grafts harvested in 1997, with the majority of these grafts being taken from the iliac crest.[50] However, problems associated with autologous grafts have guided researchers and clinicians into the development of alternative treatments. The majority of these alternatives can be categorized as one of the following: allograft or bone graft substitute. The goal of these developments is to produce a material at least comparable to the autologous bone graft with respect to osteogeneity, osteoinductivity, and osteoconductivity. Table 1.1, below, illustrates a high-level comparison of autografts, allografts, and some substitute materials.[51] The sections following in this document will more specifically highlight some of the characteristics of these surgical options.

Table 1
Modification of Laurencin's classification

Bone Grafts and Substitutes			OG	OI	OC	SS	Cost
Autograft			+	+	+	+[a]	+++/++++[b]
Allograft			-	+	+	+[a]	+/++
Substitutes	Biologic	Coral	-	+	+	-	++/+++
		Collagen type 1	-	+	+[c]	-	(No studies on DRFx)
		Demineralized bone matrix	-	±	+	-	+/++
	Synthetic	Factor-based (TGF-β, PDGF, FGF, BMP)	-	+	±	-	+++/++++[d]
		Cell-based (mesenchymal stem cells)	+	-	+[c]	-	(No studies on DRFx)
		Ceramic-based (calcium HA, tricalcium phosphate, calcium phosphate cement)	-	-	+	+	+/++
		Polymer-based	-	-	+	-	(No studies on DRFx)

Abbreviations: BMP, bone morphogenic proteins; DRF, distal radius fracture; FGF, fibroblast growth factor; HA, hydroxyapetite; OC, osteoconductive; OG, osteogenic; OI, osteoinductive; SS, structural support; TGF, transforming growth factor.
[a] If the graft includes cortical bone.
[b] Including direct and indirect costs, data based on studies of spinal fusion and tibial nonunions.
[c] If used with a carrier.
[d] Only Rh-BMP is tested on distal radius fractures.

Table 1.1: High-level comparison of autograft, allograft, and substitute materials used for bone graft applications, with respect to osteogenesis, osteoinduction, osteoconduction, structural support, and cost.[51]

Autograft Treatments

Bone formation from autologous grafts is believed to occur in two stages, during which the burden of osteogenesis is transferred between cells from the graft material and cells from the host implant site.[2] The first phase of bone formation is dominated by the cells of the graft material, making the presence of osteogenic cells critical during this 4 week-long period. The second phase begins to contribute to the process, when endosteal-lining cells, marrow stroma, osteocytes, and free hematopoietic stem cells take over the osteogeneic portion of bone healing. Endosteal cells and marrow stroma dominate this phase, producing together nearly 30% of the new bone.[2, 52] Autografts are implemented in various forms to take advantage of the osteogenic properties conducive to bone formation at the host implant site.

Cancellous Bone Chips

Cancellous bone chips leverage the two phases of bone healing mentioned in the previous section. The osteogenic cells from the cancellous chips are osteoblasts and endosteal cells. These are the primary cells that survive harvesting and implantation.[2, 53] Because the layer of osteogenic cells remaining on the cancellous chips after harvesting is relatively small, this material serves primarily as an osteoconductive substrate for the ingrowth of bone. The osteoconductive capacity in this graft material activates phase two of the bone healing process by readily revascularizing the implant site, and promoting the influx of new osteoblasts and precursor cells to the area.[2, 54] As with other autograft materials, cancellous bone chips also possess osteoinductive properties. These characteristics stem from the release of various factors from osteoblasts and osteoclasts during the cell-mediated resorption process, and the release of cytokines from inflammatory cells

during the inflammatory response following surgery.[55] These grafts also integrate quickly into the *in vivo* environment, and may reach a strength equivalent to a cortical graft after 6-12 months in an otherwise healthy patient.[56] Cancellous bone chips serve as sufficient space fillers but do not provide substantial structural support, thereby necessitating internal or external fixation for the patient.[2, 56] Problems associated with cancellous bone chips are similar to those cited previously for autologous bone grafts in general. Figure 1.6A below shows cancellous bone chips from Biomet®.

Vascularized and Nonvascularized Cortical Bone

Cortical grafts are implemented in two varieties, those with the vasculature still intact or those with soft tissues removed. Both of these materials are primarily osteoconductive in nature, but there are small amounts of surviving osteoblasts remaining on these grafts after harvest which provide an osteogenic catalyst for regeneration at the implant site. While not inherently osteoinductive, these materials can be combined with other osteogenic treatments to induce bone cell differentiation and proliferation.[2, 57] Integration of vascularized cortical grafts is rapid at the implant site, with immediate structural support being added to the fracture area. Due to the presence of viable vascularization, these grafts do not usually undergo significant resorption or revascularization. However, internal or external fixation is still required to stabilize the mechanical environment for the remodeling of the graft bone.[57, 58] Figure 1.6B shows a vascularized fibular graft used in reconstruction of the radius in a 47-year old woman who presented with pain and immobility due to a large, osteolytic tumor at the distal radius.[59] Nonvascularized cortical grafts also integrate readily at the fracture site and offer immediate structural support. However, there is initial weakening of the graft as resorption and revascularization occur during the first 6 weeks. Eventually (6-12 months), vascularized and nonvascularized grafts have little difference in mechanical strength.[57, 58] Figure 1.6C shows a

nonvascularized fibular graft prepared for application in ankle repair for a 42-year old male, diagnosed with type II diabetes.[60] Despite the many benefits of cortical grafts, there are some problems associated with their use. Donor site morbidity continues to be an issue, along with osteonecrosis, subjective sense of instability, and numbness or weakness.[61]

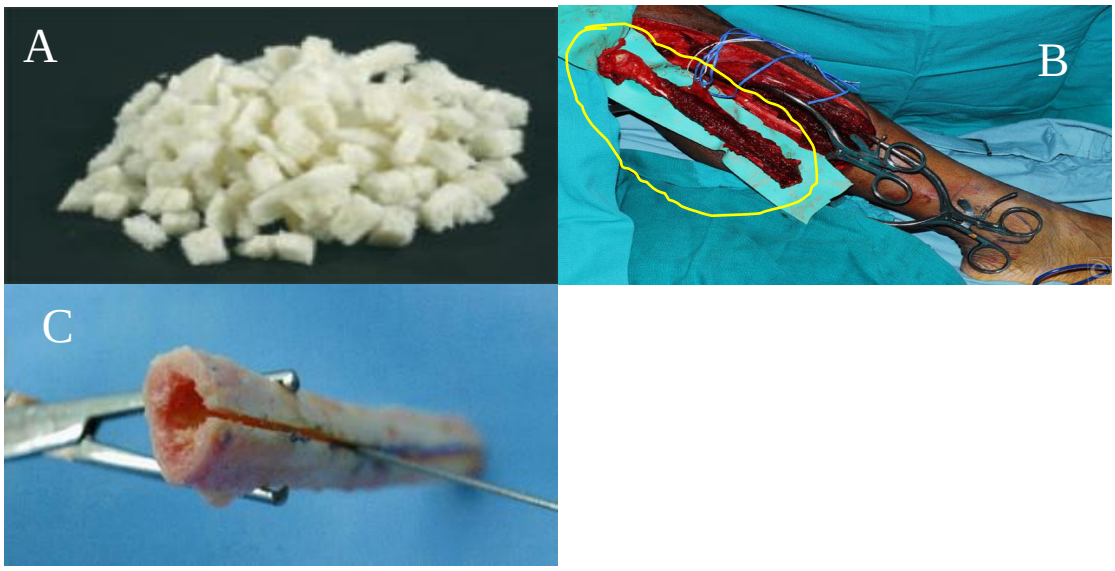

Figure 6: A) Cancellous bone chips from Biomet®, ranging from 4-10mm. (www.biomet.com)

B)Vascularized fibular graft with the muscle cuff and peroneal vascular pedicle.[59]

C)Nonvascularized fibular graft to be used in ankle repair.[60]

Autologous Bone Marrow

Autologous bone marrow, or bone marrow aspirate (BMA), is a mostly osteogenic bone graft material. This highly vascular, modified connective tissue is the source for osteoblastic stem cells when aspirate is centrifuged to concentrate the cells in solution.[2, 19] BMA may also be osteoinductive as cytokines and growth factors are released by osteoblasts during bone regeneration.[2] BMA does not provide a solution to mechanical strength issues associated with fracture healing, but it does offer the following advantages over traditional autografts: (1) Patients receiving BMA injections may leave the same day, thereby improving cost-effectiveness of the

treatment; and (2) BMA treatments are associated with fewer donor site complications than traditional autograft treatments.[62, 63] However, when not coupled with a carrier material, BMA has been shown to wash away from the desired location *in vivo*.[2] This problem has led to much research in the combination of bone graft substitutes with autologous bone marrow. Some of those combinations will be discussed later.

Allograft Treatments

Allograft bone grafts serve as alternatives to autologous grafts, ideally avoiding donor site morbidity and the lack of suitable donor bone. Allograft tissue is employed in 35% of all bone transplantations.[43] There are several preparations of allograft bone, such as: demineralized bone matrix, cancellous chips, cortical segments, collagen, and bone morphogenic proteins (BMPs).

Demineralized Bone Matrix (DBM)

Demineralized bone matrix is primarily osteoconductive, as any osteogenic properties are lost during the processing. The osteoconductive properties are mostly attributed to the favorable revascularization environment provided by DBM. Applications include bone void filling, long bone non-union repair, acute fracture treatment, or autograft extension.[2, 3] However, the osteoinductive properties of DBM have been shown to decrease as a result of the extensive sterilization process.[43, 64] This sterilization issue, along with a lack of mechanical support without fixation, and donor-to-donor variability, are among some of the drawbacks to DBM use as a bone graft substitute.[2, 43] Table 1.2 below shows some of the DBM products currently in use, and their associated advantages and disadvantages.[3]

Product	Company	Type	Indications	Advantages	Disadvantages
Grafton DBM	Osteotech	Glycerol carrier Available in gel, flexible sheet, putty and semisolid ("Crunch") forms	Nonunion, delayed and potentially delayed unions	• Potentially osteoinductive • Solid formulations provide some structural support • Large clinical experience	Human derived: • Variable starting material • Potential for disease transmission • Potential immunogenicity Gel offers no structural support • Recent concerns about toxicity of glycerol (Bostrom)
Allogro	Allosource	Particulate DBM, reconstitute with saline	Nonunion, delayed and potentially delayed unions	• Potentially osteoinductive	• Human derived, as above
DBX	Synthes USA, Musculoskeletal Tissue Foundation (MTF) & Lifecore	DBM + hyaluronic acid + collagen carrier	Nonunion, delayed and potentially delayed unions	• Potentially osteoinductive	• Human derived, as above • Critics claim hyaluronic acid carrier (xenograft coxcomb) is untested
DynaGraft, Accell, OrthoBlast	GenSci Regeneration Sciences	DBM + polymer carriers	Nonunion, delayed and potentially delayed unions	• Potentially osteoinductive	• Human derived, as above • Relatively less clinical experience compared to Grafton
Osteofil	Regeneration Technologies Inc (RTI)	DBM (24%) with gelatin carrier (17%) and water	Nonunion, delayed and potentially delayed unions Metaphyseal fractures	• Potentially osteoinductive • Moldable but hardens at body temperature • Does not wash away with irrigation • Potential for remodeling exists (not yet proven)	• Human derived, as above

Table 1.2: Allograft DBM, potentially osteoinductive materials.[3]

Collagen

Collagen is primarily used as a delivery vehicle for other osteogenic, osteoconductive, and osteoinductive materials because of its poor function as a graft substitute independently. It offers minimal structural support and also is potentially immunogenic.[39] However, collagen does possess some inherent osteoinductive and osteoconductive capability due to its *in vivo* contribution to mineral deposition, vascular ingrowth, and growth factor binding.[39, 65] When combined with BMPs, osteoprogenitor precursors, or HA, collagen becomes particularly effective as a bone graft substitute material.[39, 46]

Bone Morphogenic Proteins (BMPs)

BMPs are popular additions to bone graft composites, but may also be used independently.[2] In either case, BMPs are employed to provide osteoinductive properties to a fracture site. BMPs induce osseous cell differentiation at the fracture site.[43] More specifically, TGF-β and related families of BMP 2-10 have been shown to differentiate mesenchymal stem

cells into chondroblasts and osteoblasts.[51] Besides their combination with other bone graft substitute materials, BMPs are implemented in acute defects and nonunion in long bones, such as the tibia. BMPs are often delivered to areas where traditional bone grafts have failed or are not feasible. Cost effectiveness is one of the major limiting factors of this material.[43]

Bone Graft Substitutes

The ideal bone graft substitute is osteogenic, osteoconductive, osteoinductive, and allows rapid osseointegration.[5] Most substitutes attempt to take advantage of any bone cells, growth factors, or bone-favorable environment present at the fracture site present before implantation. This need for exogenous substitute materials to coordinate with the *in vivo* environment requires that the implant be biocompatible, and possibly absorbable. Generally, bone healing involving a bone graft substitute requires at least three surrounding surfaces of bone.[45] The surrounding tissue helps stimulate revascularization and bone cell influx throughout the graft. Bone graft substitutes are divided into a number of categories, such as ceramics, polymeric materials, and composite designs. Each material may use a slightly different mechanism to achieve bone ingrowth, but the primary function is the provision of an osteoconductive matrix. The sections following outline several bone graft substitutes, along with potential advantages and disadvantages.

Calcium Phosphates

Calcium phosphate (Ca-P) bone graft substitutes are a staple in many composite grafts, but are also used independently to influence bone ingrowth. These materials are implemented primarily for their osteoconductive capacity, which stems from the direct deposition of bone onto calcium phosphate surfaces.[2, 66] Direct deposition of bone onto the surface of Ca-P materials causes rapid incorporation *in vivo*, and has been attributed to the polycrystalline ceramic structure[67]. Pores produced during processing have been shown to provide favorable environments for osteoid formation, with pore sizes exceeding 100 μm being most effective.[2, 43, 68]

Although Ca-P may create a favorable osteoconductive environment, some reviews in the literature state that Ca-P alone has not been shown to induce differentiation of stem cells to osteogenic pathways.[43] Researchers therefore have used these materials as carriers for osteoinductive materials. However, work by researchers such as Yuan and colleagues, or Le Nihouannen and colleagues suggest that specific chemical structure and microstructures may develop osteoinductivity in Ca-P materials.[69-71] Characteristic of ceramic materials, Ca-P is mechanically weak in tension, but stronger in compression.[2] The brittleness of Ca-P means that these implants provide little structural support, with compressive and tensile properties being contingent on pore size.[46] Injectable Ca-P preparations do initially provide compression strength comparable to cancellous bone, and are completely replaced by new bone after undergoing long-term remodeling.[39] Researchers have taken advantage of the nearly natural mineral phase of Ca-P in the development of two other materials: hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP).[43]

Hydroxyapatite (HA)

HA is used as a bone graft substitute because of its excellent osteoconductive properties; the interporous geometry attributes to this characteristic.[46] Pores may provide structural similarity to cancellous bone structure. HA may be derived from coralline sources when coral calcium phosphate is converted to coralline hydroxyapatite.[2] Pore sizes for coralline HA range between 200-500 μm, exceeding the minimal 100-μm threshold necessary for osteoid formation.[2] In a study comparing bone formation with coralline HA and cancellous autograft, Bucholz and researchers reported that performances were equivalent when the substances were used to fill bone voids in tibial plateau fractures.[72] Pore formation on the surface of HA materials allows direct apposition of new bone to the graft surface.[40] Kitsugi and colleagues also performed work exploring the osseointegration of HA in bone applications. [73] Upon implantation, the expected

immunogenic response ensues and macrophages phagocytize dead cell debris and attack the surface of the HA material. This creates a roughened surface (beyond the initial porous structure) and an exposed layer of apatite that is biologically indistinguishable from native apatite. In turn, pre-osteoblasts are programmed to differentiate into osteoblasts, which deposit bone on the graft material.[40]

The mechanical properties of HA are consistent with those of Ca-P materials and other ceramics. HA is a highly crystalline material, resulting in brittleness and decreased tensile strength. [46] Researchers have also investigated the resorbability of HA, hypothesizing initially that HA does not resorb at all. However, after many different studies, it has been shown that HA does resorb, it does so at an exceptionally slow rate.[40] This slow degradation may possibly be attributed to resistance to osteoclastic resorption for HA, despite the structural similarities of HA to natural bone.[51]

Al Ruhami and colleagues conducted a study to compare the resorption of three bone graft substitutes employing HA as the principal component. The products were Laddec (Transphyto SA; Clermont-Ferrand, France), Dembone (Pacific Coast Tissue Bank; Los Angeles, CA), and Osteograf LD (CeraMed; Lakewood, CO), which have pore sizes of 600, 500, and 250-420 um, respectively. Laddec showed satisfactory bioresorption characteristics, enhanced by osteoclasts, and new lamellar-like bone formation. Dembone showed minimal resorption and chronic inflammation due to large areas of mononuclear cells (monocytes). The Osteograf material underwent resorption via hydrolysis and there was significant bone growth.[45]

Beta-Tricalcium Phosphate (β-TCP)

Beta-tricalcium phosphate (β-TCP), similar to other Ca-P materials, is also highly osteoconductive due to the formation of a porous scaffold from small crystals (70-100 μm) or larger crystals (>100 μm). The combination of crystals result in pores between 100-1000 μm that

develop the favorable osseous environment discussed previously.[46, 67] β-TCP is more porous than HA, and this increased porosity causes a decrease in compression strength and a faster resorption time.[2, 40, 46] The increased porosity leaves β-TCP more exposed to cell-mediated degradation and hydrolysis. The resorption characteristics of TCP materials are intended to match the course of natural bone healing.[46] In a year-long study comparing Vitoss and ProOsteon 500R in dogs, Erbe and colleagues confirmed that β-TCP resorption paralleled bone ingrowth. Vitoss results for the study included 76% resorption at week 6, 86% resorption at week 12, and over 98% resorption at week 52.[74] Figure 1.7 demonstrates significant resorption of a β-TCP implant in a study similar to Erbe's work.[75] Although this faster resorption rate may be favorable in some applications, researchers have not found the degradation of β-TCP to be predictable, thereby making these grafts somewhat unfavorable.[2, 76] As new bone is formed, β-TCP is removed from the implant site. As the surface of the implant is removed, osteoclastic resorption is stimulated, which transitions into osteoblastic deposition of more new bone.[39]

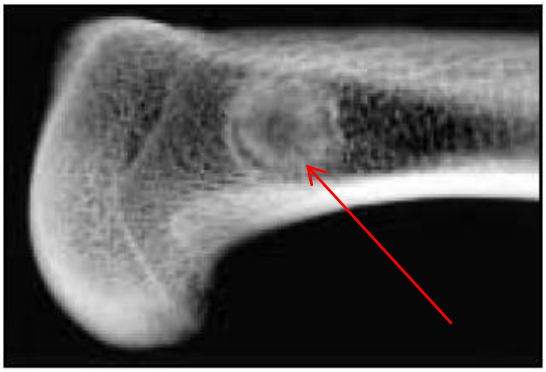

Figure 1.7: Resorption (indicated by arrow) of a β-TCP bone graft substitute in canine femur at week 3.[75]

Calcium Sulfates

Plaster of Paris, the first attempt at a bone graft substitute and a derivative of calcium sulfate, has a long history in the literature as both an independent bone graft substitute and a

carrier for other bone healing therapies.[40, 77] Plaster of Paris was first applied as bone void filler; calcium sulfate materials are still used in this osteoconductive capacity.[2, 40] In addition to the putty-like preparation of calcium sulfate materials, osteoconduction is also achieved through scaffold formation by calcium sulfate pellets. Osteoblasts attach directly to the calcium sulfate pellets, allowing osteoid deposition onto the scaffold.[46] As with β-TCP, calcium sulfate materials are crystalline in structure, but the uniformity of the crystalline structure in calcium sulfates yields a predictable resorptive rate not present in other ceramic bone graft substitutes.[78] Osteoclasts are readily able to resorb calcium sulfates *in vivo*.[46] Examples of calcium sulfate-based materials currently implemented in the clinic include OSTEOSET (Wright Medical Technology, Arlington, TN) and AlloMatrix (Wright Medical Technology). OSTEOSET is marketed as a bioresorbable and osteoconductive bone graft material. AlloMatrix also claims bioresorbability and osteoconduction, and adds limited osteoinduction through the addition of DBM.[5] Wilkins and Kelly performed a study evaluating the efficacy of AlloMatrix in putty form as bone void filler in long bone applications. Out of 76 patients, 41 (54%) received surgical intervention for removal of benign bone tumors, and 35 (46%) had long bone nonunions. Results showed that the average percentage of bone healing was 85.1% for nonunion patients and 93% for benign tumor patients. These percentages suggest that AlloMatrix may be used as bone void filler in these indications due to the consistency with autograft outcomes.[79] The figure below displays bone healing for a patient suffering femoral fracture treated with an intramedullary rod and AlloMatrix.

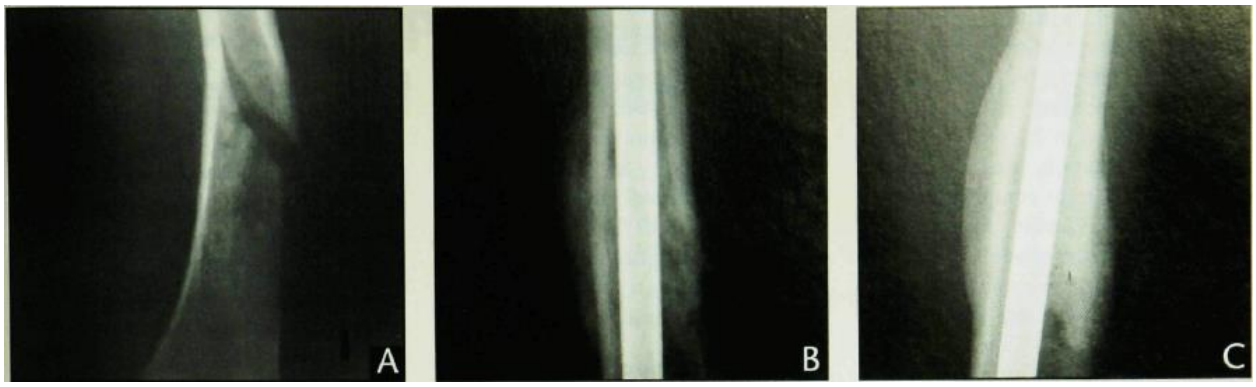

Figure 1.8: A) 24-year old male with femoral fracture treated with an intramedullary rod and AlloMatrix B) after 3 weeks, bone healing is evident with callous formation at fracture site C)after 3 months, significant fracture healing is evident and patient returns to normal function[79]

Bioactive Glasses

The definition of a bioactive material is one that elicits a response at the material-tissue interface resulting in the formation of a bond between the two.[80] Bioactive glasses consist of various combinations of SiO_2, Na_2O, and CaO, with a constant percentage of P_2O_5. The various combinations of the first three molecules have been shown to adhere to bone with variable affinity.[40, 80] Generally, bioactive glasses are used as osteoconductive scaffolds due to their ability to bind collagen, growth factors, and fibrin to form a porous matrix.[2] The presence of growth factors, collagen, and other proteins may lead to the influx of potentially osteogenic cells.[81] In the literature, bioactive glass binding to bone has been most closely compared with that of apatite. But despite the direct apposition of bone to these glasses, the lack of substantial mechanical strength and the threat of brittle failure under torsion, tension, or other mechanical stresses limits application to non-load bearing situations.[2, 40] Al Ruhaimi conducted a study measuring the osteoconductive capacity of Biogran (Orthovita; Implant Innovation, Palm Beach Gardens, FL). Biogran incorporates bioactive glass granules approximately 300 μm in diameter. Histologic evaluation revealed dissolution of granules, along with significant new lamellar-like bone

ingrowth. Bone formation was fairly dense, occurring at multiple ossification sites. The author also reported several instances of neovascularization with minimal signs of inflammation.[45]

Bone Graft Substitute Composites

The next step in the advancement of bone graft substitute materials was the combination of different osteogenic, osteoconductive, and osteoinductive substitutes, consolidating several individual material benefits to form a more complete bone graft replacement. The sections following provide a general overview of common material combinations resulting in composite bone graft substitutes. While many different medleys of these materials have been investigated, the overarching goal has been the development of an implant comparable to autologous bone graft.

Calcium Phosphate, Type I Collagen, and Bone Marrow Aspirate

One such combination of bone graft substitute materials leverages aspects of calcium phosphate, Type I and Type III collagen, and autologous bone marrow aspirate to achieve bone healing.[2, 46] The calcium phosphate, along with fibrillar collagen derived from bone, provides an osteoconductive base upon which bone precursor cells can differentiate and proliferate.[82] The addition of autologous bone marrow aspirate provides osteogeneity through proteins, growth factors, marrow cells, platelets, and other bone progenitor cells.[46] A more specific example of this type of composite is Collagraft (Zimmer Corporation; Warsaw, IN). Collagraft consists of Ca-P granules approximately 0.5-1.0 mm in diameter, where granule composition is 65% HA and 35% TCP.[5, 46] Preclinical trials conducted by Moore and colleagues showed Collagraft to be effective in bridging segmental long bone defects in dogs. Granule incorporation was rapid, with appositional bone formation directly on the Ca-P surfaces.[83] Although research has shown bone healing with Collagraft to be comparable to that of autologous graft with respect to new bone formation, the granular structure of the material limits immediate mechanical support upon

implantation.[46] However, a study performed by Zardiackas and associates showed mechanical strength in torsion after one year was at least comparable to autologous bone when implanted in canine femurs.[84] Clinical trials, conducted by Cornell and Lake showed bone healing comparable to autograft in 303 patients treated with open reduction, internal fixation, and autograft.[85] This study has been criticized in the literature, however, for its lack of control groups for patients treated with just bone marrow aspirate alone and patients treated with internal fixation alone).[46]

Demineralized Bone Matrix and Autologous Bone Marrow

Demineralized bone has been shown to be a satisfactory composite material when combined with osteogenic bone graft materials.[2, 3, 43, 86] The osteoconductive capacity of this composite can contribute to rapid revascularization of DBM and scaffolding for new bone formation. DBM may also contribute osteoinductively with cytokines and growth factors released by the matrix. Osteogenic stem cells are readily available in autologous bone marrow aspirate.[2, 86] Autologous bone marrow in combination with 10 mg DBM, forming an injectable sand-like material, has been successfully used to fill bone void defects.[63, 87] The injectable preparation of this composite has a number of advantages. One such advantage is the ability to deliver graft material to a defect site without exposure of the fracture.[2] Connolly and coworkers recorded 90% (18 of 20) union of delayed unions in open tibial fractures using this injectable material.[63] Figure 1.9 below shows similar work by Tiedeman and colleagues in a preliminary investigation of the role of DBM/bone marrow composites in osseous defects.[87] The figure illustrates fracture healing of an acute depressed lateral tibial fracture repaired by screw fixation and DBM/bone marrow composite.[87]

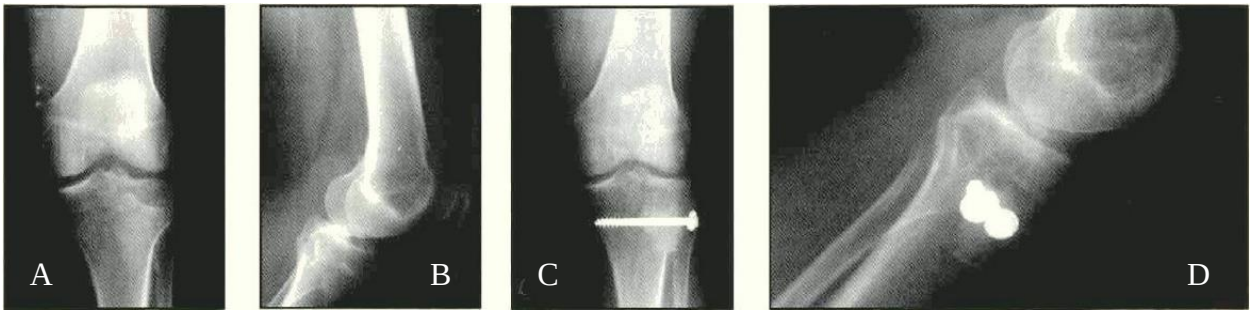

Figure 1.9: A and B) Acute depressed lateral tibial fracture, anteroposterior and lateral views, respectively; C and D) Fracture healing after 6 months following screw fixation and DBM/bone marrow treatment, anteroposterior and lateral views, respectively.[87]

Demineralized Bone Matrix and Porous Hydroxyapatite

DBM in combination with porous HA has been used by researchers and clinicians, to combine the benefits of different bone graft substitutes. For example, work has been conducted to take advantage of the osteoinductive potential of DBM, due to the presence of cytokines and growth factors released from the matrix, and the osteoconductive qualities of HA. The porous geometry of HA lends itself to *in vivo* responses similar to those of cancellous bone.[2, 46, 67, 72] Lee and coworkers performed a study evaluating the efficacy of porous HA and DBM as an inducer of bone formation *in vitro* and *in vivo*. Their combination of porous HA granules and DBM putty or DBM powder formed an injectable material.[41] This material was injected in rats and analyzed at weeks 4 and 8 for indicators of cell differentiation, such as alkaline phosphatase (ALP). Results are shown below in Figure 1.10.[41] The researchers compared DBM/HA putty to an HA preparation, as well as a positive control (osteogenic media) and a negative control (conventional media). After analysis of the results, Lee and associates concluded that the DBM/HA putty was a viable bone-inducing material due to the evidence of osteoblastic differentiation *in vitro* and the ectopic mineralized tissue formation in the rats.[41]

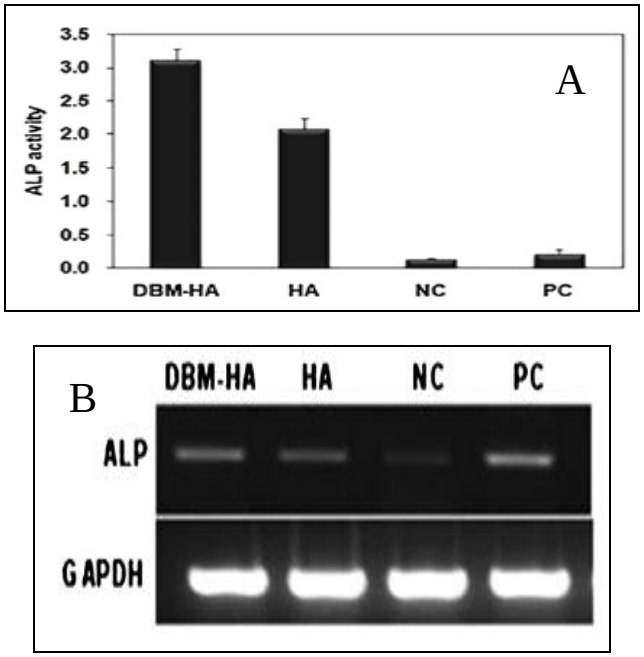

Figure 1.10: ALP activity from in vitro results. A) ALP in DBM-HA group was significantly higher than that in both control groups. B) PCR results showed significant ALP expression in DBM/HA groups.[41]

Tricalcium Phosphate and Bone Morphogenic Protein

Similar to the composite materials described previously, BMPs combined with β-TCP are geared to ameliorate the development of new bone at fracture sites. In this formulation, BMPs are the osteogenic component, while porous β-TCP serves as the osteoconductive scaffold.[40, 48] BMPs are often used independently for bone graft applications; however, a carrier such as β-TCP alleviates issue with migration of the treatment, as well as the proper structural environment for bone ingrowth.[48] Years ago, Urist and coworkers added BMPs to β-TCP and achieved positive results. A 12-fold increase in amount of bone formation was reported when compared to samples with BMP alone.[40, 88]

Polymeric Bone Graft Substitutes

Researchers and clinicians have also explored the use of polymers as scaffolds for the ingrowth of new bone. The advantage of a polymeric system is the ability to customize scaffold formation and response to particular situations.[89] Degradation rate, geometric structure, and mechanical properties are among the major variables of a polymer-based bone graft substitute system. Polymers may be natural or synthetic, or degradable or non-degradable.[89, 90] The sections below describe two of the major polymer groups implemented in bone graft substitution.

Polymethyl Methacrylate

Polymethyl methacrylate (PMMA) is polymeric cement with mechanical properties similar to natural bone. Because of the exceptional mechanical strength, this material is considered the gold standard to which other injectable polymer substitutes and bioactive glasses are compared.[3] PMMA was first cited as a bone graft substitute in work by Charnley, where he cites this "cement" material as an improvement on previously unsuccessful cement materials assessed *in vivo*.[91] Charnley, in this initial work, highlighted one of the major issues with PMMA. He reported that the methyl methacrylate liquid monomer is highly cytotoxic, and this limitation still keeps PMMA from use in more applications, especially when other materials are available.[3, 91] Furthermore, PMMA is associated with tissue necrosis due to its exothermic polymerization reaction. The heat produced during this reaction not only damages bone tissue, but has also been shown to damage soft tissues surrounding the implant area.[92] MMA has also been copolymerized with other materials in hopes of improving its toxicity issue.[3] Al Ruhaimi and colleagues explored the osteoconductive potential of BOP ("biocompatible osteoconductive polymer"; Diversified Tech International SA; Brussels, Belgium) in a histologic comparison study. BOP comprises methyl methacrylate copolymerized with 1-vinyl-2-pyrorolidone (NVP) in the form of powder with 30-100 μm crystals. As PMMA is a nonresorbable polymer implant material, the

researchers did not see significant bone growth due to the blockage of hydrolysis by the large unresorbed polymer crystals.[45]

Absorbable Polymers

Researchers have also invested time in the production of synthetic materials for *in vivo* applications. These materials can be customized to capitalize on various aspects of the polymeric implant. One of the principal requirements of polymeric implants is dimensional stability over the early stages of bone healing to allow stable deposition of osteoblasts or precursors.[90] Implant surfaces are also critical for the provision of other cell attachment and growth, enhancement of implant fixation, or implant function as a scaffold. Implant surface interaction with the *in vivo* environment largely determines the early osseointegrative results of the graft substitute.[90] Along with surface stability, mechanical characteristics are also manipulated by polymer composition, synthesis method, and implant dimensions. For example, the initial strength and stiffness of reinforced, absorbable lactide polymers can match those of cancellous bone. However, the thickness of the implant must be balanced with the geometric and anatomic restrictions at the fracture site, as well as the degradation rate of poly-L-lactide (PLL).[90, 93] Polymer reinforcement has been attempted in order to decrease implant thickness and maintain needed mechanical strength; however, each fiber type must be carefully considered for biocompatibility testing. For example, carbon fibers were employed in PLL scaffolds; however, these fibers did not degrade but rather disintegrated and began to cause mechanical irritation around the implant site.[90, 94, 95] Absorption rate manipulation is one of the main motivations for using absorbable polymers in bone applications. Figure 1.11 details degradation rates for different polymer compositions in a study by Coombes and coworkers comparing various gel-cast absorbable polymers.[96] Absorption rate is influenced heavily by morphology. Porosity facilitates fluid influx through the material,

thereby increasing the rate of chain scission by hydrolysis.[90, 97] Porosity and density also affect the release and transport of growth factors from bone graft materials.[98]

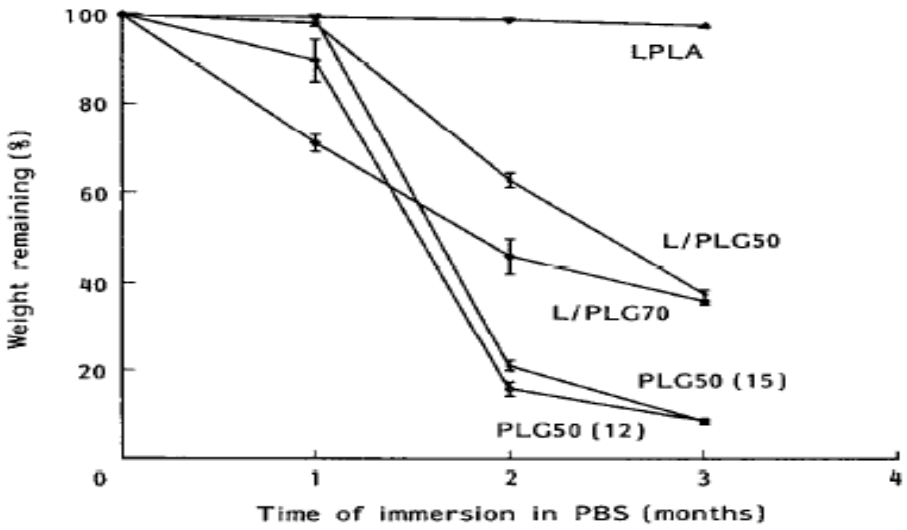

Figure 1.11: Degradation rate of gel cast absorbable polymers soaked in phosphate buffer solution (PBS) with variable compositions of each polymer.[96]

Bone Tissue Engineering Scaffolds

The previous section discussed some of the options available to clinicians when presented with bone trauma resulting in critical-sized defects. Historically, bone grafting (autograft or allograft) has been considered the gold standard in these cases. However, bone graft substitutes were developed because bone grafting is not effective in every case. Bone tissue engineering, involving some of the treatments discussed in the "Bone Graft Substitutes" section of this review, has been heralded as one of the potential bone graft alternatives able to address cases in which traditional bone grafting is not suitable.

Aside from bone grafting treatments, patients suffering bone tissue loss as a result of trauma or disease have few options other than artificial prostheses or amputation.[31] The limitations of their treatment options are compounded by comorbidities such as osteoporosis or osteoarthritis, which both limit the patient's natural bone healing capability.[31, 33] Bone tissue

engineering may help to fill this void by offering a way to restore or replace damaged bone without highly invasive donor bone harvesting, potential immunogenic responses, or mechanical property discrepancies resulting in implant failure. [34]

In their review of biomaterial developments, Burg and colleagues define bone tissue engineering as the use of scaffolding material to either induce formation of bone from the surrounding tissue or to act as a carrier or template for implanted bone cells or other agents.[34] They maintain that bone tissue engineering constructs must rely on four requirements of bone regeneration: i) morphogenetic signals, ii) responsive host cells that will respond to the signal, iii) suitable carriers that will deliver the signal to a specific site and then act as a scaffold for the growth of responsive cells, and iv) viable, well vascularized host beds.[34, 99, 100] More specifically, researchers and clinicians aim to expand osteoblasts, chondrocytes, and other MSCs obtained from the patient. These cells are then to be placed onto a scaffold that degrades or absorbs slowly in concert with tissue remodeling.[9, 34, 101] The scaffold serves to provide the necessary environment for the proliferation and maintenance of cells of a specific phenotype. Ultimately, the architecture of the scaffold helps define the geometry of the remodeled tissue, as well as its function.[101]

Scaffold Design Considerations

The basis of work focused on scaffold development is the coordination of osteoinductive, osteoconductive, and osteointegrating properties associated with each design.[2, 34] In his review of the literature, Hutmacher concludes that the ideal scaffold should: i) be three-dimensional and highly porous with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste; ii) be biocompatible and bioabsorbable with a controllable degradation and absorption rate to match cell/tissue growth *in vitro* and/or *in vivo*; iii) have suitable surface chemistry for cell attachment, proliferation, and differentiation and iv) have mechanical

properties to match those of the host tissue at the implant site.[101] However, to date, a vascularized, mechanically efficient, osteoinductive/conductive construct has yet to be developed.[31] Difficulty in realizing this achievement centers on the fundamental problem that a scaffold must have sufficiently high interatomic and intermolecular bonding to achieve the necessary mechanical strength, but must simultaneously have a physical and chemical structure amicable to hydrolytic attack or breakdown.[101] These two contradictory requirements have made achievement of the "ideal" scaffold difficult thus far.

Scaffold Geometry

Geometry is a key to scaffold design because of the three-dimensional structure of natural skeletal tissue.[102] Geometry contributes to other important scaffold characteristics such as mechanical properties or vascular ingrowth. The creation of an interconnected macro-porous structure yields triangles, hexagons, and pentagons which equally distribute mechanical forces throughout the scaffold in a manner termed tensegrity, as described by Ingber and coworkers.[103, 104] The porous structure and 3-D design also contribute to the diffusion of nutrients into the scaffold, but are not enough to independently promote cell viability in large scaffolds.[100]

Porosity can be characterized as the ratio of pore volume to scaffold material volume.[100] The resulting percentage has been reported in the literature as an indicator of transport ability for tissue engineering scaffolds, or measure of affinity for neovasculature. Some researchers have claimed that porosity should be as high as 90% to ensure satisfactory cell-material interaction.[105] Other researchers have focused on mechanical strength, only implementing porosities near 30%.[106] One of the negative aspects of introducing porosity into scaffold design is the weakening of mechanical properties with increasing porosity.[31, 33, 34] One such example is that of hydroxyapatite (HA), for which increased porosity results in decreased malleability and the inability to conform to the irregular surfaces of the host bone, thereby negatively affecting the

mechanical stability and osseointegration of the implant.[107-109] In contrast to physiological conditions, the majority of scaffolds created today have a uniform distribution of porosity throughout the construct. Anatomically, porosity is higher in the cancellous center of skeletal tissue, and is gradually decreased as the outer cortical layer is approached.[33] Future researchers should strive to create a scaffold or a fabrication method more suitable to modulated porosity.

Different from porosity, but related, is permeability. Permeability has been described as the degree to which fluid can flow through a construct.[99] This design consideration is highly related to porosity, and has often been used interchangeably in the literature. However, permeability and porosity differ in the inclusion of construct tortuosity in their empirical calculations.[100] The degree to which scaffold pores are interconnected is directly proportional to the tortuosity of fluid flow through that scaffold. A scaffold may have similar porosity, but transport, mechanical properties, or degradation characteristics may be altered due to a difference in permeability. Li and colleagues proposed a porosity/permeability ratio as opposed to porosity alone to determine the degree to which inner voids of a scaffold could be reached by fluid.[101]

Pore size has also been shown to be critical due to association with cell type specificity in culture.[34] Along with specific cell types, pore size has also been shown to affect the amount of cell/tissue growth in a construct. One baseline for researchers in the search for optimal pore size was proposed by Holmes nearly 30 years ago while studying bone regeneration with a coralline hydroxyapatite implant. Holmes suggested that the optimal pore size was between 200-400 μm due to an average human osteon size of approximately 223 μm.[110] Tsuruga and colleagues suggested that the optimal pore size of HA scaffolds is between 300-400 μm as evidenced by ectopic bone growth in a rat model.[111] However, as mentioned earlier, pore size should not be uniform throughout a scaffold. Both macro (>100 μm) and micro (<20 μm) pore structures are needed for an effective scaffold.[33, 112] Macro-sized pores are thought to contribute to osteogenesis

by facilitating cell and ion transport.[113] Micro-sized pores are thought to improve bone growth into scaffolds by increasing surface area for protein adsorption and subsequent cell attachment.[114] Micropores are also thought to act as attachment sites for osteoblasts depositing bone.[113] In a study using directed deposition of HA rods, Woodard and coworkers compared the bone growth capability of non-microporous (NMP) scaffolds with that of microporous (MP) scaffolds. Implantation of scaffolds impregnated with recombinant human bone morphogenetic protein-2 (rhBMP-2) into Yorkshire pigs yielded more significant bone growth for the MP case after 8 weeks.[112] Figure 1.12 below illustrates results from this study.

Figure 1.12: Scanning electron micrograph of explanted scaffolds after 8 weeks. Scaffolds were treated with dilute bleach and heated to 427°C to remove soft tissue. A) Shows NMP scaffold with no bone formed in macropores. B) Significant bone growth shown in macropores, with 10-30 μm pores inside the formed bone, most likely from vasculature. C) Detailed view of formed bone, with 2-8 μm pits (indicated by arrows), most likely from cell-mediated remodeling. Scale bars are 500 μm (A,B) and 20 μm (C).[112]

Pore shape optimization and cell-specific pore topography are also critical components of scaffold geometry due to the profound effects they can have on cell or protein attachment, which translates to the long-term survival of cells on the construct. [34] This assertion is reinforced by the observation that bone differs in structure based on location and function, suggesting that pore shape optimization should take into account those parameters. Similarly, order versus disorder in polymer pore geometries may also have an effect on the quality and quantity of bone formed

around a scaffold.[33] In a study focused on the differences between bone formations on a preordered HA scaffold with collagen fibers concentrated around its pores and a more random nanofibrous collagen-based sponge, Scaglione and colleagues found that the ordered scaffolding produced compact lamellar bone, while the disordered scaffold produced woven bone. These results suggest that pore shape and configuration play a key role in the resulting formation of bone.[115] Likewise, pore interconnectivity has been shown to positively influence bone deposition rate both *in vitro*[113] and *in vivo*[116]. Regularly connected pores offer the spacing needed for vasculature responsible for the nourishment of new bone and removal of waste products.[114, 117] For this reason, interconnectivity has also been associated with increased depth of bone formation in implant sites.[113] The perfect combination of pore size, pore shape, and interconnectivity for osteoconduction has yet to be discovered[112]; therefore, having the ability to modulate these characteristics in the fabrication step would be advantageous to researchers.

Promotion of Vascularity

One of the principle functions of a bone tissue engineering scaffold is the encouragement of vascular ingrowth. Although an interconnected macropore structure ranging from 200-500 μm may improve diffusion rates throughout the scaffold, simple transport of nutrients and by-products is not sufficient for large defect sites.[101] In these cases, a highly vascularized bed at the defect site may ensure the survival and function of the seeded cells until sufficient proliferation has occurred.[118] In the body, the distance between MSCs and blood vessels is less than 100 μm.[19] Because of this short distance, *in situ* vascularization may be compromised without special design consideration for vascularity, beyond the expectation of capillary ingrowth resulting from the inflammatory wound healing response.[33, 101] Researchers and clinicians have begun to embed angiogenic factors into scaffold materials with the goal of controlling the rate and degree of vascularization at the implant site. VEGF is one of the growth factors researchers are using to

induce a more substantial vascular network at the implant site.[33, 119] The release of VEGF, in coordination with the degradation of the scaffold material, serves to maintain the appropriate temporal relationship between cell proliferation and removal of scaffold material.[120-122] Figure 1.13 displays a schematic of an experiment by Keeney and colleagues depicting a CaP/Collagen scaffold that acts as a gene delivery system for a nonviral vector carrying angiogenic genes. Transfection of the complex resulted in the expression of angiogenic factors such as VEGF.[123]

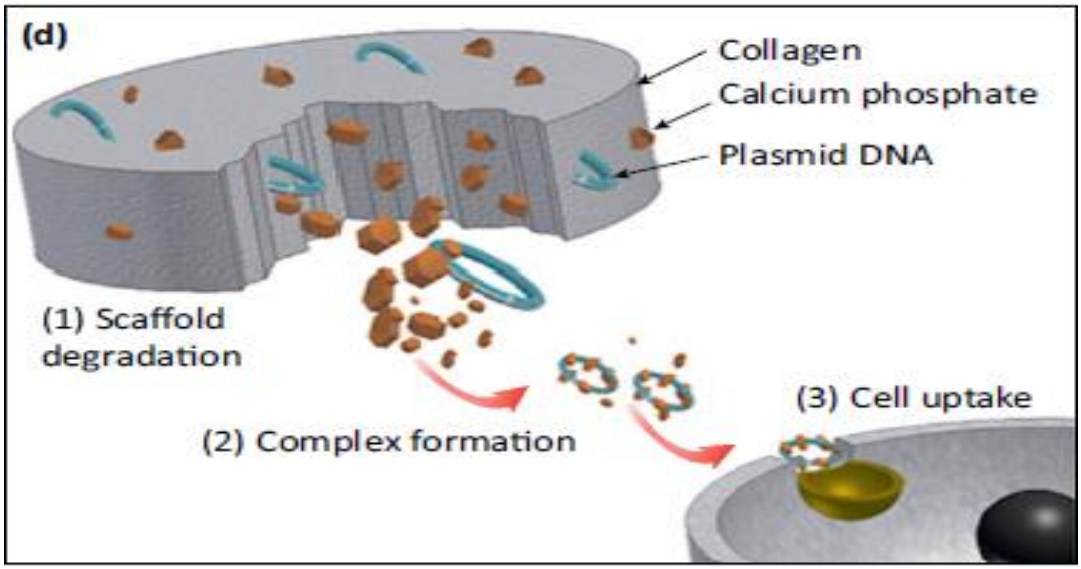

Figure 1.13: Schematic of the degradation of a CaP/Collagen scaffold containing plasmid deoxyribonucleic acid (DNA) for angiogenic factors. Upon degradation, a complex forms and is taken up by the cell, resulting in expression of angiogenic proteins and the development of vasculature.[123]

Vasculature and fluid flow does not only affect nutrient and waste transport. Fluid shear stress of the interstitial fluid surrounding the implant may also result in the remodeling of bone or the lack thereof.[124] Reich and colleagues hypothesized that bone fluid flow, by means of shear stress, is the mechanism mediating the signal in mechanical loading-induced and injury-induced remodeling.[125] The results of this study suggested that, in addition to enhancing the transport of

nutrients to cells deep within the scaffold, bone fluid flow also affects bone cell function and remodeling as it relates to the growth and differentiation factors sensitive to mechanical stimuli.[125]

Mechanical Properties

Researchers and clinicians have long agreed that the mechanical properties of a tissue engineered construct play a significant role in the efficacy of an implant both *in vivo* and *in vitro*.[2, 7, 9, 20, 34, 101, 103, 126] While this concept is accepted for all tissue engineering applications, researchers have paid particular attention to the role of mechanical properties in musculoskeletal applications. The primary concern is that the scaffold design accommodate the mechanical properties observed *in vivo* until tissue ingrowth of the implant allows the assumption of its proper functional role.[101] The absorption and degradation rates of the scaffold material are important in this strategy, as they must mirror the remodeling of host bone tissue. Lack of coordination between these phenomena may result in implant failure under physiologic mechanical stresses, or the failure to produce sufficient bone tissue as a result of stress shielding by the implant.[34] Researchers have used *in vitro* testing to understanding how dynamic mechanical conditions seen *in vivo* may affect the degradation rate, and subsequent release of factors used to promote bone deposition. Thompson and coworkers studied a poly(D,L-lactide-co-glycolide) matrix under cyclic compressive loading similar to stresses observed during slow walking during rehabilitation.[127] Their results showed that, when compared with a non-loaded control, the rate of molecular weight loss decreased and protein release from the scaffold (modeling growth factor delivery) increased in a linear fashion proportional to the rate of molecular weight loss.[127] Information gathered from *in vitro* testing, has served to help researchers develop material that may more closely parallel *in vivo* conditions. Figure 1.14 shows the results from this study.

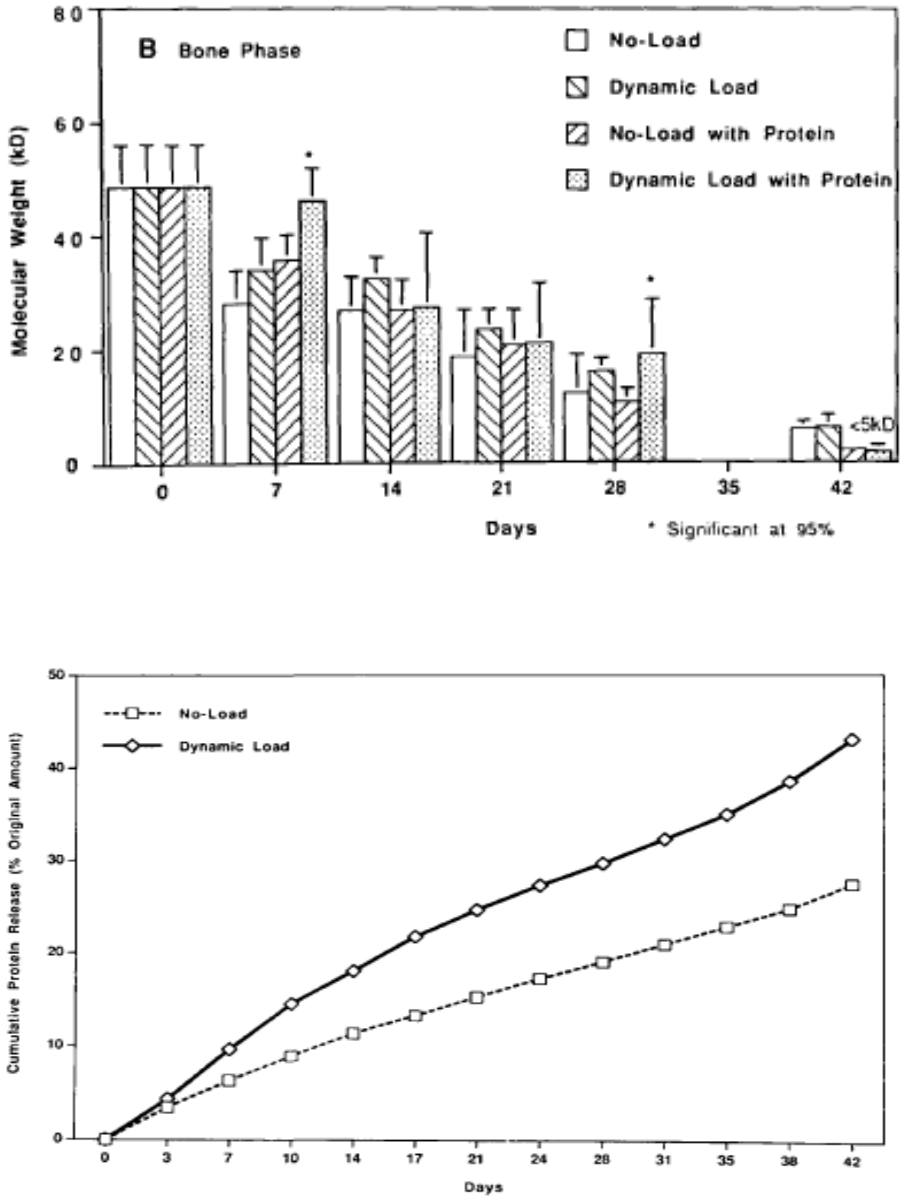

Figure 1.14: The graph on the top displays the molecular weight loss with time. The loaded conditions can clearly be seen to decline at a rate lower than that of the non-loaded conditions. The bottom figure shows the corresponding protein release with time. The loaded case shows a release rate faster than that of the non-loaded conditions.[127]

Research has also centered around the idea that dynamic mechanical loading contributes to cell differentiation and the determination of cell phenotype.[34, 103] For these researchers,

mechanical properties and flow dynamics are a means to achieving the desired cell type or phenotypic expression of harvested MSCs. However, upon implantation of a construct developed using this thought process, issues with mechanical property compliance are still present.[101, 126] A mismatch of mechanical properties at the implant site may still lead to implant failure, stress shielding leading to nonunion, or increased inflammatory response.[9, 21, 34, 47] Researchers, such as Meinel and colleagues, have leveraged mechanical conditions to attempt to assist in differentiation of harvested MSCs.[128] Meinel used a combination of dynamic flow conditions and scaffold structure to compare the osteoblastic differentiation and calcium deposition in the collagen scaffolds. The harvested MSCs were cultured *in vitro* for 5 weeks under dynamic (bioreactor) and static (control) conditions. An alkaline phosphatase (ALP) assay, along with calcein staining and histology, was used to assess efficacy of the scaffolds. Results showed that the 2-D films and 3-D scaffolds had similar calcium deposition in static culture, while dynamic culture resulted in significantly higher values than those determined in static culture. Figure 1.15 shows the results for this study.[128]

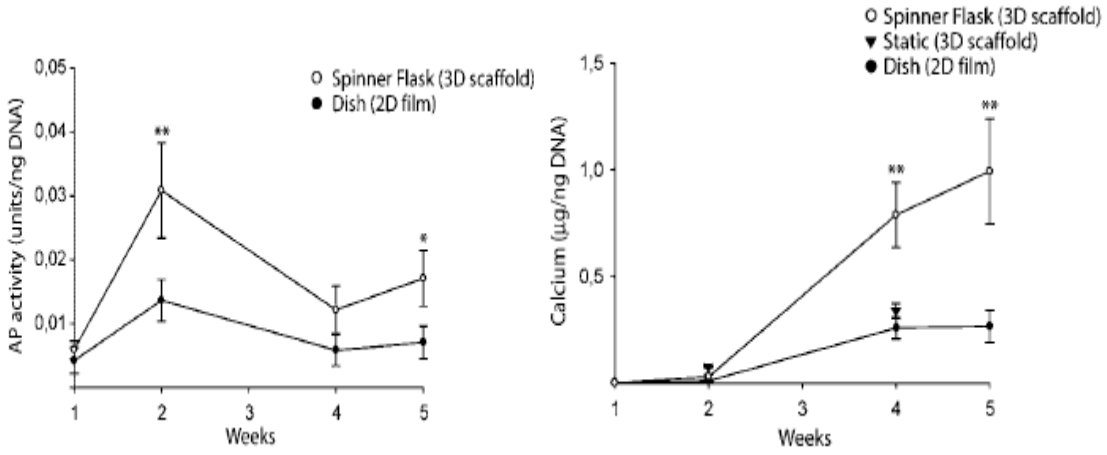

Figure 1.15: A) ALP activity of the cells in the spinner flask (dynamic) conditions versus that of cells in the dish (static) condition. ALP activity is higher for the dynamic condition, suggesting

more osteoblastic differentiation. B) Calcium deposition via calcein staining shows similar results for both static conditions, but improved output for the dynamic condition.[128]

Scaffold Biocompatibility

As with any medical device coming in direct contact with the body, biocompatibility is also a major focus for bone tissue engineering researchers. The general premise surrounding implantable constructs is that more reactive foreign material implanted yields a larger inflammatory and immune response.[126] Using this logic, highly porous implants, as suggested by Yoon and colleagues[105], would be ideal. However, bone tissue engineers also have to take mechanical strength into account. There is a balance between the need for reactive material minimization and the need for enough material to provide adequate mechanical strength. This balance has yet to be determined by researchers and clinicians.[31] Researchers have however, explored the biocompatibility of several scaffold materials. Upon degradation, the remaining by-products may be basic or acidic. The increase or decrease of local pH at the defect site may lead to cell death and eventually tissue necrosis.[9, 126] This is especially true with bulk degrading materials (most aliphatic polymers) that may exhibit a burst release of acidic by-product at a critical value.[34] To combat this change in pH researchers have sought to characterize release profiles, and understand more about degradation by-products.[26] However, some researchers, such as Shikinami and Athanasiou, have sought to take advantage of shift in pH and composite technology to create a self-buffering system at the implant site.[129, 130] Shikinami used a HA/PLL composite scaffold to demonstrate the effectiveness of HA basic degradation product buffering the lactic acid by-product of PLL.[129] Composite scaffold designs, along with elements to control release and degradation rate, are much of the focus in bone tissue engineering research.

Surface Modification

Cell-biomaterial interactions are some of the chief variables in implant integration. Researchers have focused on the improvement of these interactions at the scaffold surface by manipulating surface chemistry and topography.[131, 132] While both natural and synthetic materials have been used in bone grafting and bone tissue engineering applications, synthetic materials have the disadvantage of lack of biological recognition. Hydrophobic materials, specifically polymers, are not readily integrated due to incompatibility with the hydrophilic outer region of the phospholipid bilayer component of the cell membrane.[133] Engineering the hydrophobicity/hydrophilicity of the material surface has been attempted to change cell interactions on the micro- level. Researchers have attempted to make surfaces more hydrophilic, with an increasingly negative charge due to the relative positive charge of the extracellular surface of the plasma membrane.[19, 134] Oh's group was able to demonstrate improved bone ingrowth by increasing the hydrophilicity of a poly-L-lactide-co-glycolide (PLG) implant by blending polyvinyl alcohol (PVA) in a melt-molding, particulate leaching process to create a porous scaffold.[135]

In bone tissue engineering applications, cell affinity for the scaffold material surface may also be critical in the mechanical stability of the implant, especially with degradation of the scaffold occurring in parallel with tissue ingrowth.[132] Mechanical stability can be tied closely to the level of osseointegration shown by the scaffold.[136] The amount of bone contact and appositional growth is directly related to scaffold topography and surface chemistry, where cell morphology and differentiation depend on the ability of the cell to attach, spread, and communicate on the material surface.[137, 138] Without the attachment of new bone to the scaffold, instability and subsequent micro-motion may lead to an increased inflammatory response such as a chronic foreign body reaction.[137, 138] Some researchers have investigated coating composite

scaffolds involving bioactive ceramics. Development of methods to coat scaffolds with a uniform layer of bioactive ceramic material has proved difficult, but has been mildly successful.[139] In one study by Barrère and colleagues, which aimed to combat mechanical instability surrounding dense and porous Ti6Al4V and Ta implants, researchers used a coating of calcium phosphate to improve direct bone contact and ingrowth. The calcium phosphate was added in one uniform 30 μm layer to create a surface topography more closely related to natural bone.[140] The disadvantage of these systems is the additional variability of adequate bonding between the coating and the underlying material, especially during degradation of the scaffold.[141]

Polymeric Scaffold Considerations

While much of previous discussion has been centered around the osteoinductive, osteoconductive, and osteogenic capabilities of each system, this section will highlight some of the materials more specific to bone tissue engineering. There are two general approaches to the development of a scaffold for the ingrowth of host bone tissue. The first is the acellular approach in which no extraneous biological components are added to the scaffold prior to implementation. These materials may be solid absorbable bone void fillers or porous scaffolding for the ingrowth of vasculature and new host bone.[34] The second approach is a cellular one in which a cellular component is added to the scaffold to encourage bone ingrowth or the recruitment of other biological signals to augment bone healing.[34] Both cellular and acellular designs have incorporated drug delivery[34] as well. The following section will focus on polymeric scaffold designs, including both natural and synthetic polymers, which have been employed for tissue engineering scaffolds. Natural polymers such as collagen or chitosan have been used as the basis for designs and are able to avoid issues with biological recognition.[142] However, mechanical properties suffer without the researcher's ability to hone the characteristics of the natural polymer to a specific application.[142] Synthetic polymers, on the other hand, give researchers the

opportunity to fine tune material characteristics by creating copolymers and polymer blends that more closely accommodate the cell culture/implantation environments.[143] In the sections following, bone tissue engineering qualifications as well as recent developments will be discussed for synthetic polymers.

Poly(glycolide)

Poly(glycolide) (PG) may be considered one of the most popular synthetic polymers explored by bone tissue engineering researchers.[143] This aliphatic polyester is often synthesized through ring-opening polymerization of the glycolide monomer catalyzed by antimony, tin, or zinc.[144] The resulting high molecular weight polymer is considered to be generally biocompatible[100, 143, 145], with glass transition temperature (T_g) ranging from 25°C-65°C and melting temperature (T_m) ranging from 185°C-225°C, as characterized by differential scanning calorimetry (DSC).[146] The chemical structure for PG is shown in Figure 17. PG is highly crystalline in structure, and also hydrophilic in nature.[145] The hydrophilicity of this polymer contributes to a rapid absorption rate (complete absorption in 4-6 months) and the subsequent loss of mechanical strength in an aqueous environment.[145] Degradation generally occurs through hydrolysis of ester linkages or by nonspecific esterases *in vivo*, resulting in the release of glycolic acid.[147] Glycolic acid is naturally eliminated from the body, usually via urination.[147] Many studies have been conducted using PG as a scaffold material for bone tissue engineering applications. Recently, Cao and Kuboyama explored the application of PG in a composite PG/β-TCP scaffold with a 1:3 ratio.[148] These researchers assessed the formability and degradation characteristics of PG in combination with the appositional bone growth encouraged by β-TCP. The composite scaffold was compared to a hydroxyapatite (HA) implant and no implant in a rat model.[148] Significant mineralization and degradation rates, corresponding with osteogenic rates, were shown via histology and image analysis.[148]

Poly(lactide)

An equally popular synthetic polymer highlighted in the literature is poly(lactide) (PL). PL is also an aliphatic polyester, most often synthesized through the ring-opening polymerization of lactide monomers.[144] PL is similar in structure to PG, except that PL has a methyl side group which significantly changes the degradation profile and chemical and physical characteristics of the material.[145] This methyl group allows chirality on the alpha carbon of PL (structure shown in Figure 1.17).[145] D, L, and D,L variations are available, with PLL being used most often in tissue engineering research.[149] PLL is generally less crystalline than PG due to the added methyl side group, and has a T_g around 65°C and a T_m between 170°C-180°C.[150] Degradation mainly occurs via cleavage of ester linkages during hydrolysis, resulting in lactic acid, which is also naturally eliminated from the body. Lactic acid is most commonly eliminated through respiration in the form of CO_2.[151] Bone tissue engineering literature has numerous works regarding the use of PL as a scaffold material. In one of the more recent works, Seyedjafari and colleagues compared electrospun PLL nano-fibers in a nonwoven mesh configuration with HA-coated PLL nanofibers of the same configuration.[152] Each scaffold was tested for alkaline phosphatase (ALP) activity, mineralization, and ectopic bone growth. Results showed that the coated fibers directed stem cells toward osseous lineages more frequently.[152]

Poly(lactide-co-glycolide)

The similarity of structure combined with the ability to modulate scaffold response motivated researchers to combine PL and PG into a PLG copolymer. PLG has been highly implemented by researchers over the history of bone tissue engineering scaffold development, most often due to a researcher's ability to control degradation rate by adjusting the concentration of each polymer component.[153] Figure 1.16 below illustrates the relationship between PL content, PG content, and material half-life *in vivo*.[154] The changes in degradation rate by the methyl

pendant group on the alpha carbon of PL are a significant factor in the determination of degradation rate for a PLG copolymer.[143] Mechanisms for degradation are similar to those of the homopolymer components.[143] Figure 1.17 shows the chemical structure of a PLG copolymer.

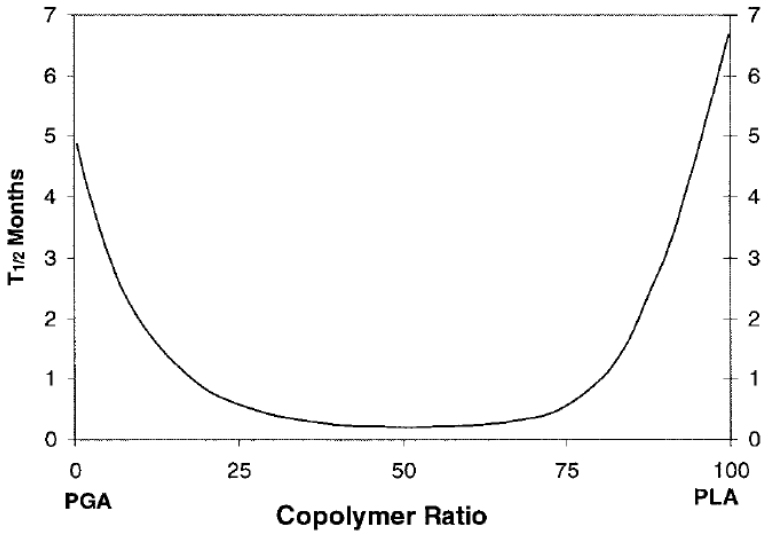

Figure 1.16: Half-life of PLG copolymer based on homopolymer concentration.[154]

Poly(ε-caprolactone)

Due to the extended degradation of time, poly-ε-caprolactones (PCL) have become increasingly popular in drug delivery applications but have also been sparingly implemented in orthopedic applications such as those involving fixation devices.[155] The focus on PCL as a drug delivery vehicle stemmed from research regarding the extended degradation rate of the polymer. Degradation of the polymer by hydrolysis, allows a much slower rate than that of PG, PL, or PLG.[145] PCL is also synthesized by the ring-opening polymerization of the ε-caprolactone monomer.[145] The series of methylene bonds yield a polymer much softer than PG or PL, with T_g of -60°C and T_m between 58°C-63°C.[156] For this reason, many of the attempts to implement PCL as an independent load-bearing device have been unsuccessful.[143] However, researchers have created composite scaffolds and copolymers to improve the resiliency of a scaffold or to modify

degradation rate.[157] One such example is the work of Canillo and coworkers. This experiment combined the appositional bone growth capability of Bioglass ® 45S5 with the soft qualities of PCL to produce a bone tissue engineering scaffold.[158] *In vitro* testing explored mechanical properties, cytotoxicity, and scaffold morphology in preparation for further cell-based studies.[158]

Polyanhydrides

Unlike all other synthetic polymers mentioned in this section, polyanhydrides degrade by surface erosion and not bulk erosion.[143] The high sensitivity to hydrolysis results in rapid and predictable degradation that has motivated researchers to explore delivery applications for these materials.[143] Furthermore, issues with burst release of acidic degradation products are avoided through the surface degradation characteristics of these materials.[145] Polyanhydrides are able to maintain implant structure and shape over an extended period, also due to the surface degradation properties.[159] Ibim and colleagues conducted a study comparing the biocompatibility and bone regeneration capability of poly(anhydride-*co*-imide) and PLG.[160] The similarity of polymer mechanical properties to that of cancellous bone in load-bearing applications was investigated by implanting each polymer into rat tibias and evaluating, through histology, the presence of inflammatory cells and bone formation.[160] Results showed that poly(anhydride-*co*-imide) was similar in response to PLG, indicating potential viability of the material.[160]

Poly(carbonate)

These materials are also aliphatic in nature and are able to degrade under physiological conditions.[143] However, between 40°C-60°C, these materials become extremely soft and unfit for load-bearing applications.[143] As a result of this softening, poly(BPA-carbonate) was developed as a stable, essentially non-degradable material with a high degree of processability, high mechanical strength, and exceptional shatter resistance.[161] In order to increase biocompatibility, hydrolytic stability was decreased by replacing a carbonyl oxygen with an imino group.[162] The

resulting fibers are hydrolytically degradable and similar in strength to those made of poly(BPA-carbonate).[162] These fibers were also found to be biocompatible *in vivo* due to the chemical proximity of the degradation products to amino acids.[162]

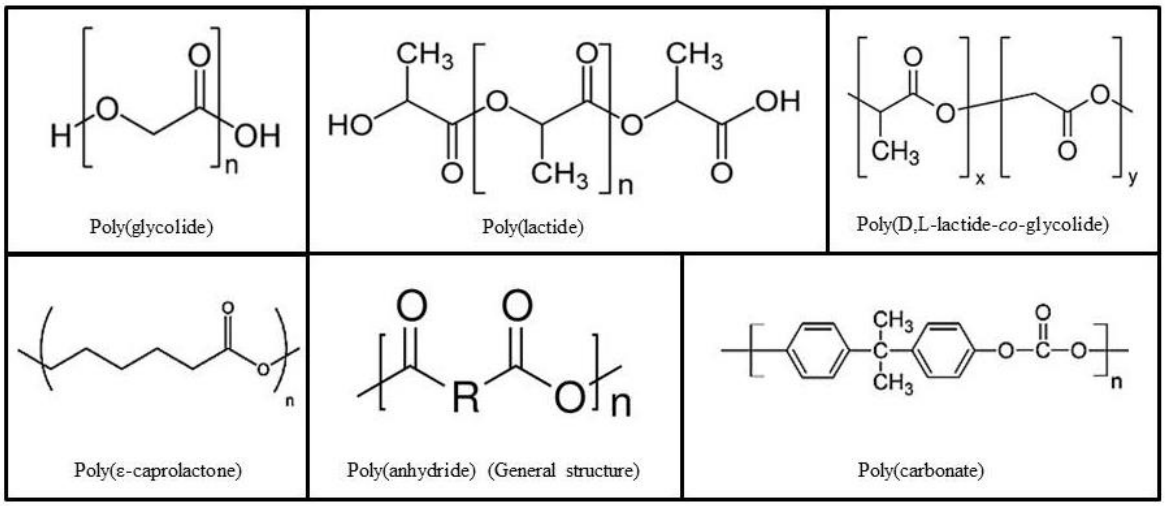

Figure 1.17: Chemical structures for synthetic polymers mentioned in the section above. (www.sigma.com, www.wikipedia.com)

Scaffold Fabrication Methods

Each one of the design considerations discussed previously is highly linked to the method of fabrication. Different fabrication techniques allow researchers to highlight certain material characteristics and augment specific design considerations based on the requirements of that device.[145] Researchers have used various fabrication techniques to modulate pore size, porosity, mechanical properties, and pore interconnectivity.[100, 143] By manipulating variables such as the use/or nonuse of solvents, heat, pressure, and pore-creating additives, researchers have been able to develop increasingly complex 3-D scaffolds for bone tissue engineering applications.[143] This review highlights several fabrication techniques currently used, as well as some methods that have served as a foundation for more recent advances in scaffold development.

Solvent Casting and Particulate Leaching

Solvent casting and particulate leaching (salt leaching) is one of the fundamental techniques for the development of porous tissue engineering scaffolds. Through previous advances in processes such as extrusion and injection molding, the development of solid orthopedic implantable devices (plates, screws, rods, etc.) has become relatively straightforward. However, these methods present problems when there is a need for porous macrostructure, or when additives cannot withstand the high heat and pressure associated with these methods.[143] Many reviews have cited Mikos and colleagues with one of the earliest demonstrations of scaffold development via solvent casting and particulate leaching.[34, 143, 145, 163] This technique involves mixing a water soluble particulate/salt (i.e. sodium chloride, sodium citrate) with a biodegradable polymer solution. The solution is placed in a mold of the desired shape, and the solvent is removed via evaporation or lyophilization. Finally, the salt is leached from the remaining polymer structure to yield pores.[163] Mikos and coworkers accomplished this by dissolving PLL and PLG in chloroform and adding particles of sodium chloride. The chloroform was removed via vacuum evaporation, and deionized water was used to dissolve out the sodium chloride particles.[164, 165] These steps resulted in the formation of a porous scaffold, which served as the basis for a later experiment by Thomson, Mikos, and colleagues in which trabecular bone was engineered using these scaffolds.[166]

Solvent casting and particulate leaching is one of the most employed methods of scaffold fabrication because of its simplicity and ability to produce consistent pore sizes.[100] Pore size and porosity can be readily controlled by particle size of the salt added and salt/polymer ratio. However, pore shape is confined to the cubic shape of the salt crystal added.[163] Further issue arises with the difficulty in removing salt particles embedded within the polymer matrix. This has limited the thickness of scaffolds created in this method, which must be laminated together to

achieve adequate thickness for orthopedic applications.[145, 163, 167] Liao and colleagues claim that scaffolds fabricated by this method range in thickness from 0.5 to 2.0 mm generally.[167] Beyond the issues with scaffold thickness, limited pore interconnectivity also contributes to inconsistent results for testing in bioreactors and *in vivo*.[168]

Gas Foaming

Another fabrication technique used in scaffold construction is gas foaming technology. Gas foaming is often employed to create highly porous polymer scaffolds without having to use organic solvents.[143, 163] Organic solvents, such as chlorofluorocarbons (CFCs), have been shown to be toxic, presumably because their hydrophobicity disrupts the cell membrane and alters permeability characteristics of the cell.[169, 170] For this reason researchers have shied away from these materials, and looked to the use of compounds such as carbon dioxide (CO_2) as a solvent. Gas foaming is often accomplished through the saturation of solid polymer discs with CO_2 under high pressure. Thermodynamic instability within the embedded gas is created when the applied pressure is lowered to atmospheric conditions. The result is the rapid escape of gas from the polymer, thereby creating bubbles that become pores within the polymer matrix.[171] Mooney and colleagues report in one of their gas foaming experiments the creation of pores approximately 100 μm with porosities approaching 93%.[172] However, there have been disadvantages reported with this technique, including the lack of pore interconnectivity[170] and the absence of surface porosity.[172] Some researchers, such as Murphy and coworkers, have attempted to augment pore interconnectivity in these scaffolds by adding a salt-leaching component to the design. The fusion of NaCl crystals at 95% humidity within the polymer matrix prior to gas saturation, followed by the foam creation and leaching of the salt, has yielded increased pore interconnectivity with similar porosity measurements (94%).[173] Figure 1.18 demonstrates the change in crystal structure during fusion, and the resulting pore interconnectivity using this approach.[173]

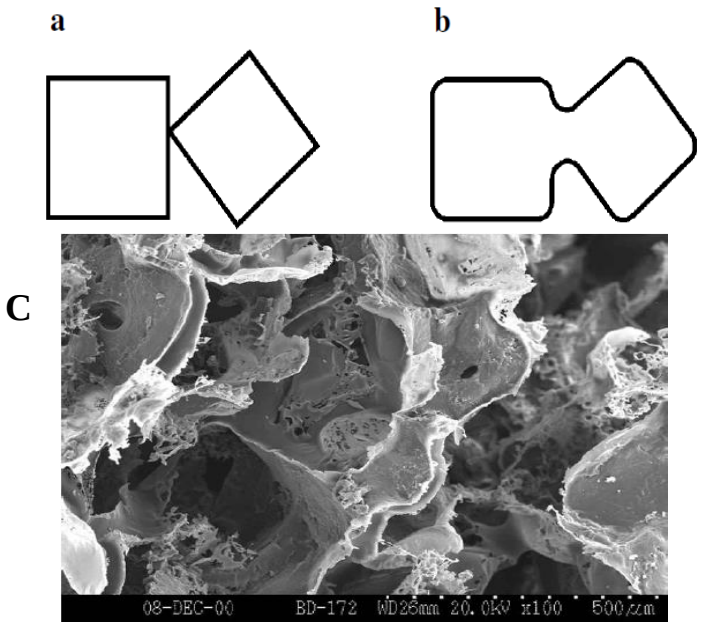

Figure 1.18: (a) and (b) show the effect of fusion at 95% humidity on salt crystals. (a) The intersection of two crystals is a sharp edge with limited connectivity. (b) Connectivity and intersection is greatly improved. (c) Electron micrograph showing the results of a gas foaming-salt leaching approach after 24 hour salt fusion. Interconnectivity of pores is significant.[173]

Rapid Prototyping

One of the most popular and versatile methods of scaffold development is a group of technologies collectively referred to as rapid prototyping (RP), or solid free form fabrication (SFF).[174] RP technologies build 3-D objects using a layering technique in which a computer-aided design (CAD) system is used to create a series of cross-sections. Each cross-sectional layer of the scaffold is deposited onto the previous layer resulting in the completed design.[100, 143, 163] The advantage of RP systems is the potential for fine control of the microstructure and macrostructure of the scaffold due to use of a CAD file. However, physical system limitations and material type limitations make the realization of this precision difficult currently.[175] The umbrella of RP techniques includes several specific techniques such as: fused deposition modeling (FDM), selective laser sintering (SLS), and 3D printing (3DP). [100] FDM uses a moving nozzle to extrude a

polymer fiber in a horizontal pattern specified in the CAD file. Once that layer is complete, the platform is lowered so that the next layer can be deposited.[176] SLS builds scaffolds by employing an infrared laser to selectively raise the local temperature between two powders.[177] The laser provides enough energy to raise the powder to its glass transition temperature where the particles can then fuse to each other and to adjacent layers.[175] 3DP is a method that prints an ink jet binder onto the surface of a ceramic, polymer, or composite powder surface.[178-180] The printer head is controlled by cross-sectional directions via the CAD file. Particles of the powder surface fuse together as they are dissolved by the binder.[181] 3DP is currently limited by the resolution capability of the system which is dependent on the size of the ink jet, making the design of small microstructures difficult[175] Figure 1.19 shows the results of applying 3DP technology to a 2-D system. Researchers using these systems hope to stack multiple 2-D constructs together to create a 3-D scaffold.[182]

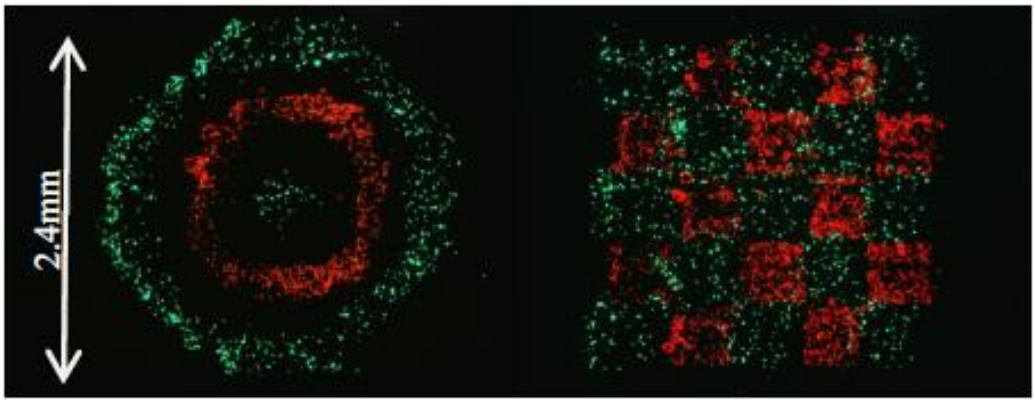

Figure 1.19: 2-D printing of D1 murine MSCs (green) and 4T07murine mammary tumor cells (red) in co-culture. Magnification is 2.5x.[182]

Thermally Induced Phase Separation

Thermally induced phase separation was first used in the creation of porous membranes, but has now been used to create porous polymer scaffolds.[163] The general process for this technique begins with the polymer being dissolved in solvent at a high temperature. Phase

separation (solid-liquid, liquid-liquid) is induced by lowering the solution temperature. The resulting precipitate is then removed, yielding a porous polymer scaffold.[183] Pore size and shape can be modulated by adjusting the solvent used, temperature gradient during phase separation, polymer used, and concentration of polymer solution.[163] Advantages of this technique lie in the ability to change scaffold characteristics based on processing variables and phase separation type. Examples include the improvement of mechanical properties for a poly-L-lactide (PLL) scaffold prepared by phase separation versus salt leaching[184], or the creation of a microtubule pore configuration based on solid-liquid phase separation and a uniaxial temperature gradient during processing.[185] However, the major disadvantage of this method is that pore sizes generally range between 10-100 μm, which is not sufficient for osteoblastic differentiation and proliferation.[183]

Electrospinning and Fiber Weaving

Other than porous foams and large solid constructs, researchers have also explored the use of fibers in the creation of tissue engineering scaffolds for orthopedic applications. One way that researchers have looked to implement fibers is through electrospinning. Electrospinning is the process by which polymer nano-scale fibers are formed when the application of an electric field to the surface of a polymer solution creates forces large enough to overcome the surface tension of the solution. The result is an electrically-charged polymer jet, which solidifies into a nano-fiber as it is ejected, and can be manipulated by alternating electrical forces to form various shapes and constructs.[186, 187] Much of the work surrounding scaffold design using fibers has been focused on the engineering of cartilage.[100, 188] However, some researchers such as Yoshimoto and colleagues have looked to use electrospun non-woven scaffolds for bone engineering. Despite reporting somewhat irregular fiber diameter and surface texture, Yoshimoto and coworkers successfully cultured neonatal rat MSCs to osteoblastic differentiation on poly-ε-caprolactone (PCL) 400 nm (±200 nm) fiber diameter scaffolds.[189] Kim and coworkers sought to improve the

performance of materials such as poly-(D,L-lactide) (PDLL), which have limitations due to lengthy degradation time, mechanical stiffness, and hydrophobicity. Kim's group used electrospinning as a method to create miscible blends of PL with more compatible polymers, such as PLG, to improve the overall mechanical, degradation, and biological characteristics of the nonwoven construct.[190]

The random nature of pore sizes produced from nonwoven constructs has been, to some researchers, a point of advantage in the development of orthopedic tissue engineering scaffolds.[191] But the random nature of nonwoven systems has led other groups to explore the effects of a highly regulated fiber-based system resulting in predictable pore sizes and porosities. Some of these researches have looked to use textile technology to create woven fiber-based systems from a variety of polymers. Groups such as those of Moutos and Valonen have used PCL yarns to create woven 3-D scaffolds for the culture of chondrocytes.[192, 193] Their ability to create these scaffolds is attributed to previous work of Moutos and coworkers in the development of a custom-built loom designed to create textile scaffolds of orthogonally-arranged, micro-scale polymer yarns with variable "tightness" of the weave.[194] In the future, similar technology may be used to modulate other characteristics of textile-based designs, such as material type, fiber structure, pore size, and weaving configuration. Figure 1:20 below compares the nonwoven scaffolds often created via electrospinning, with woven designs created with custom looms.

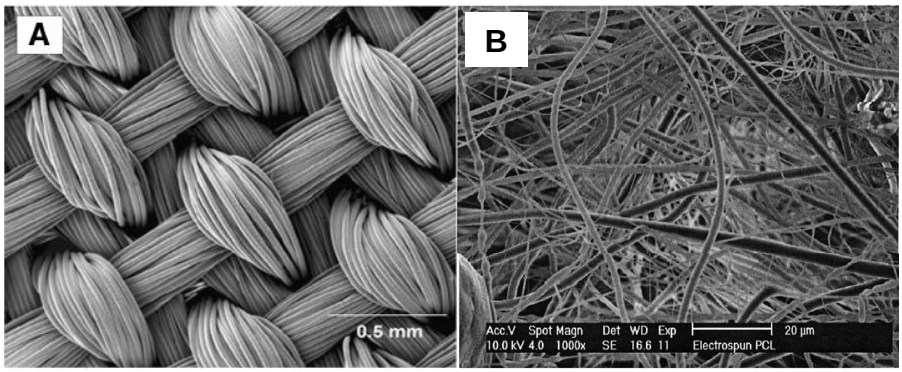

Figure 1:20: (A) Electron micrograph of woven scaffold constructed from PCL yarns on custom-built loom, with a "loose" weave configuration.[192](B) Electron micrograph of nonwoven electrospun PCL nano-fibers.[195]

Biomedical Mesh Characteristics and Applications

Mesh designs in particular have been used to address issues where scaffold architecture must match multi-faceted *in vivo* environments. For example, hernia environments require elasticity, strength, and compliance with the abdominal wall and porous scaffolds for tissue ingrowth. Pore size, material type, surface topography, and porosity all can be manipulated to suit conditions in the body. Mesh mechanical properties and degradation characteristics also come into play. The advantage of meshes is the ability to quickly customize a scaffold beyond high level factors such as material type and surface properties. Although not discussed in this review, meshes have also been employed for drug delivery applications in a similar manner as other scaffold materials such as films.

Typically, surgical meshes are designed with the following considerations. Material type is selected based on the proposed implant site, with mechanical and biocompatibility properties being considered.[196] For example, titanium meshes are used in bone applications, and not in soft tissue applications, such as hernia repair where the difference in modulus may result in adverse reactions.[196, 197] Similarly, a researcher may choose not to design a system with potentially toxic degradation products to be implanted in an area with low vascularity and the inability to remove the material from the implant site.[196] Porosity and pore size of the material are also considered. Meshes may have random pore sizes (non-woven meshes), or regular pore sizes and porosities (woven meshes).[194] Again, porosity facilitates tissue ingrowth by allowing revascularization through angiogenesis, improved transport of nutrients and waste, and cell attachment.[198] Many studies aim to validate or to further explore the ideal porosity and pore size for particular

applications.[199, 200] Degradation rate of mesh materials, in the case of bioabsorbable scaffolds, is also critical. As with other scaffold types, potentially harmful degradation products and degradation rate in concert with tissue ingrowth are of primary concern.[196] The combination of all design criteria is included in the biocompatibility requirements for surgical meshes, with the goal of minimizing an extended inflammatory response that results in chronic foreign body reaction.[196] Generally, increased amounts of scaffold material lead to larger inflammatory and immune responses.

Soft Tissue Surgical Mesh Applications

The most common application for mesh scaffolds is hernia repair. The first use of a mesh for hernia repair was reported by Usher and colleagues using a polyethylene scaffold.[201] Since the initial use of this polyethylene mesh, there has been much advancement with meshes being constructed from poly(propylene) (PP), poly(glycolide) (PG), and expanded poly(tetrafluoroethylene) (ePTFE).[202] Beyond material type, densities, pore sizes, and elasticity have also been modulated. One of the major debates currently in the area of mesh hernia repair is the argument between "heavyweight" and "lightweight" meshes.[202, 203] Unfortunately, there has been no official consensus on a particular definition for either one of these terms. Rather, physicians and researchers have characterized "heavyweight" meshes as those having relatively smaller pore sizes, usually with more material stiffness.[202, 204] These meshes are meant to provide maximal mechanical strength through the production of maximal scar tissue.[205] Due to the smaller pore sizes, there is more polymer material present, resulting in a larger surface area and increased foreign body reaction.[202] For this reason these meshes are associated with a higher incidence of complication and shrinkage.[204, 206] Conversely, "lightweight" meshes are traditionally considered to have relatively larger pore sizes, generally being more flexible.[202] Figure 1:21 contrasts the two hernia meshes. The more flexible "lightweight" meshes are not necessarily weak in mechanical

strength due to material properties, and tend to cause a lessened inflammatory response due to the reduced surface area.[203] The flexibility and elasticity of these meshes allows them to be more associated with return to normal abdominal wall function.[207]

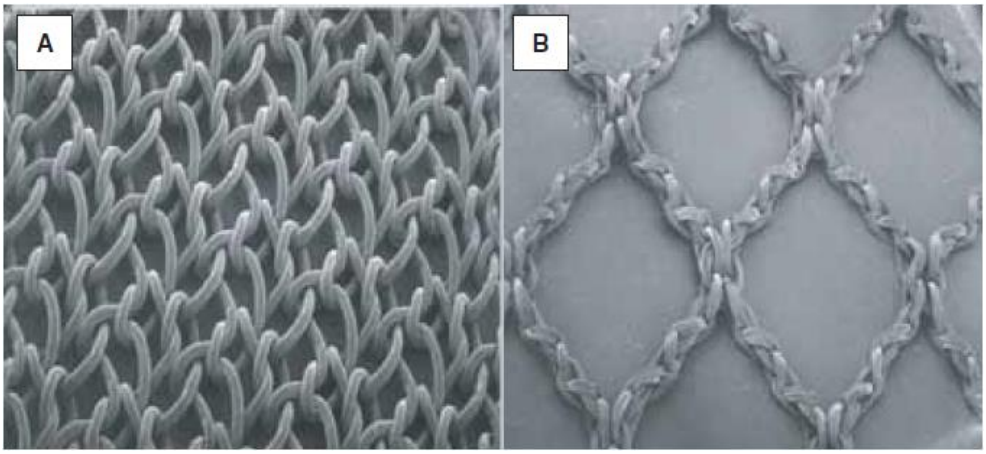

Figure 1:21: A) "Heavyweight" Marlex® mesh (Bard Inc., USA) compared with B) "Lightweight" Vypro® mesh (Ethicon GmbH, Germany) using scanning electron microscopy (127x).[203]

Other soft tissue applications for surgical meshes include the repair of pelvic organ prolapse (POP) using vaginal mesh kits. The majority of these meshes are constructed of PP monofilament.[208] Kits such as Prolift® (Gynecare, Ethicon, USA) have become popular with surgeons due to their proposed ability to be applied to a variety of pelvic floor defects.[209] The implantation of these meshes has shown positive short-term results over the past 10 years.[210] However, recent studies have shown increased complication rates for transvaginal mesh (TVM) procedures. Complications include recurrence of POP, mesh erosion resulting in infection, dyspareunia, stressful urinary incontinence, and pain.[211, 212] In a multicenter study by Elmér and colleagues, it was found that 21%, 18%, and 19% of women undergoing transvaginal anterior, posterior, or total prolapse repair experienced recurrence, respectively.[213] High complication rates such as these have led to the recall of some TVM products such as, ObTape (Mentor Worldwide,

USA, 2006), Gynecare TVT Secur system, Gynecare Prosima, Gynecare Prolift and Gynecare Prolift + M (Johnson and Johnson, USA, 2012) (www.drugwatch.com). To remedy the high occurrence of complications, researchers and surgeons have looked to improve the tension-free vaginal mesh surgical technique.[214]

Hard Tissue Surgical Mesh Applications

In hard tissue applications for surgical meshes, the employment of titanium scaffolds for bone growth has dominated the literature. Titanium implants for bone applications are popular because of the excellent biocompatibility of the material with bone tissue.[197] Titanium fiber meshes have been used in a number of studies to serve as scaffolding for osteogenic tissue engineered systems.[215] Osteoblast differentiation and ectopic bone growth have been shown both *in vitro* and *in vivo* in several different culture conditions, respectively.[215] Ectopic bone growth *in vivo* has been the result of the combination of osteoprogenitor cells with the titanium mesh.[197, 216] Surface modification by the addition of calcium phosphate was also shown to improve bone formation with these meshes.[217] Researchers have extended surface modification of titanium meshes to other surface coatings to direct stem cell differentiation and proliferation. Van den Dolder and coworkers explored the coating of titanium fiber meshes with fibronectin and/or collagen type 1.[218] Their hypothesis was that the ECM proteins fibronectin and collagen type 1 would enhance osteoblast attachment and proliferation on the mesh due to the affinity of cell binding domains to these proteins.[218] The results showed that these coatings did not provide additional osteoblast differentiation due to conformational change of the proteins when attached to the titanium, which may have altered the binding domains.[218]

The engineering of cartilage has also been the focus of researchers working with meshes. Cartilage tissue engineering has been driven by the need to provide mechanically viable scaffolds for chondrocyte seeding.[219] PL, PG, and PLG are easily formed into design shapes that will

accommodate the mechanical needs of a cartilage construct, but the hydrophobic nature of these materials makes cell seeding more difficult than naturally-derived polymers such as collagen.[219] Synthetic polymers have been formed into fibers using various fabrication techniques, with electrospinning being the most prevalent.[220] These fibers are often organized into nonwoven meshes which serve as the structural framework for tissue engineered cartilage designs.[219] Some researchers have also taken the approach of creating woven meshes for cartilage growth. Moutos and Guilak used a 3D orthogonal weaving approach to create PCL woven scaffolds, which were then encapsulated in a fibrin hydrogel.[192] This construct was seeded with human adipose-derived stem cells and mechanical testing was performed to evaluate the properties of the scaffold under chondrogenic culture conditions over 28 days.[192] Their results showed that these woven scaffolds were able to maintain biomechanically viable properties throughout culture.[192]

Mesh Material Considerations for Bone Tissue Engineering

Focusing in on bone tissue engineering applications, mesh material considerations often determine the effectiveness of proposed implant. Meshes are being explored as potential solutions to the dynamic environment around natural bone.[221] The composite structure of natural bone composed of calcium phosphate and collagen offers direction for researchers hoping to mimic the *in vivo* conditions. The changing composition of bone at the bone-cartilage interface, transition from cancellous to cortical bone, and during the remodeling phase of healing all present challenges to researchers attempting to use a material with uniform architecture.[222] Material type has been a significant part of the research in this area, with most attention being focused on titanium systems and polymer-based systems. Numerous studies concerning titanium or titanium-based composites have been explored to understand tissue engineered bone growth. Titanium and its alloys are generally better received than other metallic implants such as 316-L Stainless Steel or Co-Cr-Mo alloys due to reduced modulus, superior biocompatibility, and corrosion

resistance.[223] Titanium is generally considered to be biocompatible, mostly due to the ability to readily absorb proteins to its surface.[224] However, even in mesh applications, titanium implants suffer from interfacial instability, elastic modulus mismatch, and production of wear debris (as with all metallic implants).[225] Recent developments in titanium implants have focused on creating alloys that minimize elastic modulus, such as Ti-12Mo-6Zr-2Fe (TMFZ) or Ti-15Mo-5Zr-3Al.[225] Despite these efforts porous titanium implants still have elastic moduli higher than the natural cancellous bone researchers aim to mimic.[225]

Both natural and synthetic polymers have been used to form meshes used for bone tissue engineering. Collagen fibers have been used in cartilage and bone engineering, and can be associated with increased cell aggregation, resulting in increased calcification.[226] Many researchers have explored chitosan as a supplement to calcium phosphate scaffolds and other fiber types.[227] Chitosan has been shown to promote growth and matrix deposition by osteoblasts.[228] Xu and colleagues combined chitosan with a calcium phosphate mesh and observed a significant increase in strength of the implant, despite the presence of interconnected macropores.[229] Synthetic polymers have the advantage of producing predictable and reproducible physical, chemical, and degradation properties. These polymers are easily processed into different shapes and structures, and are able to be modified to address specific properties.[221] For example, PLL, PLG, PG, and PCL have been explored using electrospinning to create non-woven mesh designs.[220, 230] The disadvantage of these synthetic materials is the absence of natural signals that may promote desired cell responses.[221] For this reason, many researchers have used simple aliphatic polymers such as PCL to serve as the base for composite designs that include natural osteogenic capability.[221] Erisken and coworkers combined β-TCP nanoparticles with electrospun PCL fibers in a non-woven mesh configuration to enhance collagen deposition and mineralization from mouse preosteoblasts to simulate the cartilage-bone interface.[231]

Mesh Configuration Effects

Much of the motivation behind surgical mesh designs stems from previous advancements in the textile field.[232] Meshes may be woven or non-woven, with knitted designs included in the non-woven category.[233] Porosity, pore size, and morphology are the main considerations when mesh configuration is explored.[198] Researchers have implemented 2D or 3D, orthogonal or non-orthogonal, and many other design variations to achieve different results *in vitro* and *in vivo*.[198]

Non-Woven Meshes

Non-woven meshes have been employed for numerous bone tissue engineering applications. In the literature, most of these meshes comprise electrospun nano- or micro-fibers from synthetic semi-crystalline polymers.[234] Some researchers focused on the tissue engineering of bone have used non-woven meshes of nano-fibrous PLL to enhance calcified matrix deposition *in vitro.*[235] Electrospinning is perhaps the most popular method for developing seemingly disorganized polymer fibers that are then used as a mesh with variable pore size.[236] PCL is the most popular synthetic polymer in terms of non-woven mesh applications because of its linear aliphatic structure polymer chains which are closely packed. Furthermore, the semi-crystalline hydrophobic properties make the diffusion of water into the bulk of the macromolecule difficult.[221] For this reason, meshes made with PCL are generally used for slow-degrading applications or composite applications, where PCL serves as the base material. Li and fellow researchers used electrospun PCL in a non-woven configuration, in combination with TGF-β, to differentiate marrow stromal cells into chondrogenic cells.[237] PG and PLG have also been used for this application, although they are much faster in degradation rate.[238]

The biological significance behind non-woven meshes is that they possess a highly variable pore size. It is hypothesized that the relative disorganization of fiber alignment allows

cells to push fibers into the position most conducive to their growth.[191] This idea was explored in a study by Li and colleagues. The study concluded that cells were supported with phenotypic stability when presented with scaffolds of varying pore size.[191]

Knitted Meshes

Knitted meshes are a subset of the non-woven mesh configuration. However, there is much more order and consistency with pore size using a knitted design.[145, 239] Knitting by definition, is the construction of a fabric or cloth from the interlocking of threads by forming loops.[240] Because fibers used in knitting are curved, and not oriented unilaterally as in weaving, the resulting constructs are much more flexible and elastic than woven fabrics.[241] Knitted meshes serve to fill the need for biomaterial scaffolds that can handle mechanical stresses, such as strength in tension and compression, and maintain porosity for cell ingrowth.[239, 242] In the case of surgical meshes, threads should comprise biocompatible fibers, which may also be absorbable. Fibers may be created through melt-processing, extrusion, or other techniques.[239] In many cases, knitted meshes have been used as a basis for composite scaffolds, in which a natural polymer may be added to increase osteogenic properties of the scaffold.[243]

Knitted meshes are often implemented in applications requiring elastic properties, such as tendon or ligament repair.[244] However, knitted materials have also been used in cartilage applications. The most common method for developing these scaffolds is by combining a copolymer knitted mesh, such as PLG, with a natural polymer, like collagen.[239] Research groups such as Chen and colleagues, Dai and colleagues, and Kawazoe and colleagues implemented this method when they created knitted PLG/collagen sponge composite scaffolds seeded with MSCs to explore the differentiation capability of this approach in cartilage development.[219, 243, 245] Chen and coworkers showed successful differentiation of MSCs to chondrocytes, with results being consistent with native cartilage both histologically and mechanically.[243]

The ordered structure afforded by using knitted meshes has been an area of focus for many researchers seeking to control cell response, mechanical properties, and biocompatibility by adjusting mesh structure.[239] Theoretically, these different shapes may be used to suit target tissues/organs more specifically.[239] The basic structure of a knitted fabric consists of courses and wales. Courses are rows running across the width of the fabric, while wales are columns running across the length of the fabric. When the wales are perpendicular to the course of the fiber/yarn, this is called weft knitting. When the courses and wales are approximately parallel to the direction of the fiber/yarn, this is called warp knitting.[246] Figure 1:22 is included for clarification.

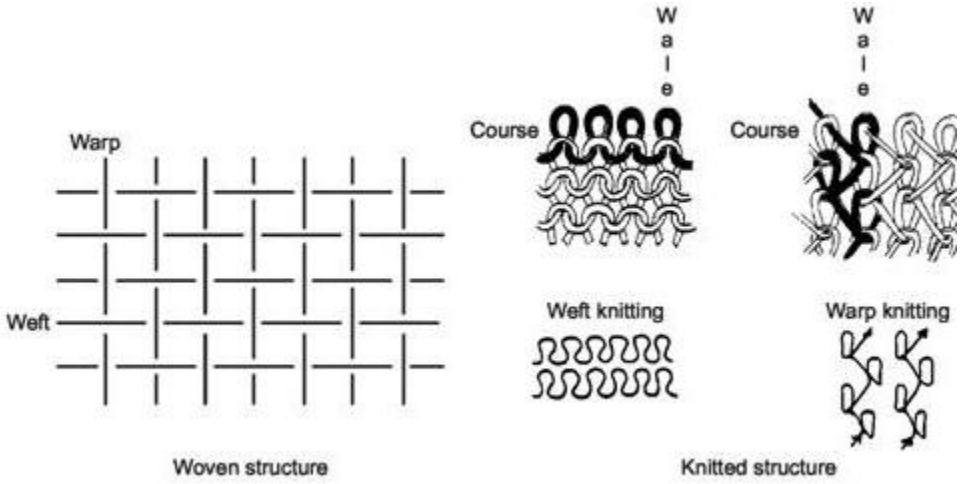

Figure 1.22: Schematic of woven and knitted structures. (www.textile2technology.com)

Researchers have varied knitting type and scaffold structure to explore cartilage development *in vivo* and *in vitro*.[239] Dai and coworkers varied the composition and structure of a knitted PLG mesh/collagen sponge scaffold to develop articular cartilage. The group tested a thin, semi-, and sandwich configuration (pictured in Figure 1.23), and discovered that all scaffolds showed significant cartilaginous deposition and morphology, but the semi- and sandwich configurations demonstrated mechanical properties more similar to that of natural cartilage.[219]

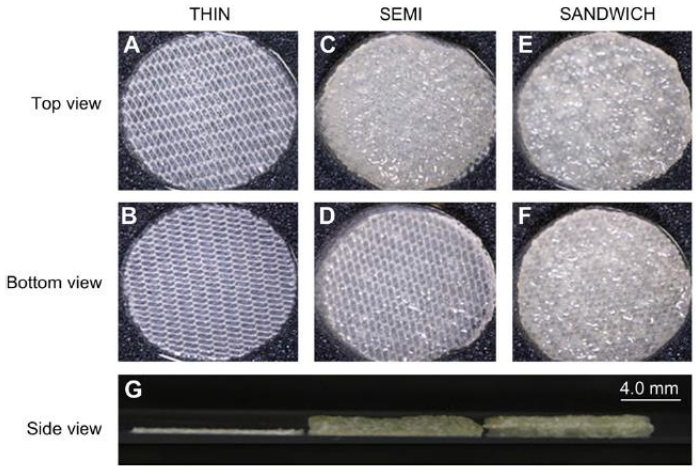

Figure 1.23: Three scaffold configurations used in articular cartilage engineering.[219]

Woven Meshes

Woven meshes are unique in that the final scaffold comprises multiple fibers interlocked oriented unilaterally and arranged in a particular weaving design.[247] Fibers are created generally the same way as knitted meshes. However, fabrication of these materials is mostly constrained to the use of custom-designed looms capable of developing 2D meshes, or 3D meshes. Conventional, or 2D, weaving is accomplished by interlacing two orthogonal sets of fibers in a process known as shedding.[247] A technique known as 3D orthogonal weaving[248] was patented by Nandan Khokar and the Mibrous Material Group, and is used to create 3D meshes.This technique is characterized by the use of a multi-layer warp with horizontal and vertical sets of weft fibers.[247]

The majority of the literature surrounding woven meshes concerns the engineering of a viable cartilage implant. Work by Moutos and Guilak has been focused on the potential for implanting human adipose-derived stem cells on orthogonal woven scaffolds comprising PCL fibers.[192] The chondrogenic culture conditions, along with the 3D mesh, resulted in a PCL construct that maintained mechanical properties similar to native cartilage throughout the 28-day culture period.[192] The foundations of this study were based on results from a previous study in

which a 3D orthogonal PCL mesh was used to reinforce a 2% agarose construct used to model the mechanical properties of articular cartilage.[194]

With the application of woven meshes to cartilage engineering, bone meshes may be the next focus. The custom looms used to construct scaffolds for cartilage applications may also be implemented for meshes in hard tissue applications. Bone meshes may require the ability to address a gradient of pore size or weave architecture, which may require the expansion of 3D weaving beyond orthogonal patterns. Researchers in industrial textile applications have used 3D angle interlock woven meshes for mechanical reinforcement purposes.[249, 250] A schematic of one of these meshes is included below. Tissue engineering researchers moving forward may look to incorporate a similar design with the increasing mechanical considerations of bone tissue.

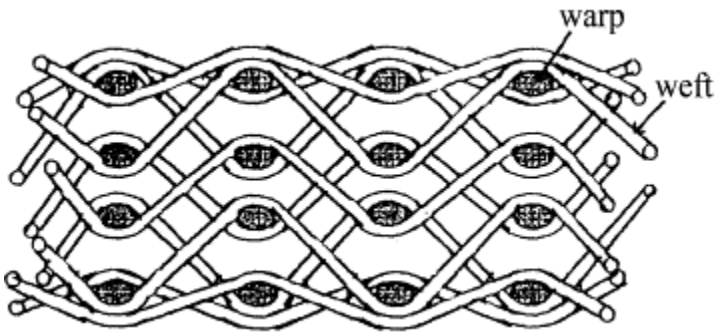

Figure 1.24: Schematic of 3D angle interlock mesh.[249]

Future Directions

This summary of the literature regarding bone tissue engineering and surgical meshes has highlighted information that points to the need for further work in several areas. One such area is the more comprehensive treatment of critically-sized traumatic (high-energy) fractures in patients unable to accommodate autologous grafting. While autologous grafting has been shown to be the gold standard of care, the issues of donor site morbidity and lack of adequate bone tissue have pushed researchers and clinicians to the development of other treatments. Allograft treatments are cited as the next most viable option for patients, however, problems with material variability and

potential immunogenicity have motivated the engineering of bone graft substitutes. Using concepts highlighted, in part, by researchers such as Giannoudis and colleagues, substitute materials have been developed with mostly osteoconductive and mechanical properties. The combination of multiple substitute materials has led to improved osteoinduction, vascular promotion, and absorption properties. Table 1.3 summarizes the progression of material considerations in the attempt to mimic autologous grafting. From the table it can be seen that polymer materials as well as ceramic materials (under certain conditions) offer the most potential for fulfillment of bone healing requirements.

Table 1.3 - Material Considerations for Bone Healing

	Autograft	**Allograft**	**Ceramic**	**Biologic**	**Polymer**
Examples	Bone Marrow Cancellous Chips	Cancellous Chips DBM	$CaSO_4$ β-TCP	BMPs 2 and 7 VEGF	PLL PCL
Osteogenic	+	±	-*	+	-*
Osteoinductive	+	+	+*	+	+
Osteoconductive	+	+	+	-*	+
Vascularity Promotion	+	+*	±*	+	+*
Mechanical Stability/Strength	+*	+*	+*	-	+*
Absorption Properties	N/A	N/A	±	-*	±

Table 1.3: Evaluation of material considerations for bone healing based on a modification of the diamond concept by Giannoudis and colleagues. Scaling is as follows: (+) indicates the promotion of property labeled on each row; (-) indicates the absence of the selected property; (±) indicates the possible presence of the selected property given the selection of certain examples within the material group (i.e. PG may absorb more readily than PLL, or another polymer); (x^*)

indicates the possibility of property fulfillment given coupling with certain other materials (i.e. a β-TCP scaffold may promote vascularity with sufficient pore size and combination with VEGF).[47]

However, if design considerations for scaffolds are considered according to the desired bone healing requirements, balance must be found between material type and fabrication methods able to accomplish the desired scaffold. Figure 1.25 evaluates bone scaffold fabrication methods by the parameters used to develop the bone healing requirements mentioned previously. For example, a combination of design for porosity/pore size and geometry may lead to a scaffold that is osteoconductive and promotes vascularity. This analysis of viable fabrication methods (although not comprehensive) suggest a fabrication technique previously only considered for soft-tissue applications.

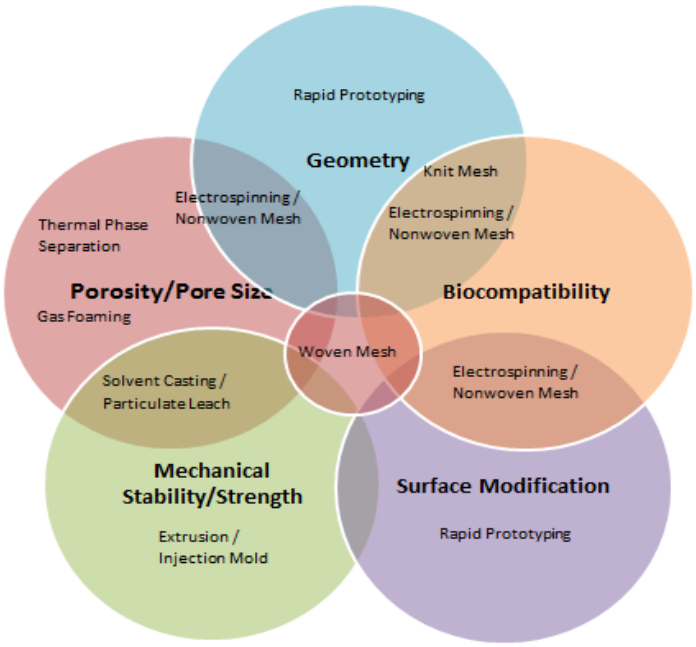

Figure 1.25: Schematic of relationship between scaffold fabrication techniques and design considerations for development of bone tissue engineering scaffolds. Techniques are placed into the category for the design consideration most addressed by the specific technique. Techniques in overlapping circles or in more than one circle indicate a technique with multiple foci.

Figure 1.25, as well as this review, point to the potential viability of polymeric woven surgical meshes for bone tissue engineering, particularly in the case of critically-sized traumatic fractures to long bones. Ceramic materials, although versatile according to Table 1.3, cannot be readily adapted to address all of the design consideration presented in Figure 1.25. Therefore, absorbable polymer materials may potentially be formed into fibers or yarns with bone-growth-specific surface characteristics and woven together in weaving configurations conducive to porosity development, mechanical strength, and 3-D geometry. Biocompatibility may be improved by the reduction of the amount of reactive material to be implanted *in vivo* (when in comparison to a ceramic material). It is concluded that polymeric woven surgical meshes may not only be implemented for soft tissue applications, but also for bone tissue engineering as a bone graft substitute with significant potential for modulation and fulfillment of fracture healing requirements.

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CHAPTER TWO

FABRICATION OF WOVEN POLYMER FIBER TISSUE ENGINEERING MESHES WITH VARIABLE PORE SIZE AND CONFIGURATION USING AN AUTOMATED BIO-LOOM

Introduction

Traumatic bone injuries are one of the leading causes of hospitalizations and emergency room visits in the United States each year. It has been estimated that nearly 3.5 million emergency room visits and 887,679 hospitalizations occur each year due to fracture.[1] Generally, these injuries can be successfully treated through bone realignment and sufficient fracture fixation. However, some cases of bone healing are frustrated due to complexity and location of the fracture or the pathology of the bone tissue. These cases result in the formation of delayed unions, nonunions, malunions, and other bone loss problems.[2] Prolonged frustration of fracture healing is diagnosed by clinicians as a critical-sized defect, or a defect that will not heal naturally due to size, anatomical location, patient fracture healing capacity, or a combination of these variables.[3] For patients presenting with a critical-sized defect the gold standard of treatment is an autologous bone graft, reinforcing the defect site with excised bone from the patient, usually from the iliac crest.

Bone graft procedures are one of the most common and profitable surgical interventions today, bringing in approximately $2.5 billion per year.[4, 5] The United States Center for Disease Control (CDC) estimates nearly 1.5 million musculoskeletal procedures in the United States involve the grafting of either an autograft or allograft, with almost 2.2 Million procedures involving these treatments worldwide.[6] However, complication rates (20.6% minor, 8.6% major) stemming mainly from donor site morbidity and lack of supply in autografts and immunologic response in allografts, have pushed researchers and clinicians to develop new orthobiologic solutions.[7] Among these new approaches, tissue engineering with the use of polymer scaffolds

has emerged as a promising area of development. Fiber based scaffolds of the nonwoven, knitted, and woven confirmations have been highlighted in the literature due to their ability to create highly specific scaffold conditions, while maintaining mechanical, biocompatible, and geometric considerations important to the growth of new bone.[8] Particularly, woven fiber scaffolds have been lauded for their ability to take advantage of dynamic 3-D geometry and material combinations to influence stem cell differentiation and proliferation in orthopedic applications.[9, 10]

Tissue Engineering Approaches with Woven Scaffolds

Woven scaffolds have a number of advantages as tissue engineering scaffolds over more traditional bone scaffolds. Meshes add an additional level of parameter flexibility when constructed from the already versatile material properties of bioresorbable polymers. In the context of the tissue engineering framework, meshes are advantageous because they provide the ability to improve cell-biomaterial interaction through rapid iterative design changes. Researchers are able to manipulate pore size, pore shape, and overall porosity quickly to create a more targeted cell response *in vitro* and *in vivo.*

Not until recently have researchers began to consider surgical meshes as a viable scaffold for bone tissue engineering. Surgical meshes had traditionally been consigned to soft-tissue applications, namely hernia and vaginal meshes.[11] These applications illustrate much of the current surgical mesh paradigm, in that clinically meshes have only been applied to situations in which elasticity and strength are the primary design constraints. However, in bone tissue engineering, other parameters such as scaffold stiffness and pore geometry are of equal importance. The increased stiffness of woven meshes, along with the ability to change the cross-section, surface characteristics, size, and spacing of individual warp and weft fibers led researchers to employ woven meshes in applications where compressional, tensile, and shear

strength are important. One such example is the use of woven meshes for the development of a tissue engineered cartilage system.[9, 12]

In the area of bone tissue engineering, researchers have not yet explored the potential for woven scaffolds. The current focus is largely associated with electrospinning and the new capacity to build nonwoven scaffolds, or fiber mats, with nano-scale fibers. Nonwoven meshes are characterized by a randomized pore structure.[13] However, cell affinity and differentiation capability of these meshes is often attributed to the size of the fibers, and may not be characteristic of the nonwoven conformation alone. These meshes have been employed as cartilage scaffolds, but several researchers have also used these scaffolds to grow bone tissue *in vitro* and *in vivo.*[14, 15]

A focus of this work was to explore the development of a tissue engineering test system with the ability to produce woven mesh scaffolds accommodating the design parameters pertinent to bone tissue engineering. There are several factors important to development of a bone tissue engineering scaffold, including geometry, surface modification, porosity/pore size, biocompatibility, and mechanical stability/strength. Given the large number of potential combinations from these five design areas, this work, as a proof of principle, focuses only on geometry, by way of weave configuration, and porosity/pore size.

Porosity, Pore Size, and Scaffold Geometry through Configuration

Geometry is a key to scaffold design because of the three-dimensional structure of natural skeletal tissue.[16] Geometry is connected to other important scaffold characteristics such as mechanical properties or vascular ingrowth. Porous structure also contributes to the diffusion of nutrients into the scaffold and cellular affinity.[17]

Porosity

Porosity can be characterized as the ratio of pore volume to scaffold material volume.[17] The resulting percentage has been reported as an indicator of transport ability for tissue engineering scaffolds, or measure of affinity for neovasculature. Some researchers have claimed that porosity should be as high as 90% to ensure satisfactory cell-material interaction.[18] Others have focused on mechanical strength, only implementing porosities near 30%.[19] Unlike natural tissue, most scaffolds created today have a uniform distribution of porosity throughout the construct. Anatomically, porosity is higher in the cancellous center of skeletal tissue, and is gradually decreased as the outer cortical layer is approached.[20] This study seeks to develop a system capable of modulating porosity and accommodating more physiologically relevant porosity gradients.

Pore Size

Along with specific cell types, pore size has also been shown to affect the amount of cell/tissue growth in a construct.[21] Holmes proposed the initial baseline for optimal pore size to be between 200-400 μm due to an average human osteon size of approximately 223 μm.[22] Later, Tsuruga and colleagues suggested that the optimal pore size of HA scaffolds is between 300-400 μm.[23] However, both macro (>100 μm) and micro (<20 μm) pore structures are needed for an effective scaffold.[20, 24] Macro-sized pores are thought to contribute to osteogenesis by facilitating cell and ion transport.[25] Micro-sized pores are thought to improve bone growth into scaffolds by increasing surface area for protein adsorption and subsequent cell attachment.[26] Micropores are also thought to act as attachment sites for osteoblasts depositing bone.[25]

Weave Configuration (Pore Shape)

Weave configuration or weaving pattern has been manipulated in the textile domain for centuries to produce fabrics differing in appearance, strength, thickness, and performance. In the

tissue engineering domain, changes in weave configuration for meshes most directly affects pore shape. Pore shape optimization and cell-specific pore topography are critical components of scaffold geometry due to the effects on cell or protein attachment, which translates to the long-term survival of cells on the construct.[21] This assertion is reinforced by the observation that bone differs in structure based on location and function, suggesting that pore shape optimization should take into account those parameters. Similarly, order versus disorder in polymer pore geometries may also have an effect on the quality and quantity of bone formed around a scaffold.[20] In a study focused on the differences between bone formations on a preordered HA scaffold with collagen fibers concentrated around its pores and a more random nanofibrous collagen-based sponge, Scaglione and colleagues found that the ordered scaffolding produced compact lamellar bone, while the disordered scaffold produced woven bone. These results suggest that pore shape and configuration play a key role in the resulting formation of bone.[27]

The Need for Woven Scaffolds in Bone Tissue Engineering

Woven scaffolds are of interest for bone tissue engineering applications due to the current advances in bone tissue engineering focusing on the application of porous scaffolds for bone regrowth. Bone tissue engineering offers a significant challenge for researchers and clinicians due to the compromise required between osteointegration, osteoconduction, osteoinduction, and osteogenic qualities. These qualities require the manipulation of biocompatibility, porosity, mechanical strength, surface modification, and transport properties (amongst other considerations).

Past and current developments in bone tissue engineering and bone graft substitutes have focused on creating biologic material composites, usually consisting of a hydroxyapatite scaffold with a osteogenic or angiogenic growth factor-containing entity. These combinations have had moderate success in the clinic, but fabrication methods and sources for allogenic biologic

materials such as growth factors have resulted in variable outcomes and the inability to adjust substitutes to specific patient defects. While cortical bone is a highly regular tissue, defects that occur across the cancellous-cortical interface or compound fractures resulting in multiple fragments or loss of vasculature, may cause complications in healing that are rarely able to be accounted for using the current treatments. Potential implementation of scaffolds from the described bio-loom is detailed in Figure 2.1. Both from an in vitro and in vivo perspective these scaffolds may be used to first highlight specific design characteristics that result in favorable bone growth outcomes. For example, stacking 2-dimensional woven scaffolds in to 3-dimensional constructs of variable thickness in an in vitro culture environment may provide information regarding the effect of 3-dimensional pore geometry with implications for nutrient/waste transport and cell attachment. In vivo, this construct might be employed to explore the degree to which scaffold thickness effects the angiogenic process. The rolled scaffolds shown in Figure 2.1 provides examples of how these scaffolds might be used in the clinic, either to wrap around, press-fit into, or supplement other treatments of fixation devices. The gradient approach offers a suggested solution to the clinical issue of defects across interfaces. The ability to design modular treatments for specific defects is the keystone to this woven scaffold approach.

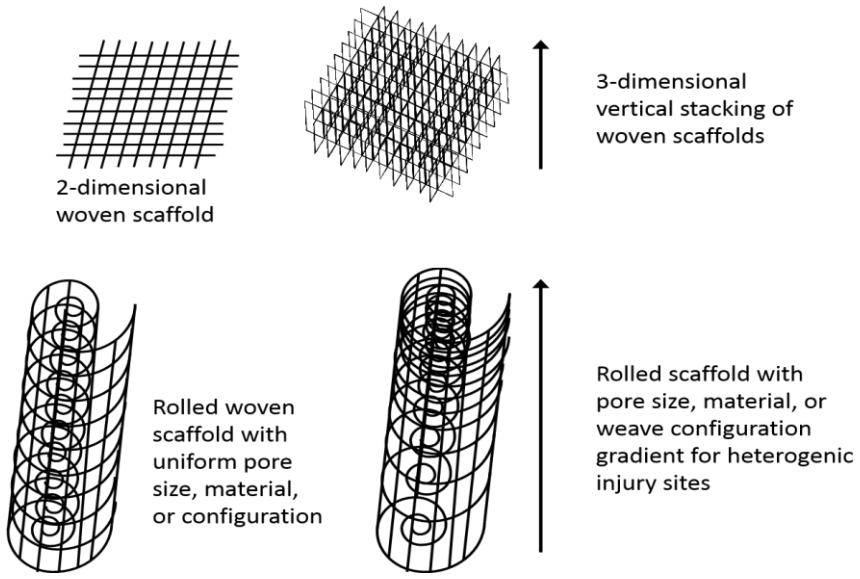


Figure 2.1: Potential in vitro and in vivo applications of the woven scaffolds made via bio-loom

Advantage of Modular Structures

One of the major proposed advantages of tissue engineering has been the idea of individualized medicine. Researchers have heralded the ability clinicians will soon have to design interventions for individual patient cases. Critical-sized defects with their heterogeneous environment present a case for individualized medicine. They require a graft substitute system to be capable of rapid modulation of scaffold parameters. Through imaging and pre-surgical evaluation clinicians may be able to specifically design scaffolds and biological cocktails for individual situations.

Woven surgical scaffolds offer this ability to modulate scaffold parameters due to their individual weft-warp structure. Figure 2.2 highlights some of the degrees of freedom within woven scaffold design implementing this bio-loom. The potential variations that a researcher or clinician could incorporate include varying the material type, weave configuration, fiber size, fiber geometry, or scaffold spacing of warp or weft fibers. This variation would facilitate the manipulation of mechanical properties, fluid transport properties, cell behavior, and

biocompatibility of surgical scaffold scaffolds. In addition to the parameter variability afforded by weaving, there is also a distinction between the mechanical stiffness of woven scaffolds and nonwoven or knitted scaffolds. This stiffness is desired in bone tissue engineering applications due to the mechanical loading seen in bone tissue.

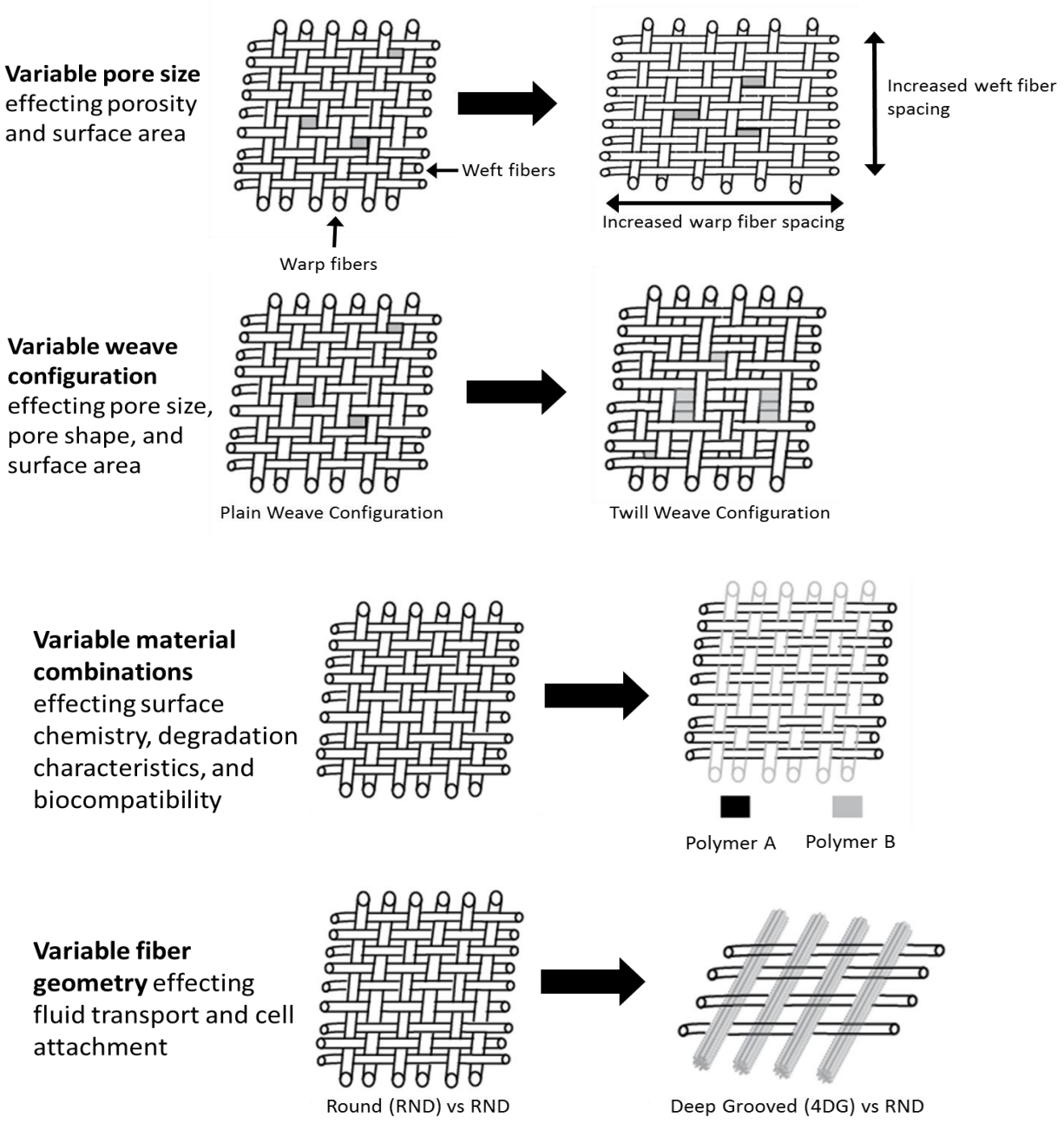


Figure 2.2: Potential bio-loom design degrees of freedom with woven scaffolds.

The Bio-Loom Concept

Many researchers focusing on the development of woven fiber scaffolds have employed textile technology to efficiently manufacture scaffolds and modulate their parameters. The most popular strategies have included the modification of various types of loom technology.

While the modern meaning of the term "loom" is relatively new (1838), the concept of using machines to intersect longitudinal warp fibers with transverse weft threads, otherwise known as weaving, has been implemented since around 100 AD.[28] In order to use this old technology to create new woven scaffolds for orthopedic tissue engineering applications many researchers have looked to developing customized biologically-focused looms (bio-looms). These looms are based on textile weaving technology such as shedding, picking, and battening, but most have been significantly scaled down to accommodate the differing material qualifications for biologically viable materials. The majority of current bio-looms are based on a dobby loom design in which a set of warp fibers is controlled by pulling down a harness to control the movement of all of the attached fibers.[29] Weft fibers are inserted by a variety of methods including air jets, water jets, and rapier systems. The result of these variations is a change in weave configuration. Weave configuration affects the shape of scaffold pores, strength of the scaffold, and may affect cell or protein attachment in some applications.

The novel bio-loom mentioned in this study was designed to produce bioresorbable polymeric meshes with variable porosity, pore size, and weave configuration in order to create a tissue engineering test system capable of producing defect-specific mesh scaffolds for future *in-vitro* work. This study was focused on the evaluation of this bio-loom in its ability to create these variable meshes. Differences in mesh porosity, pore size, and weave configuration were analyzed

via cell affinity and viability studies to understand the biological significance of differences created by the bio-loom.

Research Objectives

Specific research objectives for this work included the following items:

1. Construct a bio-loom capable of weaving meshes and modulating properties consistent with bone tissue engineering scaffolds (i.e. pore size, weave configuration, material variability, etc.).
2. Determine the degree to which the bio-loom is capable of modulating the parameters that are said to effect bone tissue engineering scaffolds.
3. Determine the extent to which changing these parameters make a biological difference in an *in vitro* environment?

Materials and Methods

Bioresorbable Fiber Production and Characterization

The first qualification of the fibers used as weaving filaments in this work was the documentation of biocompatibility and bioresorbability, given the immediate *in vitro* applications and the *in vivo* design considerations of the future. The polymers selected included poly-l-lactide (PL; Natureworks LLC, 2003d biopolymer, ~ 228,000 Da), poly-l-lactide-co-ε-caprolactone (PLCL; Purac, Purasorb PLC 7015, ~ 154,500 Da), and CAPA poly-ε-caprolactone (PCL, Zeus Inc, Perstorp CAPA 6500, ~ 50,000 Da). Each of these polymers has been used widely in tissue engineering scaffold fabrication in the literature, both *in vitro* and *in vivo*.[30, 31] PL was selected due to its extensively documented application in scaffold fabrication methods involving heat processing.[32] This relatively hydrophobic polymer has been shown to be biocompatible with favorable cellular affinity and degradation rates between 1-2 years.[33] PLCL was selected as a secondary scaffold material due to the increased hydrophobicity of the polymer, given the length of carbon chains associated with the caprolactone monomer. This material is a 70:30 mixture of

PL to PCL and has degradation rates shown to be similar to PL.[34] PCL was temporarily employed in this work as a plasticizer for non-compliant PL fibers, and also as a less-expensive alternative to PLCL in early proof-of-concept studies. PCL has had a broad use in tissue engineering applications in the form of films and electrospun fibers. Most work conducted on meshes applied to orthopedic applications has employed electrospun PCL fibers arranged in a nonwoven configuration.[35, 36] PCL has been shown to degrade beginning at 16 months, but not completely degrade until 3 years.[37]

Fibers were produced during this work via melt-spinning. Initial experimentation with this process was conducted on a single tube plunging extruder. While the resulting extruding fibers were too large for bio-loom application (~ 1 mm diameter), this test provided insight into heat and pressure effects induced on fibers as a result of melt-spinning. These early fibers were tested for thermal degradation, crystallization differences, and glass transition (T_g) properties via differential scanning calorimetry (DSC). This DSC analysis was run in concert with as-received polymer pellet samples to explore any changes induced via processing. Results from this preliminary work yielded no significant difference in polymer materials as a result of the melt extrusion process.

Preliminary validation of processing method inertness was followed by extrusion of fibers on a lab-scale double screw melt extruder driven by a series of direct current (DC) motors. This extruder (constructed by Alex James and Associates, Greer, SC) was used to fabricate fibers for the remainder of this work. The thermal characteristics and molecular stability of PL, PLCL, and PCL were tested before and after extrusion via DSC, thermogravimetric analysis (TGA) for degradation temperature, and gas permeation chromatography (GPC) for molecular weight distribution. In each case, polymers were shown to be not significantly changed by the melt

extrusion process. This validation informed the decision to proceed with using these polymers and extruder for bio-loom fibers.

The initial procedure for the melt extrusion process was as follows: 1) as-received pellets were roughed via blending in a food processor to ease the gripping of the extrusion screw to pellets as they were pulled into the initial heating zone; 2) melt temperatures were set according to T_g and melt temperature (T_m) results acquired from DSC; 3) pellets were loaded into the extrusion hopper which was sealed under a nitrogen gas purge to minimize oxidative degradation of the polymers when exposed to heat; 4) pellets were then pulled through the four heating zones via the extrusion screws until the polymer was turned into a homogeneous semi-solid melt; 5) this melt was then forced through a spinneret die of various cross-section (round (RND) or deep-grooved (4DG)) under compressive stress of the extrusion pump; 6) the extruded fiber forced through the spinneret die head was wound around a series of cylindrical mandrels rotating at various speeds to accommodate drawing ratios consistent with the desired fiber diameter (< 300 μm); and 7) the drawn fiber was collected on a spool and stored under vacuum for further analysis and use.

The 4DG cross-section was selected due to its documented facilitation of fluid transport.[38] It was hypothesized that this fiber cross-section would augment transport characteristics of the created woven scaffolds. The 300 μm fiber diameter was set as a benchmark due to the previous work focusing on fibers near 100 μm or less. These fibers were fabricated using electrospinning techniques so the 200 μm allowance was included to accommodate both the difference in fabrication method and the expected strength requirements of the bio-loom.

The extrusion procedure listed above was the baseline approach, with several other variables being included during the development of "weavable" fibers. These variables included the option to pre-dry via vacuum oven, the as-received pellets to remove pre-adsorbed moisture.

This process also briefly included a moisture content analysis step using a Computrac Vapor Pro moisture analyzer (Arizona Instruments LLC) with a threshold of 50 parts per million (ppm) to validate pellet drying. However, this process was aborted due to the difficulty with transferring dried pellets from the location of the vacuum oven to the moisture analyzer before moisture was reabsorbed. Additional options included the passing of the fibers through a column of air immediately after extrusion to quench cool fibers before drying, thereby lessening the effects of the drawing process. This option was implemented in the hopes of consistently creating larger fibers. The cooling apparatus is pictured below. The option of extruded fiber passing through a water bath was also included as a quenching tool. A small-scale water bath was fabricated (pictured below) and arranged at the outlet of the spinneret to quench cool fibers as they were produced. The results of a study characterizing the effects of all of the extrusion variables on fiber diameter, tensile strength, elastic modulus, and thermal transitions via DSC are described below. This fiber characterization preliminary study included 18 treatment conditions. Samples were varied by material combination (PL, PLCL, or a 70:30 w/w PL-PLCL blend), whether or not they were quenched via water bath, whether or not they were vacuum dried prior to extrusion, and whether fibers were extruded with the 4DG or the round cross-section. Two additional samples were added to include the as-received PL and PLCL pellets. This gave a total of 18 treatment conditions. These samples were treated tested for changes in thermal transitions via DSC (n=1 for each treatment group). Tensile strength was also tested, with elastic modulus and tensile stress at maximum load being recorded via an Instron Mechanical Tester with a 50 N load cell (n=8 for each treatment group). Average fiber diameter was recorded by stereoscopically imaging a sample from each treatment group and using ImageJ image analysis software to measure the fiber diameter (n=1 for each group). GPC analysis was conducted on each treatment (n=1) to measure

in molecular weight changes as compared with the as-received pellet samples. Results for these tests are included in the information below.

Table 2.1: ANOVA Results - Fiber Characterization

Parameter Test	Metric of Interest	Extrusion Parameter	Statistical Significance (p-value)
DSC	Glass Transition Temperature (T_g)	Cross-Section Geometry	RND > 4DG (0.011)
		Water Bath Quench	No Significant Difference
		Vacuum Dried	No Significant Difference
		Material Combination	PLCL > PL > PL-PLCL (0.0001)
	Melt Temperature (T_m)	Cross-Section Geometry	4DG > RND (0.0155)
		Water Bath Quench	No Significant Difference
		Vacuum Dried	No Significant Difference
		Material Combination	PL_PLCL > PL > PLCL (<0.0001)
Tensile Testing	Elastic Modulus	Cross-Section Geometry	4DG > RND (0.0001)
		Water Bath Quench	NWB > WB (0.0056)
		Vacuum Dried	No Significant Difference
		Material Combination	PL = PL-PLCL > PLCL (< 0.0001)
	Tensile Stress at Maximum Load	Cross-Section Geometry	4DG > RND (0.0002)
		Water Bath Quench	NWB > WB (0.0143)

		Vacuum Dried	No Significant Difference
		Material Combination	PL = PL-PLCL > PLCL (< 0.0001)
Fiber Diameter	Fiber Diameter	Cross-Section Geometry	4DG > RND (0.0011)
		Water Bath Quench	No Significant Difference
		Vacuum Dried	No Significant Difference
		Material Combination	No Significant Difference
GPC	Molecular Weight (M_n)	Cross-Section Geometry	4DG > RND (0.0024)
		Water Bath Quench	No Significant Difference
		Vacuum Dried	No Significant Difference
		Material Combination	PL = PL-PLCL > PLCL (0.0247)
	Molecular Weight (M_w)	Cross-Section Geometry	4DG > RND (0.0029)
		Water Bath Quench	No Significant Difference
		Vacuum Dried	No Significant Difference
		Material Combination	PL = PL-PLCL > PLCL (0.0267)
	Polydispersity (PDI)	Cross-Section Geometry	No Significant Difference
		Water Bath Quench	No Significant Difference
		Vacuum Dried	No Significant Difference
		Material Combination	No Significant Difference

Table 2.2: Fiber Characterization Results

Fiber Type	Diameter (mm)	Tensile Stress at	Elastic Modulus	Glass Transition	Melting T_m (°C)	M_n (Da)	M_w (Da)	Poly-dispersity

		Max Load (N/mm^2)	**(N/mm)**	**T_g (°C)**				**Index (PDI)**
PL_PLCL-D-4DG-NWB	0.453	34.848	1165.592	54.833	151.833	72586	155602	2.144
PL_PLCL-D-4DG-WB	0.393	26.215	749.037	54.5	153.833	68226	152234	2.231
PL_PLCL-ND-4DG-NWB	0.333	40.308	993.257	55.333	151.666	81819	174146	2.128
PL_PLCL-ND-4DG-WB	0.398	25.487	697.977	55.166	151.833	75453	170086	2.254
PLCL-D-RND-WB	0.432	22.264	623.602	65	137	54411	119484	2.196
PLCL-D-RND-NWB	0.295	8.614	4.324	58.166	148.166	52317	119180	2.278
PLCL-ND-RND-NWB	0.299	5.678	2.861	62	137.833	51136	116382	2.276
PLCL-ND-RND-WB	0.396	13.732	6.910	62	135.666	47962	116477	2.429
PL-D-4DG-NWB	0.48	28.513	951.992	59.166	149.166	53899	124442	2.309
PL-D-4DG-WB	0.513	28.640	838.524	59.333	149.333	91475	217357	2.376
PL-D-RND-NWB	0.248	33.423	854.010	58.333	149.333	70703	151802	2.147
PL-D-RND-WB	0.251	31.032	881.962	58.333	149.166	65623	146198	2.228
PL-ND-4DG-NWB	0.52	26.906	771.736	56	151.666	88222	176461	2

PL-ND-4DG-WB	0.474	27.944	586.776	58.5	148	95207	191693	2.013
PL-ND-RND-NWB	0.284	38.033	1092.138	58.333	149.166	60544	131858	2.178
PL-ND-RND-WB	0.232	29.275	961.949	58.166	149.333	60044	141969	2.364
PLCL_Pellet_As Rcvd				52.666	150.833	121647	256230	2.106
PL_Pellet_As Rcvd				69.166	149	77066	150145	1.948

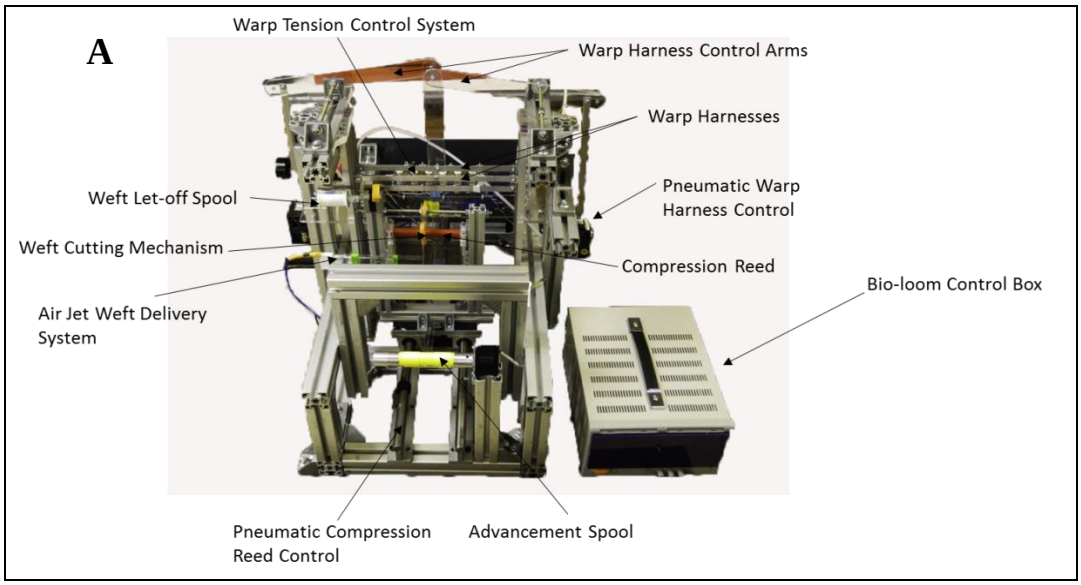
A number of fiber issues were presented as a result of the extrusion and preparation process and the determination of "weavable" fiber parameters. One of the primary issues was processing-related degradation to the point that fibers became brittle. Given the nature of bio-loom operation, compliance of fibers to bending and tensile forces is required to facilitate weaving. To remedy this fiber brittleness, extrusion temperatures were reduced and pre-drying times were increased to reduce moisture content. Another issue was a spring-like behavior in some of the fibers produced due to the cooling of fibers around the collection spool on the lab-scale extruder. Once removed from the spool, fibers coiled and were unable to be implemented on the loom. A heater was added to the bio-loom air jet to break some of the short unstable covalently bonded polymer chains thereby straightening the fiber. This approach worked sparingly for smaller fibers (< 200 μm) but not for larger fibers. Given the incorporation of excessive plasticizer, such as PCL, fibers were also sometimes too compliant to be used on the bio-loom. Excessive strain of the fiber under relatively small loads made fibers of this type unusable.

From the results in Tables 2.1 and 2.2 above and from trial and error implementation on the bio-loom, it was determined that the most critical parameter for fibers was tensile strength. Tensile strength was significantly higher in 4DG fibers than in round fibers, but this trend is combated by the need to reduce fiber diameter. 4DG fibers also had a significantly higher diameter on average than round fibers. There was, therefore, a balance to be achieved between strength and size. The requirement for higher tensile strengths was also addressed by slowing the operation of the bio-loom to reduce the likelihood of rapidly occurring high stresses on the fibers during the weaving process. Though vacuum drying did not yield any statistically significant results across fiber type, this process was highly suggested by experts in this area in the Clemson University Material Science and Engineering Department. A primary concern for this process was the ability to transfer recently dried fibers to the extruder without allowing reabsorption of water vapor. The water bath quench yielded larger fiber diameters in most fiber conditions, suggesting the ability to specifically control fiber size through quenching. However, there was also a significant reduction in tensile strength with quenched fibers making them difficult to use on the bio-loom. Material combination results yielded more favorable outcomes for PL containing fibers in strength. GPC results confirm that the extrusion process did not change the overall structural makeup of the polymer. Molecular weight measurements suggest that lower molecular weight chains were lost during the extrusion process given the difference in M_w for extruded fibers versus the as-received pellets.

Bio-Loom Development

The current bio-loom was developed from the previous work of Mersereau, who focused on a dobby loom concept employing a rapier as a means of shuttling weft fibers across the warp shed.[39] This work focused on using a series of servo motors controlled through a Matlab script to direct the movement of the warp fiber harnesses and to maintain warp tension and completed

mesh collection/advancement. This work employed silk fibers as a biocompatible weaving material. This work improved on that design through replacing the rapier system with a heated air jet that pushes the weft fiber across the warp shed as opposed to pulling it across. This process greatly increased the speed and accuracy of the loom weaving process. Additionally, servo motors equipped with counter weights were replaced by pneumatic air cylinders and stepper motors with accompanying drivers allowing for the software of the bio-loom to be controlled through digital logic system designed in LabView. This change allowed for the addition of a graphical user interface (GUI) that allowed the user to easily change scaffold design parameters. The figures below detail the components of the bio-loom in its current state. The schematic below offers a summary of current bio-loom operation and the fabrication of woven mesh scaffolds.



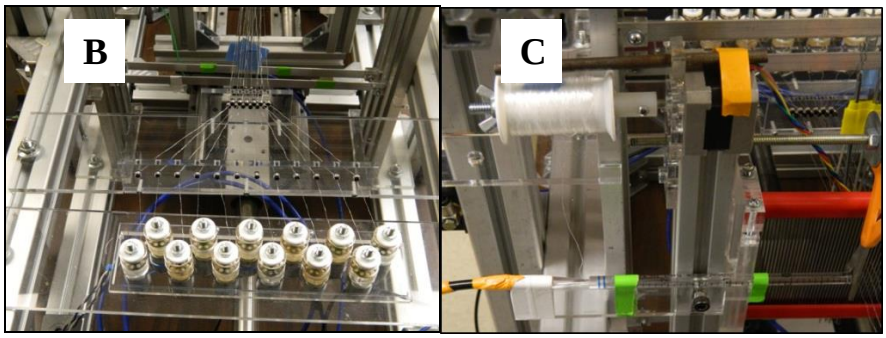


Figure 2.3: A) Bio-loom birds-eye view, pictured are bio-loom hardware and control box. Not pictured is the computer displaying the user interface; B) Array of motorized bobbins comprising the warp tension control system; C) Air jet system with weft fiber fed via stepper motor into a heated air stream

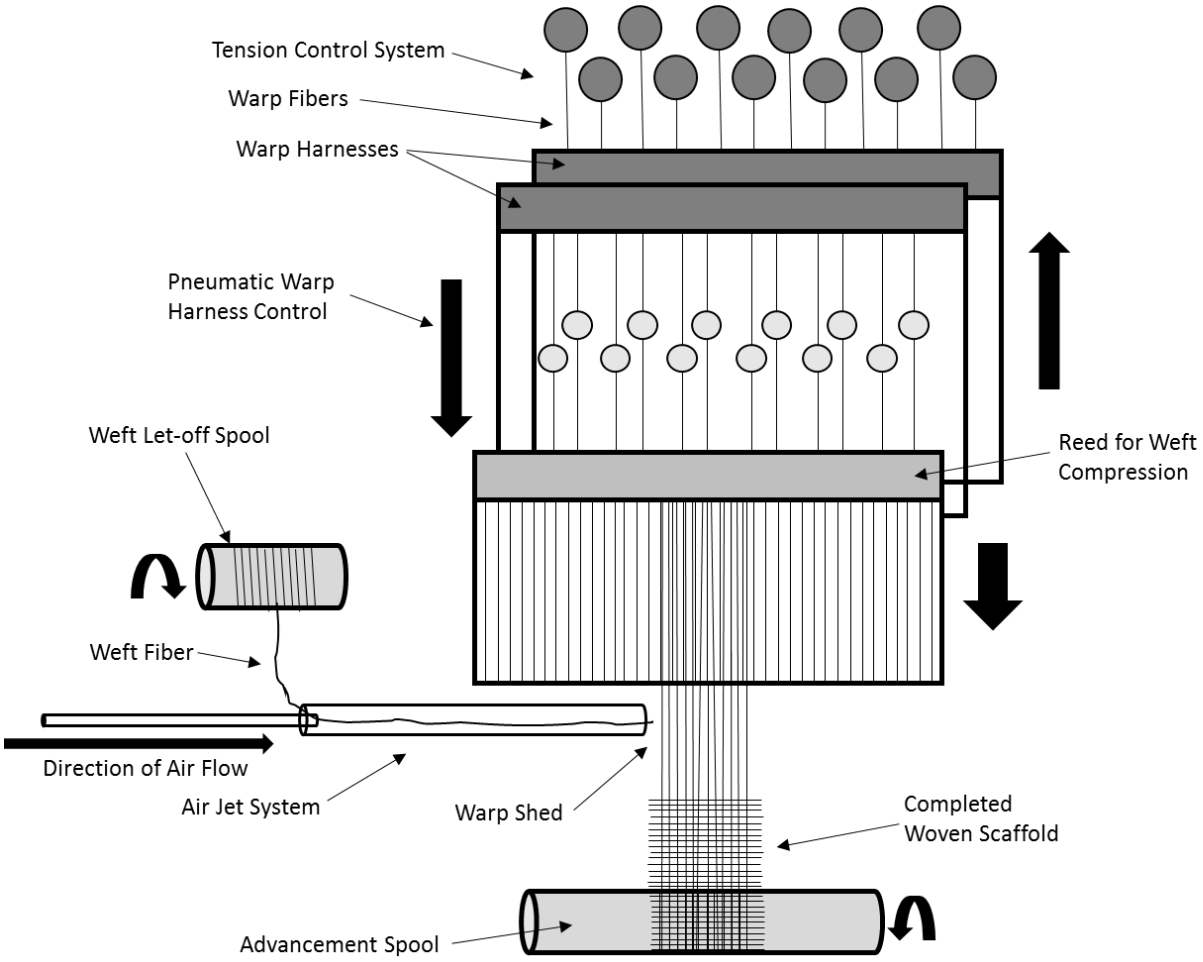


Figure 2.4: Bio-loom schematic demonstrating basic operations of the weaving process

Tension Control System

Warp fibers are arranged along an array of twelve motorized bobbins, pictured in Figure 2.3B and diagramed in Figure 2.4. The twelve direct current (DC) motors controlling the torque of each warp fiber are configured in two groups of six motors connected in series. The constant

current is applied across each six-motor array to maintain a constant torque across all twelve motors. A voltage control system uses a variable resistance controlled through the LabVIEW® user interface which adjusts the percentage of the maximum 6 V voltage that will be distributed across the 6-bobbin arrays. The result of this system is the ability to control the magnitude of torque placed on all twelve bobbins simultaneously giving the researcher the ability to employ warp fibers of different tensile strengths for weaving.

Warp fibers are anchored to a machined aluminum spool. The spool is 8 inches long with a 0.75 inch diameter. There are 1/16" slots machined into the spool stock to create grooves for each of the twelve warp fibers. These grooves help to maintain even spacing of warp fibers during weaving and advancement of completed scaffold. The advancement spool is attached to a 10 V, 500 mA bipolar stepper motor which incorporates stepping and micro-stepping to advance completed scaffold. This advancement serves to maintain proper pore spacing and is employed to manipulate pore size and porosity. The advancement spool setting on the user interface allows for adjustment of the rate at which the advancement spool rotates. The user interface uses a picks/cm value (denoting how many complete weaving iterations must occur within a 1 cm length of fiber) to correspond to a number of micro-steps on the stepper motor. The stepper motor has an excitation voltage of ±5 V which is controlled through the LabVIEW software.

Weave Configuration System

The ability to manipulate weave configuration enables researchers to develop scaffolds with variable pore shape and differing mechanical properties. The bio-loom employs two aluminum harnesses equipped with six stainless steel heddles per harness. A representation of the harnesses is included in Figures 2.3A and 2.4. Heddles have an enlarged hole or "eye" in the center through which a warp fiber is passed. Each harness is connected to a custom, laser-cut acrylic arm. This acrylic arm is attached to an aluminum rod which is fastened to an active-low

air cylinder. A solenoid valve is coupled with each air cylinder converting an electrical pulse (controlled via LabVIEW) into the mechanical extension of the air cylinder, and thus the raising of the attached harness. The pattern by which signals are sent to each solenoid valve is dictated by a simulated LED array on the user interface. Researchers may select specific weave patterns by selecting the corresponding LEDs on the array.

Weft Fiber Placement

As the warp fiber harnesses are alternated in a one-up-one-down fashion a gap between the warp fibers threaded through each heddle is created. This gap is referred to as the warp shed. The location of the warp shed can be seen in Figure 2.4. The air jet system of the bio-loom is used to pass a weft fiber through the warp shed during each pick (or iteration) of the weave. The air jet system uses a stream of air passed through a plastic tube to push the polymer fiber through the warp shed. The outlet tube is nested within the inlet of a slightly larger tube, thereby creating a concentrated stream of air through the center of the larger tube. This concentrated stream of air may also be heated as the air through the inlet tube is passed through a metal coil placed inside of an oven. This small amount of added heat serves to improve the compliance of some extruded fibers with increasingly coiled secondary and tertiary bonds. Using the heat to break some of these loose bonds, occurring from rapid cooling after extrusion, allows the fiber to become straighter and pass more easily through the air jet system. Weft fiber is fed into the inlet tube from a spool operated by a stepper motor functioning in the same way as the advancement spool motor. A photo is included above in Figure 2.3C for clarification.

Weft fibers are secured into place during the switching of warp harness position as well as with compression of the reed. The reed is a 7” wide stainless steel block with 1/8” slots that each warp fiber is passed through. The reed is secured to a large air cylinder operating via

solenoid valve in a similar fashion as the warp harnesses. The reed compresses the weft fiber into place after each weft fiber is passed through the warp shed.

Automation

In order that the system not have to be reset each time a weft fiber is passed through the warp shed, a cutting mechanism was instituted to automatically trim the outlet of the air jet system. The result is a proper positioning of the next segment of weft fiber to pass through the shed. The cutting mechanism consists of a spring-loaded pair of scissors mounted to the edge of the reed. As the reed compresses the recently passed weft fiber, the scissors of the cutting mechanism are compressed against an extruded aluminum bar mounted above the advancement spool. The scissors are aligned directly at the edge of air jet system so that the weft fiber, once trimmed, is ready for the next pick. This mechanism is pictured in Figure 2.3A.

The bio-loom is operated through a user-interface controlled through LabVIEW® software. This software allows the researcher to change scaffold parameter on an interactive screen while the bio-loom is running. Default values are set to ensure a regular starting point for each weaving session. The ability to set parameters without the requirement of constantly adjusting bobbin power, harness height, or advancement spool speed serve to make the bio-loom more automated, requiring only the occasional reset for displaced weft fiber alignment or damaged warp fibers.

Bio-loom Woven Mesh Evaluation

Successful construction of a bio-loom that produced woven meshes was then followed up with validation of the ability to change mesh parameters. The demonstration of predictable and repeatable mesh properties due to changes in bio-loom parameters was conducted in this study via analysis of pore size and porosity changes. Also, in alignment with the tissue engineering paradigm, stem cells were seeded onto these woven scaffolds to explore to biological efficacy of

the bio-loom. The overarching goal was to evaluate the extent to which creating scaffolds that modulated mesh parameters enacted a change in cell behavior.

Production of Meshes

Mesh pore size and porosity were modulated through the adjustment of bio-loom advancement rate. A slower collection of completed mesh allowed for an increased number of weft fibers to be packed into a specific length of mesh. This rate was adjusted via the "Picks/cm" setting of the bio-loom, in which one "Pick" was equivalent to one complete bio-loom cycle. This completed cycle consisted of one weft fiber being passed through a warp shed and then pressed securely into place by the reed. To create smaller pores or a less porous scaffold, the "Picks/cm" setting was increased to produce a tighter, more compacted weave.

Weave configuration was varied through the alteration of the "Weave Pattern Array" contained on the control screen. This array allowed for the adjustment of the timing for each warp fiber harness. Each harness is connected to a pneumatic cylinder which raises or lowers the warp fibers attached. The alternation of which harness is in the "up" position results in an over-under pattern for the weft fibers being passed through the shed created by the warp fibers when one harness is up and the other is down. Two classic weaving patterns were used to assess bio-loom efficacy in creating meshes with significantly different weave configurations. These patterns were the Plain weave and the Twill weave, both pictured below in Figure 2.5.

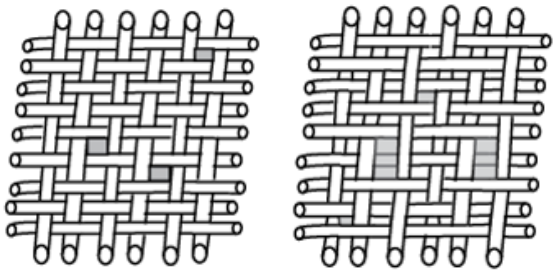


Figure 2.5: Left – Plain Weave, Right – Twill Weave

Porosity Calculation and Pore Size Measurement

Melt spun fibers were woven into meshes using 125 picks/cm, 100, 75, 50, and 25 bio-loom advancement settings. Stereoscopic imaging was used to characterize mesh pore size by averaging the dimensions of five random pores from each mesh image. Porosity was calculated by two methods. Mass porosity was calculated by encasing each mesh in a 1% agarose gel (Figure 2.6). Excess gel was removed via scalpel and the remaining mesh and gel were weighed to obtain a wet mass (M_w). Equation (1) was then employed to obtain the percentage represented by the change in mass. The void spaces of the each mesh were filled with the 1% agarose gel, thereby increasing the difference between wet and dry masses (M_d) for meshes with increased porosity. A density based porosity measurement was obtained by calculating the mesh density from a calculation of mesh total volume (l x w x h) and dry mass. This density (ρ) was then used in Equation (2) in combination with initial material density (ρ_m) from the material data sheet.

$$(1)\quad P_m = \frac{M_w - M_d}{M_w} \qquad (2)\quad P_d = 1 - \frac{\rho}{\rho_m}$$

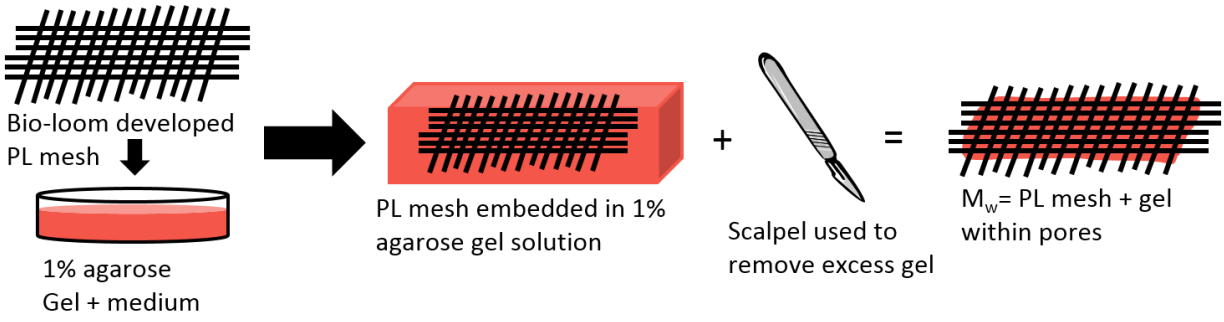


Figure 2.6: Schematic of mass method for determining mesh porosity

Pore size measurement was accomplished through stereoscopic imaging of each mesh. Images were then analyzed using ImageJ software to calculate the area of each measured pore. Not every pore was measured on each mesh. Rather, a random sampling of ten pores from each mesh was used to determine an average pore size for each mesh. Pores from the cut edges of the

mesh (after removal from the collection spool) were not included for analysis. Figure 2.7 below provides as example of this analysis.

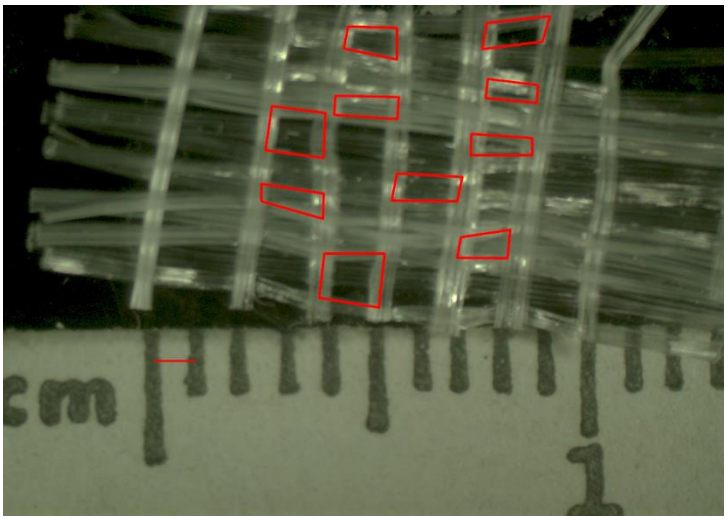


Figure 2.7: Example of stereoscopic image with pores highlighted via ImageJ for measurement

Cell Culture

Meshes were cleaned by a three cycle wash alternating between phosphate buffered saline (PBS; Sigma) and 70% ethanol. Each wash cycle lasted one minute. The wash was followed by a soak in culture medium with ultraviolet radiation for 24 hours. Meshes were then seeded with murine bone marrow stromal (D1) cells (ATCC, passage 5-8). Culture conditions consisted of 24 hours of dynamic culture on an orbital shaker at 100 RPM, followed by static culture for 5-7 days in non-treated 12-well plates (Corning). Culture medium and incubation conditions were consistent with previous work by Mersereau and coworkers.[39] Meshes were prevented from floating in the culture medium by the placement of a poly-tetrafluoroethylene (PTFE) ring.

Cell Affinity Analysis

All cell affinity analysis was completed at the conclusion of the culture period (day 5 or day 7 based on confluency of the control well). After the culture period but before analysis,

meshes were removed from the initial culture dishes and placed in a new non-treated 12 well-plates. This was done so that only cells attached to the mesh would be analyzed. Cell metabolic activity was assessed using a fluorescent (530 nm excitation, 590 nm emission) alamarBlue® assay (Life Technologies). The alamarBlue® reagent was added to each well after new culture medium had been added following the transfer of meshes to new well plates. Following the prescribed incubation period, samples of each condition were transferred to a 96-well plate for fluorescence analysis via a Synergy MX plate reader (Biotek).

Each cell metabolic activity experiment was normalized by DNA concentration through a Quant-iT™ PicoGreen® assay (Life Technologies). DNA concentration was used as a measure of the amount of cells present in each sample. Following alamarBlue® experiments, medium was removed and each mesh was washed with PBS. Cells were then lysed in 1X TE Buffer (Invitrogen). Cells were ultrasonicated at 20% amplitude with 15 second pulses. These samples were combined with the PicoGreen® reagent and briefly incubated. The plate reader was used to measure fluorescence (485 nm excitation, 528 nm emission). DNA standards were used to develop a standard curve for each data set. This standard curve was used to relate DNA quantification via fluorescence to the corresponding cell metabolic activity samples.

Qualitative analysis of cell attachment in the weave configuration experiments was accomplished through a Live/Dead® Cell Viability/Cytotoxicity assay (Life Technologies). Once moved to clean 12-well plates, cell-seeded meshes were subjected to a Live/Dead® reagent consisting of PBS, Calcein AM, and Ethidium homodimer-1. Well plates were incubated at room temperature for up to 4 hours before being imaged using fluorescent microscopy. Living cells were noted by an intense green fluorescence as a result of Calcein AM being retained. Dead cells were noted by an intense red fluorescence as Ethidium interacted with the exposed nucleic acids of cells with damaged membranes. To ensure representative images were taken of each mesh, the

outer 5 mm of each mesh was excluded (due to being covered by the PTFE ring during culture). Meshes were divided into two halves with three images being taken on each half. Magnification information is included in the figures below.

Statistical Analysis

Statistical significance for all cell affinity experiments was determined by analysis of variance (ANOVA) testing, with level of significance $\alpha = 0.05$. JMP Pro 10 software was used to run each statistical test and develop a simple mean statistical model representative of the experimental set up. Statistical outliers were removed from the data only in the case that the value exceeded a two standard error difference away from the sample mean.

Results and Discussion

The following set of images (Figure 2.8) is representative of subsequent studies done by the authors demonstrating the efficacy of the bio-loom discussed here. The images confirm the meeting of size and material objectives with respect to cell attachment and viability of the scaffolds *in vitro.* The viability of cells attached to scaffolds was assessed via Live/Dead® Cell Viability Assay (Life Technologies, Grand Island, NY). A variety of fiber geometries can be seen in the images as well, with scaffolds being successfully constructed with both RND and 4DG fibers. The ability to create a woven scaffold of heterogeneous material combination was confirmed through the weaving of poly-l-lactide (PL; Natureworks LLC, 2003d biopolymer, ~ 228,000 Da) warp fibers and poly-l-lactide-ε-caprolactone (PLCL; Purac Purasorb 7015, ~ 154,500 Da). The ability to weave scaffolds of differing weave configurations was confirmed through the successful development of the Plain weave configuration and the Twill weave configuration. Figures 2.8B and 2.8C represent the Plain and Twill weave configurations, respectively. Although 4DG fibers were used in both scaffolds, the interlocking of fibers is much

tighter in the Plain weave configuration. This change demonstrates the ability of the bio-loom to effect pore size, porosity, and cell affinity.

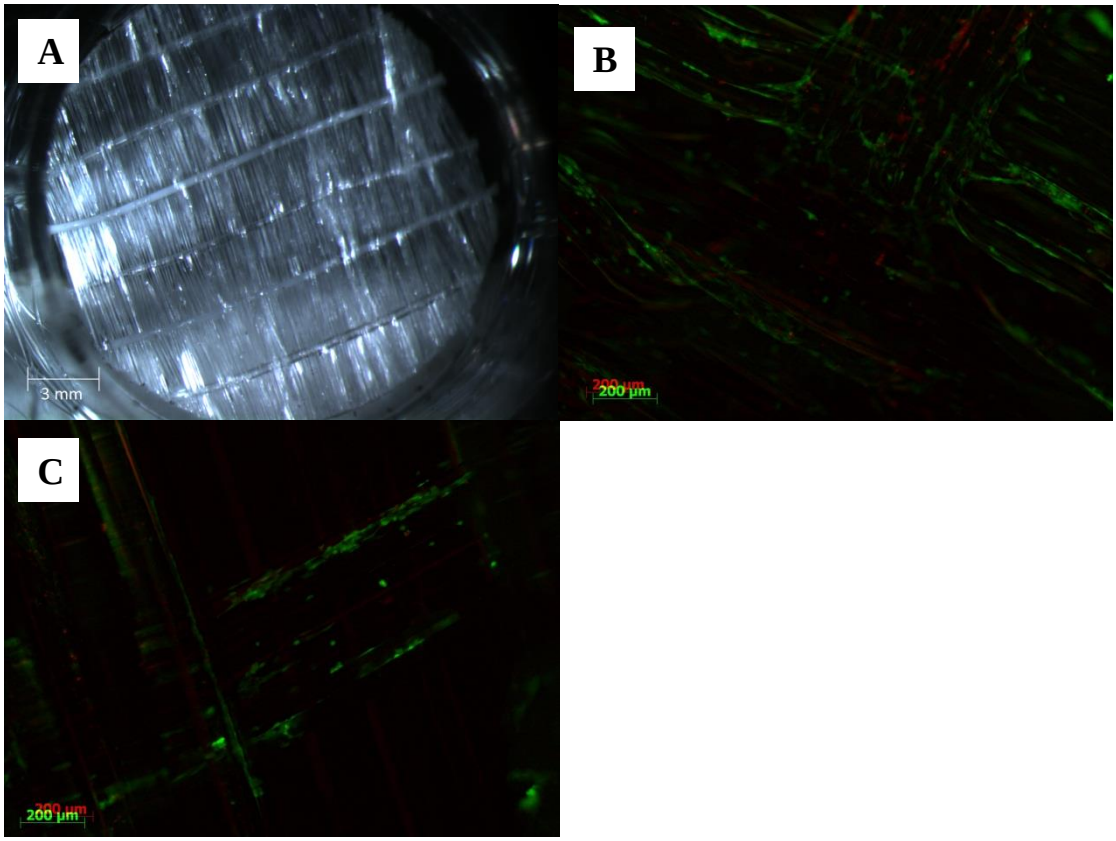


Figure 2.8: A) Stereoscopic image of woven scaffold cut and prepared for in vitro culture. Warp fibers are shown to be RND geometry with 4DG weft fibers packed together in a Plain weave configuration; B) Merged Live/Dead® images of PL warp and PLCL weft Plain weave scaffold with significant cellular attachment (green); C) Merged Live/Dead® images of PL warp and PLCL weft Twill weave scaffold with significant cellular attachment.

Porosity

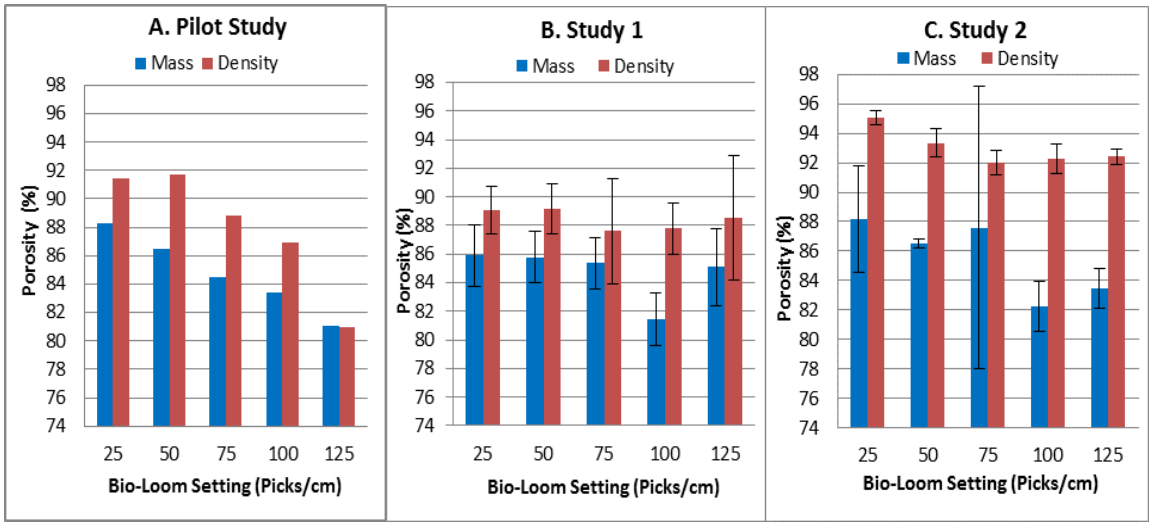


Figure 2.9: Porosity with varying bio-loom advancement spool setting. Both density and mass methods for porosity calculation are displayed. For 9A, n = 1 for each bio-loom setting. For 9B and 9C, n = 5 and n = 2, respectively.

Woven meshes were tested for porosity via mass and density methods with results shown in Figure 2.9. In each case density methods of measurement yielded a significantly higher porosity values than the mass method ($p = .0005$). This may have been due to experimental error associated with removing excess agarose gel from each mesh during the mass method, or inaccuracy of the 1 mm height assumption made during the calculation of total mesh volume for the density method. Results from the pilot study (Figure 2.9A) demonstrate a fairly linear decrease in porosity for both methods as porosity settings were increased. This pattern was the expected result as the study was repeated on several iterations of the experiment. Results shown from the follow-up experiments (Figures 2.9B and 2.9C) show a slight decrease in porosity as advancement spool settings were increased. However, the distinct linear relationship characterized in the pilot study was lost within sample variation.

Pore size

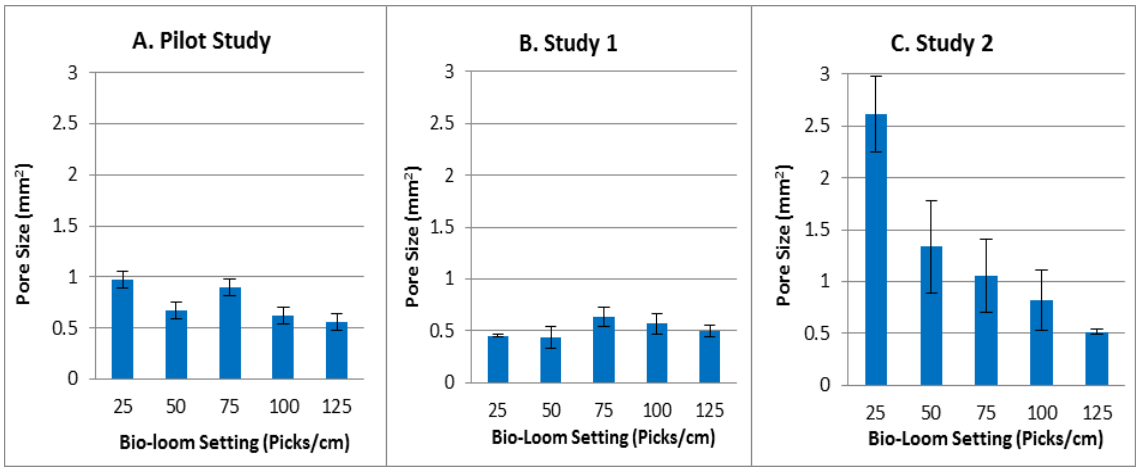


Figure 2.10: Pore size with respect to changes in the advancement spool setting on the bio-loom. For all data pictured, n = 2 meshes, with 10 random images taken on each mesh.

Pore size measurements were hypothesized to closely parallel results derived from porosity experiments. The results above show a statistically significant decrease in average pore size as the advancement spool bio-loom setting was increased ($p = .0002$, $p = < .0001$, and $p = < .0001$, respectively). These results demonstrate that as the number of weft fibers inserted within a 1 cm distance increased the spacing between each weft fiber was decreased accordingly. Differences in the magnitude of pore sizes between studies is most likely due to differences in overall fiber diameter of the meshes used in these experiments. For example, fibers from the pilot study were around 280 µm, whereas fibers from the subsequent studies were 360 µm and 200 µm, respectively.

Results described above seem to indicate several characterizations of the woven meshes produced by the bio-loom. Pore size and porosity variables do respond as hypothesized to changes in the bio-loom settings designed to affect those parameters. However, the range of settings at which the bio-loom was tested yielded results diluted by sample variation. This

variation is particularly an issue when considering varying bio-loom efficiency while weaving where a weft fiber may miss passing through the warp shed during a pick. This missed fiber results in a counted pick within a centimeter without the presence of an actual fiber. The result may be that a setting of 125 Picks/cm may result in anywhere from 80-115 actual correctly placed weft fibers. Additionally, the range at which the porosity and pore size were tested may also need to be expanded to as high as 200 Picks/cm to understand bio-loom porosity and pore size limitations.

Metabolic Activity

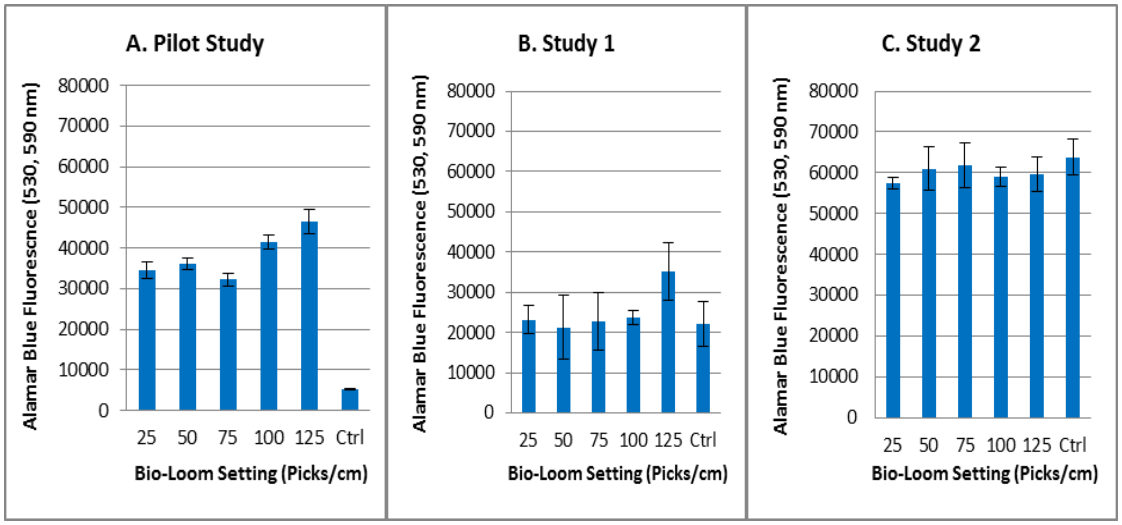


Figure 2.11: AlamarBlue® fluorescence reading in response to cell metabolic activity as it relates to advancement spool bio-loom setting. Sample size (n) for each bio-loom setting is equal to 1, 4, and 2, respectively, with three repeats for each sample in every study.

Figure 2.11 displays results for cell metabolic activity with respect to the bio-loom setting effecting pore size and porosity. Cell metabolic activity was used as a measure of cell viability and affinity. Future studies will seek the differentiation of these MSCs on woven meshes, therefore high metabolic activity during this proliferative phase of cell growth may indicate a

favorable cell response to the material and structure of the scaffold. It was hypothesized that cell metabolic activity would increase as bio-loom pore size/porosity setting increased. This is due to the smaller pore sizes produced by these settings during weaving. Smaller pore sizes have been shown to positively affect cell attachment and viability. Results demonstrate the gradual increase in cell metabolic activity as porosity/pore size is decreased. The majority of differences in cell metabolic activity were statistically significant with p-values of <.0001, <.0001, and .401, respectively.

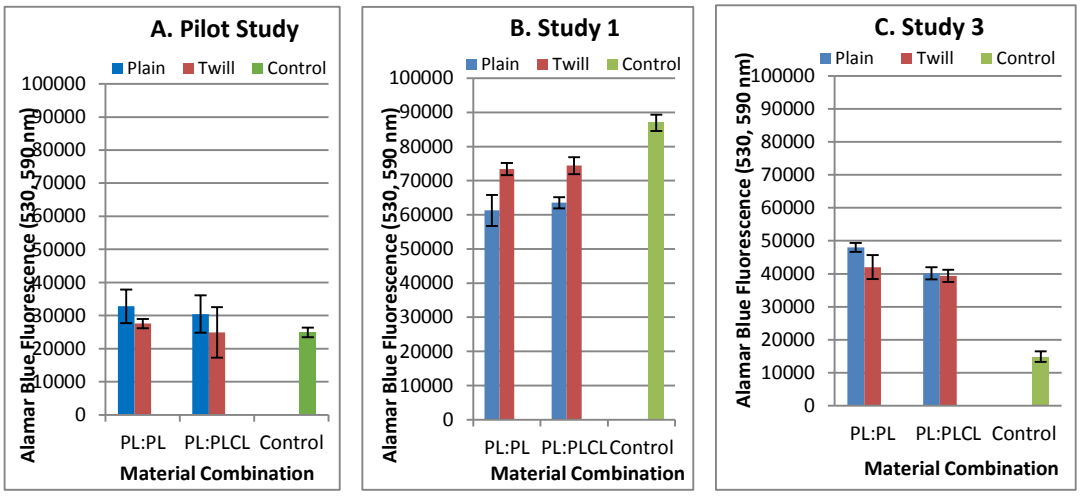


Figure 2.12: AlamarBlue® fluorescence reading in response to weave configuration and material combination. Sample size (n) for each bio-loom setting is equal to 2, with three repeats for each sample in every study.

Cell metabolic activity was measured against variable weave configuration and material combinations. Weave configuration most directly affects pore shape and material combination may elicit a different cell-biomaterial surface interaction between differing conditions. It was hypothesized that the Plain weave configuration would be more favorable for cell attachment and viability to the smaller pore size, despite the need for heterogeneous pore shape distribution throughout scaffolds. The Twill configuration was anticipated to display more variation in pore

shape distribution, but pores were also expected to be larger. The PLCL containing meshes were anticipated to increase cell attachment due to previous studies demonstrating increased cell attachment for PCL and PCL-containing fibers. However, these fibers were not arranged in a mesh configuration when tested previously. Pilot study results demonstrate a significant difference in weave configuration ($p = .0083$) where the Plain weave configuration is shown to be more favorable. Material combination results for this study were not significantly different. Study 1 yielded a significant result for material combination where PLCL containing meshes were seen to be more favorable with respect to cell viability ($p = < .0001$). Study 2 results report that meshes containing PL-only are more favorable ($p = .0134$) for cell viability. This conflicting response may be related to the need to melt-spin a blend of PLCL-PL "as-received" pellets instead of strictly PLCL material. This blend needed to be created to prevent the development of non-usable fibers due to excessive elasticity. Additionally, this blend allowed for a decrease in the amount of the more expensive PLCL material. PLCL melt characteristics were confirmed through DSC analysis so no significant thermal characteristics were affected.

DNA Concentration

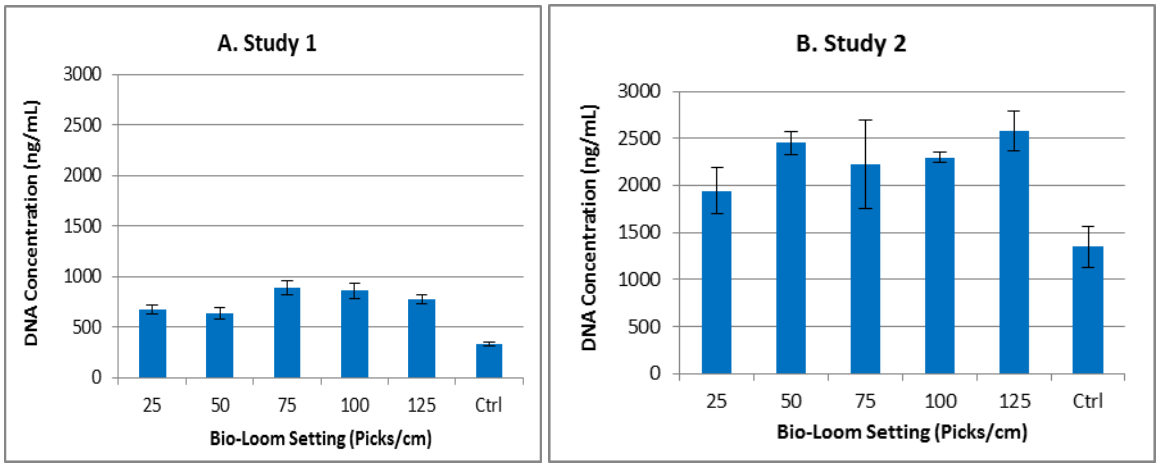


Figure 2.13: DNA concentration measured via PicoGreen® fluorescence with respect to advancement spool setting. Sample size (n) was equal to 3 with 3 repeats for each sample. A pilot study was not performed for this experiment.

DNA concentration was measured through a PicoGreen® assay in order to semi-quantify cell number at the change of each bio-loom variable. These results may be paired with AlamarBlue® results to elucidate the level to which attached cells are not only present (DNA concentration) but also viable (metabolic activity). Expected results for these tests were a close parallel with metabolic activity results, meaning an increase in DNA concentration as porosity/pore size was reduced (bio-loom setting increased). Results loosely agree with this hypothesis with DNA concentrations of low Picks/cm settings (25, 50) being significantly lower ($p = .0002$ and $p = .0801$, respectively) than those of high settings (75, 100, or 125).

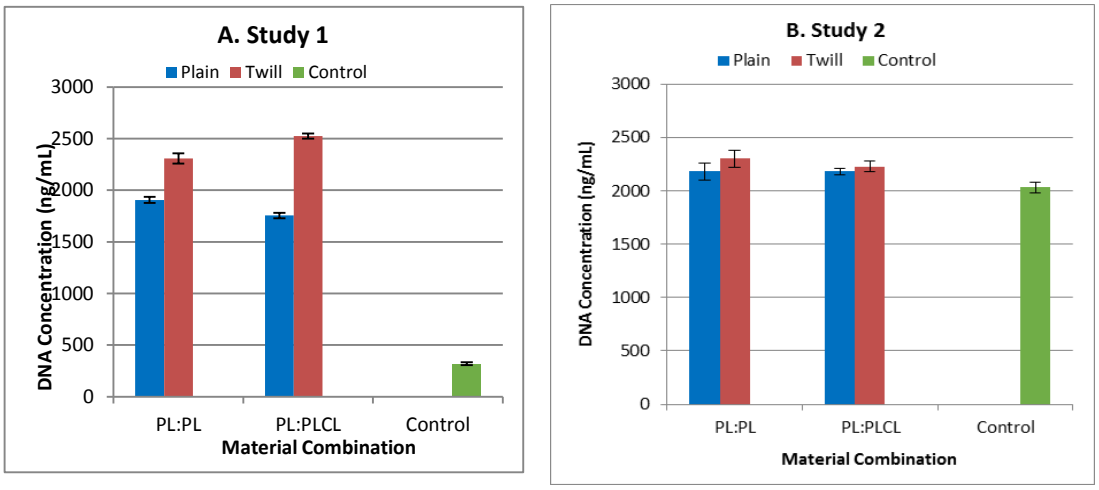


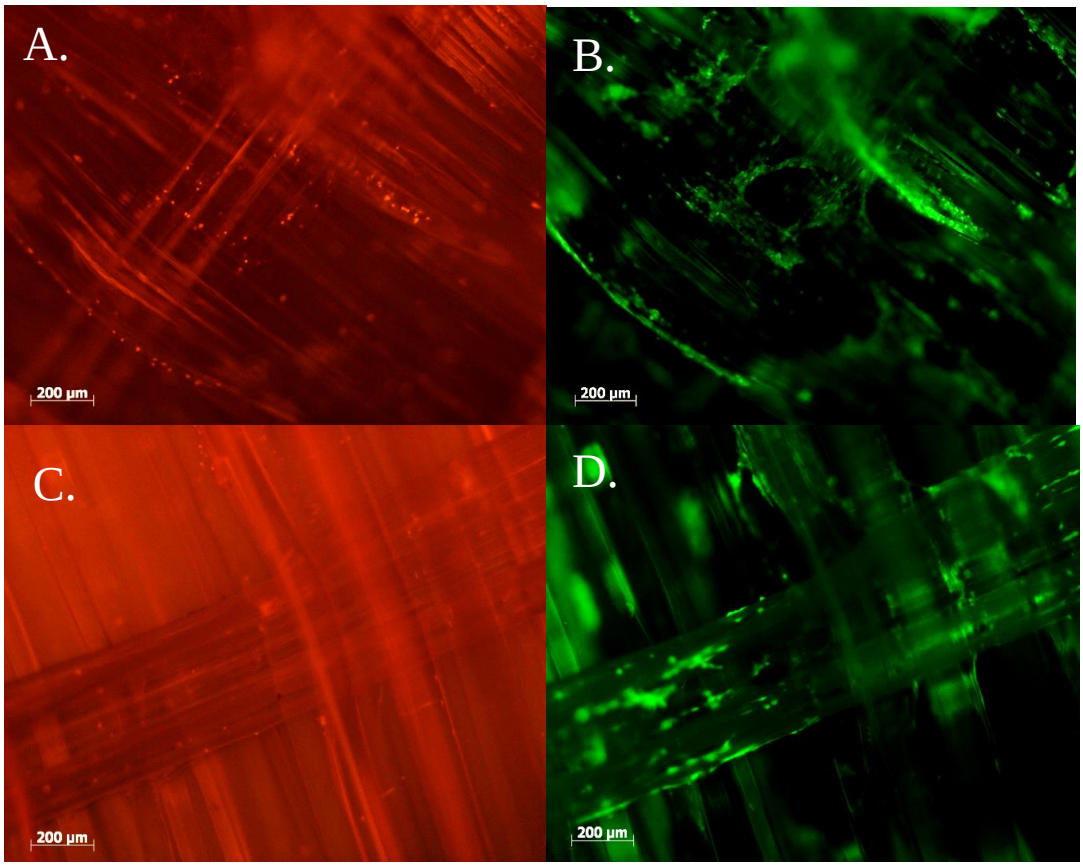
Figure 2.14: DNA concentration with respect to weave configuration and material combination. Sample size (n) was equal to 3 with 3 repeats for each sample. A pilot study was not performed for this experiment.

Similarly, DNA concentration was used to support findings from cell metabolic activity experiments in relation to material combination and weave configuration. The significantly higher

DNA content (p=<.0001) in the Twill cases for Study 1, along with results from Figure 2.12, suggests that this configuration may be more favorable to cells. Results for Study 2 are more even across both conditions.

Qualitative Cell Viability Measures

To characterize cell affinity on woven meshes qualitatively fluorescent staining and imaging were used through the calcein and ethidium-based Live/Dead® assay. Images were taken to understand where cells were attaching along the fibers and what effect proximity of nearby fibers (pore size) had on cell attachment. The images shown below are a representation of all images captured. Images show the living cells and dead cells at the same location within the mesh. Figures 2.15A-D focus on locations at the intersection of warp and weft fibers, where large concentrations of live cells can be shown. These images suggest that the interface between multiple fibers may be a favorable location for cell attachment, reaffirming the importance of reduced pore size. Figures 2.15E-H focus on areas where fibers are aligned in parallel to one another. Fair amounts of living and dead cells are pictured along the axis of the fiber. However, living cells seem to be congregating at areas where parallel aligned fibers are close together. In the case of figure 2.15H fibers are close enough for cells to be attached to both fibers. This result is also further evidence for the efficacy of the bio-loom in creating scaffolds that can sustain cell life and also alter variables that play a significant biological role, such as the distance between fibers.


A.
200 µm
B.
200 µm
C.
200 µm
D.
200 µm

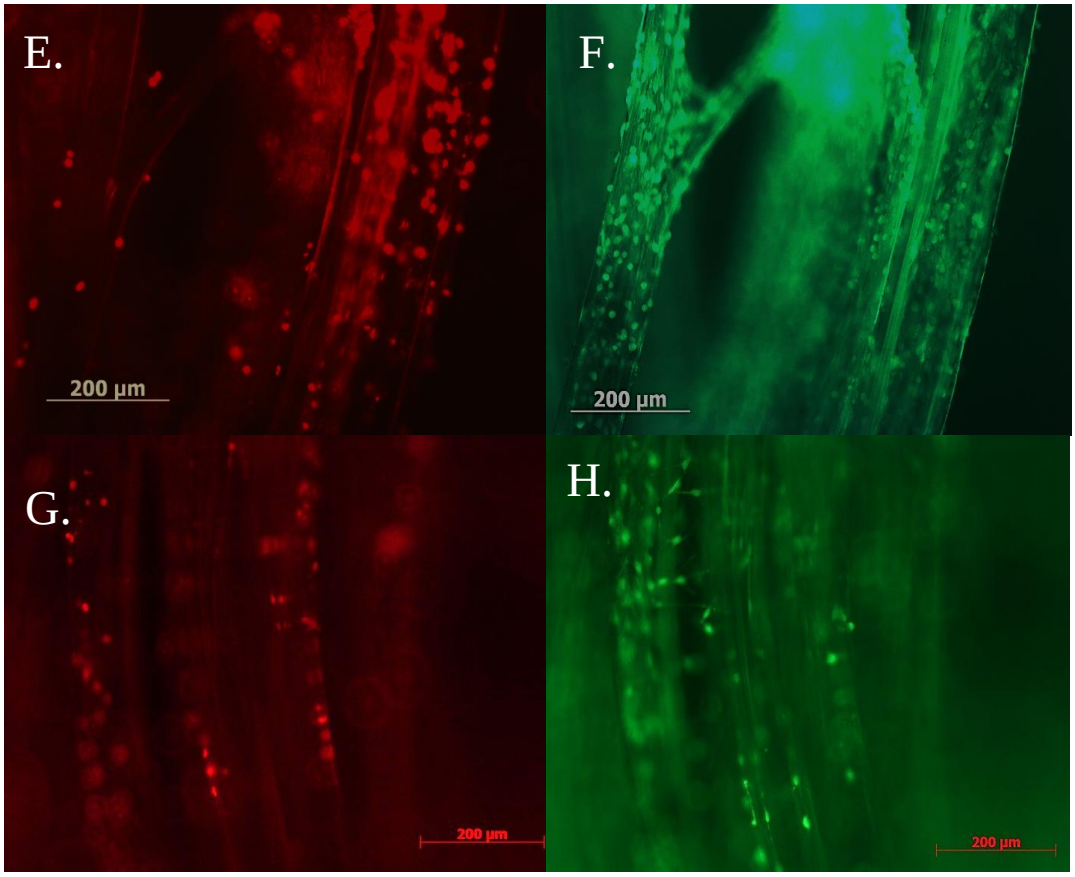


Figure 2.15: Live/Dead® fluorescent images of various cell-seeded meshes. Red denotes dead cells and Green denotes living cells. 2.15A-B show a Plain woven PL mesh at 5x magnification (50x total). 2.15C-D depict a Twill woven PLCL-containing mesh at 5x magnification. 2.15E-F show a Plain woven PL mesh at 10x magnification (100x total). 2.15G-H show a Twill woven PLCL-containing mesh at 10x magnification. All images were contrast enhanced +40% for publication purposes.

Cell affinity results were as expected with respect to porosity and pore size with cells preferring the smaller pores. Interestingly, weave configuration and material combination variations resulted in conflicting data concerning cell metabolic activity and DNA concentration. This conflict may indicate the need for a mixed set of variables to achieve ideal cell affinity conditions. Cells may favor the diversity of pore size offered in Twill meshes, but also the

material interaction of a PLCL-containing mesh. The small, regular pore size and high permeability of a PL Plain woven mesh may also be favorable.

Cells attached and proliferated on a variety of mesh types, indicating that the bio-loom, while able to create different meshes, is also capable of creating scaffolds consistently amicable with cell culture.

Fluorescent images show favorable cell attachment and survival at the intersections of warp and weft fibers, suggesting that moving warp fibers closer together in the design on the bio-loom may result in more tightly woven meshes and more favorable cell attachment. The deep grooves of the 4DG fibers implemented here also seem to serve as conduits for cell migration to areas of intersection of closer proximity to multiple fibers. This fiber geometry may be further implemented to direct cell movement to specific regions of the mesh in future developments.

Conclusions and Limitations

Currently, the bio-loom is limited in a number of parameters restricting the types and complexity of scaffolds created. There are currently only two warp fiber harnesses which restrict the complexity of weave configurations and pore shapes. Space for up to four harnesses has been included on the bio-loom and with the addition of these harnesses configurations such as 3D orthogonal weaving may be attempted. The current number of warp fibers (12) included on the motorized bobbin array serves as a size limiting factor for scaffolds. The reduction of the number of warp fibers, along with tighter spacing of the reed and advancement spool grooves, would result in a tighter, more compact woven scaffold. The addition of more warp fibers would require the application of a higher voltage across the series-connected DC motors. Currently, warp fibers towards the end of the series configuration receive less voltage due to losses along the array. These losses result in scaffolds with loose edges, thereby reducing the portion of scaffold able to be employed as a cell culture scaffold in *in vitro* experiments. Additionally, bio-loom efficiency

could also be increased by fine-tuning the air jet system to accurately place each fiber through the warp shed each time. Currently, the air jet misses the warp shed approximately 30% of the time, with this percentage increasing as the compliance of the weft fiber material decreases.

Operational speed optimization of the bio-loom and tunable pneumatic control of components are proposed strategies for future development to reduce the tensile strength required for fibers to be "weavable". Currently, the system requires stronger fibers to resist forces exerted during operation. These stronger fibers are mostly produced through an increase in fiber diameter. Smaller diameter fibers are hypothesized to provide more favorable biological results so accommodations such as reducing bio-loom speed and damping the pneumatic response of the solenoid valves is necessary to accommodate weaker fibers. Strategies for increasing strength while decreasing diameter have also been explored through various melt-spinning techniques, including adding a variable heated-draw step to the extrusion process.

Future aims include the weaving of meshes with different materials and more complex configurations to characterize the stability of the general relationships shown here for pore size, porosity, and cell affinity. Additionally, an expansion of the range of tested porosity/pore size settings should be tested to understand the manufacturing limits of the bio-loom. Efficiency of the loom during weaving should improve with the narrowing of warp fibers creating a tighter weave. This change may decrease the sample variability during the permeability and pore size tests.

The current state of the bio-loom employs an automated tension control system, weave configuration system, weft fiber placement system, and interactive user interface to weave melt-spun polymeric fibers into tissue engineering scaffolds for bone tissue applications. The manipulation of these integrated systems results in the ability of the bio-loom to effect biologically significant change in pore size, porosity, permeability, and weave configuration (pore shape). The ability to change these parameters may allow future researchers to implement such a

system to develop scaffolds for specific critically sized defects and customizable solutions to presentations of frustrated bone healing in the clinic.

The cell studies done in this work suggest that a combination of the factors manipulated by the bio-loom parameters is needed for ideal cell affinity. Future work will support the findings here, through differentiation studies and the characterization of a more diverse group of meshes, to truly indicate biologically significant results.

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CHAPTER THREE

EVALUATION OF VARIABLE WOVEN MESH FLUID-FLOW PROPERTIES THROUGH PERMEABILITY AND VERTICAL WICKING CAPABILITY

Introduction

There continues to be a clinical need in strategies for bone replacement in cases of segmental bone defects and nonunions. These defects, often the result of traumatic fractures or tumor resections, contribute to the development of critical-sized defects (CSD). CSDs present a significant clinical challenge, to which clinicians and researchers have initially developed bone grafting procedures.[1] Autologous grafts of the iliac crest have been the standard of care.[2] However, these grafts present significant drawbacks given the extensively documented complications (20.6% minor and 8.6% major complication rate, respectively) associated with donor site morbidity and lack of viable donor tissue in patients with other bone pathologies such as osteoporosis or bone cancer.[3-7] As a solution, bone graft substitutes, implementing the cell-seeded scaffold concepts of tissue engineering, have emerged as an important area of research. In developing appropriate scaffolds for clinical purposes there are many variables that must be investigated. This work is focused on the characterization of mesh fluid flow properties as they relate to the transport functionality of the woven meshes created by the bio-loom. Transport properties in tissue engineering scaffolds have been shown to affect diffusion of nutrients and waste and are modulated by scaffold porosity and permeability.[8] The transport of nutrients and waste is critical in the promotion of angiogenesis, tissue ingrowth, prevention of adjacent tissue necrosis, and MSC proliferative or differentiative capabilities.[9, 10]

In describing the ideal scaffold, Hutmacher claimed that a scaffold should be three-dimensional, highly porous with interconnected pore network for cell growth and flow transport of nutrients and wastes.[11] To address this requirement some researchers have focused on developing woven or knitted scaffolds due to their ability to combine the effects of several

parameters through varying scaffold materials, structure, and geometry. Two variables often investigated are porosity and permeability. Porosity and permeability have often been incorrectly used interchangeably in the literature. Porosity refers to the amount of void space within a structure, and can be related to varying the surface area exposed to cells and fluid.[12] Previous studies showed strong correlation between high surface area - to - mass ratios and cellular attachment and differentiation.[13] Permeability is a measure of the degree to which fluid flows through a structure and can be related to fluid-biomaterial and fluid-cell interaction.[12] Both porosity and permeability affect nutrient/waste transport, biomechanical cellular response, and material degradation. The two parameters are related through the degree of pore interconnectedness in a scaffold.[14] Two scaffolds may have the same void space, but their measures of permeability may be different due to varying levels of pore interconnectivity and tortuosity.[9] Within the realm of musculoskeletal tissue engineering, it is important to include a passageway for nutrients and wastes for the seeded cells. In living tissue, vasculature supplies this function, directing both diffusive and convective transport. However, there is a lack of this blood supply immediately after implantation or *in vitro* leading to tissue necrosis, arthrodesis formation, or failed healing.[9]

This work focuses on the characterization of permeability across a set of woven polymer meshes with variation in material combination, weave configuration, and fiber geometry. To further explore the fluid transport characteristics of these scaffolds, wicking fibers were included as a variable in the scaffolds and an analysis of the vertical wicking capability was conducted. Wicking (deep-grooved, 4DG) fibers have grooved cross-sections and parallel continuous channels that run the length of the fiber.[15] Grooves and spaces were hypothesized to enhance and increase transport properties in this experiment's scaffolds. Previous studies showed that the rate of diffusion of proteins is greatly enhanced in hydrogels containing wicking fibers compared to

those without wicking fibers.[15] By weaving melt-spun polymer fibers of specific cross-sectional geometries, the permeability and wicking properties of these scaffolds were modulated and tested using an adaptation of Darcy's law and a constant head hydraulic conductivity test.[16, 17] Experimental measures of permeability and volumetric fluid transport were used to determine the relative transport capacity of variably constructed woven meshes. Bio-loom parameters modulated for Chapter 2 were also modified here to determine the effect of those parameters on transport. The four different scaffold combinations investigated here explore optimal scaffold parameters and inform future bone tissue engineering studies.

Research Questions

The following questions were used as a guide in the study of fluid flow properties in the previously developed woven meshes. In addition to answering these questions, there was also focus on understanding the implications of any significant change on the proposed use of theses meshes as bone tissue engineering scaffolds.

1. To what extent does changing bio-loom parameters affect overall fluid flow through the scaffold as reported by the hydraulic conductivity coefficient?
2. To what extent does changing bio-loom parameters affect the scaffold directional fluid flow properties?

Materials and Methods

Scaffold Development

Scaffolds were constructed using the custom-built bio-loom discussed in Chapter 2. Using this bio-loom, scaffolds can be woven in a variety of material and geometric configurations. The efficacy of the loom in modulating these parameters enables the ability for specific testing of various scaffold properties, with this work focusing on the change in the permeability and fluid transport as characterized by wicking rate.

Given the proportional relationship between porosity and permeability, scaffold pore size was modulated by changing the configuration with which each scaffold was constructed. Previous work (detailed in Chapter 2) validated the ability of the loom to affect porosity through changing the collection speed of completed woven scaffold material. The aim of this work was to explore the differences in scaffold permeability based on changes in weave configuration effecting pore size and pore shape. Scaffolds were weaved in two configurations for this study. Both the Plain and Twill weave configurations are pictured in Figure 3.1. Weft fibers are those in the horizontal plane (left-to-right), and warp fibers are those in the vertical plane (top-to-bottom). Increased space between warp or weft fibers resulting from changes in configuration was hypothesized to produce changes in pore shape and size, as well as changes in permeability.

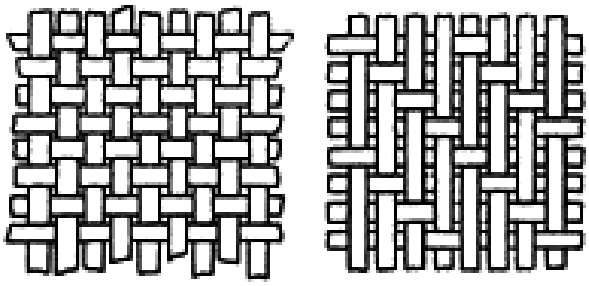

Figure 3.1: Left – Plain Weave, Right – Twill Weave (www.rapra.net)

The Plain weave configuration can be described as a simple over-under pattern in which warp and weft fibers are passed over and under each other in an interlocking fashion with each pass of the loom. This configuration was hypothesized to produce a tighter weave, with smaller, more constricted pores, thereby producing a less permeable scaffold. The Twill configuration varies in that weft and warp fibers are passed over and under only after every fourth pass of the loom. It was hypothesized that this design would create a more loose weave, resulting in larger, more open pores, giving way to increased permeability of the scaffold overall.

In addition to permeability and wicking changes being induced by the macro-pore structure created through weave configuration, this study also aimed to modulate permeability

through weaving fibers of different geometries. The two fiber geometries tested here were round fibers (RND), with a circular cross-section, and deep-grooved wicking fibers (4DG), with the cross-section shown below in Figure 3.2. The wicking fiber geometry has been designed for the transport of liquid through the channels of fibers by the Eastman Chemical Co. This geometry has been employed in various textile and transport applications[18-20], but was introduced to our research group through the work of Burg and colleagues.[21] This capability will be employed to transport liquid throughout the scaffold. Given the additional spaces for fluid to travel and potentially become trapped in the wicking fibers, it was hypothesized that adding wicking fibers would decrease the permeability of the scaffolds due to the increased tortuosity of the fluid path through the scaffold.

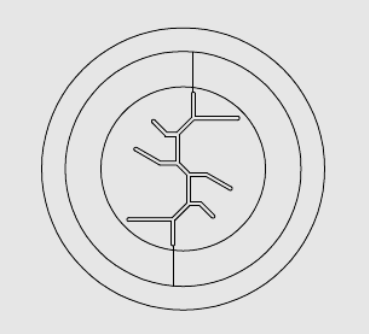

Figure 3.2: Deep-grooved (4DG) wicking fiber cross-section

The two materials used in this study were melt-spun polymer fibers consisting of Poly-L-lactide (PL; Natureworks LLC, 2003d biopolymer, ~ 228,000 Da) and a PL: Poly-l-lactide-co-ε-caprolactone (PLCL; Purac, Purasorb PLC 7015, ~ 154,500 Da) blend. The PL:PLCL blend was a 70:30 weight to weight ratio of polymer chips as received from the manufacturer. This combination is denoted PLCL throughout this work. Fibers had an average diameter of approximately 260 μm post melt-spinning.

Permeability Test

Testing the permeability of the scaffolds of interest initially proved challenging in that there is currently no standardized method for this type of analysis on a tissue engineering scaffold. One of the challenges associated with developing such a standard is the variability of geometry, materials, and mechanical properties that may be present in a bone tissue engineering scaffold. Other forms of analysis, such as Brunaeur, Emmett, and Teller (BET) surface area analysis by gas absorption were investigated for use in this work. However, BET analysis proved to be an inefficient method given the requirement of at least 1.0 gram of material for analysis. The average mass of a mesh woven on the bio-loom, cut to size for a 24 well plate, was approximately 60 mg. Therefore, performing a BET analysis would have required more than 10 woven meshes, with the remaining issue of deriving permeability from this indirect measure confounded by sample variability between meshes. Similar complexity and variance issues were present in the consideration of imaging techniques for indirect measures related to permeability as well.

These difficulties led to the adaptation of permeability calculation techniques from soil science concepts. The constant head hydraulic conductivity test and the falling head hydraulic conductivity test were of particular interest.[22] Both tests are based on the application of Darcy's Law to fluid flow through a porous medium. Darcy's Law states that the rate at which a fluid flows through a porous medium is based on two factors, 1) the size of the hydraulic gradient of the water head (dH/dx), and 2) the permeability of the porous medium as described by the permeability coefficient, k [m^2].[23] Additionally, there are three conditions to be met in the application of Darcy's Law, 1) flow is assumed to be laminar, with no turbulent flows, 2) the porous medium is fully saturated, and 3) the flow is in steady state with no temporal variation.[23] The constant head test was more suitable to the testing of these scaffolds given its basis on the

application of a constant head pressure across the sample throughout the measurement. Soil test using this method often employ a spring or screw-loaded system to apply pressure, however, for the purpose of this test, gravity was considered the constant pressure on the scaffold.

Fellow researchers have also taken the approach of building custom permeameters and modifying Darcy's Law to measure bone tissue engineering scaffold permeability. Work by Dias and coworkers focused on validating the use of their custom system against a computation finite element model of fluid flow through a porous scaffold wax model of varying height and porosity.[24] The custom system compared in this work was first described by Kemppainen and Hollister and focused on passing fluid through a porous scaffold at a constant rate and tracking the change in weight over time of the outlet reservoir collecting the fluid. This change was then used to calculate permeability.[25] While this design achieved results linearly related to the finite element analysis model, overly complex equipment requirements motivated the search for a simplified method.

A pilot study was conducted focusing on measuring permeability of a scaffold through recording the time required for a fixed volume of fluid to pass through a test sample. The apparatus described in Figure 3.3 below featured a gravity-fed system in which fluid loaded into the reservoir was passed through the mesh at a constant rate. This initial design featured a valve release system operated by hand, with a camera recording the time of valve opening until the time that 40 mL of colored water had passed through the mesh. The mesh was held in place by a custom-built fitting threaded to accommodate the ¾" valve outlet. The fitting was designed in SolidWorks (Dassault Systèmes SolidWorks Corporation, Waltham, MA) and 3-D printed using a Cubify Cube 3D Printer (3DSystems, Rock Hill, SC). A representation of the fitting is pictured below in Figure 3.3.

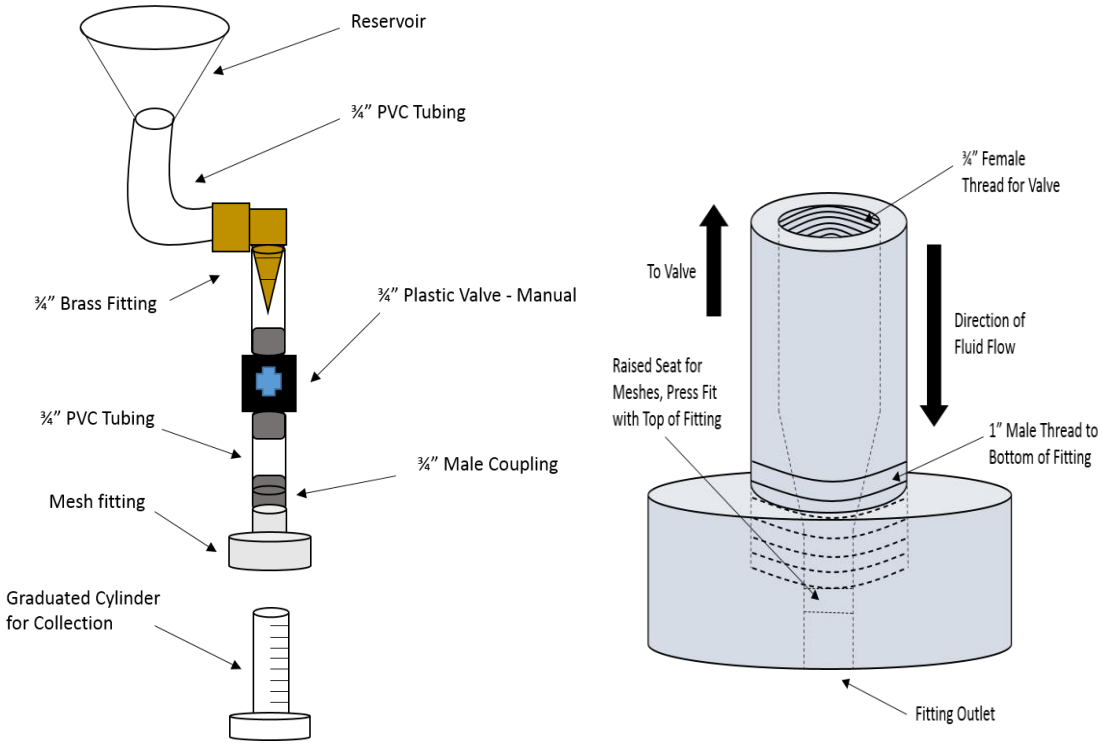

Figure 3.3: Left – Schematic of permeameter set up for pilot study, Right - Permeameter mesh fitting used to house the mesh during testing of permeability

The outcome of the pilot study revealed that this method for calculating permeability was a viable option. Results (Figure 3.4) demonstrated that differences in mesh permeability based on bio-loom parameters could possibly be detected using the developed system. There were no statistically significant differences ($p = 0.301$) for this pilot. However, it was hypothesized that automation of the system by controlling the release valve electrically, as opposed to manually, would eliminate some of the within sample variance. There was also a goal to automate the collection of time data by digitally monitoring the state of the valve (open/closed).

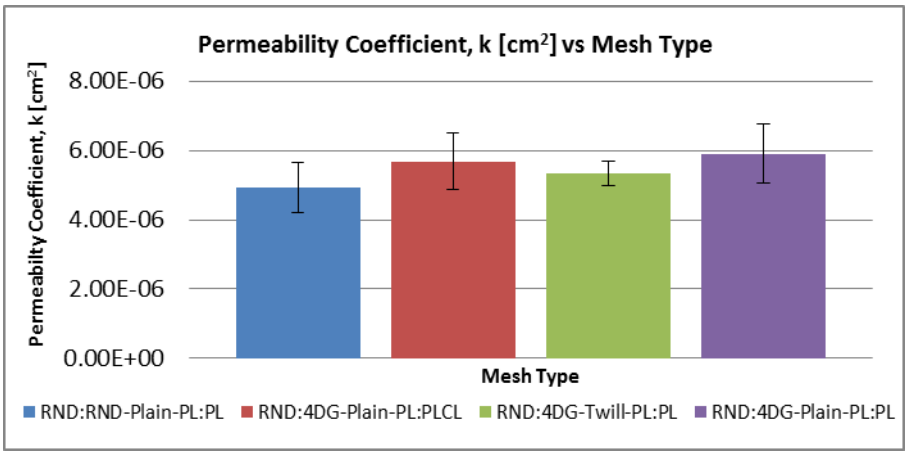

Figure 3.4: Permeability pilot study results demonstrating efficacy of custom permeameter strategy based on adaptation of Darcy's Law. N = 3 for each mesh type.

The final design of the permeameter was the result of several design iterations. The primary focus of the design changes was increased automation of the system. A solenoid valve (triggered by 12 VDC) replaced the manual valve to remove the human error from opening and closing the valve during testing. This valve was triggered by a 5VDC relay. Initially, this relay was connected in series with a two-position toggle switch to the 5VDC power supply. The manual triggering of this toggle switch would then open and close the solenoid valve. However, to fully automate the system the relay was to be triggered through the level of fluid in the collection cylinder. To incorporate this control, a water level sensor was designed to measure the level of water in the cylinder as it passed through the mesh. The conductive water would serve as a switch, replacing the toggle switch, and triggering the activation of the 5V relay. Additionally, the goal of automating the measurement of time it took for the fluid to pass through the mesh was addressed by creating a LabView (National Instruments, Austin, TX) program that monitored the voltage across the water level sensor. The threshold voltage of 1.2 V was employed to trigger the timing program to start and stop. The timer in LabView was pulled from the internal computer clock with timestamps being created for the start and stop times. The difference between these

two time stamps was considered the pass through time for the particular mesh. This time was then stored in an array that could be printed and exported to Microsoft Excel for further analysis and calculation of the permeability coefficient. Figure 3.5 displays a schematic of this iteration of the design, along with a photograph.

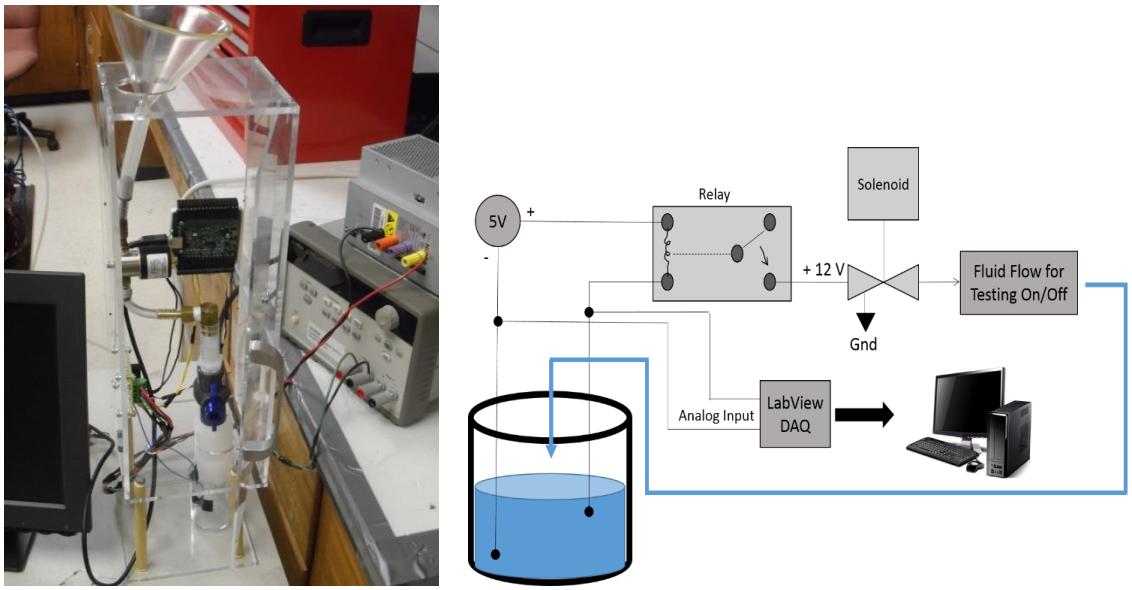

Figure 3.5: Left – Photograph of permeameter, Right – Schematic detailing water level sensor incorporated into control of permeameter system

There were several issues with the previous design that resulted in further development and the eventual simplification of the design for data collection purposes. The first issue was the conductivity of the fluid being passed through the mesh. Initially, water was used, but it was discovered that the impedance of this medium needed to be greatly reduced to register a voltage high enough to trigger the relay. Other, water soluble ionic compounds and solutions were employed, such as phosphate buffered saline (PBS, Sigma), ammonium chloride (NH_4Cl), and sodium chloride (NaCl). None of these solutions brought the voltage to the level that was needed to trigger the solenoid. There was a voltage difference present when both leads from the water level sensor were submerged in liquid. However, this difference was not significant enough to

cause a logic change in the LabView program. To remedy this, the next strategy was to manipulate the threshold triggering a logic high or low in LabView. A series of Schmidt triggers were used to accomplish this task. Schmidt triggers have classically been used as rudimentary analog to digital converters, taking a rapidly changing analog signal and outputting a more stable digital signal based on the changing voltage level with respect to a tunable threshold. A signal must pass over or under the threshold to institute a logic level change. This change moved the LabView data acquisition card from capturing an analog signal input to capturing a digital signal input, which changed the program to being based solely on Boolean logic. A change in the data structures required to accommodate this Boolean logic proved difficult to change. Rather than continue rewriting the LabView code, the decision was made to reduce the system back to the toggle switch state in order that data could be collected in a timely manner. The results included in the section following were collected using the toggle switch based system. The circuits employed in these design iterations are included below. In each case, a printed circuit board was made using the peroxide/muriatic acid etchant method, with circuits designed in CadSoft Eagle PCB Design Software (CadSoft, Inc., Ft. Lauderdale, FL).

Permeability of each mesh was calculated through the adaptation of Darcy's Law shown in Equation 1 and a constant head hydraulic conductivity test.[22] Scaffolds were placed into the fitting chamber and secured with tape along the edges outside of the testing area. 40 mL of fluid was then passed through the scaffold by engaging the gravity-fed automated valve system. Videos were recorded of fluid being passed through each scaffold, with the pass-through time of 40 mL of fluid being recorded for each scaffold. These times were then applied to the equation below, with the following variables being constant: fluid viscosity, μ, approximated as 0.001 Pa·s (viscosity of water); length from fluid reservoir to scaffold, L, equal to 45 cm; hydraulic head pressure, h, approximated as 4414.5 Pa given the gravitational constant and neglecting the

changing height of the reservoir level during the test due to insignificant distance change; and surface area of the scaffold exposed to fluid during the test, A, calculated as 0.4418 cm^2. The flow rate, Q, was calculated through dividing the fluid volume of 40 mL by the time measured via video. All of these variables were combined according to Equation 1 to yield the permeability coefficient, k [cm^2].

Equation 1: $$k = \frac{Q * L * \mu}{A * h}$$

Three iterations of this study were completed, each categorizing the permeability of four meshes from four different material and configuration combinations. The combinations are reported in the following format: *Weft Fiber Geometry: Warp Fiber Geometry – Weave Configuration – Polymer Material.* The four combinations of scaffolds are as follows: 1) 4DG:RND – Plain – PLCL; 2) 4DG:RND – Plain – PL; 3) 4DG:RND – Twill – PLCL; and 4) 4DG:4DG – Plain – PLCL. The total number of scaffolds analyzed for each condition was 12, giving a total of 48 scaffolds analyzed overall.

Wicking Test

The method for testing the vertical transport of fluid through a scaffold was adapted from work by Tabbaa and Burg regarding the wicking of cells through 4DG constructs.[15] Figure 3.6 illustrates the set-up of scaffolds being analyzed for this study. Scaffolds were held in the upright position in 35mm x 10mm polystyrene petri dishes with custom slotted inserts. The bottom of each mesh was positioned in such a way that only the tips of the fibers contacted the liquid in the bottom of the dish. Prior to the addition of the scaffold, each dish was weighed both before and after the addition of 2 mL of water. The water was also colored to facilitate the visualization of wicking in real time. Aside from dishes with scaffolds in them, an evaporation control was also established by treating a dish exactly as the dishes with scaffolds except for the addition of a scaffold. This evaporation control was weighed before and after a 30 minute period to determine

the amount of fluid lost to evaporation. The same process was followed for each scaffold-containing dish. The 30 minute wicking period was determined from a previous preliminary time study focusing on the time after which scaffolds no longer vertically wicked fluid. The time resulting from this preliminary study was approximately 30 minutes, therefore this time period was used for this study. Pre- (M_{t0}) and post-mass (M_{t30}) differences were taken and the evaporation control (E_i) and empty dish mass (M_e) were subtracted out of all results, according to Equation 2, giving the total volume of fluid wicked (V_{wicked}).

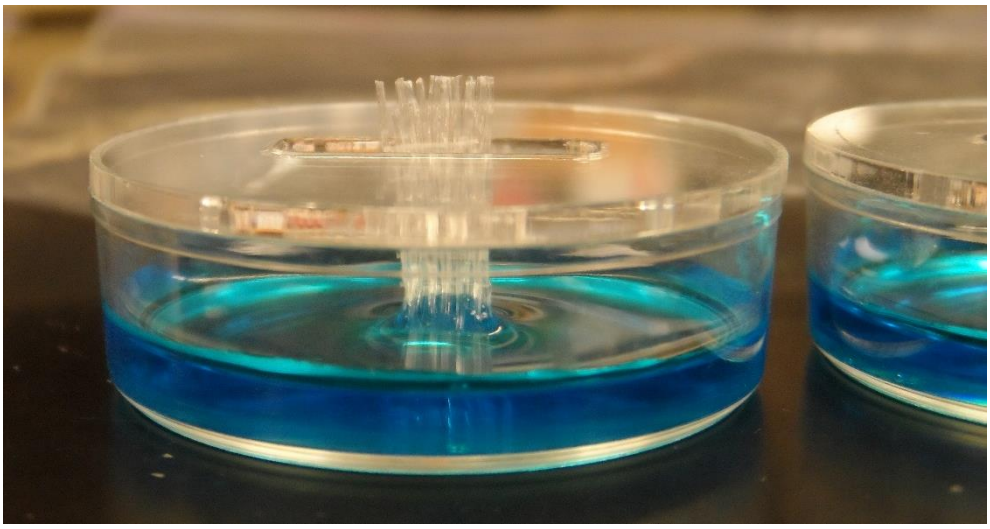

Figure 3.6: Wicking test set-up, with 2mL colored water and scaffold supported by slotted insert

Equation 2: $$V_{wicked} = ((M_{t0} - M_e) - (M_{t30} - M_e)) - (M_{t30} * E_i)$$

This method was validated through the conduction of a pilot study highlighting the differences in meshes subjected to the wicking test protocol. This preliminary study (Figure 3.7) pointed to potential differences in mesh wicking rate based on weave configuration, material combination, or fiber geometry. However, a limited number of samples (n=2) made it difficult to draw conclusions. To follow up on this study the same number and combination of scaffolds was used for the wicking test as was used for the permeability test, resulting in a total of 48 scaffolds analyzed.

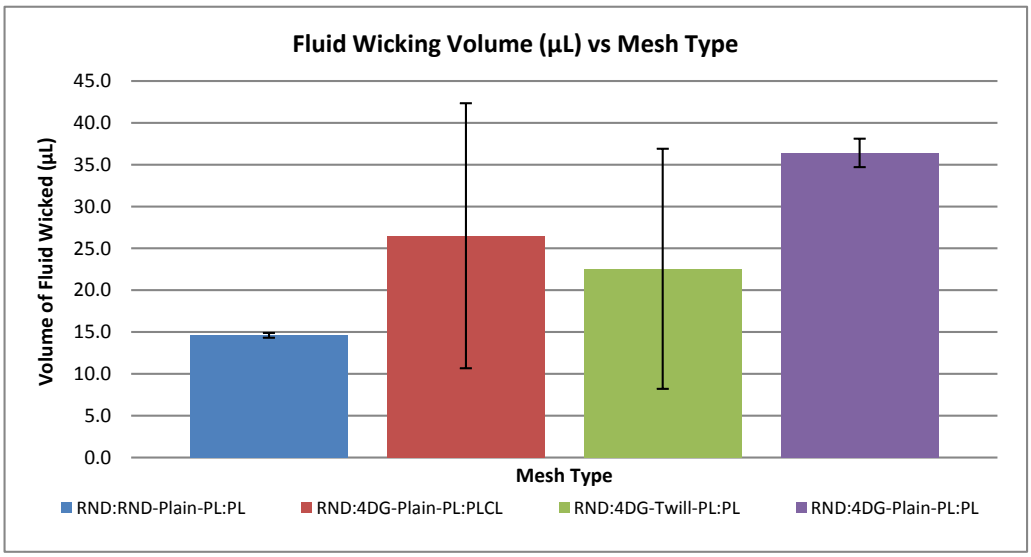

Figure 3.7: Wicking test pilot study results showing potential differences in mesh type ($p = 0.359$). $N = 2$ for each mesh type.

Statistical Analysis

Given the factorial nature of this study, all of the 32 potential variable combinations were not tested. The four combinations that were selected serve as a representative sample of these variable combinations. Analysis of the results from both the permeability and wicking studies was conducted through Analysis of Variance (ANOVA) testing for the simple means model, including effects for material combination, fiber geometry, and weave configuration. Subsequent analysis focused on isolating specific effects within the data. Both analyses included a level of significance, α, of 0.05. Statistical analysis was conducted through the use of JMP Pro 10 Statistical Analysis Software.

Results and Discussion

Permeability Test Results

The overall results presented in Figure 3.8 suggest that all scaffold types yielded similar permeability coefficients across all three experiments. However, further examination of results through analysis of isolated variables (Figure 3.9) reveals that there were some differences in

scaffold permeability that were potentially confounded due to combination of variables. Figure 3.9A shows a statistically significant difference in permeability for 4DG fiber-based scaffolds and RND fiber-based scaffolds. The 4DG fiber geometry was reported to be more permeable than the RND fiber geometry, which was contrary to the initial hypothesis for this case. While it was expected that the channeled fibers would transport fluid more quickly across the scaffold, it was thought that fluid being wicked away from the direct fluid contact points on the scaffold would lead to an increased pass-through time for the test fluid. This increased time would then lead to a decreased flow rate, thereby decreasing the directly proportional permeability coefficient. However, these results suggest that the opposite scenario is true. It is possible that the channeled transport of the 4DG fibers facilitated transport so much more efficiently than the RND fibers that the increased time for fluid spreading across the scaffold was insignificant, thereby increasing the flow rate and permeability coefficient.

Figures 3.9B and 3.9C did not reveal statistically significant results but suggested that the Twill weave configuration was more permeable than the Plain configuration (p-value = 0.2758). This result was expected as the Twill configuration was meant to increase the overall pore size of the scaffold resulting in reduced resistance to the passing through of liquid. Scaffolds that incorporated PLCL into the material combination also demonstrated higher permeability than those only containing PL (p-value = 0.0816). While the composition of the fibers was fairly similar, the increased hydrophobicity of PLCL over PL may have contributed to a higher contact angle for the fluid hitting the scaffold surface, resulting in faster beading of the fluid and saturation of the scaffold. This explanation would contribute to a faster pass-through time, and therefore a higher permeability.

The statistical models based on one isolated variable reveal fiber geometry as the most significant variable effecting permeability. But the combination of variables, shown below in

Table 3.1, also reveal that the interaction between these characteristics also directly affect permeability. A statistical model based on a combination of all of the variables revealed a significantly different permeability coefficient ($p = 0.0313$) across all three experiments with the 4DG:4DG – Plain – PLCL scaffold type being the most permeable. An analysis of fiber geometry and weave configuration with respect to the permeability coefficient of all scaffold types yielded a significantly higher k for 4DG:4DG scaffolds with a Plain weave configuration ($p = 0.0103$). These results suggest that weave configuration may be the poorest predictor of permeability information, with fiber geometry and material combination demonstrating significant influence over permeability performance of scaffolds.

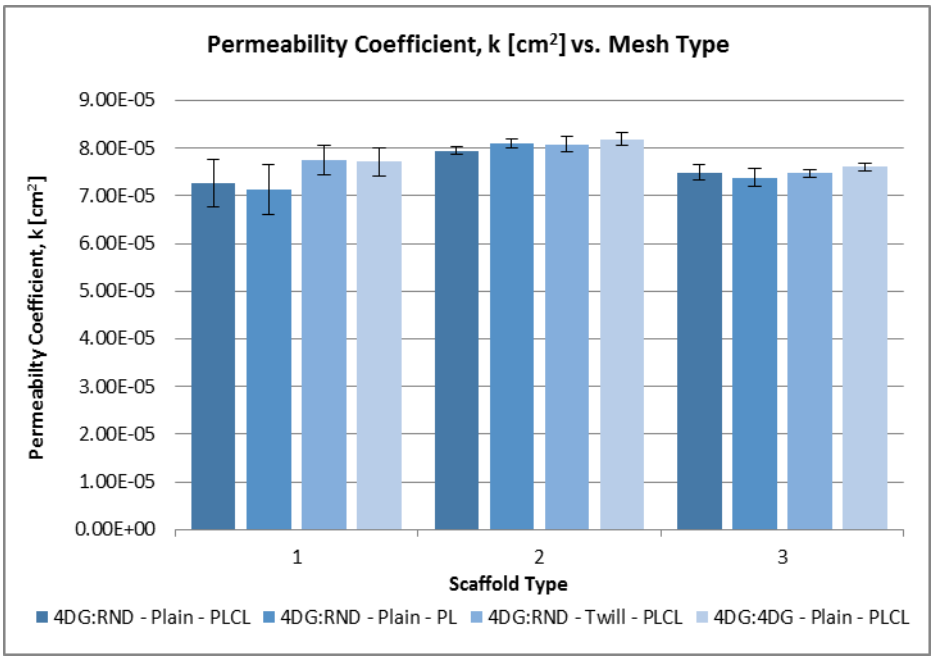

Figure 3.8: Permeability testing results detailing the calculated permeability coefficient with respect to each scaffold type that was tested. N = 4 for each scaffold type over all three experiments, giving an overall N = 48. No statistically significant scaffold types were indicated.

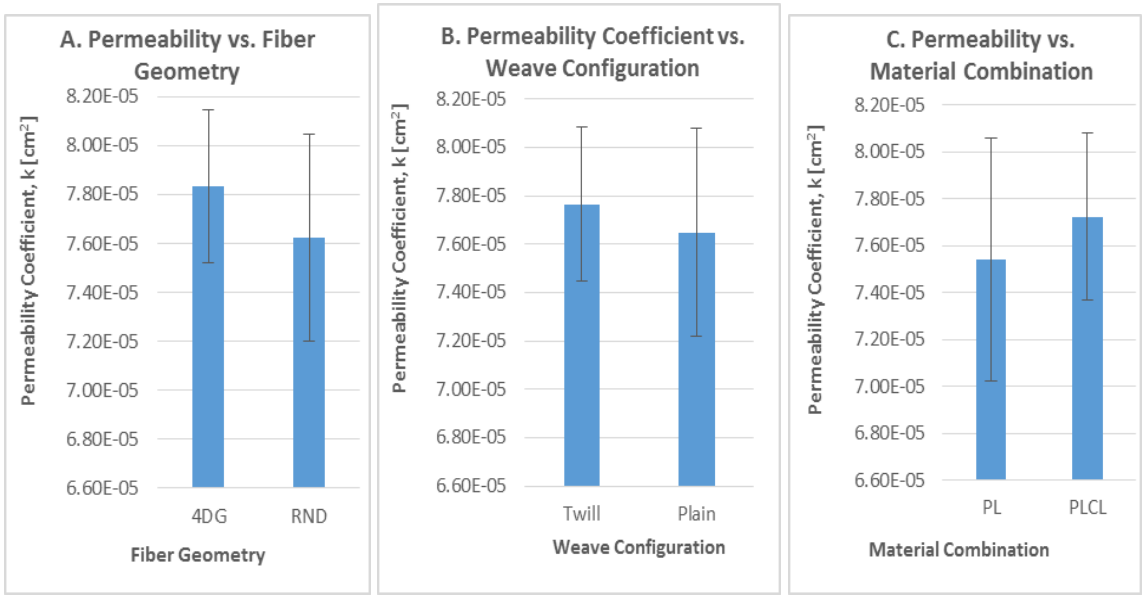

Figure 3.9: Permeability test results with calculated permeability coefficient, k. Figure 3.9A represents these results with respect to fiber geometry. N = 12 for 4DG samples, and N = 36 for RND samples. Figures 3.9B and 3.9C represent weave configuration and material combination, respectively. N = 12, N = 36, N = 12, and N = 36 for Twill, Plain, PL, and PLCL samples, respectively. 4DG fiber-based scaffolds were significantly more permeable than RND fiber-based scaffolds, p-value = 0.041 (Figure 3.9A).

Table 3.1: Permeability Test - ANOVA Statistical Analysis

Permeability Coefficient vs. Fiber Geometry x Weave Configuration x Material Combination	
Effect Combination	*P-Value*
Whole Model	0.0313
Fiber Geometry x Weave Configuration	0.0103
Fiber Geometry x Material Combination	0.0598
Weave Configuration x Material Combination	0.1936
Fiber Geometry	0.041
Weave Configuration	0.2758
Material Combination	0.0816

There are several implications for the permeability results of this study towards bone tissue engineering. More permeable 4DG-based scaffolds may yield increased distribution of

nutrients throughout an *in vitro* culture environment, when compared with a RND-based scaffold of similar size. Additionally, the increased tortuosity created by the grooved fibers facilitates the creation of a multi-scale pore structure, with micro- (< 20 μm) and macro- (< 200 μm) sized pores combining to create an ideal bone healing environment.[3, 26] Similarly, in an *in vivo* environment channels may help direct diffusive transport of nutrients and wastes for cells anchoring themselves to the scaffold. The increased surface area of the 4DG fibers will also facilitate scaffold degradation as fluid-scaffold contact will be enhanced. Material combination is expected has been demonstrated in previous work by the authors to play a significant role in cell attachment *in vitro.* The combination of biomaterial interactions with transport properties will allow for the tuning of scaffold parameters to an ideal configuration. While a significant difference in permeability solely based on weave configuration was not indicated in this study, previous work has demonstrated that configurations with more drastic differences, such as woven scaffolds versus nonwovens, play a major role in *in vitro* and *in vivo* musculoskeletal tissue engineering applications.[27-29]

Wicking Test Results

The results in Figure 3.10 depict the volume of fluid transported vertically through each scaffold type. These results indicate that 4DG:RND – Twill – PLCL scaffolds transported the most fluid (experiments 1 and 3 of Figure 3.10), followed by 4DG:RND – Plain – PL, 4DG:4DG – Plain – PLCL, and 4DG:RND – Plain – PLCL, respectively. However, due to the high degree of variance between samples these results are not statistically significant.

There were also no significant differences indicated when results were analyzed isolating individual and combined sets of variables. Figure 3.11 and Table 3.2 display these results.

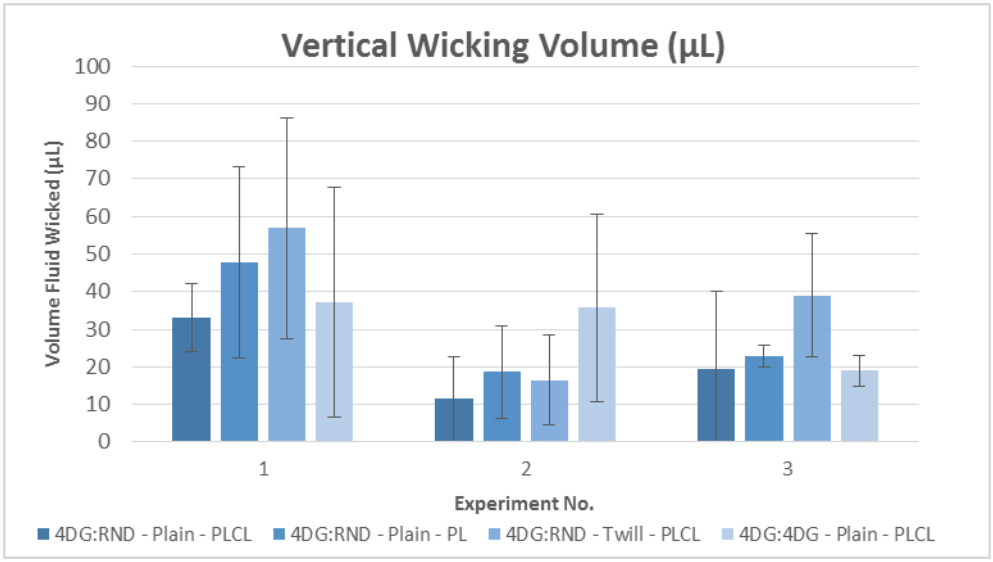

Figure 3.10: Vertical wicking test results across all experimental groups over three iterations of the study. N = 4 for each scaffold type in each of the three experiments.

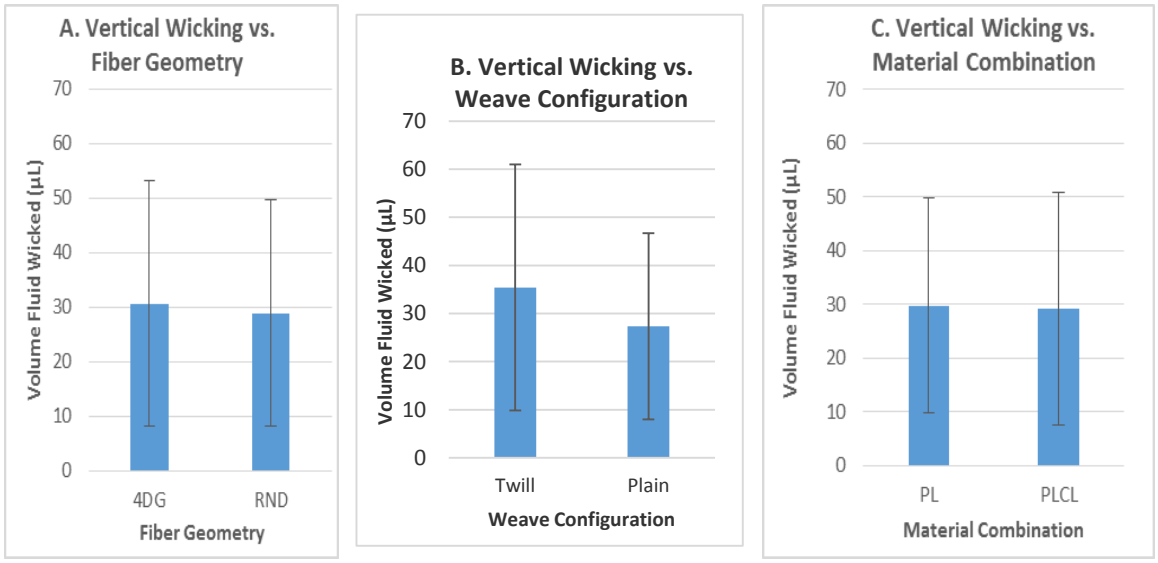

Figure 3.11: Vertical wicking test results with volume (µL) of fluid transported over the 30 minute interval. Figure 3.11A represents these results with respect to fiber geometry. N = 12 for 4DG samples, and N = 36 for RND samples. Figures 3.11B and 3.11C represent weave configuration and material combination, respectively. N = 12, N = 36, N = 12, and N = 36 for

Twill, Plain, PL, and PLCL samples, respectively. None of these results were statistically significant.

Table 3.2: Fluid Wicking Test – Statistical Results	
Volume Wicked vs. Fiber Geometry x Weave Configuration x Material Combination	
Effect Combination	*P-Value*
Whole Model	0.305
Fiber Geometry x Weave Configuration	0.4573
Fiber Geometry x Material Combination	0.1431
Weave Configuration x Material Combination	0.5549
Fiber Geometry	0.3177
Weave Configuration	0.3201
Material Combination	0.4775

It was hypothesized that the 4DG:4DG fiber geometry combination would transport the most fluid due to the grooved fiber structure and previous results seen in the literature. However, this experimental set-up, being adopted from Tabbaa and Burg[15], may not have accurately evaluated the ability of scaffolds to transport fluid due to the nature of the test itself. Figure 3.12 shows the experimental set-up and the hypothesized flow of fluid through the scaffold. Potential causes for highly variable data include different numbers of fibers contacting the fluid initially and differing distances from the surface of the water to the first horizontal fiber. It was thought that the horizontal movement of fluid in Figure 3.12 would have increased the capacity for volume of fluid being taken up. Results did not agree with this assertion and visual inspection of the scaffolds after testing appeared to restrict fluid movement only to the vertical fibers below the first horizontally oriented fiber.

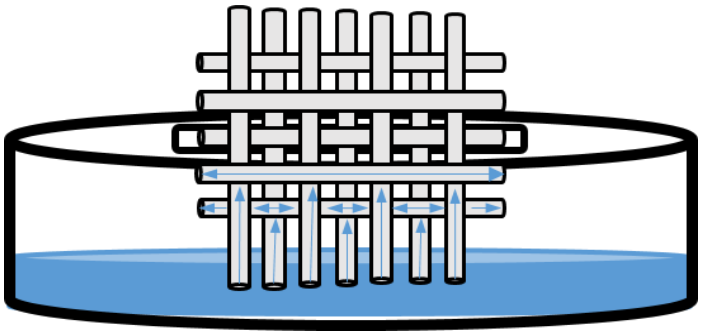

Figure 3.12: Experimental set-up and hypothesized fluid flow during testing. Fluid was expected to travel up the vertical fibers, then across the horizontal fibers, before fluid continued vertically.

The bone tissue engineering implications of this part of the study are difficult to interpret due to lack of comparable data. These results suggest that implementing a woven scaffold with any of these configuration changes would not result in a significant discrepancy in the amount of fluid, nutrients, or waste able to be transported. This study should be repeated, comparing these woven scaffolds to other types of scaffolds currently used in research (i.e. nonwoven, knitted, films or sheets) to determine the overall efficaciousness of woven scaffolds for modulating transport properties in bone tissue applications. The potential advantages to a scaffold able to accomplish this task may be the ability to move nutrients from a highly vascularized region to a less vascularized one, selectively move waste or pathogens away from an injury site, or serve as a customizable construct able to focus concentrations of nutrients in particular areas of a scaffold.

Conclusions

As researchers and clinicians continue to seek solutions for large segmental bone defects the application of tissue engineering concepts remains a viable option for inducing bone healing. With the complexity of the fracture environment it is important to consider a scaffold system that can be modulated to address varying properties pertinent to bone regrowth. This work reveals that the weaving configuration, fiber geometry, and material combination play key roles in the variation of scaffold permeability for woven PLCL and PL constructs. This scaffold system may

be able to be successfully tuned to address multiple factors *in vitro* and *in vivo*. Results for wicking fluid through scaffolds remain unclear. But previous work and literature suggest the viability of these scaffolds as systems to be implemented for specialized transport needs. The ability of a scaffold to transport nutrients and waste, along with promoting vascularization is a development toward sustaining bone healing over large, complex, or poorly vascularized injury sites. The further development of this work will serve to build a system capable of construction of patient-specific bone graft substitutes and reducing the need or complications with autologous bone grafts.

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CHAPTER FOUR

EVALUATION OF WOVEN MESHES AS A BONE TISSUE ENGINEERING SCAFFOLD FOR MESENCHYMAL STEM CELL ADHESION AND DIFFERENTIATION

Introduction

One of the primary design considerations for any tissue engineering construct is cell-biomaterial interaction. It is this interaction, on the micro-, and even nano-scale that dictates biocompatibility, cell affinity, differentiation, and a cascade of other factors leading to factors such as angiogenesis and mechanical strength. In the body, the process of cell attachment begins with the material surface being coated with proteins introduced via blood, plasma, or serum. These materials contain proteins produced by the extracellular matrix (ECM) of surrounding tissue, and it is this layer of proteins that provides an interface for which the biomaterial may interact with cells.[1] There are several factors contributing to the extent of protein attachment. Protein-surface interaction has been shown to vary with surface topography, surface chemistry modification due to processing, and mechanical properties.[2] Researchers have attempted to engineer surfaces that are increasingly hydrophobic in order to induce the displacement of water molecules from the material surface, making room for more protein adsorption and spreading. Other researchers have focused on synthesizing polymers with highlighted functional groups such as the arginine-glycine-aspartic acid (RGD) sequence that selectively adsorbs cells to protein-coated surfaces.[3-5]

Following the adsorption of proteins to the surface, cells selectively adhere to proteins attached to the biomaterial surface by a number of mechanisms. Researchers have attributed the dominant mechanism of adhesion of mesenchymal stem cells (MSCs) to integrin binding between cell membrane proteins and extracellular matrix (ECM) proteins adsorbed onto the polymer surface.[6, 7] Proteins adhered to the biomaterial surface serve as ligands to multiple integrin

receptors. Cells regulate their attachment to surfaces through integrin binding by expressing heterodimeric transmembrane proteins consisting of an α and β integrin subunit.[3] There are 16 different α subunits, and 8 β subunits combining in a variety of ligand combinations for cell adhesion, spreading, and proliferation.[8]

ECM protein adhesion and integrin binding are dynamic processes that may rapidly change or gradually transition over time. When considering the characterization of cell adhesion on a biomaterial surface it is important to consider the multiplicity of sources from which proteins adhere to the surface. Initial protein adhesion may be dominated by serum based proteins such as fibronectin or vitronectin. However, this layer of proteins may be quickly adsorbed or remodeled by the attaching cells. These cells may then express proteins such as collagen or laminin that may then attach to the biomaterial surface.[3] The change in proteins represented on a material surface or expressed on an attached cell not only direct adhesion, but also activate various intracellular signaling pathways that help regulate transcription, cell growth, and differentiation along with other growth factors.[3, 9]

After cells attach to a surface, a combination of biological, mechanical, and chemical factors contribute to the subsequent behavior of the cell. In an environment conducive to proliferation, cells will rapidly grow and divide. When conditions favorable to differentiation are present, stem cells may greatly decrease the rate of proliferation, but instead progress down a number of phenotypic paths specified by the environmental conditions. In the case of osteoblast differentiation, stem cells transition toward the bone cell lineage in the following process: 1) proliferation, 2) ECM maturation, and 3) mineralization.[7]

This work was separated into two complimentary phases focusing on the fundamental viability of these woven meshes as bone tissue engineering scaffolds and graft substitutes. Viability was tested through the characterization of MSC adhesion to the meshes as bio-loom

parameters are manipulated. MSC differentiation toward osteoblasts comprised the second phase of this viability testing. This work investigated the factors contributing to favorable MSC adhesion on woven meshes. MSCs have successfully been differentiated to osteoblasts on nonwoven, electrospun meshes, both *in vitro* and *in vivo* in an ectopic model.[10-13] Researchers focused on cartilage tissue engineering have employed 3-D orthogonal woven meshes as scaffolds. However, this work has only focused on the differentiation of MSCs to chondrocytes.[14, 15] The second phase of this work focused on the use of woven meshes from the bio-loom as scaffolds for osteoblast differentiation. Bio-loom parameters were varied to characterize conditions more favorable to differentiation. Results from this study give implications into the successful differentiation of osteoblasts on these meshes, leading to further bone graft substitute research focusing on modeling specific defect conditions *in vitro*.

Research Questions

1. How does a woven scaffold created via the bio-loom affect adhesion of ECM proteins Fibronectin (Fn), Vitronectin (Vn), Type I Collagen (Col-I), and Laminin α-2 (Lam-α2) over 28 days?

2. How does protein adhesion relate to expression of integrin subunits β-1, α-2, α-5, and α-V over the same 28 day period?

3. How do changes in woven mesh parameters affect the differentiation of MSCs to osteoblasts over 28 days in *in vitro* culture?

4. To what extent do protein adhesion, integrin subunit expression, and osteoblast differentiation complement one another given variable woven scaffolds as culture substrates?

Materials and Methods

Cell Culture

The mesenchymal stem cells used in this study were murine bone marrow stromal D1 cells. Cells were first cultured in T-150 polystyrene culture flasks (Corning) to confluency in a growth medium cocktail consisting of Dulbecco's Modified Eagles Medium (DMEM, Atlanta Biologics), fetal bovine serum (FBS, Life Technologies), antibiotic-antimycotic (Life Technologies), and fungizone (Life Technologies) at 37°C. Growth medium was changed every three days with 1 mL being used per well. Cells were then removed from their initial flask via trypsinization and split into 24-well non-treated, low-attachment polystyrene culture plates with the number of cells seeded per well consistent with the amount of samples needed for a particular study. The maximum number of cells per well was 190,000 due to confluency limits of 24-well plates. Cells cultured on the 24-well non-treated plates were seeded directly onto the woven mesh scaffolds described below. These meshes were held to the bottom of the culture dish by polytetrafluoroethylene (PTFE, Teflon®) rings with inner diameter of 11 mm and outer diameter of 15 mm. A diagram of the culture set-up is included in the figure below. Cells were cultured on meshes in the growth medium cocktail for 7-10 days. This period was implemented as an attachment and proliferation period for the cells. The first 24 hours of this proliferative period, cells were incubated at 37°C on an orbital shaker rotating at 100 rpm. The rest of the proliferative period cells were cultured under static conditions. Following the 7-10 day proliferative period, culture medium was changed to an osteogenic differentiation cocktail consisting of growth medium, ascorbic acid (50 μg/mL), and beta-glycerophosphate (0.01 M). This differentiation cocktail was used for the duration of the studies, with media changes occurring every three days.

Woven Scaffold Development and Preparation

Meshes for this series of experiments were woven on the bio-loom described in previous chapters. The following material combinations, weave configurations, and fiber geometries were employed in each aspect of the study. For all of these studies, warp fibers were poly-l-lactide (PL; Natureworks LLC, 2003d biopolymer, ~ 228,000 Da) with a round (RND) cross sectional geometry. The other material option for this work was poly-l-lactide-co-ε-caprolactone (PLCL; Purac, Purasorb PLC 7015, ~ 154,500 Da). The weave configurations used in this study were Plain and Twill as described in previous chapters. Fiber geometry was varied between RND and deep grooved (4DG) fibers.

Table 4.1: Mesh Types for Adhesion and Differentiation Characterization

Experiment	Material Combination (Weft) - Weave Configuration - Fiber Geometry (Weft)
ECM Protein Adhesion	PL – Plain – 4DG
MSC-Osteoblast Differentiation	PL – Plain – 4DG, PL – Twill – 4DG, PLCL – Plain – 4DG, PLCL – Twill – 4DG
Alizarin Red Imaging	PL – Plain – 4DG, PL – Twill – 4DG, PLCL – Plain – 4DG, PLCL – Twill – 4DG
Alkaline Phosphatase Expression	PL – Plain – 4DG, PL – Twill – 4DG, PLCL – Plain – 4DG, PLCL – Twill – 4DG
Pico Green DNA Quantification	PL – Plain – 4DG, PL – Twill – 4DG, PLCL – Plain – 4DG, PLCL – Twill – 4DG

Following mesh weaving, meshes were cut in rectangles approximately 10 mm by 10 mm. Dimensions did slightly vary across all mesh types and samples due to variable warp fiber spacing. Cut meshes were then cleaned and prepared for cell culture via the protocol outlined below (Figure 4.1). Meshes were washed in sequential baths of 70% ethanol (EtOH), then rinsed in sequential baths of Dulbecco's phosphate buffered saline (PBS, Sigma), then soaked in DMEM for 30 seconds each. This washing process was followed by a longer soak in 50:50 FBS:DMEM under ultraviolet radiation for 15 minutes on each side of the mesh. Lastly, meshes were placed in their respective well-plates and covered with PTFE rings to prevent floatation. This cleaning and

preparation procedure was adapted over several previous cell culture studies. The 50:50 FBS:DMEM soak was added to the protocol particularly for this study due to protein adhesion results showing a significantly higher amount of protein adhering to the mesh when compared with a plain DMEM soak. This increased amount of protein adhesion was used to augment cell attachment to each mesh.

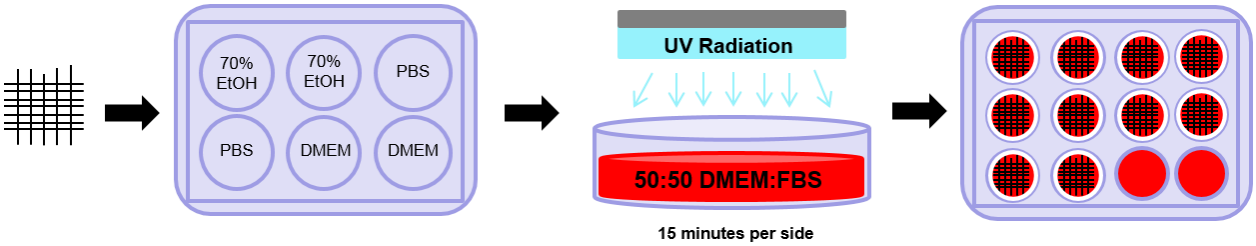


Figure 4.1: Schematic of woven mesh cleaning and preparation procedure, along with cell culture set up in 24-well non-treated plates with PTFE rings to prevent mesh floatation

ECM Proteins of Interest

The ECM proteins used in this study were selected based on their respective roles in cell adhesion, proliferation, and differentiation. Fibronectin (FN) is a high molecular weight (~450 kDa) glycoprotein found in the ECM that binds not only to integrins, but also to other ECM components such as collagen, fibrin, or heparin.[16] Because of its multiple binding capability, fibronectin has been shown to play a key role in cell adhesion, growth, migration, differentiation, and the wound healing process. Vitronectin (VTN) is a lower molecular weight (~ 75 kDa) glycoprotein abundantly found in serum, ECM, and bone. This protein has been shown to promote cell adhesion and spreading through the RGD (45-47) sequence which is a binding site for intergrins.[17] Both FN and VTN are hypothesized to be present in the serum-containing DMEM. These proteins are expected to adhere and detach dynamically over the course of the 28-day study. Both have been shown to be involved in the adhesion of osteoblasts *in vitro*.[8] ECM proteins being explored that are expected to be present largely due to production by adhered cells include Type 1 Collagen (COL1, ~ 235 kDa) and Laminin α-2 (LAMA2, ~ 400 kDa). Both of

these extracellular proteins have been shown to be involved with cell-cell adhesion, as well as attachment to other ECM components.[18] All of these proteins were shown to have a strong and rapid (30 minutes) adhesive attraction with human MSCs, except for Laminin which showed adhesive interaction after 2 hours.

Integrin Subunits of Interest

The integrin subunits selected for this study are all only a small subset of those that are active in cell-biomaterial adhesion and osteogenic differentiation. Several integrins that are associated with the previously mentioned ECM protein ligands (FN, VTN, COL1, and LAMA2) have been shown to regulate the mineralization process of differentiating MSCs *in vitro.* For example, the inhibition of α5 or β1 subunits by using specific antibodies showed a 20% and 45% reduction in mineralization, respectively.[6, 19] Similarly, antibodies blocking αVβ3 and α2β1 integrins reduced mineralization by 65% and 95%, respectively.[6, 19] These results suggest that adhesion modulated via these integrins is a necessary criteria for osteoblast differentiation *in vitro.* However, conflicting studies, such as work by Cheng and colleagues suggest that the over expression of these same integrins may also negatively impact mineralization.[6, 20] These conflicting results lead to the selection of the following subunits for analysis of the osteodifferentiation on these woven meshes: β1, α2, α5, and αV. Table 2 below shows these intergrin subunits and the ligands associated with them as noted in the literature.

Table 4.2: Integrin subunits and associated ligands[8, 21]

β-subunit	α-subunit	Associated Ligands
β1	α2	COL1, LAMA2, possibly FN
	α5	FN
	αV	FN, VTN

Osteodifferentiation Markers of Interest

The differentiation of MSCs to osteoblasts is a progression along a pathway regulated primarily by Runx2. MSCs differentiate to preosteoblast cells, expressing high levels of

osteopontin. Osteopontin is also expressed in the later stages of differentiation. But as these cells mature along the differentiation pathway, high levels of the enzyme Alkaline Phosphatase (ALP) are expressed. Later, osteocalcin (OC) and COL1 are highly expressed as tissue mineralization begins and osteoblasts reach the most mature stages of differentiation.[22, 23] This study examined the expression of ALP and OC as osteodifferentiation markers. ALP was considered an early stage marker of maturing osteoblasts, while OC was associated with the later stages of differentiation and tissue mineralization.

Immunofluorescence Adhesion Analysis

To understand how ECM proteins from the serum, and later from MSCs, were adhering to woven meshes a 28 day study was conducted. This study employed immunofluorescence (IF) techniques to highlight the adhesion of FN, VTN, COL1, and LAMA2 at each time point. A control well of only D1 MSCs was also included in the analysis. Prior to the conduction of this experiment, pilot studies were conducted to understand the adhesion of these proteins without the presence of cells, and to gauge the general level of protein adsorbed to the biomaterial surface during soaking step of the preparation protocol outlined above.

Two concentrations of FBS were used in the exploration of general protein adhesion during the soaking step. A 10% and a 50% FBS:DMEM solution were used as soaking medium on prior to the conduction of a Bicinchoninic Acid assay (BCA, Pierce BCA Protein Assay Kit, Life Technologies) for protein concentration. This assay is colorimetric in nature, correlating the color change in a sample solution from green to purple in proportion to the amount of protein present in the sample. This colorimetric change was calculated via absorbance with a Synergy Mx Micro Plate Reader (Biotek) at a 562 nm wavelength. A sample size, n =3, was used for each of six time points tracking initial protein concentration. 30 minutes, 1, 3, 5, 8, and 16 (overnight) hours were measured. PL-Plain-4DG meshes were the only mesh type employed in this pilot.

Results for this study are shown below in Figure 4.2. It can be seen that protein concentration peaks during this initial time period around 1 hour. For this reason, a 30 minute soak time (also taking into account preparation and transition time) under UV radiation was employed during the preparation of meshes for subsequent studies. These results also show that there is an approximately 5 fold increase in protein expression employing the 50% solution over the 10% solution. This solution concentration was used for subsequent studies as well.

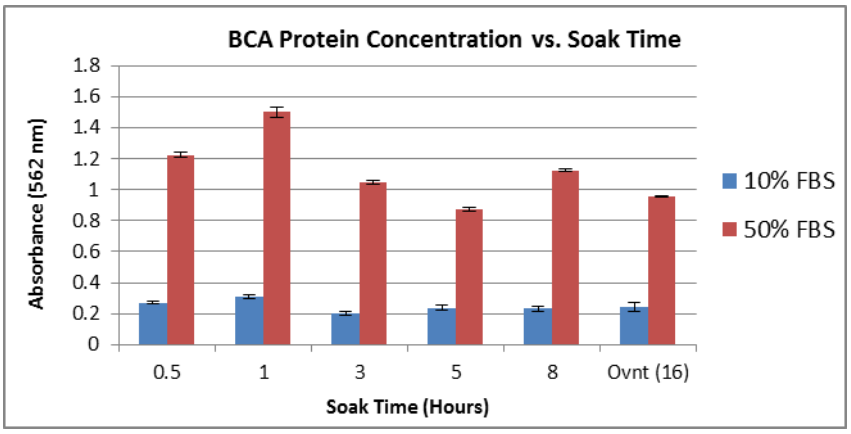


Figure 4.2: BCA protein concentration on woven meshes of variable FBS:DMEM concentration and soak time. N = 3 for each time point. Paired t-test results yield significantly higher protein concentrations for each time point for the 50% FBS case (p = 0.0001).

To begin the IF Adhesion procedure, cell-seeded meshes were first rinsed with PBS, and then transferred to a clean black-sided 24-well plate to reduce light exposure and prevent analysis of cells attached to the previous plate (and not the mesh). Once in the clean plate, the meshes were fixed with 10% formaldehyde for 30 minutes at room temperature. A 10% blocking solution of Casein protein from powdered milk in PBS was then added to each sample and incubated at room temperature on an orbital shaker at 100 rpm for 1 hour. Following the removal of the blocking solution, 1:100 primary antibody solutions in PBS were added to the samples. Control meshes were covered in 100% PBS for this step. All primary antibodies were polyclonal and are listed here: rabbit anti-laminin alpha 2 (Bioss USA), rabbit anti-fibronectin (Bioss USA), rabbit

anti-vitronectin (Bioss USA), and rabbit anti-collagen type 1 (Novus Biologicals). Samples were incubated with the primary antibody solutions for 2 hours at room temperature on an orbital shaker at 100 rpm. All primary antibody solutions and PBS (control well) were removed and a 1:100 secondary antibody solution in PBS was added. The secondary antibody employed was goat anti-rabbit IGG antibody, conjugated with Alexa Fluor 488 (Bioss USA). A FITC conjugated secondary antibody was also attempted but photobleaching of the samples led to the decision to go with the more stable Alexa Fluor fluorescent dye. After a 2 hour incubation at room temperature on an orbital shaker at 100 rpm in the secondary antibody solution, samples were rinsed with PBS and analyzed via a Synergy Mx Micro Plate Reader for fluorescence with 499 nm excitation and 519 nm emission wavelength. N=3 for each mesh antibody type in this study.

Prior to testing protein adhesion on meshes with cells, a pilot study was conducted focusing only on protein adhesion. Meshes were cleaned and prepared as previously discussed but were not seeded with cells. All components of this pilot were consistent with the methods described for the cell-seeded experiments, except the secondary antibody for this study was a FITC conjugated goat anti-rabbit IGG antibody. While this study was only conducted over one 4 hour time point and N=2, results confirmed important information about the early stages of protein adhesion on the woven meshes. Fibronectin and Vitronectin showed slightly higher adsorption than Laminin-α2 or Collagen Type 1, as hypothesized due to a significant amount of latter proteins being produced by attached cells with lesser concentration in the serum. Results are shown below in Figure 4.3.

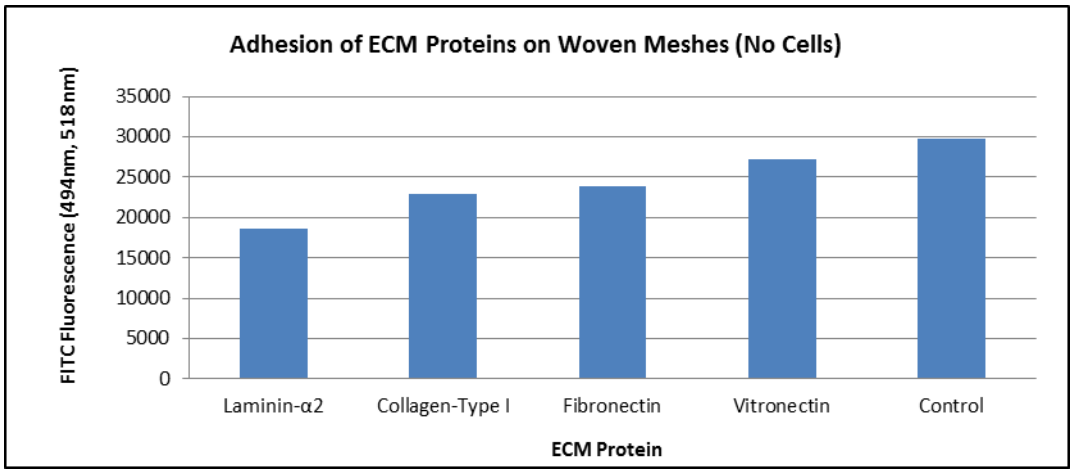


Figure 4.3: ECM protein adhesion pilot study with proteins adhering to a woven mesh without cells. N=2 for each ECM protein.

RT-PCR for Integrin Subunit Tracking and Osteodifferentiation

To complement the analysis of ECM protein adhesion onto woven meshes, a real time polymerase chain reaction (RT-PCR) was conducted focusing on the expression of integrin subunit genes: β1, α2, α5, and αV. Prior to beginning the RT-PCR sample preparation process, meshes were washed with PBS and transferred to a clean 24-well polystyrene culture plate. This process helped to eliminate cells that were not bound to the mesh, but instead were attached to the original culture plate.

Ribonucleic Acid (RNA) isolation was performed via the TRIzol® reagent (Invitrogen). However, this process proved difficult due to the added complexity of removing cells from the mesh without significantly affecting cell adhesion proteins through a process such as trypsinization. The TRIzol reagent was added to each sample with a vigorous pipetting motion to remove cells from each mesh. It was observed that the addition of TRIzol to the polymer meshes caused degradation of the mesh, particularly after vigorous pipetting. The lysed sample was then combined with 0.2 mL of choloroform (Honeywell, HPLC grade) to dissolve RNA into an aqueous phase. The RNA sample was then centrifuged to phase separate the RNA, protein, and

cellular components. The solubilized RNA was then precipitated by adding 0.5 mL of isopropyl alcohol (VWR). After removal of the isopropyl alcohol and solvent from the RNA sample, the RNA was washed with 75% ethanol (Sigma). This ethanol was removed and the RNA was air dried before resuspension in 30 µL of nuclease-free water (Promega). The RNA sample was then treated for removal of any contaminant DNA through the TURBO DNase-Free kit (Ambion). The purified sample was then analyzed for quality and quantity via a NanoDrop 1000 spectrophotometer (Thermo Scientific), then stored at -80°C until further analysis.

Purified RNA was then reverse transcribed to complementary deoxyribonucleic acid (cDNA) using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). 1.0 µg of purified RNA was used to synthesize a 20 µg/µL cDNA solution in nuclease free water. cDNA samples were diluted by half for a total volume of 40 µL to accommodate the number of samples needed for analysis. In the case that the RNA concentration (as determined by results from the NanoDrop 1000 spectrophotometer) was too low to yield 1 µg of RNA from at most 10 µL of RNA solution, an approximated cDNA solution was made using the 10 µL of RNA maximum volume.

A QuantiTect SYBR Green kit (Qiagen) was used to highlight gene expression in the cDNA samples via RT-PCR. PrimeTime® qPCR Primer Assays (Integrated DNA Technologies) were used for ALP, OC, β1, α2, α5, and αV, with sequences listed in Table 4.3 below. The StepOne Plus Real Time PCR System (Applied Biosystems) was used to run the PCR with the following protocol: 95°C holding temperature for 15 minutes, then 40 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 20 seconds, and extension at 95°C for 15 sec. Melting to check for primer dimers occurred at a ramp from 55°C-95°C over 1 minute. The cycle number (C_T) value was then used to calculate the relative expression ratio (RER) or the target genes (ALP, OC, β1, α2, α5, and αV) when compared with the internal standard, GAPDH, using the $\Delta\Delta C_T$

method shown below in Equation 1. This analysis was conducted over three iterations, with N = 2 samples for each sample across the 28 day study.

Table 4.3: RT-PCR Primers

PrimeTime® qPCR Primer Assay	Sequence	Assay ID (www.idtdna.com)
GAPDH	5'-GTGGAGTCATACTGGAACATGTAG-3' 5'-AATGGTGAAGGTCGGTGTG-3'	Mm.PT.39a.1
OC (Bglap)	5'-GCTTGGACATGAAGGCTTTG-3' 5'-ACCATCTTTCTGCTCACTCTG-3'	Mm.PT.56a.30200299.g
ALP	5'-GCCTTCTCATCCAGTTCGTAT-3' 5'-CAAGGACATCGCATATCAGCTA-3'	Mm.PT.56a.8794492
β1	5'-GTAAGCGTCCATGTCTTCACT-3' 5'-CAGTCCCAAGTGCCATGAG-3'	Mm.PT.58.7167969
α2	5'-GAGCGTTCTCATCCGAAGAG-3' 5'-TCAGTCTCACGATTCCTCTCC-3'	Mm.PT.58.43429946
α5	5'-GTCATCTAGCCCATCTCCATTG-3' 5'-CTCACCTATGGCTATGTCACC-3'	Mm.PT.58.8660452
αV	5'-CCCTTCTTATCTTCACCACCAT-3' 5'-TCCAGACTACAAAGCTGAACG-3'	Mm.PT.58.31188090

Equation 1: $$RER = 2^{(-\Delta\Delta C_T)}$$

$$\Delta\Delta C_T = \Delta C_{T(experimental)} - \Delta C_{T(control)}$$

$$\Delta C_{T(experimental)} = C_{T(target)} - C_{T(reference)}$$

$$\Delta C_{T(control)} = C_{T(target)} - C_{T(reference)}$$

Alkaline Phosphatase Fluorescence and Pico Green DNA Quantification

To bolster osteodifferentiation results a colorimetric Alkaline Phosphotase assay was conducted. Samples were first moved to clean 24-well polystyrene plates (Corning) and lysed through sonication at 20% amplitude for three 15 second intervals in a 1x Tris-EDTA (TE) buffer solution (Invitrogen). An ALP buffer solution was prepared from Alkaline Buffer (Sigma), deionized water, and Magnesium Chloride ($MgCl_2$) powder (Sigma). A 5mM para-Nitrophenylphosphate (pNPP) solution was also made from two Phosphate Substrate tablets (Sigma) and ALP buffer. The ALP enzyme solution, composed of bovine lyophilized ALP enzyme powder (Sigma) and ALP buffer, was made separately for each iteration of the study. A portion of the 5 mM pNPP solution was diluted down to a 1 mM solution and used to generate a standard curve for ALP expression when combined with the ALP enzyme solution. The

remaining 5 mM pNPP solution was combined with the lysed sample in a clear-bottom 96-well plate (Costar) along with the solutions for the standard curve. The 96-well plate was incubate at room temperature, protected from light exposure, for 1 hour. Following this incubation, a 3 M Sodium Hydroxide (NaOH) solution was added to each well to stop the ALP enzymatic reaction. The plate was then read in the Synergy Mx Micro Plate Reader for absorbance at a 405 nm. The slope of the standard curve was used to determine the concentration (µmol) of pNPP produced during the reaction. The resulting values were then used in Equation 2 to determine the ALP activity in each sample. This assay was completed three times with N = 3 for each sample in the study, resulting in 9 ALP activity values.

Equation 2: $$ALP\ Activity = \frac{\frac{A\ (amount\ of\ pNPP\ generated\ in\ \mu mol)}{V\ (volume\ of\ sample\ added\ to\ each\ well\ in\ mL)}}{T\ (time\ of\ reaction\ in\ minutes)}$$

ALP activity was normalized by dividing each calculated value by the DNA concentration of that sample. DNA was quantified through a Quant-iT Pico Green dsDNA Assay kit (Life Technologies). To formulate a standard curve, a 1 µg/mL DNA standard was prepared and serially diluted with 1x TE buffer for concentrations of 2000, 1000, 500, 250, 125, 62.5, 37.25, and 0 ng/mL. Samples were diluted from 20 µL to 100 µL with 1x TE buffer and added to each well of a clear-bottom 96-well plate. A solution of PicoGreen® reagent (PicoGreen® and TE buffer) is then added to each well, including standard wells. The plate was then covered from light and incubated at room temperature for 5 minutes. Following incubation the plate was read for fluorescence at 485 nm excitation and 528 nm emission wavelengths in the Synergy Mx Micro Plate Reader. Three repeats for each sample were collected across all three iterations of this study, resulting in 9 average DNA concentrations that were used to normalize the corresponding ALP activity results.

Alizarin Red Images

Alizarin Red staining was employed to complement OC gene expression results. The ALP colorimetric assay served to clarify early osteogenic marker expression. This staining and subsequent imaging served to clarify outcomes for late stage differentiation characteristics by highlighting tissue mineralization *in vitro.* Each cell-seeded mesh was rinsed with PBS, moved to a clean 24-well plate and then fixed with a 10% formaldehyde solution for 30 minutes at room temperature. The fixative was then removed and a 40 mM Alizarin Red S (ARS) solution was added. This ARS solution (Sigma-Aldrich) was composed of Alizarin Red S powder and deionized water titrated to pH 4.1 with hydrochloric acid (HCl, Fisher Scientific). The plate was incubated at room temperature on an orbital shaker at 100 rpm for 30 minutes. The ARS solution was then removed and the samples went through four cycles of rinsing with deionized water and orbital shaking for 5 minutes. After meshes were considered free of any unbound ARS by rinsing and visual inspection, they were imaged via light microscopy on a Axiovert 135 (Zeiss) microscope with a ProgRes C10 Plus (Jenoptik) camera with ProgRes Capture Pro v.2.8.8 software. N =3 for each mesh type in this study.

Statistical Analysis

All quantitative data was initially conducted through JMP Pro 10 software (SAS) where an analysis of variance (ANOVA) test was first run against a constructed simple means model. Following the ANOVA test, other statistical tests were employed to understand how outcomes related specifically to mesh parameters, protein ligands, and integrin subunits affected results. In the case of the ECM protein adhesion work, a contrast of the least squared means was important to discover relationships between specific antibody treatment groups aside from the results obtained considering the entire model. Gene expression, colorimetric ALP, and DNA

concentration were tested via paired t-test in MS Excel (Microsoft) to understand the differences between variables.

Results and Discussion

ECM Protein Adhesion to Woven Meshes

Results from this study (Figures 4.4 and 4.5), show a significant change in VTN expression over the course of the 28 days (Table 4.4A, $p = 0.0031$). Other proteins tend to show a more constant level of adhesion over time. These results suggests that there may be little displacement and reattachment by FN, COL1, and LAMA2 over the course of the study. Earlier work by Hayman and coworkers supports these results as their study showed that VTN had cell attachment activity 8-16 fold greater than that of FN.[24] This study also countered the thought that FN was the dominant adhesion protein active in FBS intended for cell culture.[24] The results from this study confer the findings on Hayman in that FN was not only shown to be consistently less active than VTN over the course of the study, but FN expression was also significantly lower than every other protein regardless of time point (Table 4.5).

COL1 expression did not significantly differ from that of VTN (Figures 4.4 and 4.5, Table 4.5). Also, the relatively constant expression of these proteins over the course of the study was contrary to the hypothesized increase in expression as osteoblastic differentiation was advanced. These results seem to suggest that the level to which cells were synthesizing and depositing COL1 and LAMA2 into the ECM and onto the woven meshes was not variable during the ECM maturation and mineralization phases of differentiation. Perhaps, the expected change would have been more evident in the proliferative phase of MSC differentiation. The COL1 results do confer with previous work by Chastain and colleagues where COL1 was seen to dominate cell adhesion to poly-lactide-co-glycolide (PLGA) scaffolds.[6] Given the similar structures of PL and PLGA, with glycolic acid only differing from lactic acid by one methyl

group, it is reasonable to suspect that COL1 would also be a strong promoter of adhesion on the PL-Plain-4DG woven meshes used here. Chastain also reported a strong relationship between VTN and adhesion to poly-caprolactone scaffolds, which may offer insight into the variability of cell attachment to PLCL containing meshes over the 28 day study, as shown in the DNA concentration results below (Figure 4.9).[6]

Interestingly, LAMA2 expression did not differ significantly from VTN or COL1 expression (Figures 4.4 and 4.5, Table 4.5) over the course of the 28 day study. This result suggests that many of the attached cells may have only been in the early stages of differentiation into osteoblasts. The literature has suggested that the adhesion of MSCs being differentiated toward osteoblasts is characterized by early dominance of Laminins, followed by VTN, Types 1 and 4 Collagen, and lastly FN.[7, 25] In fact, Salaszynk and colleagues strongly concluded (and others suggested) that while VTN and COL1 promote osteodifferentiation, laminin may not play a major role in osteogenesis unless it is very early or very late in the process.[7, 26] The suggestion that laminins may be active in the early preosteoblast stages on osteogenesis is consistent with the results presented here. LAMA2 is expressed equally with the two proteins cited to be strongly related to osteogenic differentiation. This suggests that there was heterogeneity of differentiation level within the cells attached to the mesh. Perhaps cells in the earlier stages were adhering primarily through LAMA2 interactions, while cells more advanced on the differentiation pathway relied more heavily on COL1 and VTN.

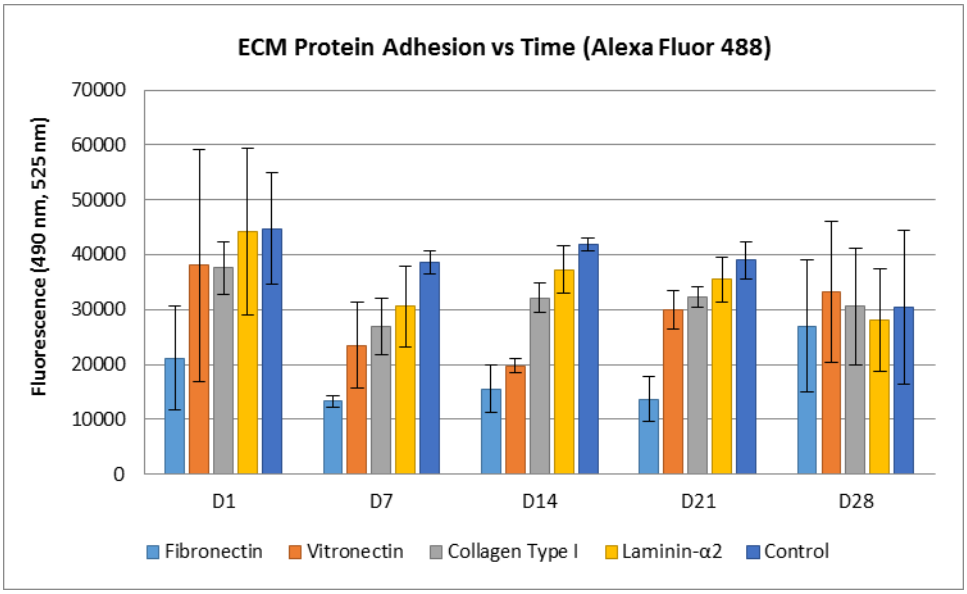


Figure 4.4: Bar chart showing comparison of ECM protein expression to other proteins across the 28 day study via fluorescence. N = 3 for each ECM protein at each time point.

Table 4.4: Statistical Significance of Protein Adhesion (α = 0.05)

A. ECM Proteins Over All Days	P-Value	B. ECM Proteins on a Specific Day	P-Value
Fibronectin	0.1318	D1	0.2661
Vitronectin	0.0031	D7	0.0025
Collagen Type-I	0.2469	D14	<.0001
Laminin α-2	0.3804	D21	<.0001
Control	0.1844	D28	0.971

Table 4.5: Comparison of ECM Proteins (α = 0.05)

ECM Protein Comparison	P-Value	Summary
Fibronectin vs Vitronectin	0.0139	Vitronectin > Fibronectin
Fibronectin vs Collagen Type-I	0.0066	Collagen Type-I > Fibronectin
Fibronectin vs Laminin α-2	0.0145	Laminin α-2 > Fibronectin
Fibronectin vs Control	0.0088	Control > Fibronectin
Vitronectin vs Collagen Type-I	0.2991	No Significant Difference
Vitronectin vs Laminin α-2	0.1556	No Significant Difference
Vitronectin vs Control	0.0722	No Significant Difference
Collagen Type-I vs Laminin α-2	0.1115	No Significant Difference
Collagen Type-I vs Control	0.0253	Control > Collagen Type-I
Laminin α-2 vs Control	0.0385	Control > Laminin α-2

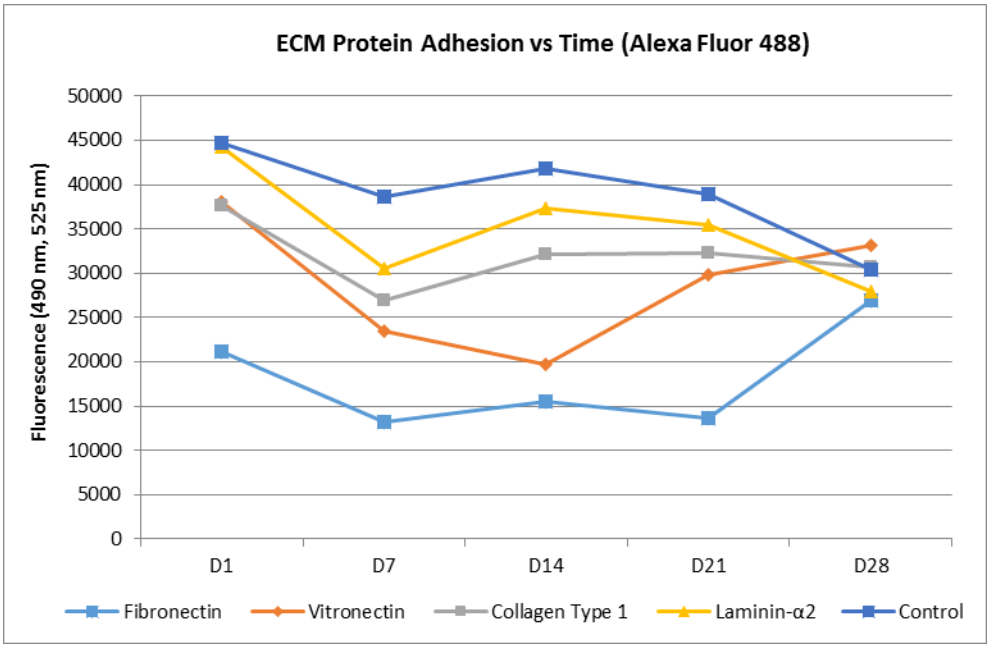


Figure 4.5: Line graph showing results highlighted in Figure 8. Illustration allows for easy comparison of protein expression over time to the Vroman Effect.

As with any biological interphase it is also important to consider the competitive nature of protein adsorption onto the surface. The Vroman Effect describes the competitive displacement of earlier adsorbed proteins with those of stronger binding affinity.[27] It has been noted that several factors are important to the competitive binding of proteins to a surface: size, charge, and structure stability.[28] The relatively small size of VTN (75 kDa) may have contributed to the increased mobility and changing concentration present on the scaffold over time. Hirsh and colleagues describe a dynamic process in which there is an initial layer of protein adsorbed to the surface, which is then embedded with a second layer of protein that may not have been high enough in concentration or affinity to arrive first. During the conformational changes of the protein structures during adhesion and cell binding the adsorbed protein layer is "turned" and eventually the initial proteins may become displaced from the surface during the spreading of the secondary and tertiary protein layers.[29, 30] This classic explanation of the Vroman Effect may have

contributed to the significant variability (Table 4.4B), and eventual dominance of VTN on the woven mesh surface (Figure 4.5).

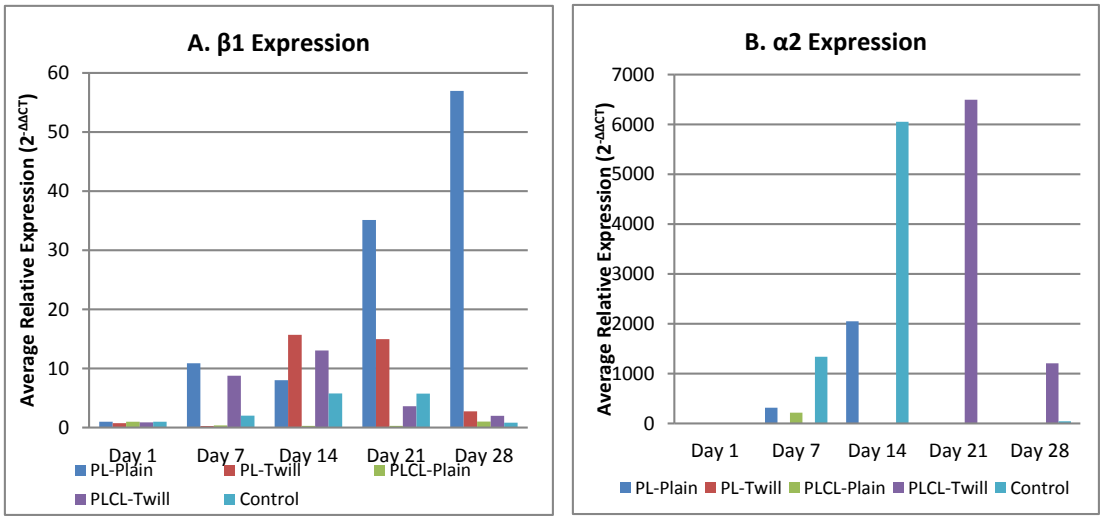
RT-PCR for Integrin Subunit Expression and Osteodifferentiation

Outcomes for the PCR aspects of this study were highly variable due to challenges with RNA isolation and non-specific amplification of integrin subunit PrimeTime assays. The challenges in RNA isolation included of inconsistency in removing cells from the woven meshes via the TRIzol® reagent, which resulted in lower quality RNA (as determined through NanoDrop analysis) and lower concentrations of RNA. Lower concentrations of RNA contributed to low amounts of the target gene present in reverse transcribed cDNA samples. This shortage in some cases led to little or no expression of the housekeeping gene GAPDH, rendering analysis and any derived result via the $\Delta\Delta C_T$ unreliable. Non-specific amplification in the samples make some results unreliable as it cannot be proven that the expression shown is indeed the gene of interest. This non-specific amplification was denoted by examining the melt curves of the respective genes after amplification. Some primers showed the amplification of multiple products, indicated by double-peaked melt curves. Smaller double-peaks may have indicated the presence of primer-dimers, but for the samples indicated with non-specific amplification the melt curves showed two distinct products being amplified. For these reasons, statistical analyses for the following data are not included below as to prevent misrepresentation of the significance of any results.

Expression of the β1 integrin subunit (Figure 4.6A) can be considered a control across all samples as it can serve as a ligand to all ECM proteins examined in the previous adhesion study. β1 is shown below to be increasing across all mesh types over the course of the first 21 days. PL-Plain meshes continued to show up regulation into day 28, but all other meshes show down regulation here. This result is consistent with previous results in the adhesion study given that PL-Plain meshes were used for all conditions exploring protein adhesion. It can be concluded that the

β1 integrin subunit, being able to bind with all ECM proteins, is up regulated throughout the 28 day study regardless of the dominant ECM protein. While similar results would be expected for similar material or configuration meshes, it is not clear how ECM proteins would adhere to these specific mesh types, therefore a direct comparison is not feasible.

The expression of α2 integrin subunit has been associated with COL1, LAMA2, and potentially FN (Table 4.2).[21] Therefore, this subunit was expected to express later in the study as cells matured toward the osteoblast phenotype. Results for collected α2 expression (Figure 4.6B) confirm this hypothesis, although the magnitude of the fold change expressed here may be indicative of non-specific amplification during the PCR.



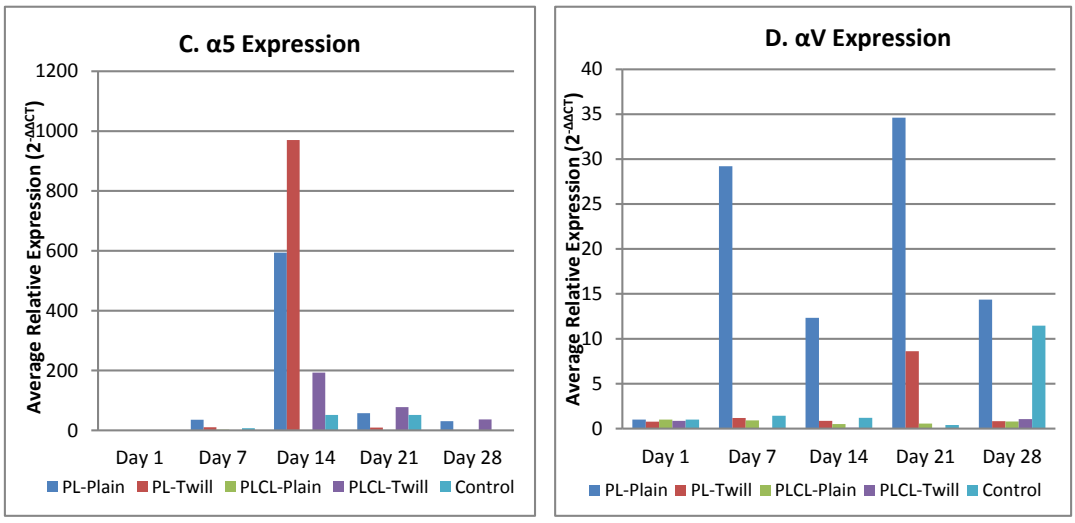
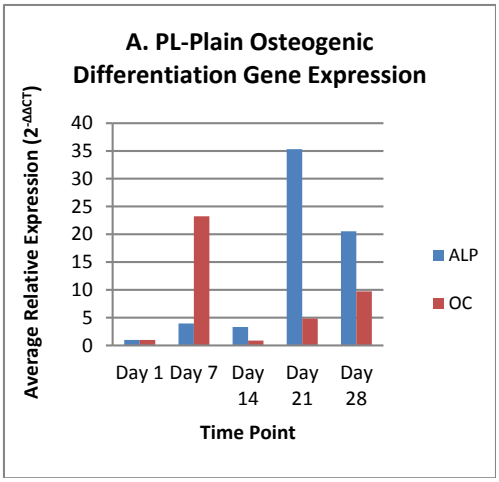


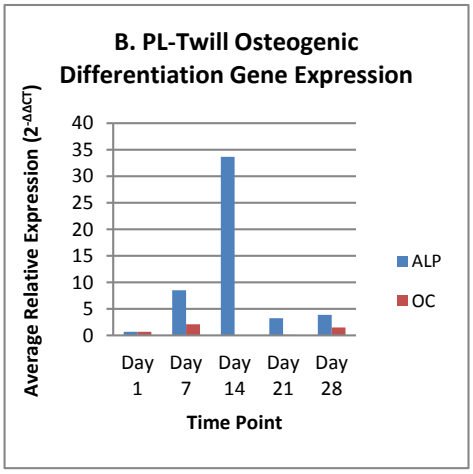
Figure 4.6: Integrin subunit expression across mesh type over 28 days. N = 4 for each time point.

The α5β1 integrin has been shown to strongly associate with FN adhesion during osteoblast differentiation and bone formation.[3, 31] The literature has offered that FN dominated cell adhesion is indicative of late stage osteodifferentiation.[25] Figure 4.6C does show slight up regulation of this subunit starting at day 21. The large spike in expression at day 14 for PL meshes was ignored due to non-specific amplification indicated in the melt curves of these samples during the PCR. This result is consistent with Alizarin Red results discussed in a later section where control wells show significant mineralization at the same time points, indicating late stage differentiation.

The αV integrin subunit has been shown to associate with both FN and VTN ligands in *in vitro* differentiation osteoblasts (Table 4.2).[8, 26] Interestingly, PL-Plain meshes as were used in the ECM protein adhesion study, display a highly variable expression of this integrin subunit in a similar fashion to the variability shown in Figure 4.5 of VTN adhesion. The control wells, showing the most consistent evidence of late stage osteogenesis, also show up regulation of αV at day 28. This expression may be due to integrin binding with FN ligands during the late stages of osteogenesis.

Osteogenic differentiation markers ALP and OC were also tested. These results indicate the expected expression of ALP as an early stage marker only in the case of PL-Twill meshes (Figure 4.7B). PL-Plain meshes (Figure 4.7A) do show expression later during the study, which is a theme confirmed through the ALP analysis via colorimetric assay in the following section. Interestingly, there is no expression of ALP for PLCL containing meshes. This result is not confirmed in the ALP colorimetric study. This data may have been lost due to insufficient RNA quantity. Control meshes also strongly indicate the expected early up regulation and subsequent (after day 14) down regulation of ALP. OC results did show the expected up regulation late in the PL-Plain meshes. The spike in expression on day 7 is due to non-specific amplification, as indicated by the sample melt curve. For PLCL-Plain meshes, OC is the only osteodifferentiation gene to be expressed during the study at day 28.





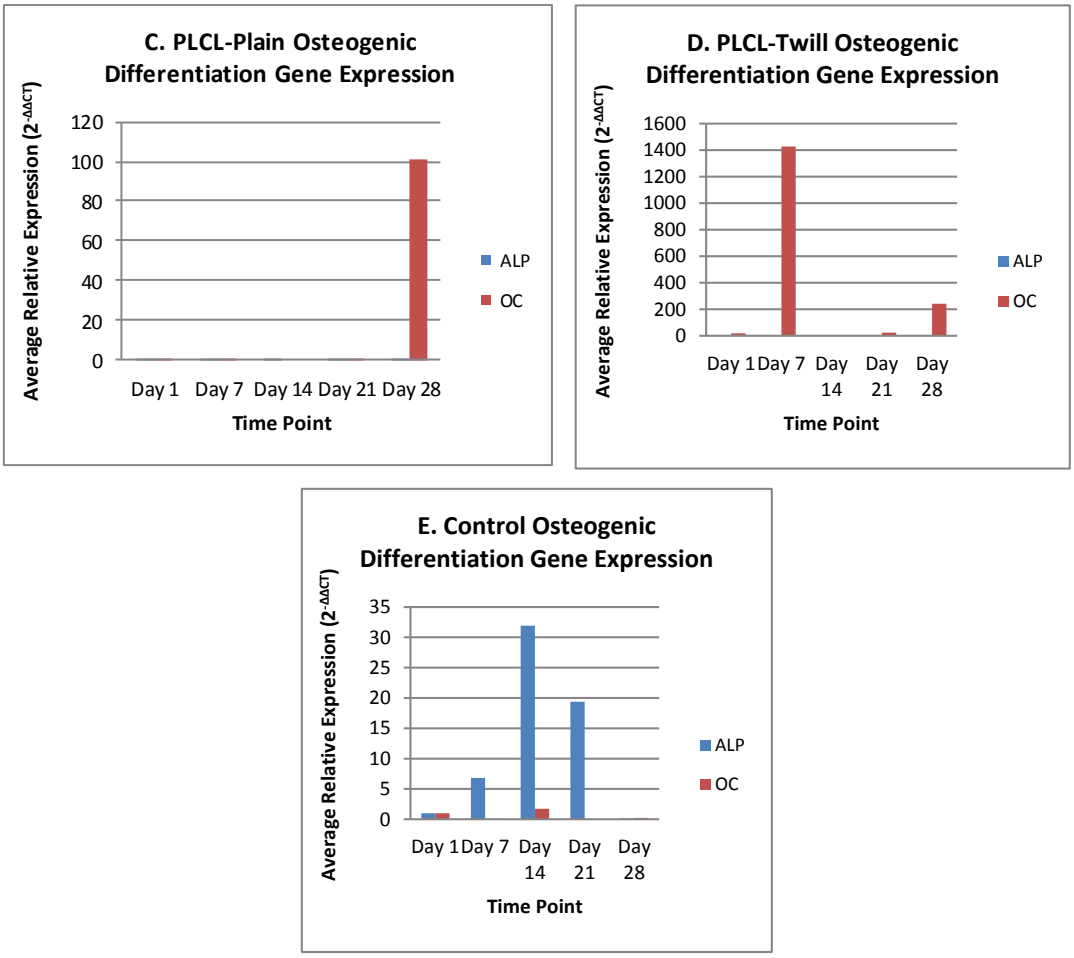


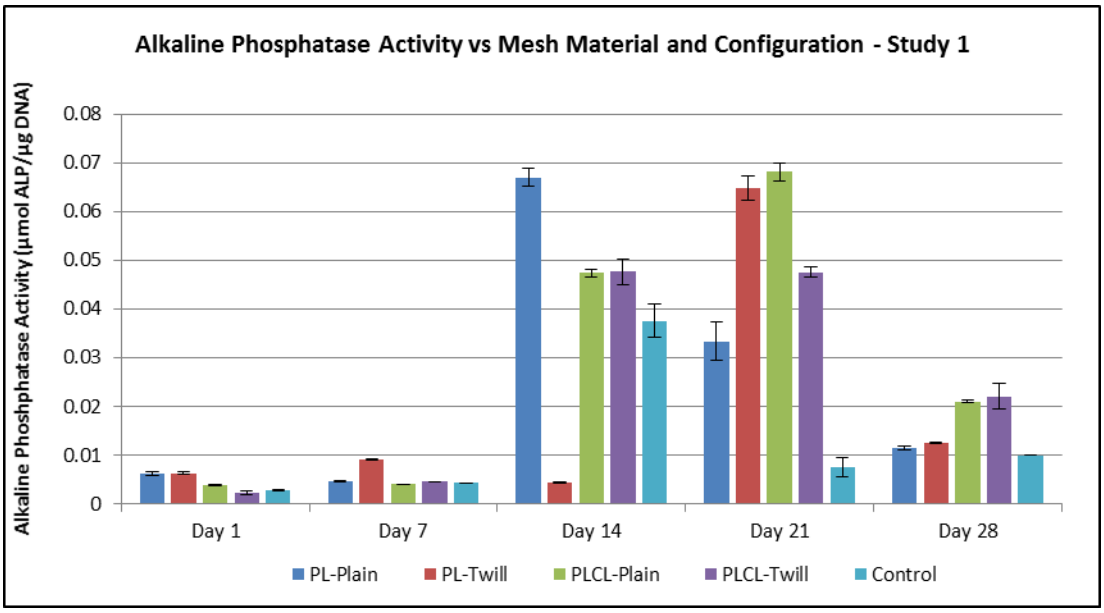
Figure 4.7: Gene expression of osteogenic markers on various scaffolds over 28 days. Osteogenic markers were ALP (early) and OC (late). N =4 for each time point. Error bars not included to maintain scale sizes.

Alkaline Phosphatase Expression and DNA Quantification via PicoGreen® Assay

The results shown below further evaluate the early stage osteodifferentiation of MSCs via expression of ALP. It was expected that the ALP enzyme would demonstrate a rapid peak in expression around days 7 and 14, followed by a down regulation of expression in the later time points. This type of up regulation would indicate the early transition from proliferative to the ECM remodeling stage, but the equally important down regulation would indicate another transition to the tissue mineralization phase where late stage markers would be expressed.

These results do indicate the expression of ALP in the earlier stages of the study; however, expression was shifted to days 14 and 21. This result is consistent with other findings in this chapter, suggesting that cells were not fully progressed through the differentiation process at the end of the 28 day study.

Specifically, in Study 1 expression of ALP did not begin until Day 14. For PLCL containing meshes the expression continued through Day 21 and down regulation was evident at Day 28. For the PL-Twill mesh expression did not begin until Day 21 but there was drastic down regulation immediately following on Day 28. The PL-Plain mesh displayed a progression of expression over time consistent with the hypothesized process, except for the late onset of expression. These results suggest that the woven meshes were facilitating early stage osteodifferentiation through the expression of ALP for these MSCs.



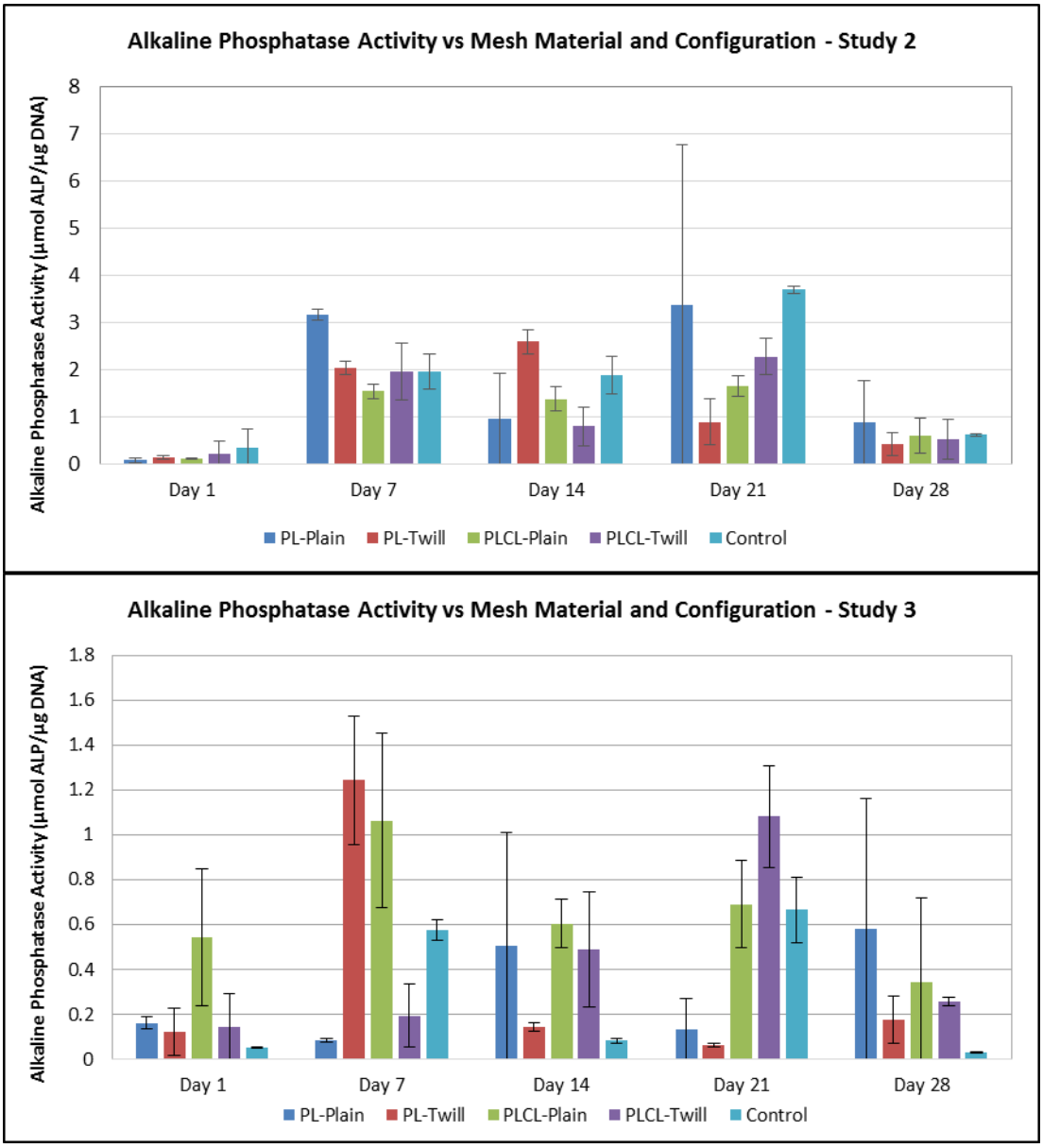
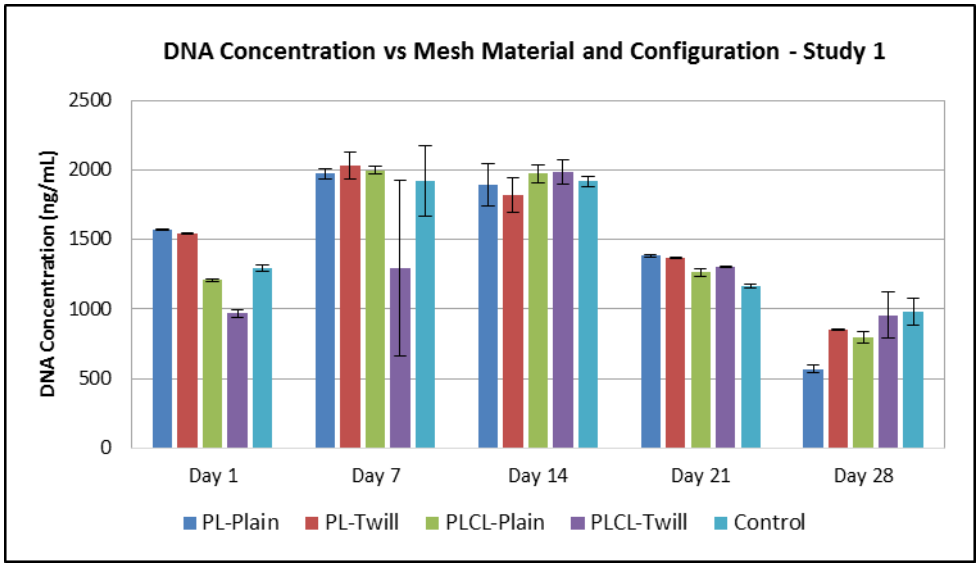


Figure 4.8: ALP activity normalized by DNA concentration (Pico Green results in Figure 7) across mesh material and weave configuration over 28 days.

Study 2 shows ALP expression beginning at the expected Day 7 but expression is sustained over 14 days, with down regulation demonstrated after Day 21. This sustained expression indicates a stagnancy in the differentiation of some of the attached cells. Study 3 results show the expected early expression of ALP for PL meshes. PLCL meshes show a similar

sustained expression over the course of the 28 days. This difference was not statistically significant however.

The ALP expression results were normalized by corresponding DNA concentration. The results for the PicoGreen® dsDNA assy are displayed below in Figure 4.9. Study 1 seems to show a constant level of DNA concentration, suggesting little proliferation of cells during the 28 day period. This may indicate the successful transition of cells to the ECM remodeling phase. In Studies 2 and 3 results are more variable indicating that there could be some proliferation still occurring within the samples.



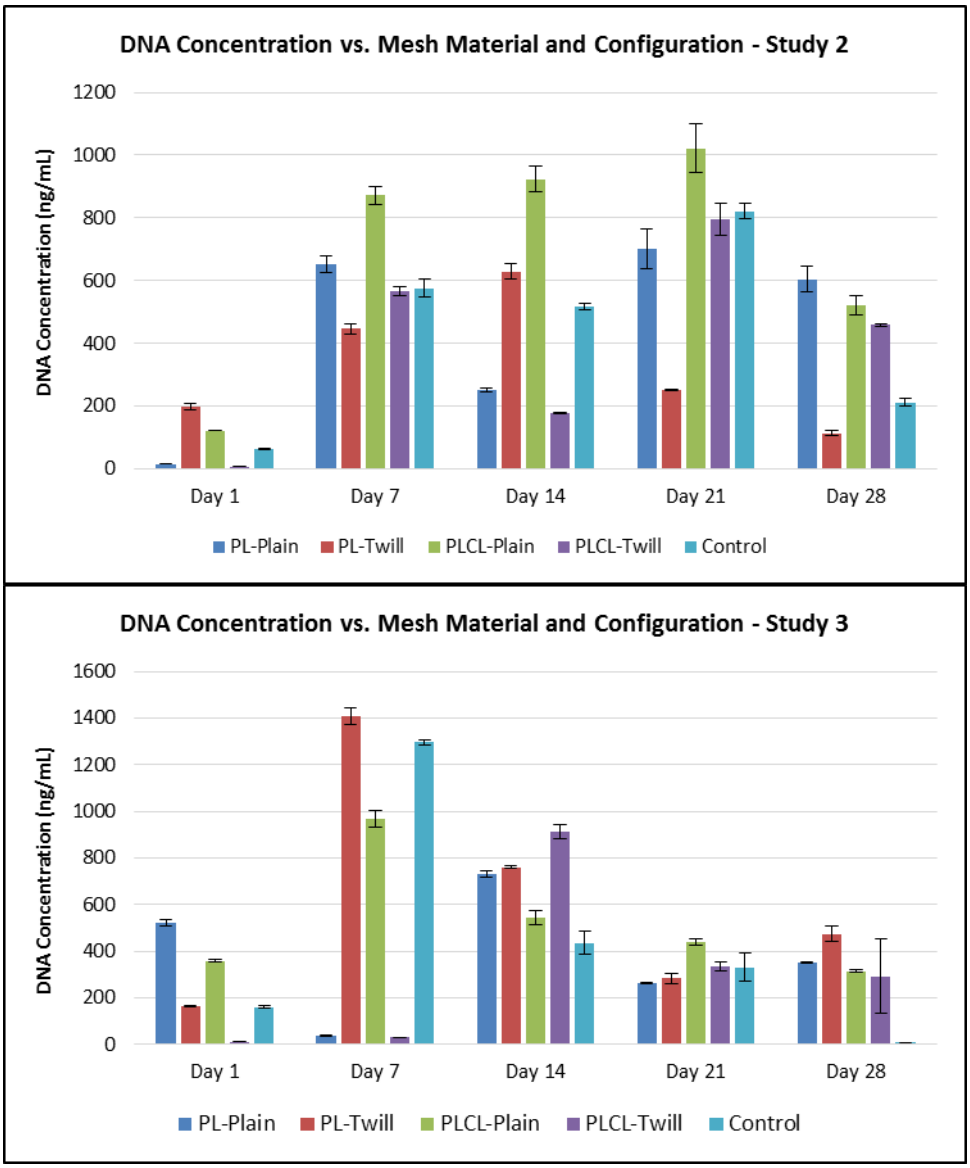


Figure 4.9: Quantification of DNA content in mesh samples used to examine ALP activity. Results were derived via fluorescence expression (485 nm, 528 nm) using the Quant-iT PicoGreen® dsDNA Assay Kit (Life Technologies). N = 3 for each study.

Alizarin Red Staining for Tissue Mineralization

Alizarin Red staining was employed to strengthen conclusions about late stage differentiation. Tissue mineralization occurs in conjunction with late stage osteodifferentiation markers such as osteocalcin.[22] The red stain from the alizarin is due to the formation of a

precipitate with ionic calcium.[32] ARS staining results were difficult to interpret due to complexities with removing excess dye from the fibers with 4DG cross-sectional geometries. The images shown below in Figure 4.10 demonstrate the excess dye retained in all mesh types over the course of the study. It is believed that cells attaching to meshes were undergoing mineralization and thus activated some of the red staining shown in the images below. However, with the presence of excess dye, even after several extra scaffold rinses, it is difficult to draw this conclusion.

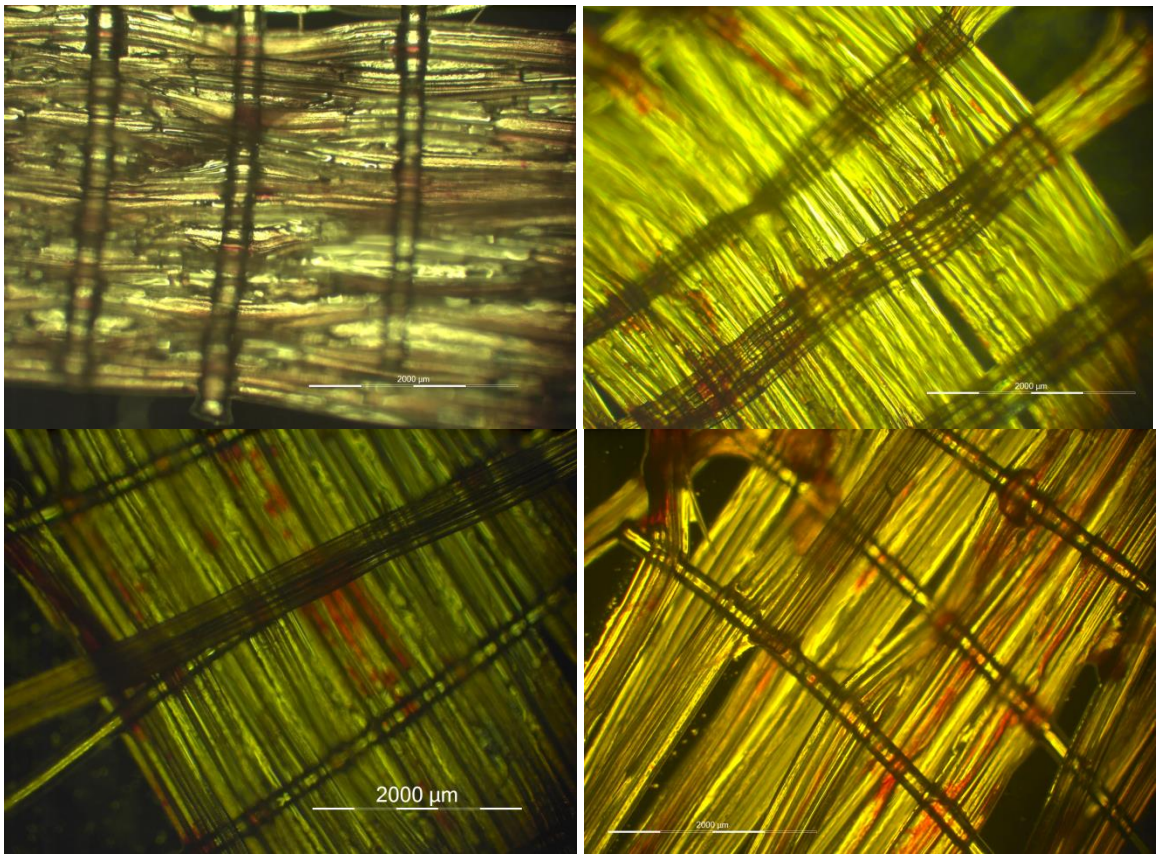


Figure 4.10: Four mesh types stained with Alizarin Red S for tissue mineralization. Top left: PL – Plain – 4DG mesh with image taken on day 1; Top right: PL – Twill – 4DG image taken on day 28; Bottom left: PLCL – Plain – 4DG image taken on day 7; Bottom right: PLCL – Twill – 4DG image taken on day 21. All images were taken at 25x total magnification.

Alizarin Red staining of the control wells provides evidence that cells were indeed undergoing mineralization, as characteristic red staining is evident across all days of the 28 day study. This result is interesting, given that many of the cells in the control wells were expected to be less viable than those adhered to meshes due to the decreased wettability of the polystyrene culture surface in the absence of the plasma treatment used to improve cell adhesion. Cells can be seen clumped together, and were observed to be detached from the surface in a film-like mass in many control wells, particularly later in the study when cells had been in culture for 28 days.

It is also interesting that tissue mineralization appears evident across all days in the study. This is contrary to the hypothesized late expression of mineralization along with OC. These results suggest that the mineralization process started shortly after the addition of the osteogenic differentiation medium cocktail. The fact that these meshes simultaneously demonstrate mineralization and ALP expression across days 7-21 further stengthen the argument that cells adhering to the meshes may have been differentiating at a different rate than those attached to the well plate, with the cells on the mesh being delayed.

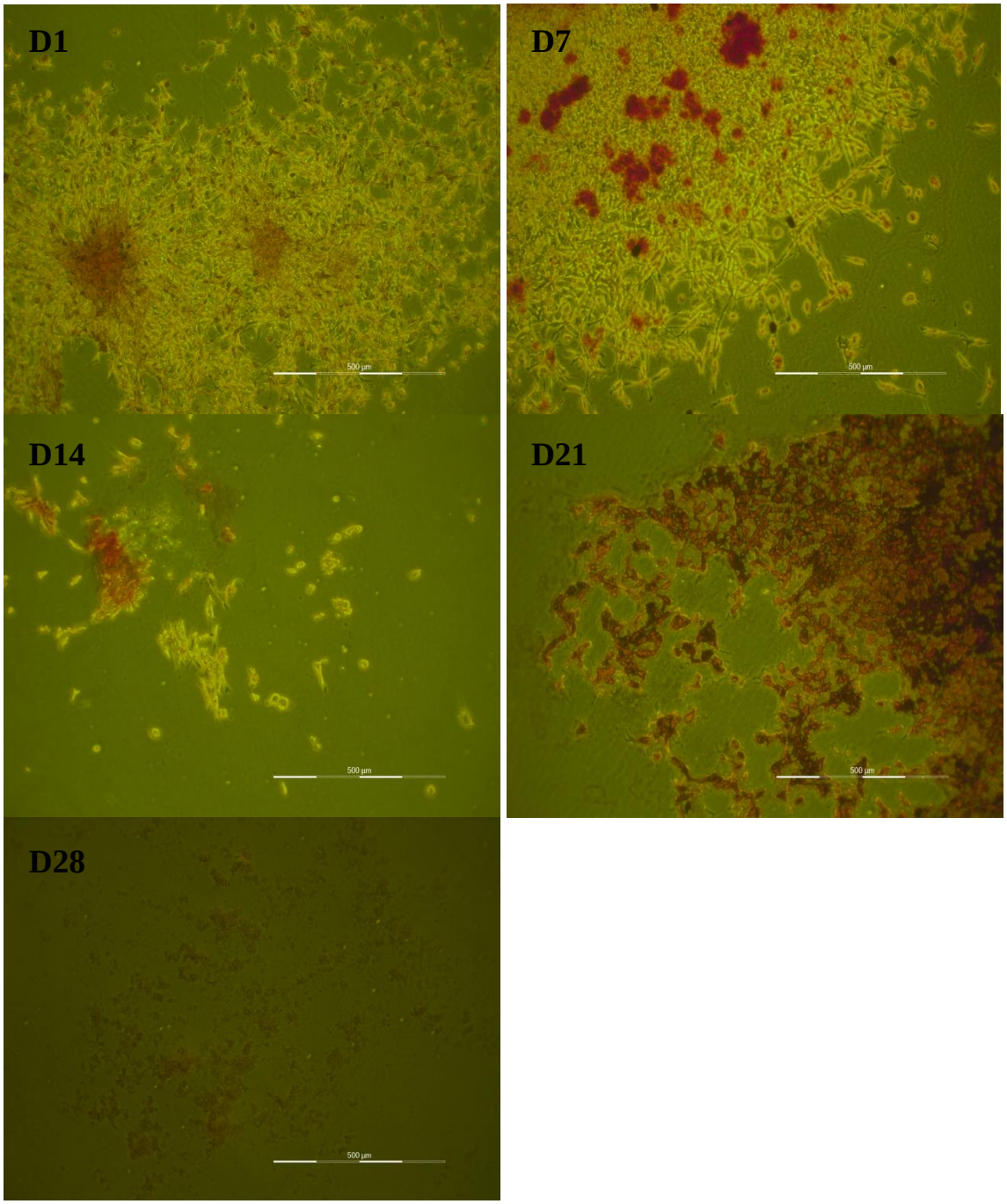


Figure 4.11: Alizarin Red S stained control wells for the third iteration of the study demonstrating mineralization of tissue. Mineralization is indicated by the red coloration maintained within each cell membrane. All images were taken at 100x total magnification.

Conclusions

The goal of this study was to first determine how ECM proteins adhered to woven meshes created on the bio-loom. The results from the adhesion study demonstrated that VTN was the most active ECM protein on the material surface. This result was consistent with the literature on ECM protein adhesion onto polymer surfaces. FN, COLA1, and LAMA2 had equal expression on the mesh surface, suggesting that there may be a variety present on each mesh for the progress of cells through the differentiation process.

Integrin subunit expression, as determined by RT-PCR, seemed to complement the adhesion study results despite challenges with RNA concentrations and primer specificity. β1 integrin subunit consistently increased throughout the study, possibly binding with different ECM protein ligands as dominance on the mesh surface was changed. α2, α5, and αV results also complemented their respective ECM protein ligands.

Differentiation results were less clear, however, the colorimetric ALP assay and Alizarin Red mineralization staining served to support the finding that cells did show evidence of differentiation towards osteoblasts. Early stage marker, ALP showed delayed expression in both the PCR and colorimetric studies suggesting that cells may have still been in the ECM remodeling phase of osteodifferentiation at the end of the study. OC results were less clear, with little mineralization being evident on meshes stained with Alizarin Red. However, control wells showed a consistent presence of mineralizing cells from the very beginning of the study. The combination of ALP and OC results lead to the thought that cells adhered to the scaffold may be in drastically different stages of differentiation.

The combination of results indicate that these woven meshes do serve as viable bone tissue engineering scaffolds. However, more work should be done to look more specifically a mesh

parameters that might change the effect of ECM protein adhesion and subsequently, cell attachment. Material type dominated the extent to which meshes could vary MSC behavior. Smaller fibers and pores may lead to conformational changes more suitable to effecting differentiation.

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CHAPTER FIVE

Select results in this chapter were generated by the Institute for Biological Interfaces of Engineering in collaboration with the Clemson University Department of Curriculum and Instruction, including Clemson University doctoral student Justin Ballenger. Results were submitted for publication in the following peer-reviewed journals: Science Scope, February 2015; and Journal of Pre-college Engineering Education and Research, March 2015.

DETERMINING THE EFFECTS OF AN INTERDISCIPLINARY ENGINEERING INTERVENTION ON STEM CAREER CHOICE IN UNDERREPRESENTED MINORITY MIDDLE SCHOOL STUDENTS

Introduction

Since the 1980's the United States has seen a steady decline in the percentage of science, technology, engineering, and math (STEM) professionals in the workforce.[1, 2] This decline has been coupled with increased ethnic homogeneity in STEM fields due to a decrease in the percentage of ethnic minorities, particularly Blacks and Hispanics, pursuing STEM careers.[3] There has been a slight increase in the percentage of females in STEM careers since the 1980's; however, the percentage of females working in STEM fields is substantially less than the percentage of females in other fields .[1] Females make up approximately 50 percent of the labor force but only account for 25 percent of jobs in STEM fields.[4] Some researchers argue that stagnation in the number of students pursuing STEM majors in college is causing the United States to lose ground in STEM innovations to developing nations such as China and India, each of which produce higher proportions of STEM professionals within their respective populations than does the United States.[5]

The shortage of minorities and women in STEM fields has dire implications for the future of the United States as a leader in innovation and an economic super-power. Population trends shifting toward larger proportions of ethnic minorities suggest heightened importance for diversification of the STEM workforce.[6] STEM professionals create new technology and discover innovations in fields such as engineering and medicine that create new sectors of the

economy and keep the economic engine of our nation and the world running.[5] STEM fields are driven by the ability of professionals to innovate and solve problems in efficient ways. Studies suggest diversity increases the level of innovation in a work force and reduces the opportunity cost of producing viable solutions.[1, 5, 7]

There have been many attempts to explain the reasons for the shortage of minorities successfully graduating in STEM fields from institutions in the United States.[8] One of the major issues limiting the diversity of STEM fields is the lack of exposure, recruitment, academic preparedness, and support for minorities early on in their educational development to encourage the pursuit of STEM majors in college.[9-16]

Studies suggest several societal constructs as potential reasons for the disconnect both minorities and women exhibit in pursuing STEM majors in college. Many minority and female students may have apprehension about aspiring to a career in a STEM field. Some researchers have suggested that those belonging to ethnic minority groups may associate such aspirations with “acting white”, which for many minority students has a negative connotation.[17-19] Females may also be discouraged from pursuing STEM careers by societal pressure to pursue careers that are traditionally associated with females.[20] Other societal factors such as low socioeconomic status (SES) and a breakdown in familial structures have also been suggested as contributing to the achievement gap for ethnic minorities, a factor which also limits students’ ability to pursue STEM majors in college.[21]

To address the decline in the production of STEM professionals in the United States there is a national focus on the promotion STEM education. The US Department of Education has recently funded collaboration between individual state departments of education and private industry to develop and promote the adoption of the Next Generation Science Standards (NGSS). Adoption of the NGSS is regarded as a means of helping to improve K-12 STEM education in the

United States. In addition to the incorporation of NGSS**,** there is also a national push to promote the pursuit of STEM careers by organizations such as the National Science Foundation (NSF) and the National Aeronautical and Space Administration (NASA).

This particular work was focused on the development of STEM career interest in underrepresented minority (URM) students. STEM education researchers have commonly defined underrepresented minorities (URM) as female, African American, Hispanic/Latino, or Native people, including Native American, Alaska Native, Native Hawaiian, and Pacific Island students.[22] Recent research has shown that URM students begin to lose interest in (STEM) around the 7th grade.[14] Furthermore, research has been presented indicating that the majority of students that major in STEM during college make their decision in the early parts of high school.[10] This study was an integrated approach designed to incorporate the NGSS while also promoting student interest in the pursuit of STEM careers during the critical middle grades. NGSS were a focal point due to the desire to disseminate modules to practicing K-12 educators for classroom instruction. The target populations for this study were chosen by gender, ethnic background, and socio-economic status. The composition of the student groups is outlined in detail in the "Methods" section of this chapter. Students participated in interdisciplinary bioengineering modules that complimented and coordinated with NGSS pertinent to middle school grade levels. We designed and implemented the modules to include immersive pedagogy in which students were encouraged to learn through discovery.[2] The purpose of the modules was expose students to STEM related skills and careers, and then measure the impact on their indication of STEM career pursuit. Additional information was gathered in regards to parent attitudes about STEM to better understand what relationship might exist between parent attitudes towards STEM and student indication of STEM career pursuit. Previous studies have shown

parental engagement to be one of the most significant factors in the success of minority students academically, and specifically in STEM.[23]

Materials and Methods

This research was performed over the course of two summers at two different locations. During the first summer the entire program was implemented at Clemson University, and three of the four student groups participated during this time. During the second summer the program was conducted at LEAD Academy, a free, public charter middle school in Greenville, SC, which housed a summer camp for middle school students. Students from the second summer attended Clemson University on the last day of the program to tour the laboratories and campus.

Student Populations

The sample population for this study consisted of four groups of underrepresented students from a variety of backgrounds. A brief summary of group demographics is included in the table below.

Table 5.1: Program Participant Description

Group Name	Grade Range	Gender Distribution	Race/Ethnicity Distribution	Additional Factors	Sample Size	Participation Period
Project WISE (PW)	6^{th}-8^{th}	100% Female	56% Black/Af.Am. 40% White/Cauc. 4% Hispanic/Lat.	Parent, self, or teacher selected student to attend	44	Summer 1
Club LEAP (LP)	5^{th}	53% Female 46% Male	46% Black/Af.Am. 23% White/Cauc. 23% Hispanic/Lat. 8% Asian	Low socioeconomic status (SES) and Underperforming on Standardized Tests	13	Summer 1
Project Middle Passage (PMP)	6^{th}-8^{th}	100% Male	100% Black/Af.Am.	None	21	Summer 1
LEAD Academy (LD)	7^{th}-8^{th}	55% Male 45% Female	59% Black/Af. Am. 35% White/Cauc. 6% Hispanic/Lat.	Low SES	34	Summer 2

Student populations from each summer camp were engaged in modules designed to introduce students to concepts related to STEM fields, in particular engineering. Multiple sessions were held with each student population. Due to varying time constraints for each of the student groups, there was some variation in the length and number of sessions that were held with each group. The variations among student groups are noted in the narrative of this section.

The Project WISE (PW) group was a one week camp focused on the promotion of science and engineering among female middle school students. Students were invited to participate in this program based on nomination from their science teacher or personal interests. There was a cost of $650 associated with attending the camp, however, need-based financial assistance (provided internally through the Project WISE program coordinator) was made available as part of the application. Participants in this group were housed on campus for the entirety of the camp and participated in various engineering and science based activities. The research concerning the program presented in this work was one of the engineering based activities, occurring over the course of three days with two-hour segments each day. This group was split into two smaller groups, consisting of 25 and 24 students, respectively. The discrepancy in the number of program participants from this group and the sample size listed in Table 1 is due to incomplete survey data from the students not included.

The Club LEAP (LP) group was a 7 week summer day camp for local students of low SES or who were underperforming academically as indicated by report cards and standardized test scores from the previous school year. This program was housed at a local elementary school. Students were bused each day from the school to Clemson University for participation in the program. Participation occurred over the course of four days with two hours of instructional time within a three hour total exposure each day. The additional hour was used for transitions and

lunch. Other components of the LP program included recreational field trips, entrepreneurial projects, and academic support.

Project Middle Passage (PMP) was a two week residential summer camp housed at Clemson University for low SES African American males in regional middle schools. Participants in the summer program were taken from an after school program with the same name administered by a teacher training program at Clemson University. The camp was not explicitly science or engineering based, and included activities in business, leadership, composition, and recreation. The program discussed in this work was included as one of the camp activities and participation occurred over the course of three days with three hours of instructional time each day.

The LEAD Academy (LD) group consisted of students that attended the charter school during the normal school term. The summer camp served as an academic enrichment opportunity for students and participation was based on requests during the school year. We traveled to the site each day for implementation of the program, which occurred over two days with two hours of instructional time each day. The following day, students were bused from the charter school to Clemson University for laboratory tours and the conclusion of the final components of the program.

Program Design

The program was based around two Biomedical Engineering modules combined with supplementary lecture material and activities to provide necessary background information and scaffolding for the successful completion of the modules and achievement of targeted engineering outcomes. Modules and associated outcomes were initially developed using the National Science Education Standards (NSES; www.nap.edu) and the National Council of Teachers of Mathematics (NCTM) standards, there was also significant cross-over with the recently adopted

Next Generation Science Standards (NGSS; www.nextgenscience.org).[24] The process of selecting appropriate standards was coordinated by Justin Ballenger, one of the co-authors with specific expertise in this area, being a current middle school science teacher and doctoral-level graduate student in Curriculum and Instruction. Each module was designed to be readily available for use by teachers in middle school science classrooms. Engineering outcomes (as dictated by NGSS) included the ability to use formulas and analytical math skills in an engineering or science context, the ability to express to others scientific ideas through writing, and the ability to analyze or predict the results of an iterative design approach for the improvement of future designs.

The modules employed in this program included a cranial mesh module modeling the design concerns around developing a metallic cranial mesh. Biomaterial considerations, mechanical testing, patient recovery, and aesthetics were considerations for student design. Impact testing was performed on student designs to allow for evaluation and redesign which simulated the iterative engineering design process. The other module was a hernia mesh module in which students simulated a laparoscopic hernia mesh surgery. Students were prompted to consider biomaterial concerns, surgical equipment designs, and potential for implant failure. After completing their designs, students were asked about various aspects of the simulation to check for understanding of major biomedical engineering design considerations. Strong emphasis was also placed on depicting the interphase between engineering or science and real-world applications such as medicine.

The modules were implemented with an instructional plan based on *Experiential Learning Theory*.[25] Kolb's Learning Cycle, displayed in Figure 5.1, is designed to involve students in an experiential learning process. In the case of this study, the experience was creating a solution for a bioengineering problem.

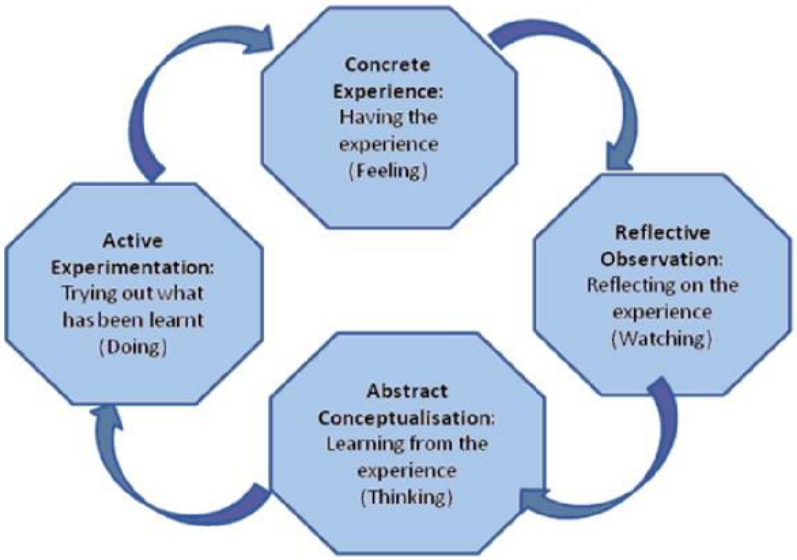


Figure 5.1: Kolb's four stage learning cycle.

http://www.jcu.edu.au/wiledpack/modules/fsl/JCU_090344.html

According to Experiential Learning Theory, the act of "doing" assists learners with grasping certain concepts more effectively than traditional methods such as lecturing. Kolb's Learning Cycle emphasizes continual collaboration and evaluation as students are involved in each part of the learning cycle. The collaborative aspect of the cycle was implemented through the continuous use of group work and feedback from peers. This strategy allowed for an increasingly difficult task but also relieved some cognitive load from the students as they were able to rely on group mates for feedback. The evaluative focus of the cycle was included in the analysis, testing, and iterative design approach of the student work. The focus for this aspect of the camp was not specifically on the level of correctness of the designs, rather it was on the strategy and incorporation of design suggestions and corrections after the first phase of testing. Kolb's Learning Cycle progresses students through four phases that are intended to be revisited throughout the lesson. The phases include: Concrete Experience, Reflective Observation, Abstract Conceptualization, and Active Experimentation.[26] The students were guided through

each of these phases during participation in the program. Concrete Experience was derived from the initial attempt to design a solution for the bioengineering problem. Reflective observation came with the students' task to record their procedures and ideas for improvement after test phase 1. Abstract conceptualization came as the students' were given the opportunity to reflect on the results of their testing and plan for a new design with the necessary adjustments. Finally students experienced Active Experimentation in their redesign and testing.

General guidelines were established for implementing the modules in the context of Kolb's Learning Cycle. Each group followed the same general guidelines for program structure. Some activities were split into different sessions based on the logistical restrictions of each group; however, each group was exposed to the components of the program in the same order each time. The program began for each group with conducting a brief pre-survey for the students along with a pre-survey for the students' parents or guardians. Survey details are outlined in the following section.

Students were then introduced to the acronym STEM, meaning Science, Technology, Engineering, and Math. Suggestions for potential careers in these areas were taken from the students, and we gave additional examples to ensure a comprehensive list. Bioengineering, as a discipline and potential career choice, was mentioned specifically to each group. A short breakdown of the term bioengineering was given, separating the "bio-"and "-engineering" components of the term. Potential applications of bioengineering were also given. Students were then asked to name some skills that would be useful to scientists and engineers. We aimed to direct the conversation to a focus on applying math and science concepts to real world problems and communicating scientific ideas to other scientists as two vital skills. To illustrate the importance of scientific communication, students were asked to create a procedure list for making a peanut-butter and jelly sandwich. We used student-generated lists to make the sandwiches, in

which instructions were explicitly followed. The goal was for students to understand the importance of specificity and organization in scientific communications. This skill was revisited in the design phases of the modules as students were asked to develop procedure lists for their designs as well.

The area of focus for the module was then introduced and students were asked background questions to check for prior knowledge. For example, before the Cranial Mesh module, students were asked the following questions: 1) What are three major functions of the skeletal system? 2) What other body systems work with the skeletal system? 3) What are some ways that the skeletal system could be damaged? These questions were used as an introduction to the module, which was constructed around a "patient case" in which a traumatic cranial injury was to be repaired by the use of a cranial mesh. Students were then given the module material "kit" which contained all necessary materials for completion of the task. Students were instructed on the representation of each item given (i.e. Plaster of Paris simulated bone cement, or yarn simulated a polymer suture material). After reiterating the goal of the module students were given the opportunity to implement their design plans. Grade-level math tasks were added to the design phase to reiterate the application of math concepts in the engineering process. Examples include the calculation of the wound area and volume before developing an implant. Students were prompted to record the procedures they followed as a way of communicating their method to other scientists.

Following the first design phase, student designs were tested via impact testing for the cranial mesh module and a burst strength test for the hernia mesh module. Students were given the opportunity to discuss the results of their design testing and begin to form a plan for improvement. All groups were able to repeat the design process a second time for the cranial mesh module. The hernia mesh design step was conducted only once for each group. Each group

concluded instruction with a game of Jeopardy to check for knowledge. Questions were based on general information about STEM, prior knowledge on the body systems involved in the modules, and math questions similar to those used in the module. Finally, students were given a post-survey concerning their opinions on STEM careers.

Module Materials

These particular areas of biomedical engineering were chosen due to clear applications to everyday life, and availability of inexpensive materials similar to actual biomaterials used in surgery. One example is Plaster of Paris, which was used to model bone cement. Historically, this material was used as a first attempt at bone cement, and its calcium sulfate composition is structurally similar to the mineral composition of poly-methyl-methacrylate (PMMA) which is currently used as bone cement.[27] A complete materials list for each module is included below in Table 5.2.

For the cranial mesh module, students were provided a variety of materials, including three different mesh material options. The Nylon mesh, plastic mesh, and aluminum sheet could all be used to simulate surgical mesh materials, where additional holes could be added to each with a hole punch. The glitter symbolized bone stem cells included in real-world bone tissue engineering applications. Other materials were included to facilitate freedom and creativity in design. The additional teacher supplies were needed for the preparation of kits and testing portion of each module. The hernia model denoted in Table 5.2B consisted of a plastic Tupperware dish with a foam sheet covering its open end. This foam sheet simulated the abdominal cavity, and a rubber glove blown up via the balloon pump served to model the ruptured intestinal wall and protruding components present during a hernia. The metal eyelets were used to facilitate the "suturing" of the wound site following the procedure. Figure 5.2 displays the hernia model as it would be placed in the hernia kit prior to student participation.

Table 5.2 Materials List (per kit)					
A. Cranial Mesh Module			**B. Hernia Mesh Module**		
Item	**Quant.**	**Source**	**Item**	**Quant.**	**Source**
Styrofoam Mannequin Heads	2	Beauty Supply Store	Yarn	4 ft.	Fabric/Craft Store
Hole Punch	1	Science Lab	Clothes Hanger Wire (cut into straight segment)	1	Fabric/Craft Store
#4, ¾" Screws	6	Hardware Store	Balloon pump	1	Party Supply Store
Sheet of Nylon Mesh	1	Fabric/Craft Store	Styrofoam Block	1	Fabric/Craft Store
Phillip's Screw Driver	1	Hardware Store	Forceps	1	Science Lab
Cut 4" x 4" Plastic Mesh	1 sheet	Fabric/Craft Store	Tweezers	1	Science Lab
Tape Measure	1	Hardware Store	¼" Plastic Tubing	1 ft.	Hardware Store
Plastic Spoon	1	Grocery Store	Rubber Gloves	2	Science Lab
Bottle of Glitter	1	Fabric Craft Store	Foam Board w/ Wire Basket Attached	1	Fabric/Craft Store
Plastic Bowl	1	Grocery Store	Tape	1 roll	Science Lab
Cut 4" x 4" Alum. Sheet	1 roll	Hardware Store	Cotter Pin (bent into crescent shape)	1	Hardware Store
Plastic Putty Knife	1	Hardware Store	Scissors	1	Science Lab
Scissors	1	Science Lab	Hernia Model	1	See Instructions
Plaster of Paris	3 tbs.	Hardware Store	5" x 5" Sheet of Nylon Mesh	1	Fabric/Craft Store
Additional Teacher Supplies			**Additional Teacher Supplies**		
Meter Stick	1	Science Lab	Hole punch	1	Science Lab
2 lb., 3lb., and 5 lb. Dumbbell	1 of ea.	Sporting Goods Store	Brass Eyelets	6	Fabric/Craft Store
3' long 6" dia. Polyvinyl chloride (PVC) Pipe	1	Hardware Store	Pliers	1	Hardware Store

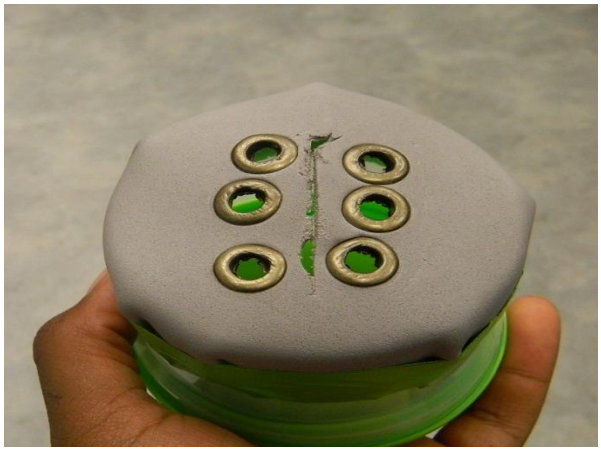


Figure 5.2: Hernia model (prepared previously by instructor) to be included in hernia mesh kit

All student materials were packaged in a plastic shoe-size box (excluding mannequin heads) for distribution to students in groups of three or four.

Instructional Format

The modules were designed to develop engineering design skills with practice, and minimal supporting lectures (Figure 5.3). Emphasis was placed on working in collaborative groups, inclusion of cross-curricular components, and scientific writing, all of which are critical components of real-world engineering. The chart below gives a brief outline of the instructional approach used in the execution of the cranial mesh module during a 3-day summer camp. The same strategy was implemented for the hernia mesh module without the second iteration of design and testing. The outline includes formative and summative assessment activities that might be used by teachers employing this type of activity in a classroom setting during the school year. The module also includes metacognitive exercises, engaging students in the evaluation of their approach as they are solving the problem.

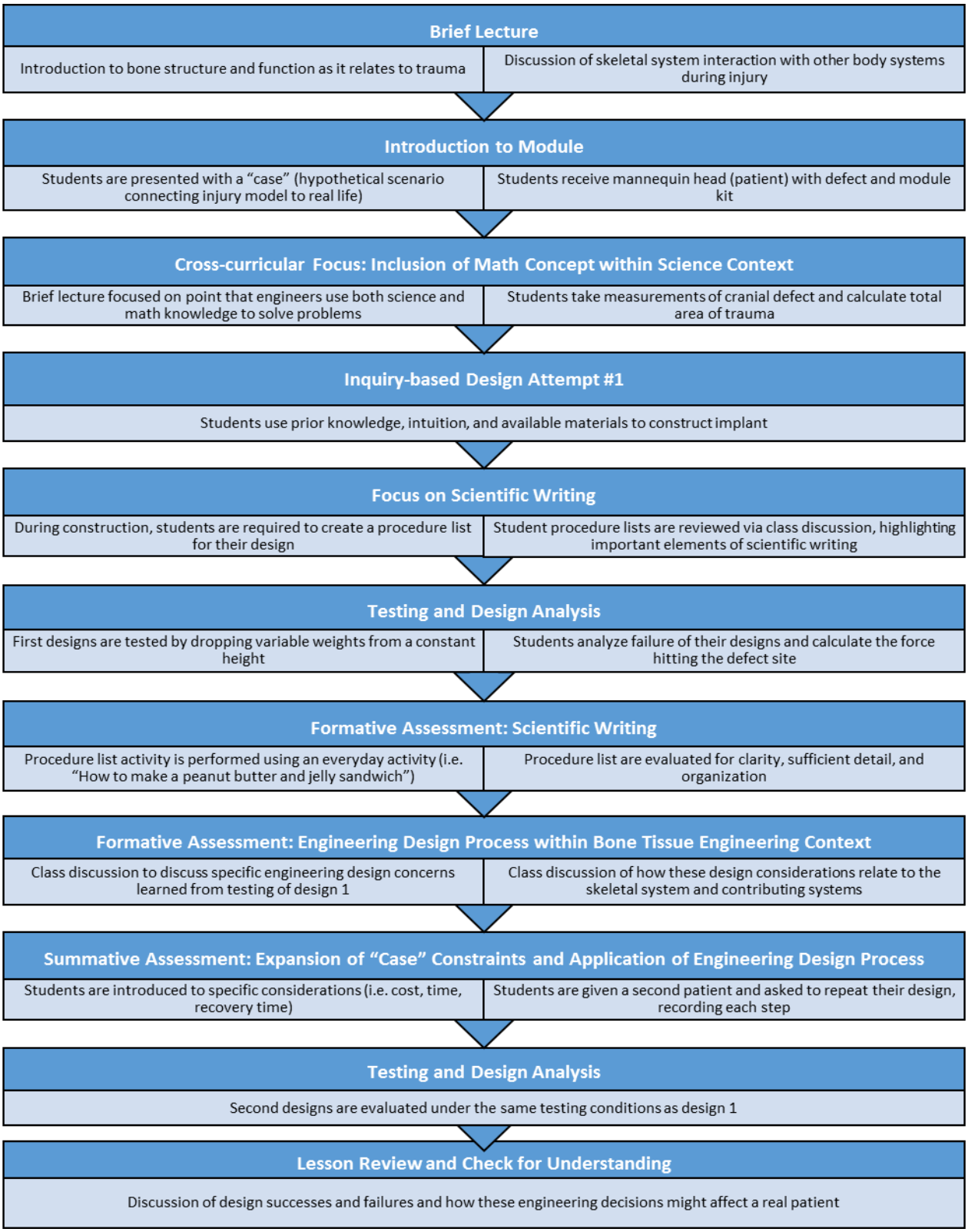


Figure 5.3: General format, including assessment and activity focus for cranial mesh module

Incorporated Science Standards

The National Science Education Standards (NSES) were used as a guideline to insure that the modules addressed topics that are relevant middle school learning objectives. This correlation may make the modules more easily adapted for use as part of the classroom curriculum during study of the human body. The recently incorporated Engineering Design portion of the Next Generation Science Standards (NGSS) also correlates with the activities outlined here. Table 5.3, shown below, displays the NSES standard for which the activity was designed, along with the corresponding standard from the NGSS. The applicable math standards from the National Council of Teachers of Math (NCTM) are also included below.

Table 5.3 – Module Activities with Corresponding Science Standards

Activity within Module	NSES (National Academies 2013) or NCTM (D'Amico and Galloway 2008) Standard	NGSS (Achieve Inc. 2013); Disciplinary Core Idea; Crosscutting Concept; Common Core State Standard Connection
Introduction: Bone structure and function, interrelated systems	**NS.5-8.3 Life Science** – Structure and function in living systems	**MS-LS1-3** Use argument supported by evidence for how the body is a system of interacting subsystems composed of groups of cells **LS1.A** Structure and Function; **LS1.B** Growth and Development of Organisms
Engineering design and everyday life via presentation as a "patient case"	**NS.5-8.6 Person and Social Perspectives** – Personal health, science and technology in society	Influence of science, engineering, and technology on society and the natural world
Defect measurement and area calculation	**NM-GEO.6-8.4 Geometry** - Use visualization, special reasoning, and geometric modeling to solve problems; **NM-MEA.6-8.1 Measurement** – Understand measurable attributes of objects and the units, systems, and processes of measurement; **NM-MEA.6-8.2 Measurement** – Apply appropriate formulas to determine measurements	**7.EE.3** Solve multi-step real-life and mathematical problems posed with positive and negative rational numbers in any form (whole numbers, fractions, and decimals), using tools strategically. Apply properties of operations to calculate with numbers in any form; convert between forms as appropriate; and assess the rationale of answers using mental computation and estimation strategies.
Inquiry-based design attempt #1	**NS.5-8.1 Science as Inquiry** – Abilities necessary to do scientific inquiry	**MS-ETS1-1** Define the criteria and constraints of a design problem with sufficient precision to ensure a successful solution, taking into account

		relevant scientific principles and potential impacts on people and the natural environment that may limit possible solutions. **ETS1.A** – Defining and delimiting engineering problems; **ETS1.B** – Developing possible solutions
Creation of procedure lists to communicate designs with others	**NS.5-8.7 History and Nature of Science** – Science as a human endeavor	**RST.6-8.3** - Follow precisely a multistep procedure when carrying out experiments, taking measurements, or performing technical tasks.
Testing of design using impact force	**NS.5-8.2 Physical Science** - Motions and forces	**MS-PS3-2** Develop a model to describe that when the arrangement of objects interacting at a distance changes, different amounts of potential energy are stored in the system **PS3.C** – Relationship between energy and forces
Calculation of force for testing	**NS.5-8.2 Physical Science** - Motions and forces	**7.EE.3** See previous items
Discussion of engineering design concerns after testing design #1	**NS.5-8.1 Science as Inquiry** – Understandings about scientific inquiry; NS.5-8.5 Science and Technology – abilities of technological design	**MS-ETS1-2** Evaluate competing design solutions using a systematic process to determine how well they meet the criteria and constraints of the problem; **MS-ETS1-3** Analyze data from tests to determine similarities and differences among several design solutions to identify the best characteristics of each that can be combined into a new solution to better meet the criteria for success
Redesign of implant considering previous design failures	**NS.5-8.5 Science and Technology** – Understandings about science and technology	**MS-ETS1-3** See previous items; **ETS1.A** – See previous items **ETS1.B** – See previous items **ETS1.C** – Optimizing the design solution
Analysis of design performance	**NS.5-8.1 Science as Inquiry** – Understandings about scientific inquiry	**MS-ETS1-2** See previous items

Evaluation of Students

Engineering design activities often become difficult for instructors to evaluate objectively, due to the subjective nature of the design process. For these particular activities, we aimed to evaluate student designs on three broad criteria, i) repair strength, ii) patient safety and

return to normal function, and iii) aesthetics. Repair strength was mainly evaluated by performance in the force impact testing for the cranial mesh module and rudimentary burst strength testing for the hernia mesh module. This evaluation was performed on a qualitative basis, as it was difficult to quantify the amount of damage that corresponded to a specific force. Patient safety and return to normal function was evaluated by analyzing a brief verbal design description given by each student group. For example, if students said "We first covered the brain with bone cement, and then we added the metal plate", the fact that students put bone cement directly on brain tissue would be problematic. Bone cement has a mildly cytotoxic effect; therefore this design may result in significant damage to brain tissue. The last criterion, aesthetics, was judged by teachers assisting with the camp. The aim of this criterion was to focus student attention on some of the issues a real patient might consider important when developing their design.

The quantitative evaluation of the students, which may be translated into a summative assessment in the classroom, was focused on the procedure lists created by students. Particular attention was paid to the lists from the second design (or proposed second design in the hernia mesh module case), since students had been exposed to direct instruction on scientific writing at that point. A number of factors were considered in this evaluation. Procedure lists were evaluated on the basis of clarity, level of detail, and organization. A sample rubric, with a breakdown of requirements and corresponding numerical values, is included in Table 5.4.

Bone Tissue Engineering (Cranial Mesh) Module Procedure List Scoring Rubric				
	Areas of Focus	**0 pts**	**10 pts**	**Pts**
Clarity	Coherence of steps	Incoherent steps, completely lacking basic ordered structure	Clear and concise outline of design steps, clear understanding of each step displayed	
	Proper spelling of materials	More than 5 misspellings	No misspellings	
	Complete sentences	Incomplete fragments or run-on sentences	Complete sentences with proper grammar	
Level of Detail	Description of materials used in the design	No description of materials given	Clear description of each material (i.e. size, color,	

			shape, etc.)	
	Sufficient description of the entire design	Absence of any of the details necessary to understand the design	More than sufficient detail to describe each step, clearly describing entire design	
Organization	Numbered/ lettered steps	Numbers/ letters absent	Numbers/ letters present, ordered, and clearly labeled	
	Complete material list	Material list absent	Complete material list with names and quantities of all materials clearly stated	
	Title/label for procedure	Title/label absent	Clear, succinct, and descriptive title with date	
Comments				
Total (out of 80)				

Table 5.4: Sample rubric for the evaluation of procedure lists created by students during the first and second design attempts.

The rubric focuses on some of the basic elements of scientific writing. Prior to beginning the first design phase, students were instructed on the significance of a collaborative scientific community in which information is shared. The procedure list rubric is meant to focus on helping students to create a document that facilitates sharing of information. This type of rubric could be used in an instructor's classroom to help evaluate student progress or track development of scientific writing skills. This rubric was shared by the authors with the teachers present with each group of students to get feedback as to how this type of assessment might be used in their classrooms. Teachers agreed that a rubric able to demonstrate desired outcomes of scientific writing (as stated in the NGSS and in correspondence with standardized testing material) and a student's progression towards such objectives would be a necessary component of any module to be implemented in their respective classrooms.

Cross-Curricular Focus

Figure 5.3 and Table 5.3 point out several elements of this module with cross-curricular focus. We aimed to include pertinent math, English – Language Arts (ELA), and physical science aspects on par with the middle school students that we targeted. Elements such as the calculation of area by measurement of the patient defect enable real-world application of measurement,

geometry, and problem-solving skills for students in a math and science context. One of the reoccurring themes of the module was that engineers use math and science knowledge to solve real-world problems. By having students calculate the force of the object hitting their implant during testing, students were exposed to how engineers might use knowledge from physical science and math to evaluate designs. We found students to be highly engaged while applying this prior knowledge to the task of creating a cranial mesh. Students were also very much engaged in working together to accomplish the difficult laparoscopic hernia repair through the hernia mesh module.

Description of Surveys

Prior to student participation in the program, parents were given a brief survey to gauge the overall home environment for students with regard to the pursuit of a STEM career. Students were also given short pre- and post-surveys to gauge interest in pursuing a STEM career before and after participation in the program, respectively.

The survey given to parents of participating students was a paper-based instrument with 18 total questions. A complete list of those questions as they were presented to the parents is included in the appendix section of this work. For the purposes of this work survey questions were categorized into the following 5 categories: STEM Encouragement, Extracurricular STEM Exposure, Perception of STEM Ability, STEM Prioritization, and Outside STEM Influences (see Table 5.5). These questions were categorized and analyzed to be used as descriptors of potential student barriers for STEM careers prior to participation in the program. STEM Encouragement items focused on the likelihood of parents to encourage their children to pursue STEM careers or educational pathways toward STEM. Extracurricular STEM Exposure items aimed to highlight the extent to which students were exposed/had access to STEM materials outside of the classroom. Perceived STEM Ability explored parent's beliefs toward their child's and their own

ability in STEM. STEM Prioritization examined how important parent's perceived aspects of STEM to be in their child's success. Outside STEM Influence items provided some insight into what other modalities (besides parental influence) might be contributing to student STEM preconceptions. For PW and PMP, parents completed surveys upon dropping their children off for the respective camps. For LP and LD, parent surveys were sent home with the students the first day of the camp. The participants were instructed to return the forms the following day. Parents were also notified by the schools to return the forms. Surveys that were completed and could be matched to a corresponding student were included for analysis.

The student surveys were conducted using Socrative®, an online survey tool. Both surveys were a combination of open-ended and multiple choice responses and all survey items can be seen in Tables 5.6 and 5.7 for the pre- and post-surveys, respectively. The pre-survey consisted of two items and the post-survey consisted of four items. Open ended questions included asking for student names and for indication of career choice in the case of a changing choice post-program (see Table 5.7). Multiple choice items directly address interest in a STEM career, and whether choice was changed after participation in the program (see Table 5.6 and 5.7). Names of students were collected on each survey to facilitate the pairing of student answers with the corresponding parent survey responses. Pre-surveys were conducted before students were exposed to any program material. Post-surveys were given at the conclusion of the program, not at the conclusion of the respective summer camps.

Table 5.5: Categorized Parent Survey Items

STEM Encouragement
How likely are you to encourage your child to pursue a STEM occupation? ___ Very Likely ___ Likely ___ Unlikely ___ Very Unlikely
How likely are you to encourage your child to pursue a STEM major in college? ___ Very Likely ___ Likely ___ Unlikely ___ Very Unlikely
Extracurricular STEM Exposure
Does your child have access to a computer outside of school? (Yes, No)
Do you work in a STEM related career? (Yes, No)
Do you have any friends/family that work in a STEM related career? (Yes, No)

How often does your child participate in science related activities outside of school? ___ Very Frequently ___ Frequently ___ Rarely ___ Never
How often does your child participate in math related activities outside of school? ___ Very Frequently ___ Frequently ___ Rarely ___ Never
Perception of STEM Ability
How would you rate your child's ability in science? ___ Advanced ___ Satisfactory ___ Poor ___ Very Poor
How would you rate your child's ability in math? ___ Advanced ___ Satisfactory ___ Poor ___ Very Poor
How would you rate your child's ability in technology? ___ Advanced ___ Satisfactory ___ Poor ___ Very Poor
How would you rate your own ability in science? ___ Advanced ___ Satisfactory ___ Poor ___ Very Poor
How would you rate your own ability in math? ___ Advanced ___ Satisfactory ___ Poor ___ Very Poor
How would you rate your own ability in technology? ___ Advanced ___ Satisfactory ___ Poor ___ Very Poor
STEM Prioritization
How important is your child's success in science? ___ Very Important ___ Important ___ Unimportant ___ Very Unimportant
How important is your child's success in technology? ___ Very Important ___ Important ___ Unimportant ___ Very Unimportant
How important is your child's success in math? ___ Very Important ___ Important ___ Unimportant ___ Very Unimportant
Outside STEM Influences
Which of the following people most influences your child's choice of career? ___ Parent/guardian ___ Teacher ___ Celebrity/Public Figure ___ Peers ___ Mentor
Which of the following forms of media most influences your child's choice of career? ___ Television ___ Social Media (Facebook®/Twitter®) ___ Radio ___ Newspaper/Magazine

Table 5.6: Student Pre-Survey
1. Please enter your last name, first name. (ex. West, Michael)
2. Are you interested in having a job in Science, Technology, Engineering, or Math? (Yes, No, Not Sure)

Table 5.7: Student Post-Survey
1. Please enter your last name, first name. (ex. West, Michael)
2. Are you interested in having a job in Science, Technology, Engineering, or Math after participating in this program? (Yes, No, Not Sure)
3. Did your job choice change after participating in this program? (Yes, No, Not Sure)
4. If your job choice did change, what was your first choice, and what is your choice now? If it did not change please enter "Did not change" and enter your job choice.

Statistical Analysis

Student responses to survey items were analyzed for statistically significant differences with a two-Way Analysis of Variance (ANOVA) test, with a 0.05 level of significance. A simple

regression model of the overall response to post-survey item 2 was first constructed by quantifying the survey response values to determine general differences between pre- and post-responses. Quantification was conducted as follows: Yes = 2, Not Sure = 1, No = 0. Next, different model effects were added to the model, such as group, and answer to pre-survey item 2. These models were also analyzed using two-way ANOVA testing. Parent survey data was also quantified in a similar manner and concatenated with student survey results. Analysis of this data using ANOVA testing was also conducted, but is not reported in this work due to a significant number of missing parent responses for corresponding student survey results.

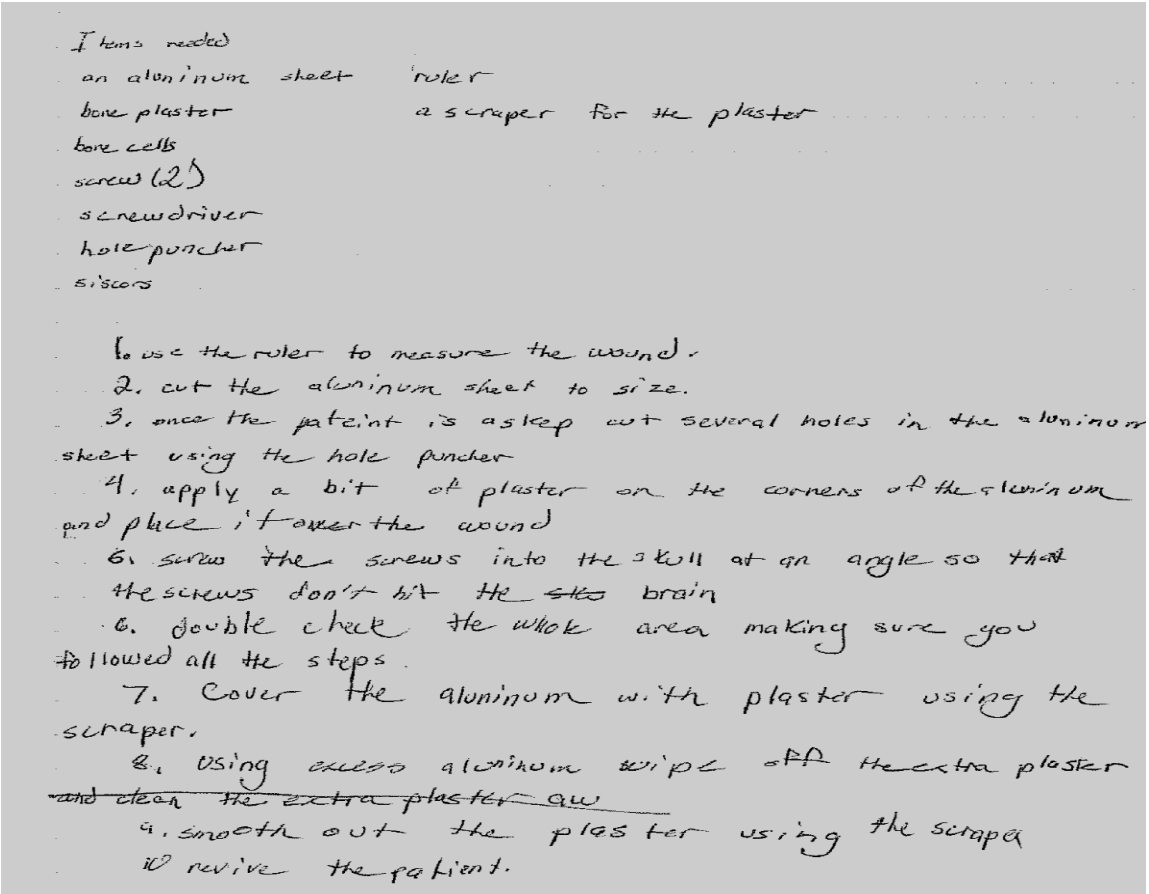
One such limitation is that the pairing of parent and student data vastly decreased the sample sizes analyzed here. Although 120 students participated overall, students that did not complete all surveys and had a corresponding completed parent survey were not included in this analysis. This limitation moved the total sample size across all groups down to 77 students. Parent survey data for this study has not been analyzed specifically for statistical significance due to variation in the number of parent surveys completed correctly. Parent survey results will be used as descriptive support for student survey findings. Future iterations of this study will include prevention of survey completion at home by parents. This issue was a particular challenge with the LEAD Academy group, in which many surveys were sent home and were returned incompletely or incorrectly filled in. The triangulation of parent survey descriptive data, multiple choice student responses, and open-ended student responses will be used to draw conclusions in this work.

Results

Samples of Student Work

This section highlights some examples of student work, particularly procedure lists from the first and second design phases (Figures 5.4 and 5.5). We observed that students paid close

attention to design test results, not just from their own designs, but also from those of their peers. These design concerns were often highlighted in explanations provided on the procedure lists for the second design phase. We also observed that most students focused their design improvements on points of failure in the previous design phase. Students focused less on aspects of design that were not specifically addressed by our method of testing. For example, several groups simply added more material to make their design stronger under the impact force test, or said that they would have added more suture material given the opportunity to repeat the hernia repair. These students were not considering the biological or aesthetic consequences of this type of design. While a performance-based approach is at the center of much engineering design, we submit that it is also valuable for students to be able to anticipate design flaws that may or may not be tested. This skill is valuable in the engineering field and is highlighted in the NGSS as a high school engineering design standard (HS-ETS1-3) (Achieve Inc. 2013). Perhaps students in a more advanced class would be more amicable to including some of these less obvious aspects in the evaluation of their design.



Items needed
an aluminum sheet ruler
bone plaster a scraper for the plaster
bone cells
screws (2)
screwdriver
hole puncher
scissors

1. use the ruler to measure the wound.
2. cut the aluminum sheet to size.
3. once the patient is asleep cut several holes in the aluminum sheet using the hole puncher
4. apply a bit of plaster on the corners of the aluminum and place it over the wound
6. screw the screws into the skull at an angle so that the screws don't hit the ~~the~~ brain
6. double check the whole area making sure you followed all the steps.
7. Cover the aluminum with plaster using the scraper.
8. Using excess aluminum wipe off the extra plaster ~~and clean the extra plaster off~~
9. smooth out the plaster using the scraper
10 revive the patient.

Figure 5.4: Design phase two procedure list from rising eighth grade students. Material list is included with a coherent list of steps and fairly specific instructions. However, dimensions of materials used are not mentioned and the group does not include a title/label for this document.

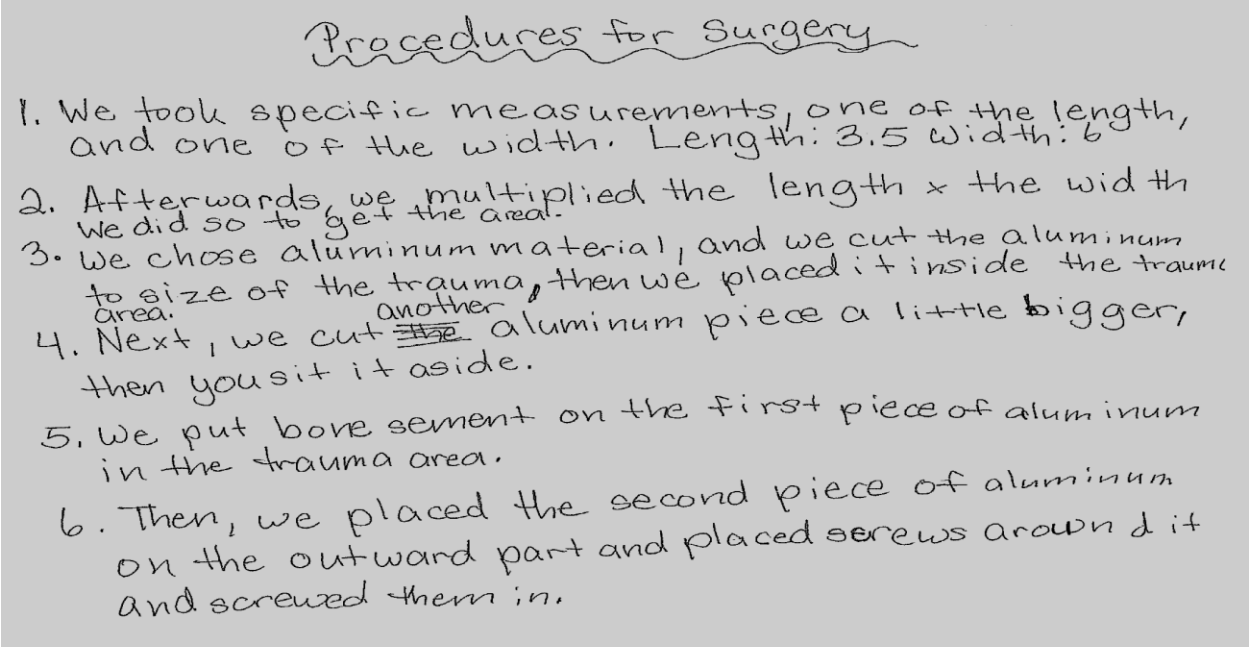


Figure 5.5: Design phase one procedure list from rising seventh grade students. Material list does include a title/label (although nondescript) with clear and coherent steps. The students do mention the dimensions of the defect. However, the students fail to include a materials list.

Survey Results

Figure 5.6 below displays the results for each of the five categories of parent survey data. Overall survey results are included in figures 5.6A, 5.6C, 5.6D, 5.6G, and 5.6I. These results convey the overall response distribution across all groups. The sample size for each survey item is included in the respective legends. Figures 5.6B, 5.6E, 5.6F, 5.6H, and 5.6J display parent survey results separated by program. These figures complement the overall results by displaying the distribution of responses across program for each survey item. These figures were developed through calculating the respective percentage of each program's responses on each question. These percentages were then combined to yield the graphs below. The total number of student participants turning in parent surveys was 77 students. However, due to different survey administration methods, responses for parent surveys across the first and second summers were

inconsistent. In particular, eight survey items were either not answered or incompletely answered for the LD group. These responses were not recorded in the overall results. Given, that there were 12 completed responses from both parent and student in this group, the sample size for those eight items was 65. This information is included in the legends for overall data and in the horizontal axis labels (denoted "No LD") for responses by program.

Results for the 'Likelihood of Parental Encouragement' items demonstrate that parents report strong likelihood for encouraging their child to pursue both a STEM career and a STEM major (Figure 5.6A). Groups characterized by low SES had higher representation in the answer choices showing lower levels of likelihood of encouragement (Figure 5.6B). While the overall number of parents selecting the answer choices is low, it is interesting that representatives from this small subset of parents are most likely associated with students from the low SES groups (PMP, LD, and LP).

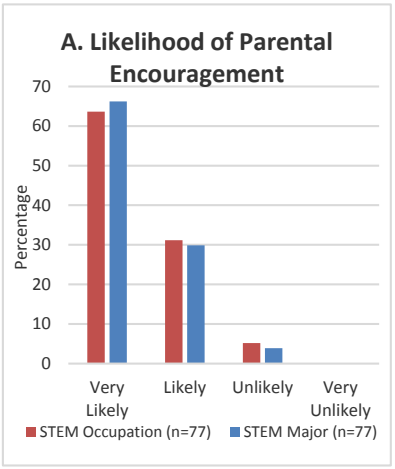
'Extracurricular STEM Exposure' (Figure 5.6C) measures demonstrated that most often students participate in extracurricular math activities as opposed to science activities. It was reported that a large majority of students had access to a computer outside of school, giving students access to many resources associated with STEM extracurricular activities. It was much more likely that students were exposed to a family member or friend that was employed in as STEM career as opposed to having a parent in such an occupation. Figure 5.6E clarifies overall results by reporting that only students in lower SES groups did not have access to a computer outside of school. Additionally, the students from these lower SES groups were much more likely to have a parent not employed in a STEM career. However, all students seemed to have equal likelihood of being exposed to a STEM professional through a family member or friend. Parent data in Figure 5.6E also shows that students from the PW group were much more likely to

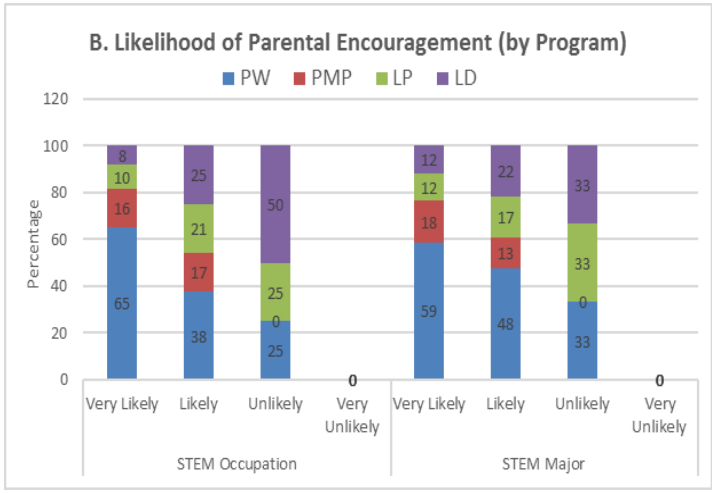
participate in extracurricular math or science activities. Rare or nonexistent extracurricular participation was much more associated with the lower SES groups (PMP, LP, and LD).

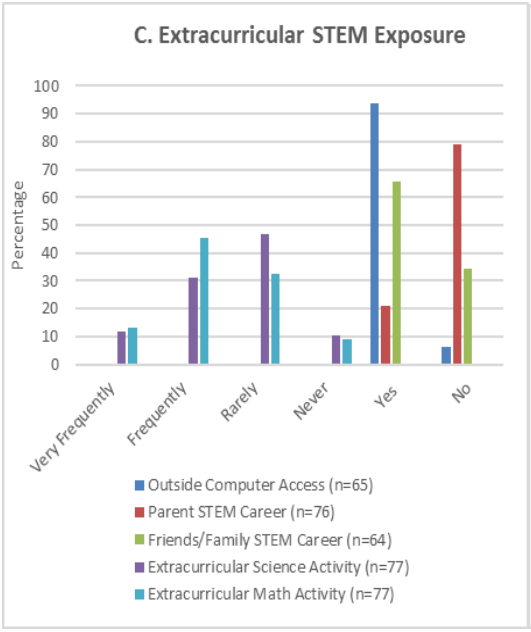
Figure 5.6D reports 'Perceived STEM Ability' responses from parents. Interestingly, parents were much more likely to rate their own skills as lower than those of their children. Parents rated their child's ability in science, math, and technology as advanced more often than rating themselves at this level. Advanced abilities revealed math as being the most developed STEM area. Among satisfactory perceptions, science and technology were the most indicated areas. There were few parents willing to rate their child's or their own ability as poor or very poor. Figure 5.6F adds that PW parents were the most likely to indicate their child's abilities in science, math, or technology to be advanced. The distribution among parent groups for the perception of satisfactory abilities was more even across all areas. However, it is clear that for the groups of parents selecting a less-than-satisfactory perception, these parents were much more likely to come from LP or LD, groups characterized by a lower SES.

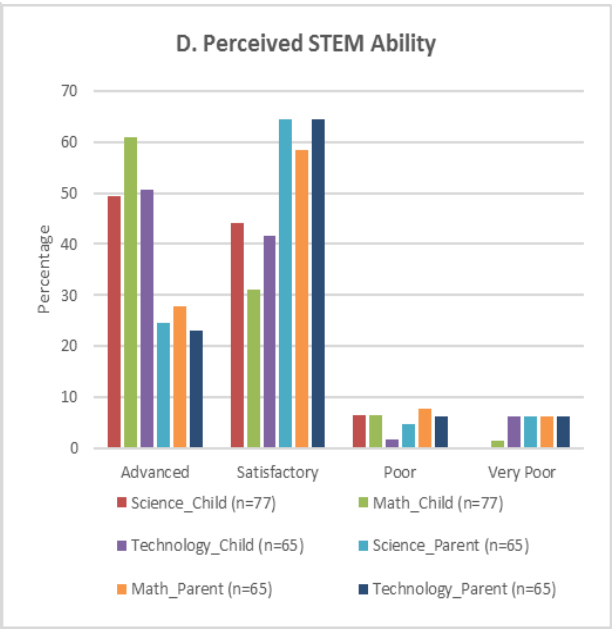
Figure 5.6G and 5.6H demonstrate that most parents thought that science, math, and technology were all very important to their child's success. Math was considered the most important. Results separated across groups showed that this feeling was consistent across all groups, with even distributions of parent responses across each group for answers of "Very Important" or "Important".

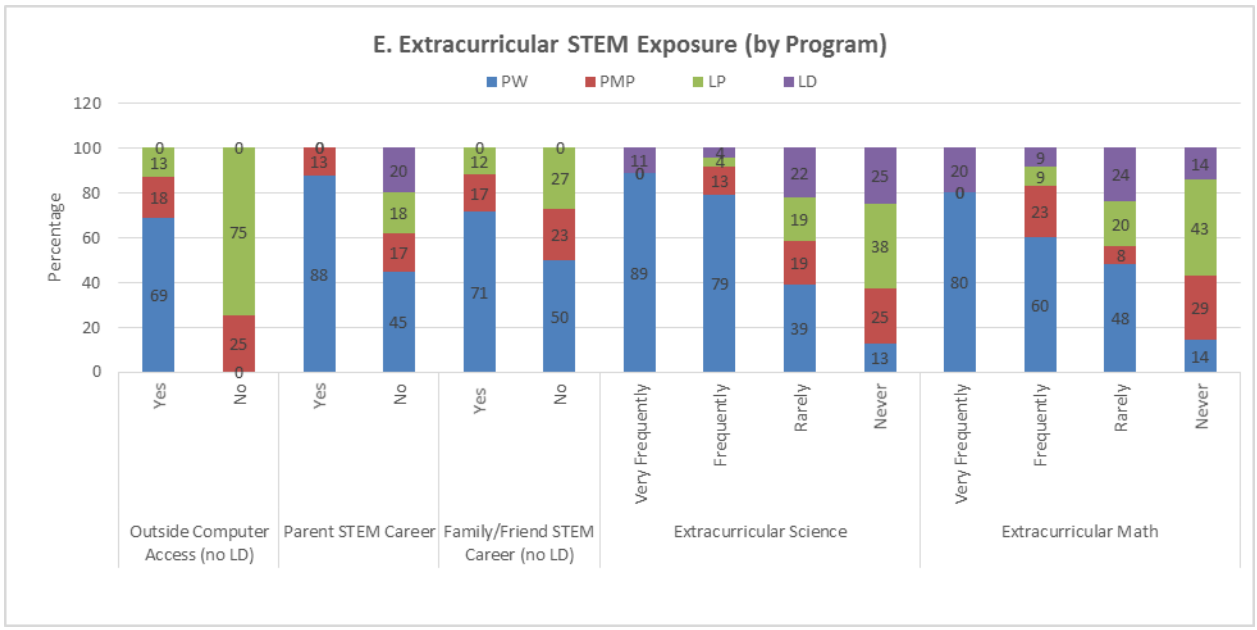
Figures 5.6I and 5.6J report that parents report themselves as the most influential person for their child in matters of career choice. Interestingly, parents across groups, indicated that other influential people (while at a lesser degree) were also very influential in their child's career choice. Television was the most influential form of media, which is an interesting result considering the current prevalence of social media.

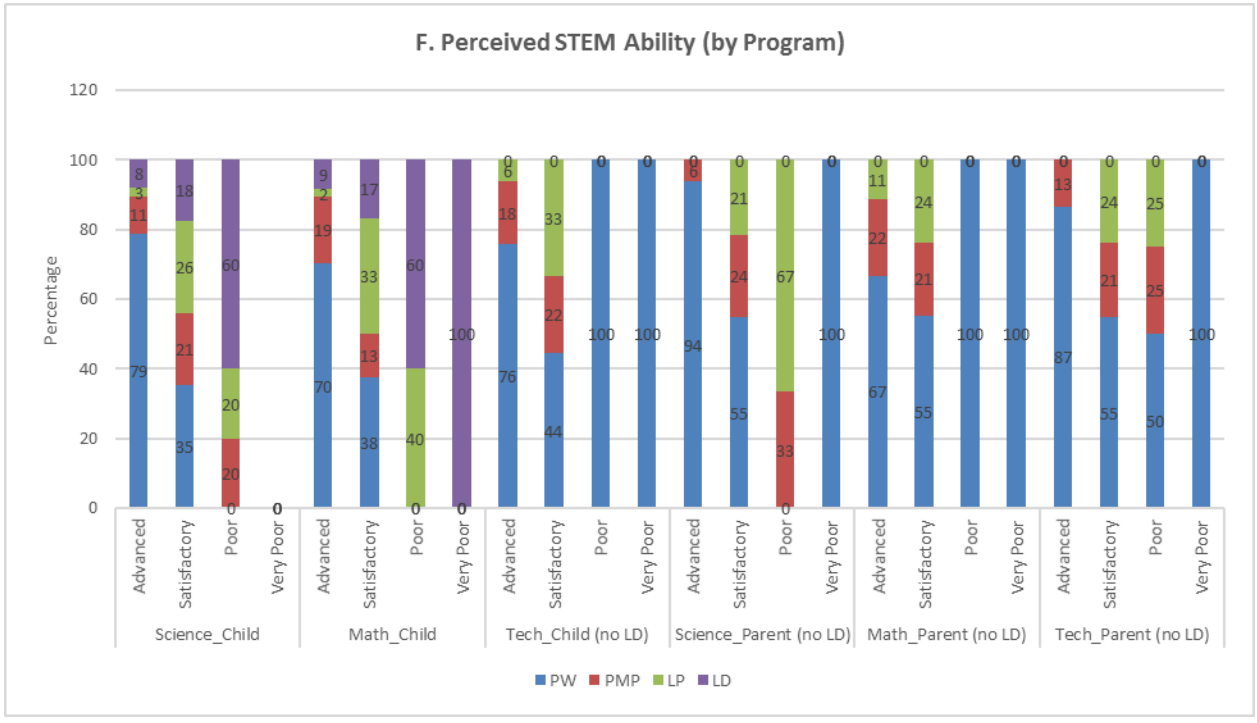

A. Likelihood of Parental Encouragement
Percentage
70
60
50
40
30
20
10
0
Very Likely
Likely
Unlikely
Very Unlikely
STEM Occupation (n=77)
STEM Major (n=77)


B. Likelihood of Parental Encouragement (by Program)
PW
PMP
LP
LD
Percentage
120
100
80
60
40
20
0
8
10
16
65
25
21
17
38
50
25
0
25
0
12
12
18
59
22
17
13
48
33
33
0
33
0
Very Likely
Likely
Unlikely
Very Unlikely
Very Likely
Likely
Unlikely
Very Unlikely
STEM Occupation
STEM Major


C. Extracurricular STEM Exposure
Percentage
100
90
80
70
60
50
40
30
20
10
0
Very Frequently
Frequently
Rarely
Never
Yes
No
Outside Computer Access (n=65)
Parent STEM Career (n=76)
Friends/Family STEM Career (n=64)
Extracurricular Science Activity (n=77)
Extracurricular Math Activity (n=77)


D. Perceived STEM Ability
Percentage
70
60
50
40
30
20
10
0
Advanced
Satisfactory
Poor
Very Poor
Science_Child (n=77)
Math_Child (n=77)
Technology_Child (n=65)
Science_Parent (n=65)
Math_Parent (n=65)
Technology_Parent (n=65)


E. Extracurricular STEM Exposure (by Program)
PW
PMP
LP
LD
Percentage
Outside Computer Access (no LD)
Parent STEM Career
Family/Friend STEM Career (no LD)
Extracurricular Science
Extracurricular Math
Yes
No
Very Frequently
Frequently
Rarely
Never


F. Perceived STEM Ability (by Program)
Percentage
Science_Child
Math_Child
Tech_Child (no LD)
Science_Parent (no LD)
Math_Parent (no LD)
Tech_Parent (no LD)
Advanced
Satisfactory
Poor
Very Poor
PW
PMP
LP
LD

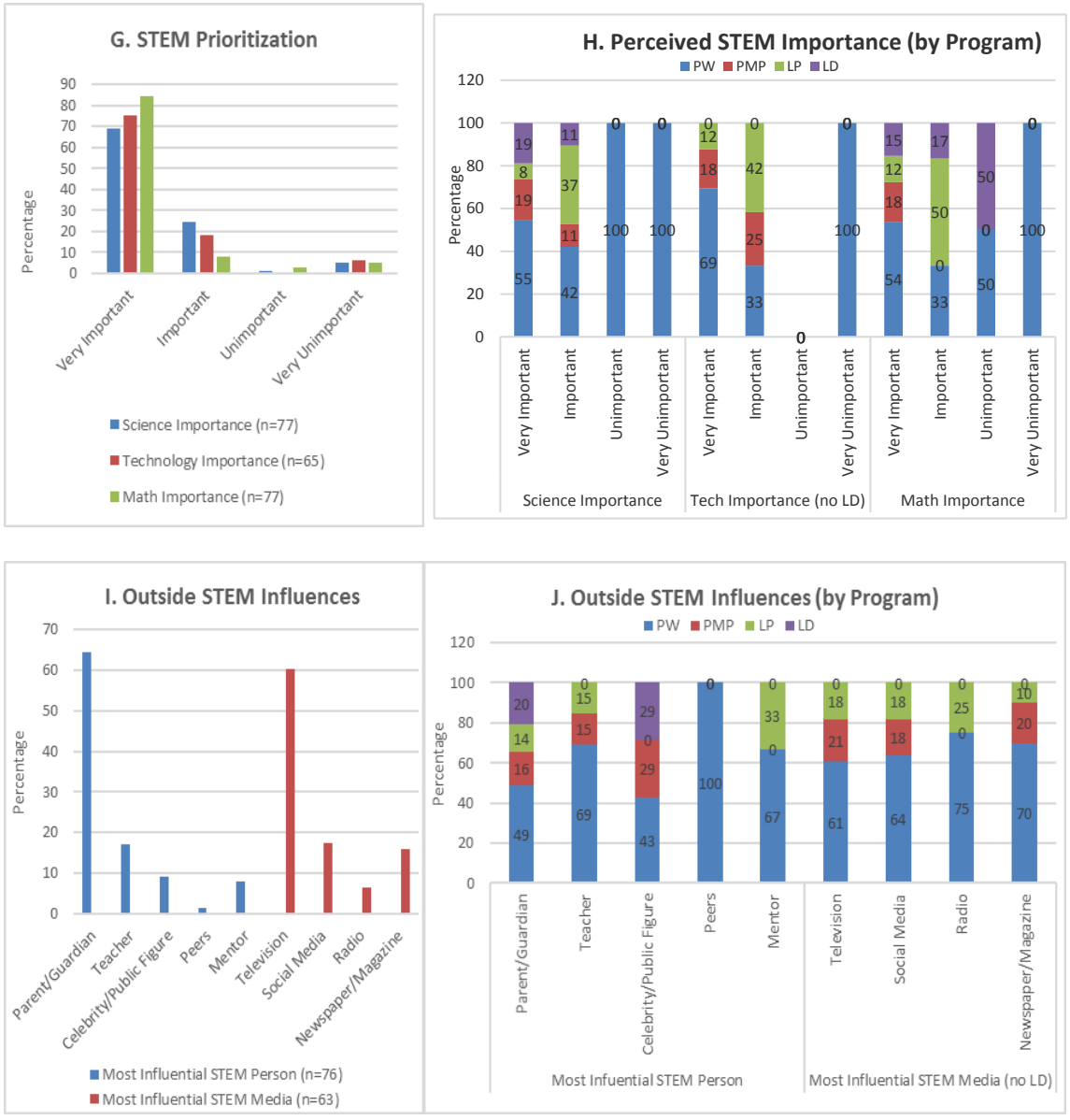


Figure 5.6: Parent survey results grouped by topic as indicated in Table 5.5. A, C, D, G, and I cover overall survey responses by question and topic based on selected survey items. B, E, F, H, and J show results separated by student group. The sample size for each survey item is included in the overall results. Data is reported as a percentage of the responses of the overall group with respect to particular questions. N = 77 for questions answered by all groups. N = 65 for those questions not answered by LD.

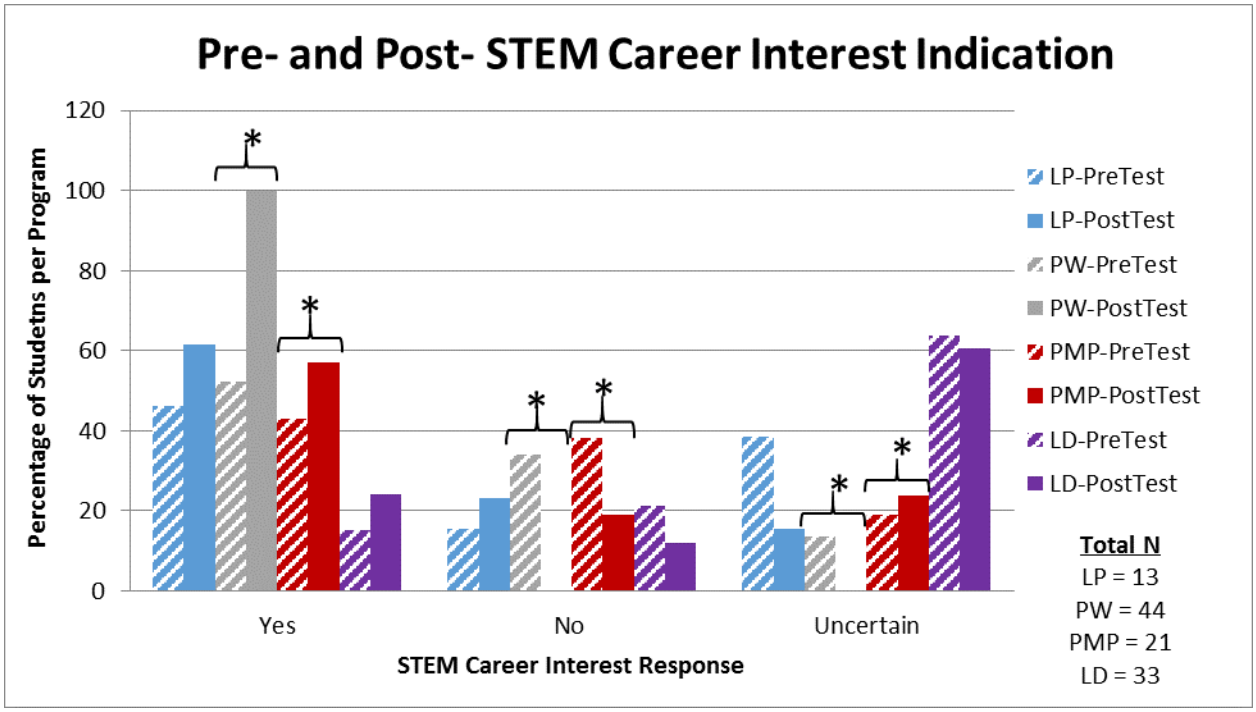


*Figure 5.7: Pre-program and post-program STEM career interest indication by students. Only students with returned parent surveys were included for analysis. * indicates statistical significance (α= 0.05) for difference between pre- and post- answer choice concerning interest in a STEM career.*

The graph in Figure 5.7 above displays results from the student pre- and post-surveys. From the results, it can be seen that in every group a significant number of students initially indicated disinterest or uncertainty in the pursuit of a STEM career prior to participating the program outlined here. The results for interest in a STEM career after participation were as follows: 100% of PW students, 61% of LP students, 57% of PMP students, and 24% of LD students. This result yields a shift to interest in STEM careers of 47% for PW students, 15% for LP students, 14% for PMP students, and 9% for LD students.

Student pre-survey answers were paired with their corresponding post-survey responses. From this combination, students that changed from initial disinterest or uncertainty to interest in a

STEM career are included in Figure 5.8 below. However, due to the differences in group structure and experience, these changes cannot be directly related to the program discussed in this work. To elucidate motives for interest change the responses to the open-ended survey question on the student post-survey were explored. Although PW students were exposed to different fields of engineering all week, about 25% of the students who changed their career choice to a STEM career specifically mentioned bioengineering as a career choice. Table 5.8 below includes example responses from the PW group. The program presentation included information about STEM careers in general with an emphasis on engineering, specifically the biomedical concentration. Figure 8 highlights open-ended responses for students that indicated interest in STEM careers that specifically mentioned the word "engineering" in their response to the open-ended survey item. The number of students that indicated STEM careers closely parallels the number of students who changed from being initially disinterested to being interested, except for in the case of LD. Engineering responses accounted for the following percentages of all STEM career responses: 56% of PW students, 28% of PMP students, 33% of LP students, and 19% of LD students. Possible reasons for differences in these groups will be discussed in the following section.

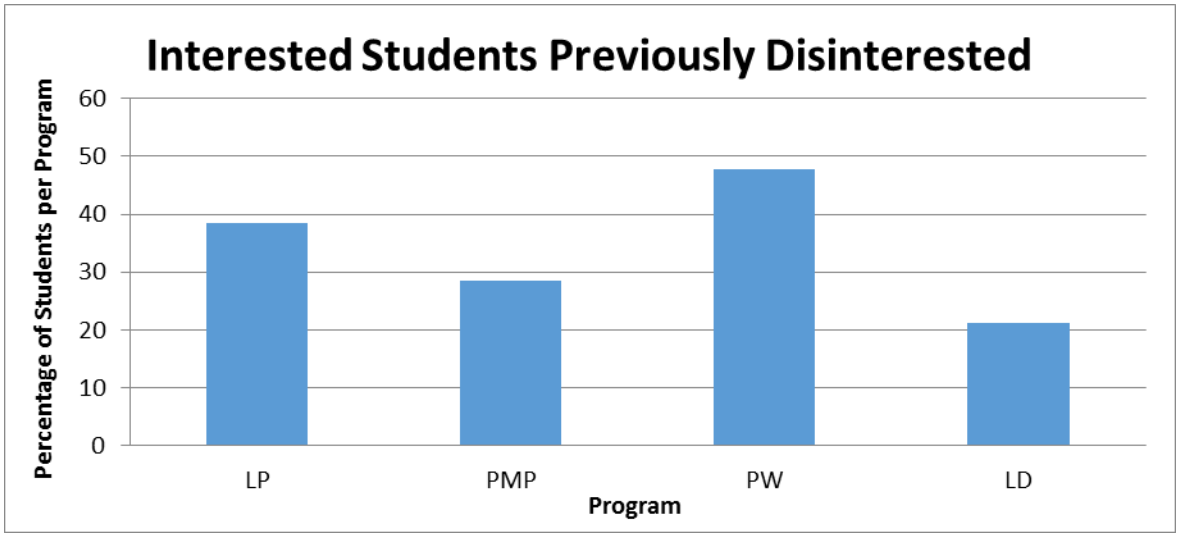


Figure 5.8: Graph displays number of students that answered "No" or "Uncertain" for the pre-survey and later answered "Yes" for the post-survey concerning interest in a STEM career. N = 13, 21, 44, and 33 for LP, PMP, PW, and LD, respectively.

Table 5.8: Selected responses from PW student's that changed their career choice, specifically mentioning "Bioengineering"	
PW	*"I have always wanted to be a pediatrician, but I am thinking about Bioengineering."*
PW	*"My career choice didn't change but if it did change I would pick Bioengineering."*
PW	*"Performer --> Bioengineer/Biologist"*
PW	*"anesthesiologist to bio medical engineer"*
PW	*"First it was real estate agent, now it is bioengineering"*

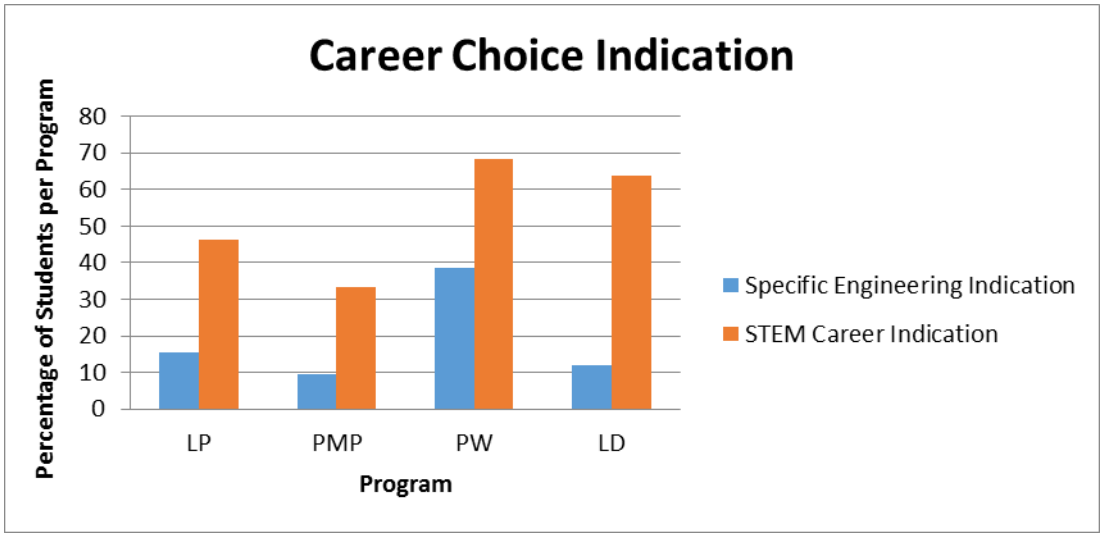


Figure 5.9: Graph compares two groups: (Red) Percentage of students that simply indicated interest in a STEM career on the post-survey by answering "Yes" to the post-survey item inquiring into their STEM career interest after participation (Q2, in Table 5.5) or by specifically listing a STEM career in the open-ended post-survey item asking students to list their career choice (Q4, in Table 5.5); (Blue) Percentage of students that specifically mentioned "Engineering" as a career choice in their open-ended post-survey response (Q4, in Table 5.5). N = 13, 21, 44, and 33 for LP, PMP, PW, and LD, respectively.

Discussion of Survey Results

The likelihood of parents encouraging STEM career or STEM major was significantly higher for the PW and PMP groups. This result may indicate some connection between STEM career and SES. Both of these groups were not characterized by low SES as the other two groups were. Although students coming from low SES environments were included in these groups, their parents reported being from a higher SES than the LP and LD groups. This argument is supported by the fact that PW parents indicated the highest amount of STEM employment for themselves and family/friends. PMP had a smaller percentage of parents currently working in STEM careers, but had significant reporting of STEM career exposure via family/friends. Both LP and LD groups, characterized by low SES, reported zero parents currently employed in STEM occupations. This data seems to support the connection between interest in STEM and SES, as stated by Mau in his work exploring the relationship between SES and persistence in STEM in underrepresented minority groups.[21]

The parent survey results also suggest prioritization of math over science. In their work on the Ecologies of Parental Engagement (EPE), Barton and colleagues provide examples of parent beliefs centered around the perceived importance of math as a life skill and math performance as an important academic marker.[28] These results seem to support this belief, but may also suggest the potential for other barriers to science participation outside of school. While parents indicated a higher frequency of extracurricular math activities than science activities, factors such as the larger infrastructure requisite for science as opposed to math activities are important to consider. This suggestion points to the focus of the EPE framework on the combination of both capital and space for significant parental engagement.[28] Extracurricular math and science activities provide opportunity for the creation of a space in which parents can participate in math or science with their children, regardless of their own domain-specific capital.

Amongst parents, STEM interest, knowledge, or career may develop the social or material capital needed to influence their level of encouragement toward STEM.[29] Without this capital, additional space (i.e. extracurricular activities such as museum visits, STEM focused television shows, family science activities) must be created to increase parental agency toward STEM, which may then increase encouragement toward STEM for their child.[28, 30, 31] This theme is repeated in the results surrounding the parent's perceived importance of math or science to their child's success. Again, parents responded with a significant bias toward math. These parent responses suggest a promotion of math concepts as more important than science concepts, which may have led to the following result. All parents rated their children's ability in math to be at a satisfactory or advanced level. PW parents, whose children were selected for participation in the program, were the only group to report comparable "Advanced" ratings for both math and science. Other groups had responses concentrated around the "Satisfactory" rating for both groups, with math being higher for every group. The consistency with which parents were more likely to report advanced STEM ability for their children rather than themselves (across all groups) also points to a perceived lack of domain-specific capital with which to engage their children in STEM activities.

These parent results speak to some of the potential barriers to STEM career pursuits faced by minority students. Researchers have long suggested that SES and parental occupation are strong contributors to STEM major selection, specifically among minority students.[32, 33] Limited personal/professional experience or confidence with STEM amongst parents may also lead to the perceived inability to participate or encourage their child to pursue a STEM vocation. Hoover-Dempsey and Sandler assert that parents become involved in their children's education when they feel they can be effective and successful in helping their children and when they perceive their participation will impact their children's education.[34] As indicated by the

"Outside STEM Influences" survey responses, across all groups parents are overwhelmingly certain that they most influence their child's career choice. If this is true, a parent's perceived personal disinterest or incompetence in STEM may have a strong relationship with their level of encouragement for their child's STEM aspirations.

A comparison of parent survey results with student pre-survey results, although not paired, may suggest that less enthusiastic parent responses in regards to STEM encouragement, importance, perceived ability, and extracurricular activities are evidenced by a significant number of students disinterested or unsure about the pursuit of a STEM career. This may be particularly true in all groups except PW, in which students were selected specifically based on their science interests or aptitude. Interestingly, however, there are also a significant number of PW students that indicated disinterest or uncertainty prior to participation. This may be related to their home environment, partially elucidated by parent survey responses. Despite STEM aptitude denoted by the selection process for PW, a lack of parental support for STEM careers may hinder interest in STEM careers for these students. Work by Constantine and coworkers buffets this argument as a sample of African American adolescents were shown to positively associate career choice certainty with perceived parental support.[35] Also possible is that despite aptitude or parental interest and encouragement in STEM, students may still have hesitation about pursuing a STEM career. This option challenges the overwhelmingly consistent parent response that they are the most significant career choice influence for their child. Lent and colleagues assert, through Social Cognitive Career Theory, that there is interplay among personal, environmental, and behavioral variables with respect to a student's career goals. These goals are a combination of self-efficacy, social supports, social barriers, interests, and outcome expectations.[36] The environmental factors presented in a home where STEM careers are not encouraged or supported, either through parental disengagement in this area or through societal barriers such as low SES, may create

significant challenges to the pursuit of a STEM career for these students. These challenges, along with a scarcity of behavioral experiences related to exposure of STEM careers may lead to a lack of interest or uncertainty regarding outcome expectations. Working together these elements may present a significant set of barriers guiding a student away from a STEM career.

Post survey data reveals several significant insights into program effectiveness. The first is the fact that all PW students left the program interested in pursuing a STEM career. This is contrasted to the pre-survey results in which 47% of PW students indicated disinterests or uncertainty. These students were exposed to other STEM activities outside of this program during the camp, therefore, the results shown may suggest other programs were responsible for STEM career changes. While this may be the case for some students, several students specifically mentioned the Bioengineering program as a contributing factor in their change to STEM career interest. Those comments are shown above in Table 5.8. Comments from the PW group specifically mentioning the word "bioengineering" were selected for this chart. Post-interest in STEM careers was also increased in every other group, with disinterest decreasing in every group as well. Uncertainty also decreased in every group except the PMP group, in which there was a small increase. These results seem to indicate the relative success of the program in promoting STEM career choice indication in these underrepresented student populations.

Specifically looking at camp set-up, results seem to indicate that the PW set-up of a week-long camp focused on various aspects of STEM resulted in more students interested in STEM careers generally, and students indicating specific interest in engineering, as denoted in the Figure 6 above. The other programs were characterized by a mixed scope of activities, which may have resulted in less enthusiastic STEM responses. Also of note is the fact that PW students were specifically selected or self-applied based on science aptitude. Students from other groups had a more diverse set of skills and academic focus. The differences in group recruitment, structure,

and camp set-up speak to the robustness of an interdisciplinary interventional approach in affecting student STEM career choice indication. While groups were significantly different in their make-up, prior experiences, and focus of summer experience, each group expressed an increase in STEM career goal aspirations following participation in this program.

The interdisciplinary structure of the program in this work combined science, math, and engineering knowledge to solve a problem. The math and science coursework presumably learned in the previous year was revisited prior to the design phase. This process, along with introductions and examples of Bioengineering and other engineering disciplines, may have inclined students generally enjoying STEM to migrate more specifically toward engineering. Figure 8 above shows that students specifically mentioning the word "engineering" in their open-ended career goals response generally follow the same pattern of students simply indicating another STEM field. This result may suggest that the focus on engineering in this program does not dissuade students from indicating interest in a variety of STEM careers.

Student survey results also indicate a difference in STEM career indication for groups that visited campus for program administration and those that participated in the program at an outside site. LD students indicate a much higher level of uncertainty than other groups in their pursuit of a STEM career, both before and after the program. It has been suggested that exposure of students to engineering contexts and environments may affect their self-efficacy toward an engineering task.[37] Students' uncertainty may have been just the result of marginal interest, but also the inability to see themselves in an engineering environment. Students participating in the program on campus were able to observe other scientist and engineers doing science and engineering work as they were learning about these areas. This experience may have provided a more concrete visualization of future career goals in STEM. Exposure of students to this community of practice was an essential part of the program due to its capacity to connect school

learning with the workplace. Lave and Wenger discussed the benefits of such a strategy in learning in their work in Situated Learning. In defining this theory of learning they asserted that "learning, thinking, and knowing are relations among people engaged in activity in, with, or arising from the socially and culturally constructed world."[38] In congruence with this theory, immersing students in a STEM career environment during the program served to contextualize both what they learned in the program, and the knowledge learned in the classroom that had been applied to program activities.

Conclusions and Limitations

Given the results and previous discussions, we conclude that this interdisciplinary engineering intervention was successful at inspiring interest in STEM careers for many of the students in our sample population. The student survey results of the PW group were particularly promising with all students leaving interested in a STEM career. Generally, results from each group showed a higher number of students that left interested after initial disinterest than students that left disinterested after initial interest or students that stayed the same. This result indicates that the program is at least effective at stimulating thoughts around STEM careers for these students, with most outcomes being positive. Responses to the open-ended survey item concerning career choice after participation, Q4 in Table 7, seem to support this argument. Many students specifically mentioned activities from the modules as the reason for their indicated career choice. Several students also cited portions of the modules they disliked as support for their disinterest in a STEM career. We believe that all of these outcomes are beneficial to fostering interest in STEM careers in middle school students. Results from this sample population suggest that the strategy and design of the program modules used here might be implemented in the classroom during the academic year or in a similar fashion to this study to introduce and engage students with a variety of STEM disciplines.

Parent survey results echoed several thoughts previously highlighted in the literature. Encouragement towards a STEM career or major, perception about their child's ability, extracurricular involvement, and perceived importance of STEM concepts have all been explored with respect to parental engagement. Parents from this sample population indicated an emphasis in math over science, particularly in extracurricular activities. Previous works have suggested a parental preference of math over science, however, further investigation is needed to make that conclusion. Encouragement toward a STEM career was fairly consistent across all groups. This encouragement was present despite perceived level of ability. We attribute this pattern to a suspected parental belief in the socio-economic opportunities provided through STEM. Being that the majority of parents, particularly in groups characterized by low SES, did not work in a STEM career, we conclude that these parents perceive a connection between STEM and academic success, leading to higher SES. Parents overwhelmingly believed in the dominance of their influence over their child's STEM career. This result is encouraging because it suggests the prioritization of a creation of space for engagement, particularly in those parents lacking the social and material capital to engage otherwise.

There were several limitations to this study which provide opportunities for future work. The sample populations selected for this study were limited by proximity to Clemson University. Participants in the first year had to be on campus or close enough to commute. Second year students had to be close enough for the facilitators to commute. This issue limits the potential diversity of our sample populations. If possible, future researchers should aim to gather a more geographically and ethnically diverse sample, potentially disseminating modules online for easy access at multiple sites. Each group consisted of a selected portion of the URM community. However, the scope of each camp comprising these groups differed significantly. While this issue could address the robustness of the program, it also presents concerns with drawing conclusions

from results given the many confounding variables. Ideally, each selected portion of the URM community (i.e. women, African Americans, Native Americans, low SES, etc.) should be tested independently to first characterize the group response to this type of program. Groups could then be intermingled to understand how results are affected by the interaction of groups within the URM community. Further investigation is needed in the area of understanding the extracurricular opportunities and barriers students face. A next development in this work should include more parental survey questions regarding their specific feelings toward science extracurricular activities, math extracurricular activities, and the barriers associated with each. Lastly, data collected for this study was only done so at one time point. Results would be more meaningful if a follow-up study was conducted to check the stability of students' indicated career interests later in their academic development.

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CHAPTER SIX

Select results in this chapter were generated collaboratively by an Institute for Biological Interfaces of Engineering interdisciplinary team, including former Clemson University doctoral student Erin McCave. Results were presented at the 2014 Northeastern Biomedical Engineering Conference (McCave et al., 2014a) and the 2014 American Society for Engineering Education Annual Meeting and Exposition (McCave et al., 2014b). These results were similarly reported, with the permission of the Clemson University Graduate School in Erin's dissertation.

ENGINEERING AND SCIENCE STUDENT PREPAREDNESS FOR RESEARCH: EXPLORING THE CONNECTIONS BETWEEN STUDENT IDENTITY AND READINESS FOR RESEARCH

Introduction

The increasingly technical global economy and rapidly changing national demographics have presented the US with a critical workforce shortage in the educational areas of Science, Technology, Engineering, and Mathematics (STEM).[1] As the country attempts to maintain its leadership position in research, development, and innovation, the literature has made clear that US production of STEM graduates needs improvement. Employment in STEM fields grew by 23% between 1994 and 2003, compared to only 17% for non-STEM fields; nonetheless, the US is struggling to meet the rapidly increasing demand for STEM workers.[2] The continued need to remain globally competitive and the fact that 39% of people in the US under 18 are persons of color (U.S. Census 2000) underlie the urgent need for colleges and universities to improve their efforts to graduate minority students in STEM disciplines.[3]

Along with an increased interest in undergraduate degree attainment, there is significant interest in increasing the number of graduate degrees awarded in STEM, particularly to underrepresented minority students.[4] STEM education researchers have commonly defined underrepresented minorities (URMs) as African American, Hispanic/Latino, or Native people, including Native American, Alaska Native, Native Hawaiian, and Pacific Island individuals.[4] The drive to increase the number of graduate degree recipients is directly relevant to research and

innovation goals and national economic interest.[4] One of the strategies employed to increase the number of URM students in STEM is the introduction and promotion of undergraduate research programs. Both federal and private agencies have committed to investing significant funding into these programs, as they have been reported to increase student intention of enrolling in graduate or professional school.[5,6]

Many researchers have explored potential causes for minority student underrepresentation in the STEM disciplines. Issues such as preparedness deficiencies, stereotype threat, familial or societal expectations, or low esteem have been presented as potential reasons of ethnic minority students for low interest, aspiration, admission, retention, and persistence in STEM.[7-14] Diminished pursuit of graduate studies for URM students was thought to be largely related to financial hardship post baccalaureate; however, further research has shown that URMs in STEM also may not see graduate or professional schools as significantly beneficial to career aspirations and interest.[15]

Undergraduate research programs have been shown to be effective in fostering the interest, skills, and aspirations that may develop into pursuit of graduate/professional school and potential research and innovation careers.[5,6] The concept of "communities of practice" described by Wenger supports the idea that participation in different communities and experiences affects participant identity development.[16] The National Science Board members, in their report "Moving Forward to Improve Engineering Education", propose participation in research experiences, specifically in the freshman and sophomore years, as a desirable means to engage URM students in the community of STEM.[17] These experiences aim at introducing students to STEM and broadening their education while improving retention. One aspect that has been highlighted by undergraduate research experts is identity development within the context of STEM.[5] Attention to identity has increased, specifically within the sciences[18] as work continues toward increasing the

STEM population and workforce. Investigators have suggested that participation in an undergraduate research program results in domain identity related to the area of participation.[19] It is this identity development process that fosters feelings of preparedness for future research and creation of a research identity integrated with a student's STEM domain-specific identity. Domain-specific identity, comprising three dimensions of student beliefs: interest, performance/competence, and recognition, has been used to observe math, physics, and general sciences identities.[20-25]

It has been suggested that engineering research is advanced by an increasingly diverse population of STEM researchers working collaboratively to accomplish interdisciplinary research objectives. Diversity of thought and perspective is a prerequisite to addressing the world's complex problems. There is a significant need for training and development of diverse populations to answer evolving research questions. To develop researchers one must understand how their identity, which is based on a researcher's belief about his/her performance, competence, recognition by others, and interest, influences his/her feelings of preparedness for research experiences. It is our assertion that students who feel more prepared for research experiences are more likely to participate in future research experiences. Our study focused specifically on a research training opportunity funded through the National Science Foundation (NSF) Emerging Frontiers in Research and Innovation (EFRI) program. Student experiences highlighted in this study pertain to their participation in an NSF-EFRI Research Experience and Mentoring (REM) program during the academic year and subsequent research or professional experiences the following summer. These research or professional experiences included Research Experience for Undergraduates (REU) programs, cooperative education (co-op) experiences, institutionally-funded research programs, and other summer employment opportunities. The purpose of this study was to understand how a student's perception of preparedness is influenced

by the student's science and engineering identity developed through their participation in interdisciplinary research.

Methods

Clemson University received NSF:EFRI funds that allowed engineering researchers from Clemson University and biology researchers from the University of North Carolina at Charlotte (UNCC) to work together to build and analyze breast cancer benchtop tissue test systems. The NSF offered a competitive supplemental funding opportunity to funded EFRI grantees, termed Research Experience and Mentoring (REM); the goal of the opportunity was to further the progress in EFRI topic areas while broadening participation of underrepresented groups in STEM fields.[26] Through the funded NSF:EFRI grant, we applied for and were awarded REM funding for the 2012-2013 academic year. The objective of our NSF:EFRI-REM program was to introduce URM undergraduate students, especially those at an early transition point in their academic careers, to a positive introductory research experience that would inspire confidence and create credentials for future research opportunities. Given the national emphasis on early exposure to research experiences for undergraduates and the success of such programs, admission to these types of research experiences has become increasingly competitive. Often students in early stages of their academic career, or students with grade point averages (GPA) below 3.0 are excluded from participation in these experiences. It is our assertion that this limitation of the research experience pipeline leads to a less diverse graduate research population, denies the opportunity for exposure to initially less informed or interested students, and reinforces the notion of academic elitism amongst those who participate in STEM research. To directly address our target population and focus on the objective of this study, we specifically sought students with no prior research experience, or students that would otherwise be less competitive for admission into an undergraduate research experience. Each student participated in 1 semester of the NSF:EFRI-

REM program, either in the fall or spring, and each had the opportunity to apply to participate in a 10-week REU program encompassing experiences at both universities. All REM participants were encouraged to apply for other REU programs across the nation, other summer research experiences, or co-operative education experiences.

During the school year, URM students were recruited through Clemson University and UNCC support offices, i.e. the Clemson University – Programs for Educational Enrichment and Retention (PEER) office and the Producing Readiness of Diverse University Cohorts in Education (PRODUCE) office, with focus on underrepresented student recruitment and retention in STEM. Students were encouraged to apply for the REM opportunity and were selected based on their interest in the program, their ability to communicate how this experience might influence them, and their academic progress (including performance in STEM classes). The principal investigator as well as graduate student and staff mentors reviewed applications; the selected undergraduate students, termed Research Participants (RPs) by NSF, were notified of their acceptance into the REM program. Each semester, the REM program began with an approximately 8-hour Research Studio before students began the laboratory experience. The Research Studio included an introduction to tissue test systems and overall EFRI project goals, completion of laboratory safety training, an introduction to research ethics, technical writing, and basic laboratory practices, participation in a team building exercise, discussion of the projects to which each student would be exposed, and discussion of the expectations of RPs. Once RPs completed the Research Studio, each RP was paired with a graduate student mentor and the mentor's project. Projects focused on the characterization and fabrication of polylactide (PL) beads, cellular response to such beads, PL fiber fabrication via melt-spinning, and development of automated components for a lab-scale loom for weaving tissue engineering scaffolds. After completion of the Research Studio, each student was required to spend 3 hours on lab/research-

related activities each week during the semester. Weekly professional development exercises introduced the RPs to a variety of research-related skills and topics. Students ended the semester with a rapid fire podium presentation and poster presentation at Networking Day, a day where all students, graduate student mentors, faculty mentors, and external support mentors gathered to discuss research activities and outcomes of the REM program.

The joint EFRI:REU began in late May for a 10-week period and included two RPs from Clemson University and two RPs from UNCC. The first 5 weeks were spent in the engineering laboratories at Clemson University, and the second 5 weeks in cancer biology laboratories at UNCC. The research focus for this REU built on the PL bead and fiber characterization work. RPs and mentors worked to incorporate beads and fibers into Collagen Type-I/Matrigel hydrogel constructs to evaluate the effect of modulating matrix stiffness on breast tissue acini and ductal structures.[27] Each REU weekday consisted of approximately 8 hours of lab/research-related activities. All EFRI:REU RPs gave poster presentations overviewing their research at the end of the REU and all EFRI:REU students were invited to apply to participate in/present at the NSF and American Association for the Advancement of Science-sponsored Emerging Researchers National Conference in STEM in Spring 2014.

Each academic semester, eight RPs participated in the REM program, four at each university. The demographics of our population were determined by information submitted in the REM applications, including gender, ethnicity, college level, major (with concentration), and minor. Of the sixteen RPs in the REM program, three were male and thirteen female. Students self-indicated their ethnicity on the application as: Hispanic or Latino (regardless of race), American Indian or Alaska Native, Asian American, Black or African American, White, or Native Hawaiian or Other Pacific Islander. Our population included two Hispanic or Latino students, thirteen Black or African American students, and one Asian American student. RPs

included thirteen sophomores, one freshman, and two juniors. Clemson University selected students seeking either engineering or science degrees while UNCC selected students pursuing science degrees. Out of the 16 participants, six were obtaining engineering degrees in industrial engineering, computer engineering, environmental engineering, and chemical engineering, while two had yet to declare an area of focus and were enrolled in the general engineering program. The other 10 students were pursuing science degrees; specifically, chemistry (1) and biology (9). Of the 16 students that participated in the REM program, two from each university were selected to participate in the summer REU program. All of the REU participants were female and three of the four were science majors. The REU RPs identified their ethnicities as Asian American (1), Hispanic/Latino (1), and Black or African American (2).

An identity survey was used in order to assess identity development after participation in the REM program. Former REM RPs were given an identity pre-survey in May before they started their summer activities. Eleven of the 16 participants completed the pre-survey. Students that completed the pre-survey were given a follow-up identity post-survey the first week of the fall semester following the various summer activities. Ten post-surveys were completed; 5 by science majors and 5 by engineering majors. The summer experiences of these 10 RPs included REU (4), co-operative education experience (2), Summer research experience (2), and non-research related activities (2). The identity survey questions were adapted from the Sustainability and Gender in Engineering (SaGE) survey.[20-22,24,25] Questions for engineering and research identity were adapted from these valid and reliable survey items with the help of experts in engineering education research. The survey items were separated into three identities: science, engineering, and scientific research. The same questions were asked to investigate each identity, substituting the word science, engineering, or scientific research in each item. Each question was evaluated on a Likert-type scale, the far left of the scale anchored as “Strongly Disagree” (1.0)

and the far right of the scale anchored as “Strongly Agree” (7.0). Questions in the survey pertaining to preparation were categorized based on the type of future experience, and included research, non-research, and graduate research questions. Statistical analysis of the data was conducted using Analysis of Variance (ANOVA, $p<0.05$, via JMP Pro 10, SAS, Cary, NC) to determine statistical differences between majors, for both pre-survey and post-survey responses, and within majors (pre- to post-response).

Results and Discussion

Results from pre- and post-surveys suggested that science and engineering identities are related to each other, as well as to the development of research identity. The analyses shown below in Tables 6.1, 6.2, and 6.3 compare survey item responses for science majors versus that of engineering majors. For example, the first line of Table 6.1 indicates that pre-survey responses for science majors yielded a mean (μ) response of 6.80, while engineering majors had a mean response of 7.0. These responses were related to the question, “To what extent do you disagree or agree with the following statement? I am interested in learning more about science.” The difference in science majors’ and engineering majors’ pre-responses yielded a non-significant p-value of 0.3466 after ANOVA testing. Similarly, post-responses also yielded a non-significant difference (p-value = 0.1720) with means of 7.0 and 6.4 for science and engineering, respectively. Analyses completed comparing pre- to post-responses within majors was conducted but is not included in table format. Only two of the survey items were significant; descriptions of these items are included below.

Two questions addressed the aspect of domain-specific interest. The questions “I am more interested in learning more about …” and “I enjoy learning …” revealed significant differences between the science and engineering majors when the topic was engineering, for both the pre-survey and post-survey responses (Table 6.1). While the science and engineering majors’

means for both the pre-survey and post-survey are nearly equal for science and scientific research identity items, the engineering identity items reveal a significant difference. Engineering students identified much more interest in engineering topics as compared to the science students.

Table 6.1: Self-Reported Interest Items Comparing Science and Engineering Majors. Symbols μ and σ represent the mean and standard deviation of the population, respectively. P-Values highlighted indicate significant differences between majors.

Table 6.1 - Interest	**Pre-Summer Experience**					**Post-Summer Experience**				
	Sci.		**Eng.**		**P-Value**	**Sci.**		**Eng.**		**P-Value**
Survey Item	μ	σ	μ	σ		μ	σ	μ	σ	
To what extent do you disagree or agree with the following statements?										
I am interested in learning more about science	6.80	0.45	7.00	0.00	0.3466	7.00	0.00	6.40	0.89	0.1720
I enjoy learning science	7.00	0.00	6.60	0.55	0.1411	6.80	0.45	6.60	0.55	0.5447
To what extent do you disagree or agree with the following statements?										
I am interested in learning more about engineering	5.00	1.73	7.00	0.00	0.0325	5.20	1.64	6.80	0.45	0.0688
I enjoy learning engineering	4.40	0.89	6.80	0.45	0.0007	5.20	0.84	6.80	0.45	0.0055
To what extent do you disagree or agree with the following statements?										
I am interested in learning more about scientific research	6.60	0.55	6.40	0.89	0.6811	6.75	0.50	6.40	0.89	0.5097
I enjoy learning scientific research	6.60	0.55	6.40	0.89	0.6811	6.60	0.55	6.40	0.89	0.6811

Questions were posed about RP competence in the three areas of science, engineering, and research. While the survey items addressed competence, performance was not included in this analysis as there were no grades assigned to student research outcomes. Regarding competence (Table 6.2), we found that science students felt significantly less confident in their ability to understand science outside the classroom after their summer experience. This could be, in part, because more in-depth research and summer experiences broadened the students' perspectives to what is required to understand science and conduct scientific endeavors outside the classroom.

The other area of significance of note within competence from Table 6.2 is in the differences of "understanding engineering", "understanding concepts studied in engineering", and "being able to overcome limitations and setback/obstacles in engineering". Significant differences were seen by science students in all of these categories, except for the statement "I am confident that I can understand engineering in the laboratory". The results may be explained, in part, by the fact that three of the five science majors participating in the joint summer EFRI:REU program completed the surveys. The summer EFRI:REU incorporated an engineering component and thus many of the science majors were exposed to engineering problems. The engineering students were significantly more confident in every one of these categories after their summer experiences. This result was expected, as all but one of the engineering RPs that completed the survey were involved in summer research that focused on some aspect of engineering, most of them in areas of their own majors. These RPs gained experience and knowledge in their specific engineering areas and thus would have strengthened identity in the area of competence. The engineering question that did not result in significant differences when comparing majors both pre- and post-summer experience was "Engineering makes me nervous". However, the science students, when comparing their pre- to post-summer experience responses with respect to science, did indicate significantly less ($p = 0.0046$, data not shown in table format) nervousness post-summer.

One of the major foci for this study was the development of feelings of preparedness for future research opportunities. Results shown in Table 6.2 below indicate that both engineering and science majors are relatively confident in their level of preparedness for future research. This is signified by means above 6.0 for nearly every preparedness item. There was no significant difference between engineering and science majors in terms of preparedness, suggesting the program helped to develop confidence in research preparedness across the spectrum of represented majors. The mean confidence level of science majors with respect to preparedness

items was also slightly higher (though not significant), again indicating that perceived research outcomes may be more closely related to the skillset students identify with science.

Table 6.2: Self-Reported Competence Items Comparing Science and Engineering Majors. Symbols μ and σ represent the mean and standard deviation of the population, respectively. P-values highlighted indicate significant differences between majors.

Table 6.2 – Competence	**Pre-Summer Experience**					**Post-Summer Experience**				
	Sci.		**Eng.**		**P-Value**	**Sci.**		**Eng.**		**P-Value**
Survey Item	μ	σ	μ	σ		μ	σ	μ	σ	
To what extent do you disagree or agree with the following statements?										
I am confident that I can understand science in class	6.40	0.55	6.20	0.84	0.6666	6.80	0.45	6.80	0.45	1.0000
I am confident that I can understand science in the laboratory	6.20	0.45	6.20	0.84	1.0000	6.60	0.55	6.20	1.10	0.4860
I am confident that I can understand science outside of class	6.40	0.55	5.20	0.84	0.0278	6.60	0.55	6.40	0.89	0.6811
I understand concepts I have studied in science	6.60	0.55	5.80	0.84	0.1114	6.80	0.45	6.60	0.55	0.5447
Science makes me nervous	4.20	1.79	3.20	2.28	0.4626	3.20	1.48	3.40	2.07	0.8651
I can overcome limitations in science	5.60	0.89	5.75	1.50	0.8565	6.20	0.84	6.40	0.55	0.6666
I can overcome setbacks/obstacles in science	5.60	0.89	6.00	1.00	0.5237	6.40	0.55	6.40	0.55	1.0000
To what extent do you disagree or agree with the following statements?										
I am confident that I can understand engineering in class	4.40	0.55	6.40	0.55	0.0004	4.80	1.48	6.40	0.89	0.0727
I am confident that I can understand engineering in the laboratory	5.20	1.30	6.40	0.55	0.0943	5.20	1.64	6.00	1.00	0.3796
I am confident that I can understand engineering outside of class	4.40	0.89	6.00	0.00	0.0039	4.60	1.52	6.40	0.89	0.0516
I understand concepts I have studied in engineering	4.40	0.89	6.40	0.55	0.0027	4.40	1.82	6.20	0.84	0.0790
Engineering makes me nervous	4.60	0.89	3.60	2.30	0.3917	3.00	1.22	3.00	1.41	1.0000
I can overcome limitations in engineering	4.00	0.71	6.00	1.00	0.0065	4.20	0.45	6.40	0.55	0.0001
I can overcome setbacks/obstacles in engineering	4.00	0.71	6.20	1.10	0.0054	4.60	0.89	6.40	0.55	0.0050
To what extent do you disagree or agree with the following statements?										

I am confident that I can understand scientific research in class	6.40	0.55	5.80	0.45	0.0943	6.60	0.55	6.20	0.84	0.3972
I am confident that I can understand scientific research in the laboratory	6.40	0.55	6.00	0.00	0.1411	6.60	0.55	6.20	0.84	0.3972
I am confident that I can understand scientific research outside of class	6.00	0.71	5.60	0.55	0.3466	6.40	0.55	6.00	1.00	0.4554
I understand concepts I have studied in scientific research	6.20	0.45	5.80	1.10	0.4714	6.60	0.55	5.80	0.84	0.1114
Scientific research makes me nervous	4.80	1.30	4.00	2.00	0.4751	3.40	1.52	3.40	1.82	1.0000
I can overcome limitations in scientific research	5.80	0.45	6.00	1.22	0.7404	6.20	0.84	6.40	0.55	0.6666
I can overcome setbacks/obstacles in scientific research	5.80	0.45	6.40	0.55	0.0943	6.40	0.55	6.40	0.55	1.0000
To what extent do you disagree or agree with the following statements? I feel prepared to participate in...										
Academic research program (e.g. REU, research experience) offered during the summer	6.40	0.55	6.00	0.71	0.3466	6.80	0.45	6.40	0.89	0.3972
Academic research programs offered during the academic year	6.40	0.55	6.20	0.45	0.5447	6.80	0.45	6.60	0.55	0.5447
Non-academic research program (e.g. scientific or engineering based co-operative education experience or internship) offered during the summer	6.20	0.45	6.40	0.55	0.5447	6.60	0.55	6.40	0.89	0.6811
Non-academic research programs offered during the academic year	6.20	0.45	6.20	0.45	1.0000	6.40	0.55	6.20	0.84	0.6666
Continued research at the graduate level	6.40	0.55	5.75	0.96	0.2381	6.40	0.55	5.80	1.10	0.3052

The third aspect of identity, recognition, revealed some of the stark differences between science students and engineering students with respect to how they and others recognize them in the communities of practice of science, engineering, and research. Recognition plays a crucial role in how people see themselves fitting into a Community of Practice and a lack of recognition has been shown to deter students from pursuing certain career paths.[28]

Before the summer experience, science students reported significantly higher ($p = 0.0039$) recognition from their mentor(s) as compared to engineering students, whereas in every other aspect of science identity (i.e. recognition of self and recognition by parents, friends, advisor(s), and faculty), there were no significant differences by major in either the pre- or post-summer experience items. Engineering identity of science majors was significantly lower (Table

6.3) compared to the engineering majors both pre- and post-summer experience, except for recognition by their mentor(s) in the pre-survey. The higher recognition by mentor(s) of the science students in this category could be due to the fact that two of the five science students who completed the surveys participated in the engineering REM program instead of the science REM program, thus their mentor(s) were of engineering backgrounds instead of biology. The last significant difference of note was between majors evaluating the survey item "Others ask me for help in scientific research". The science student responses, in the pre-survey, reveal significantly higher ($p = 0.0438$) recognition with respect to others asking their help compared to engineering majors. This difference is most likely influenced by the coursework completed by each student. Many of the engineering students, at this point in their degree progress, have just begun to enroll in science-related classes, whereas science degree-seeking students enrolled in general science classes immediately upon matriculation as they are required to take many more science classes compared to engineering students. Further, engineering students are less likely to take a biology class compared to science students, as most engineering degrees require many more physics classes and physics is not, at this point, classified as a general science class for engineering majors.

Table 6.3: Self-Reported Recognition Items Comparing Science and Engineering Majors. Symbols μ and σ represent the mean and standard deviation of the population, respectively. P-values highlighted indicate significant differences between majors.

Table 6.3 - Recognition	**Pre-Summer Experience**					**Post-Summer Experience**				
	Sci.		**Eng.**		**P-Value**	**Sci.**		**Eng.**		**P-Value**
Survey Item	μ	σ	μ	σ		μ	σ	μ	σ	
To what extent do you disagree or agree with the following statements?										
I see myself as a science person	6.60	0.55	5.80	1.10	0.1823	7.00	0.00	5.60	2.19	0.1909
My parents see me as a science person	6.60	0.55	5.80	1.64	0.3319	6.80	0.45	5.60	2.07	0.2415
My friends see me as a science person	6.80	0.45	5.40	1.34	0.0578	7.00	0.00	5.40	2.07	0.1228

My faculty advisor sees me as a science person	6.20	1.30	5.40	0.89	0.2907	6.80	0.45	4.75	2.06	0.0641
My mentor(s) see me as a science person	6.60	0.55	5.00	0.71	0.0039	6.80	0.45	5.40	2.07	0.1783
My professor(s) see me as a science person	6.00	1.22	4.80	0.45	0.0736	7.00	0.00	5.20	2.05	0.0851
Others ask me for help in science	6.00	0.71	5.20	1.48	0.3080	6.40	0.89	6.40	0.89	1.0000
To what extent do you disagree or agree with the following statements?										
I see myself as an engineering person	3.80	1.64	6.60	0.55	0.0068	4.20	1.64	6.60	0.55	0.0147
My parents see me as an engineering person	2.80	1.10	6.40	0.55	0.0002	3.80	1.30	6.40	0.55	0.0034
My friends see me as an engineering person	2.75	1.50	6.20	0.84	0.0031	3.60	1.52	6.40	0.55	0.0047
My faculty advisor sees me as an engineering person	2.40	1.14	5.80	0.45	0.0003	3.40	1.34	6.40	0.55	0.0017
My mentor(s) see me as an engineering person	3.80	2.17	5.80	0.45	0.0780	4.00	1.58	6.40	0.55	0.0125
My professor(s) see me as an engineering person	3.20	1.64	5.40	0.55	0.0218	3.40	1.34	6.20	0.84	0.0042
Others ask me for help in engineering	3.00	1.22	6.00	1.00	0.0028	2.20	1.30	5.80	1.64	0.0050
To what extent do you disagree or agree with the following statements?										
I see myself as a scientific research person	5.80	0.45	5.40	1.52	0.5871	6.60	0.55	5.20	2.05	0.1783
My parents see me as a scientific research person	6.20	0.45	5.20	1.48	0.1869	6.00	0.71	5.40	1.95	0.5358
My friends see me as a scientific research person	6.40	0.55	5.20	1.48	0.1281	6.60	0.55	5.20	1.92	0.1562
My faculty advisor sees me as a scientific research person	6.00	0.00	5.20	1.30	0.2073	6.20	0.45	5.00	2.12	0.2509
My mentor(s) see me as a scientific research person	6.20	0.45	5.20	1.30	0.1434	6.60	0.55	5.20	2.05	0.1783
My professor(s) see me as a scientific researcher	5.80	0.45	4.80	1.30	0.1434	5.80	1.30	5.40	2.07	0.7245
Others ask me for help in scientific research	5.00	1.00	3.00	1.58	0.0438	5.60	0.55	5.00	1.87	0.5108

One of the major outcomes of this analysis was the indication that science RPs did not identify as engineers, either before or after participation in various summer experiences. This result was consistent across all explored aspects of identity: interest, competence, and recognition. This result was also statistically significant across most survey items concerning engineering identity, with science RPs reporting statistically lower means than those of their engineering RP counterparts. For the RPs surveyed, this result suggests a distinction between

science and engineering for students majoring in science. When comparing science major responses with regard to science identity to corresponding engineering identity items, a significantly higher mean response (statistics not shown in table) can be seen for science responses. This further supports the assertion that these science RPs have very strong viewpoints on the components of science identity and its distinction from engineering identity components.

In contrast to these results, engineering RPs indicated comparable levels of science identity as reported by their science RP colleagues. It can be seen across each measured component of identity that engineering student and science student responses to science-focused identity items resulted in non-significant differences in most cases. It is our assertion that these results indicate an intersectionality of science identity and engineering identity for engineering students. The engineering students do not see the two fields of study as inherently different as do the science students. This idea is supported by the work of Godwin and coworkers, in which both science and physics identities were shown to support or contribute to the development of engineering identity.[22] The results suggest that, for these engineering students, the components contributing to a strong science identity are the same as, or necessary for the development of, the components of their engineering identities.

These contrasting results are interesting, considering the implications derived from the research identity items explored in this study. For the most part, research identity items yielded non-significant differences between science and engineering majors for both pre- and post-survey results. However, closer examination of the mean values of these items reveal that, although not significant, science majors consistently reported slightly higher responses than engineering majors with respect to research identity items. Because these results are not statistically significant and because of the limited sample size, we cannot definitively conclude that science majors report higher research identity than engineering majors. However, the consistency of the

responses across all areas of identity suggests that science identity may be more closely linked to research identity for these students. Interestingly, the lack of significant difference also suggests that engineering students also readily identify with components of research. We offer two explanations of this result. First, engineering students may identify with research through some set of components common to both engineering and science identity. This explanation supports the previous assertion there is significant intersectionality between the science and engineering identities of engineering students. Second, engineering students may identify with research through their identification with science. This idea supports the previous statement that the most direct link to research identity may be through a strong science identity, but science and engineering identities are indeed separate. Figure 6.1 below illustrates these two potential explanations.

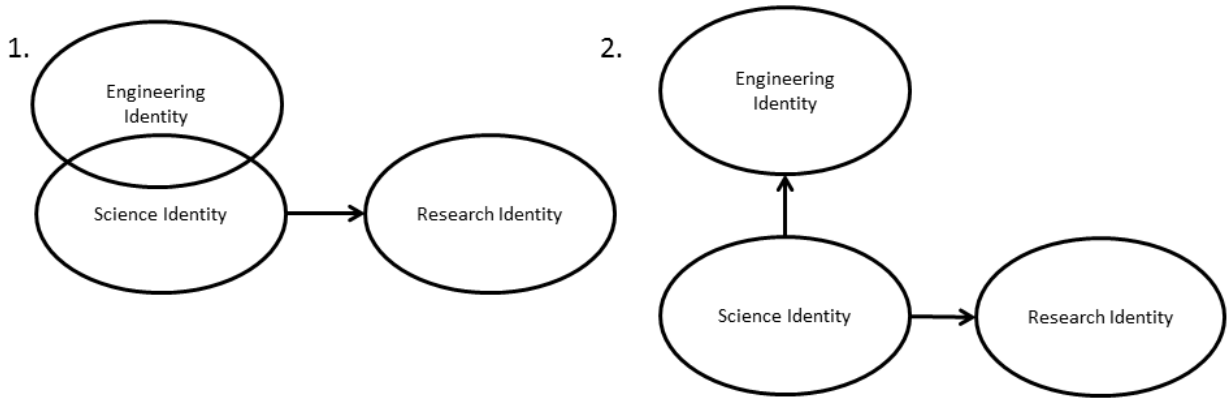

Figure 6.1: Potential explanations for research identity data. 1) Significant intersectionality between engineering and science identities, with science identity being most directly linked to research identity. 2) Engineering and science identity are distinctly different, but connected. Science identity is most directly linked to research identity.

It is also important to note some outcomes of this work not specifically related to the analysis. Science student post-survey results indicated a significantly higher response to the item, "I see myself as a research person" when compared to pre-survey results. This result indicates a significant growth in the self-recognition component of research identity for this group of students. It was the goal of this work to improve research identity development in these students; therefore, this result was a positive outcome of the study. Corresponding engineering student results for this item indicate comparable pre- and post-results without a statistically significant difference. This result reinforces the previous assertion that students of both majors more closely associate research with science at this stage in their academic development. It is our hypothesis that the differing natures of summer experiences for the engineering students responding to this survey may have played a role in research identity indication. We also hypothesize that students overwhelmingly consider research to be an academic exercise; therefore, students participating in more industry-focused experiences may not have associated their specific summer activities with research.

Our results suggest that engineering students identify lower with research compared to science students, and subsequently feel less prepared to conduct research; however, participation in an interdisciplinary experience increases their indication of academic research preparedness. Our results show, for the population studied, that participation in a research program, such as REM and summer REUs, increases URM student research identity which, in turn, could help increase diversity of the research population.

Limitations and Future Directions

While we believe that this work is a good starting point to better understand minority undergraduate students' perceptions of science, engineering and research identity and preparedness to conduct research, we understand that our program, and therefore our survey,

results are hindered by the small sample size. While this study was intended to assess how students participating in our program identify within science, engineering, and research, we believe that further, in-depth work assessing engineering and research identity is necessary to better understand how federally-funded and related programs impact students and the future of STEM fields. Some limitations of the study related to the survey items include the adaptation of items and missing data. The survey items have been validated and proven reliable for science and math identity through the SaGE study.[25] Further, missing data responses were dealt with by deleting entire responses for missing pre- or post- results.

Future work in this area of study should focus on capturing a larger, more representative population of underrepresented minority undergraduate researchers. A longitudinal study would be insightful to follow up this work in order to see how all the identities of science, math, engineering, and research change and morph over time with each RP's experiences and beyond, as each RP becomes part of the STEM community. Further work must be conducted to establish the validity and reliability of research identity survey items. Based on current literature, science, math, and physics identities play into the development of engineering identity.[22] Future research may explore the relationship of these already validated identities with research identity, or may explore the connection of engineering identity to research identity.

Conclusions

The overall motivation for this work is to increase the number of underrepresented minority students pursuing STEM careers which may lead to the fulfillment of research and innovation goals for the United States in years to come. It is our position that participation in undergraduate research programs foster the development of research identity in both science and engineering students and will allow students to feel more prepared to pursue further research opportunities. The program highlighted in this work combined "hands-on" experience with

faculty and graduate student mentoring to develop this research identity. Interest, competence, and recognition are critical factors in the development of any type of identity. Survey tools used in this study sought to explore the effect of participation in this program on those factors in identity development. Results showed that science students and engineering students may see their respective areas of study in different lights than their counterparts, but also they see research and its connection to their established academic identities as different. Science majors seemed to identify highly with only science, while engineering students identified with both science and engineering identity items. Science identity seemed to be the most direct link to the development of research identity in these students. Based on the results from this study, we consider these programs to provide a positive and impactful experience for underrepresented minority students interested in research careers.

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CHAPTER SEVEN

CONCLUSIONS

The following section outlines conclusions that can be drawn from data presented in this work. After a review of the literature and the completion of this work, it can be concluded that woven meshes are able to fulfill at least some of the ideal properties of a bone tissue engineering scaffold (Figure 1.25). This work focused on the porosity/pore size, geometry, and biocompatibility aspects of an ideal scaffold. Although surface modification was not specifically addressed here, study of protein adhesion at the cell-biomaterial surface interface provided some insight into what surface treatments might be beneficial to increasing cell affinity. Fiber geometry modulation and weave configuration variation contributed to mesh geometric evaluation, demonstrating that changes in geometry were related to cell affinity (from Aim 1) and fluid transport (Aim 2). Porosity/pore size, addressed in Aim 1, was shown to be a tunable mesh feature with clear biological implications. In this work, porosity and pore size were significantly related to cellular adhesion and differentiation. Short-term biocompatibility was confirmed through the conduction of all studies concerning cells, given the lack of cytotoxic effects from the meshes or treatments. The relationship of ideal bone scaffold parameters to woven mesh has been demonstrated through this work.

The novel bio-loom designed for this work was successfully constructed with the ability to weave meshes of variable porosity, pore size, and material configuration. These bio-loom parameters were biologically validated by cell affinity testing focusing on metabolic activity and DNA concentration. Metabolic activity was shown to increase on PL-Plain meshes, presumably due to it being the mesh condition with the smallest pores. DNA concentration results also confirmed that cells preferred meshes with smaller fibers and pore sizes as DNA concentration increased as porosity settings on the bio-loom were decreased. Live/Dead staining showed

significant cell adhesion to the polymer fibers, with 4DG fibers or fibers closely aligned in parallel. Weave configuration (pore shape and size) was also an important parameter as Twill meshes were shown to have higher DNA concentrations than Plain meshes, yet metabolic activity results showed Plain meshes as the favorable condition. Porosity and pore size was controlled via the automated bio-loom. The efficacy of bio-loom pore settings was confirmed through the decrease in both porosity and pore size as the picks/cm parameter was increased.

From the evaluation of fluid transport properties for these woven scaffolds it was discovered that 4DG based meshes were more permeable than those meshes based on RND fibers. There was not a significant difference in permeability based on weave configuration, but the difference presented did show that Twill meshes were more permeable than Plain meshes, and PLCL meshes were more permeable than PL based constructs. When looking at the interactions between various parameters it was shown that meshes that were 4DG in weft geometry, and Plain in weave configuration were the most permeable over all. All meshes wicked fluid in the wicking test but 4DG:RND-Twill-PLCL meshes wicked the most fluid. This result was not statistically significant however due to a large amount of variability in the determination of wicked fluid volume. It seems that any type of woven mesh would behave similarly under wicking conditions.

Evaluation of MSC adhesion and differentiation on the woven meshes confirmed many previous findings in the literature, but demonstrated how this previous work applies to a new woven tissue test system. VTN, known in the literature to be one of the most active ECM proteins present in FBS, did show the statistically significant changes across each time point over the 28 day study. Other proteins (FN, COL1, and LAMA2) were expressed more consistently. Constant COL1 expression suggestion maturation of the early osteoblasts, but LAMA2 expressions equal to VTN and COL1 suggests cells still in the early stages of osteodifferentiation. These two conflicting results lead to the conclusion that MSCs differentiating on meshes are not all

developing at the same rate. These may be in completely different stages of development after initial attachment. Additionally, the mobility and activity of VTN may be contributed to its relatively small size and the Vroman Effect in which proteins undergo a competitive surface adhesion competition.

No significant differences could be derived from RT-PCR data due to low RNA concentrations and non-specific amplification, but many results corresponded with other experiment results suggesting that there was a degree of validity to this data. β1 was up-regulated throughout the study when compared to the housekeeping gene GAPDH. This up-regulation is presumably due to the ability of the β1 integrin subunit to bind to all of the ECM ligands examined in this study. The α2 integrin subunit, known to bind with COL1, LAMA2, and FN ligands, was expressed through Day 21 as hypothesized signifying the presence of maturing osteoblast. The α5 integrin subunit binds with the FN ligand which showed slight up-regulation at Day 21. This result was consistent with Alizarin Red mineralization staining and maturing osteoblasts moving towards mineralization. The αV subunit, which binds with VTN and FN, showed variability across all time points. These results mirrored the expression of VTN in the adhesion immunofluorescence study, meaning that as VTN was changing conformation and adhesion sites on the mesh, the integrin subunit expression was also changing.

OC results did not indicate significant mineralization in meshes. However, in every group where OC is expressed, the up-regulation occurred late, usually between day 21 and 28. This result suggests that there may be some cell differentiating on meshes to the point of mineralization but they are in small numbers. ALP expression by PCR showed delayed expression (day 14 -21) of ALP but only PL meshes and the Control wells demonstrated any ALP expression. ALP expression by colorimetric assay confirmed the shifted expression of ALP beginning at day 14. It may be concluded that meshes were facilitating early stage

osteodifferentiation. However, the lack of mineralization present in PCR or in Alizarin Red staining suggests that cells may not have reached full osteogenic potential with mineralization.

Outcomes from this work are summarized by concluding that woven meshes may serve as bone tissue engineering scaffolds, and altering mesh parameters does promote change of biological response to MSCs *in vitro*. While full differentiation was not strongly represented, there is evidence that continuing this work for longer periods of time will allow for further progression of these cells down the osteoblast phenotype pipeline. Permeability, porosity, material type, and weave configuration are all valuable components of a woven tissue engineered test system. Further work should be done to support these findings by isolating the parameters that are most important for specific types of bone defects.

Based on the outcomes of this work there are several mesh parameters that should be furthered explored for the development of a clinical application of woven mesh to critical-sized defects. It was shown through both cell affinity and fluid flow experiments that the combination of 4DG fiber geometry and the Plain weave configuration offered a favorable environment for cell attachment and transport. For this reason, a mesh with 4DG weft and warp fibers in the Plain weave configuration would be the basis for a clinical design. Also the PLCL material would be incorporated due to its east of use and increased compliance when weaving. 3-Dimensional mesh effects would be incorporated through a rolled mesh design with a gradient of pore sizes orthogonal to the rolled axis. The gradient approach might allow for the incorporation of this mesh type in interfaces of bone and cartilage tissue.

There were several major takeaways from the study designed to encourage STEM careers in underrepresented minority students. There was a strong relationship between socioeconomic status (SES) and the level of encouragement, favorable STEM environment, and extracurricular

activities that students were exposed to. This result is consistent with the current thinking that STEM careers and exposure provide better socioeconomic opportunities for individuals and families. Parents felt overwhelmingly confident in their level of influence on their child's career choice and parental encouragement did positively relate to a child's likelihood to indicate interest in a STEM career prior to participation in our program. The STEM camp program, incorporating cross-curricular content based on the Next Generation Science Standards, was shown to be successful in retaining student interest in STEM careers, and fostering new interest in this area. The continued development of the modules presented to students, and the dissemination of these strategies to teachers in the classroom may serve as a viable tool for fostering STEM aspirations in many underrepresented students.

On the higher education level, using early research experiences for the development of research identity in underrepresented science and engineering majors proved to be an effective strategy. Significant improvements in self-recognition and competence in the research domain were documented for both engineering and science students. Interestingly, student's association with research was closely connected to their science identity, despite the area of study of the student. Both engineering and science students related research more to science, as opposed to engineering. This finding suggests that the scope of research should be redefined to include a broader picture of research opportunities for students. Additionally, the intersection of science and engineering should be emphasized in the research domain to encourage cross-collaboration and the participation of a diverse group of students.

CHAPTER EIGHT

RECOMMENDATIONS FOR FUTURE WORK

While suggestions for the improvement of each of the studies presented has been provided within each chapter, this section serves to highlight the next steps in research to improve upon this work or to advance the work from the points concluded here. General considerations for laboratory based work include the following:

1. Handling of meshes during cleaning, media changes, assay preparation, and the like lead to damage of the mesh and changes in configuration properties. Future studies should consider ways to minimize mesh handling as this damage introduces variability between meshes of the same type, making results difficult to interpret.
2. Imaging of meshes using 2-D modalities proved difficult from a focus and resolution standpoint. Future work should focus on the use of confocal microscopy to obtain better images.

Chapter 1

1. The current state of the literature lacks documentation in the description of loom designs for the weaving of tissue engineering scaffolds. This addition should be included to advance and disseminate bio-loom technology for broader research applications.
2. The literature also lacks documentation regarding cell adhesion and differentiation to woven structures. There is dearth of information regarding nonwoven and knitted structures, but few researchers have explored woven scaffolds in the tissue engineering context.

Chapter 2

1. The next stage of bio-loom development is the addition of more warp fibers and an additional warp axis for 3-dimensional weave configurations and designs. These additions would greatly increase the weave complexity capability of the bio-loom.
2. Bio-loom pneumatic operation should also be enhanced to accommodate smaller fibers, and fibers of polymer compositions with less tensile strength. This improvement would markedly improve cellular outcomes on meshes as fibers below 200 μm in a variety of materials have been shown to be efficacious *in vitro*.
3. Porosity and pore size studies should be validated through a high resolution imaging modality after the bio-loom advancement settings have been validated for efficiency (i.e. making sure that 125 picks/cm is really 125 picks/cm).
4. Fiber characterization should be revisited to include a comparison of commercially melt spun fibers. In-house-made fibers should be validated as being equivalent to the commercially available fibers given the inherent inconsistencies with the lab-scale extrusion products. This comparison could then be carried over to the fluid transport and differentiation work included here.

Chapter 3

1. A computational model of the flow of fluid through meshes would be helpful to better inform the design on scaffolds attempting to implement specific transport strategies.
2. An *in vitro* study exploring the ability of the mesh construct to transport waste and nutrients would also help to prove efficacy of woven scaffolds. Perhaps this could be accomplished by culturing cells on one side of a mesh and measuring lactic acid and glucose concentrations at the cell site and away from the cell site.

Chapter 4

1. Prior to exploring differentiation of MSCs on woven meshes, MSCs should be differentiated on cell culture plates. These mature cells can then be transferred to meshes and examined for cell attachment and efficacy. This step would provide insight into expected response of osteoblasts on these meshes.
2. More mesh types should be tested for ECM protein adhesion, particularly modulating fiber geometry and material combination. This work would provide understanding as to which ECM protein behaviors are specific to the woven confirmation and which ones are simply material or geometric responses.
3. Osteodifferentiation and integrin subunit RT-PCR studies should be repeated with extension of the test period to 36 days to understand the potentially delayed osteogenic response discussed in this work.

Chapter 5

1. Future repetition of this study or similar ones including parent surveys should ensure the completion of parent surveys on site at one designated time to facilitate more accountable data collection.
2. Future work should include a more geographically diverse sample of students, as the student participants in this work were all from South Carolina. Additionally, educational experience diversity would also be interesting to study with the recent influx of charter schools and virtual schools.
3. The effect of the STEM Camp was confounded by other activities students were exposed to during the week of the study. Next steps in validating the results of this study include

isolating the experiences of students during the study period to gain more clear reasoning for outcomes.

4. More work is also needed in understanding the extracurricular environment of students with respect to STEM. Further parental surveys are needed to understand the scope and barriers to extracurricular STEM activities. Corresponding student surveys reporting STEM career encouragement from parents and peers would also be helpful to complement or challenge parental notions of STEM career pursuit.

Chapter 6

1. More work needs to be done regarding research identity in general. The research identity, though fairly recent, could be more easily translated after researchers understand the perspectives that various groups have concerning research. Research is presumably different to a scientist than it is to an engineer. Understanding these differences will help to advance research in this area.
2. This survey and follow up interview process should be repeated with the same student participants at various career milestones (graduation, admission to graduate school, employment, etc.) to understand shifting perspectives and the lasting impact of research experiences.
3. Beyond exposing underrepresented students to research early in their academic careers, research identity development in underperforming student should also be studied. This work should include analysis of the effect of research on academic outcomes and identities. Developing research identity prior to strong association with science or engineering identities could provide explanation into the relationship of these three areas, as well as providing strategies for fostering persistence in STEM academic work.