HE, TING. Tissue Engineering 3D Textiles Scaffolds for Multiple Junction Tissue Regeneration. (Under the direction of Dr. Martin W. King and Dr. Robert Dennis).

The concept of using a heterogeneous or multiphase scaffold containing a structural and functional gradient is essential in the regeneration of interfacial tissue engineering such as a muscle-tendon junction (MTJ). The primary goal of this study was to use appropriate textile technologies to engineer an integrated and contiguous multiphase scaffold that can serve for MTJ regeneration.

The latest warp knitting technology was used to develop a multiphase spacer fabric scaffold with an appropriate gradient of structure and properties that mimic the natural MTJ junction tissue. Four knitting methods were developed to fabricate the multiple phase spacer fabric scaffolds. The spacer fabric scaffolds were evaluated in terms of their physical and mechanical properties. Murine myoblasts and fibroblasts were co-cultured on the preferred MTJ spacer fabric scaffolds under both static and cyclic stretching conditions that represented dynamic mechanical stimulation for the natural MTJ tissue. The biological properties of the co-cultured spacer fabric scaffolds were monitored by scanning electronic microscopy (SEM), routine histology and quantitative polymerase chain reaction (qPCR). A novel approach using laser scanning confocal microscopy (LSCM) together with fluorescent cell labelling was developed to create 3D images of the cell morphology for the regenerated muscle-tendon junction tissue on and within the spacer fabric scaffold. A compact bioreactor system has been developed to provide cyclic stretching stimulation during dynamic muscle tendon junction co-culture. This study also briefly investigated the isolation and culture of porcine primary muscle and tendon cells, which points to an alternative primary cell source for future studies using a pig model.
This research is innovative in designing and engineering textile scaffolds and a dynamic cyclic stretching bioreactor, as well as establishing a systematic approach for the fabrication and evaluation of MTJ tissue engineering constructs.
Tissue Engineering 3D Textiles Scaffolds for Multiple Junction Tissue Regeneration

by
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Dr. Warren Jasper              Dr. Stephen Michielsen
老爸, 我是何博士啦~ 你看到了吗?

爱你~

^_^
BIOGRAPHY

Ting He was born January 19, 1987 in Xuzhou, Jiangsu, China. She attended Xuzhou Senior High School in Xuzhou. During her undergraduate program in the College of Textiles at Donghua University in Shanghai, China, she was selected to attend the 3+X exchange program with North Carolina State University (NCSU) in 2009. Ting received her Bachelor of Science in Textile Engineering from Donghua University in 2010. Then she was admitted by the graduate program in the College of Textiles at NCSU where she undertook research on biomedical textiles under the supervision of Prof. Martin W. King. After receiving her Master of Science in Textile Engineering, she continued her research in biomedical textiles by enrolling in the doctoral program in Polymer and Fiber Science in the College of Textile, NCSU.
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CHAPTER 1

INTRODUCTION

My study focuses on the application of current textile technologies to engineer 3D scaffolds that can be used for the regeneration of multiple tissue junctions. The conventional treatments for patients who suffer tissue loss or end-stage organ failure include autografting or allografting, which have limitations due to a shortage of donor tissue or immunogenicity complications. Over the last 20 years the paradigm of regenerative medicine and tissue engineering (TE) has been developed to generate viable tissue that will replace injured or damaged tissue and eventually will replace diseased organs for those patients suffering from tissue-loss and end-stage organ failure. The latest interest in the field of tissue engineering focuses on interface tissue engineering (ITE) which addresses the problem of how to generate a multiple cell tissue junction which has integrity, continuity and consists of different yet contiguous types of cells.

Interface tissue engineering (ITE) is facing several significant challenges in designing and optimizing 1) a functional scaffold that integrates different tissues and cell lines, and 2) a biomimetic in vitro environment to promote the formation of a functional tissue interface, 3) an analytical method to monitor and measure the cellular activities at the tissue interface in three dimensions.

To answer these challenges, this study has used a muscle tendon junction tissue as an interface tissue example, and has designed and produced a multiphase scaffold with structural and mechanical gradients. It has created a practical co-culture protocol as well as a three-dimensional (3D) bioreactor system that can provide the mechanical stimuli similar to the stresses that would be experienced in the native muscle-tendon junction tissue.
GOALS AND OBJECTIVES

The primary goal of this study is to use appropriate textile technologies to engineer an integrated multiphase scaffold that can serve a specific interfacial tissue engineering (ITE) application such as a muscle-tendon junction (MTJ).

In order to achieve this primary goal, there is a demand to develop a knitting method, build a controllable co-culturing \textit{in vitro} bioreactor, and customize testing and evaluation protocol for the co-cultured scaffold. Therefore, the primary goal was divided into the following objectives:

1. to use the latest warp knitting technology to develop a multiphase spacer fabric scaffold with an appropriate gradient of structure and properties for engineering muscle-tendon junction (MTJ) tissue.
2. to co-culture muscle and tendon cell lines on the developed and fabricated multiphase scaffold under \textit{in vitro} cyclic stretching conditions that represent dynamic mechanical stimulation.
3. to develop imaging technology that uses a confocal microscope to identify and measure cell attachment, proliferation, infiltration and junction formation of viable muscle tendon junction tissue on 3D textile scaffolds.
4. to develop a 3D dynamic bioreactor with mechanical stimulation and an \textit{in situ} contractile force monitoring device for the regeneration of primary pig muscle tendon junction tissue.
5. to develop a protocol of co-culturing primary pig muscle and tendon cells so as to mimic the formation of a muscle tendon junction under \textit{in vitro} conditions.

The five objectives are related to each other. Each of them generates an individual project.

\textbf{Project 1: Development of MTJ Spacer Fabric Scaffolds}

The specific aims are:

1) to design multiphase warp knitted spacer fabrics with contiguous structural and mechanical gradients that can be used as scaffolds for muscle tendon junction tissue engineering.
2) to develop, evaluate and refine the knitting methods to fabricate the proposed prototype designs using a non-degradable surrogate polymer.

3) to identify the preferred knitting methods based on the efficiency of knitting production and to measure the properties of the non-degradable prototype structures.

4) to use the preferred knitting methods to fabricate and evaluate MTJ spacer fabric scaffolds knitted from degradable yarns.

**Project 2: Regenerate MTJ junction tissue by co-culturing muscle and tendon cell lines on the developed and fabricated scaffolds under static and dynamic stretching conditions.**

The specific aims are:

1) to study the cytocompatibility and cell proliferation of the two preferred scaffolds from Project 1 by culturing commercially available mice muscle and tendon cell lines separately under static conditions on the different regions of the scaffold.

2) to study the formation of a muscle-tendon interface zone by co-culturing commercially available mice muscle and tendon cell lines in a cyclic stretching bioreactor developed by Wake Forest Institute of Regenerative Medicine (WFIRM).

**Project 3: Develop imaging technologies that identify the regeneration of a muscle tendon junction on 3D spacer fabric scaffolds.**

The specific aim is to develop an analytical approach that uses multiple fluorescent labeling, auto-fluorescent fibers and laser scanning confocal microscopy to view the morphology of multiple cell types on a 3D spacer fabric scaffold.

**Project 4: Development of 3D Dynamic Bioreactor**

1) to develop a 3D dynamic bioreactor with cyclic stretching and recovery mechanical stimulation for muscle-tendon junction regeneration.

2) to evaluate the operation of the bioreactor by co-culturing muscle and tendon cells on 3D multiphase scaffolds and evaluating the engineered junction tissue.
Project 5: Harvesting and Isolation of Primary Porcine Muscle and Tendon Cells

The specific aim is to develop the isolation technique and culture protocol of primary porcine muscle and tendon cells for future study.

There are eight chapters in this written dissertation. They include five projects, which are presented in Chapters 3 to 7. Each project addresses a different aspect of the primary goal of the study.

Chapter 1 introduces the background to the subject, and describes the primary goals and the specific aims of the five projects.

Chapter 2 reviews the previous research on interface tissue engineering, textile scaffolds for interface tissue engineering, warp knitted spacer fabric design and construction and alternative approaches to tissue engineering a muscle-tendon junction.

Chapter 3 (Project 1) develops four types of 3D warp knitted spacer fabric prototypes with an integrated interface and multiple phase structure to mimic muscle, tendon and a muscle-tendon junction, using permanent surrogate polyester yarns. After evaluating the prototypes in terms of their knitting methods and their physical and mechanical properties, the scaffold design with the most suitable mechanical performance was fabricated using degradable poly(lactic acid) yarns. The degradable scaffold’s physical and mechanical properties were studied and compared to natural porcine and human tissues.

Chapter 4 (Project 2) develops an in vitro approach to regenerate a muscle-tendon junction by co-culturing murine muscle and tendon cells on the multiple phase degradable spacer fabric scaffold developed in Chapter 3. The muscle bioreactor developed by Wake Forest Institute of Regenerative Medicine (Winston-Salem, USA) was used to provide dynamic cyclic stretching stimulation during co-culture. The biological properties of the co-cultured spacer fabric scaffolds were examined by scanning electronic microscopy (SEM), routine histology, quantitative polymerase chain reaction (qPCR) analysis and laser scanning confocal microscopy (LSCM).

Chapter 5 (Project 3) introduces an innovative confocal microscopic method to view multiple cells cultured on and within a multiple phase fabric scaffold in three dimensions.
Chapter 6 (Project 4) develops a compact bioreactor system, which is customized for the regeneration of muscle tendon junction tissue. This NCSU developed MTJ bioreactor system has a number of superior features. One such advantage is the incorporation of sensors to monitor the applied load compared to the cyclic stretching bioreactor developed by WFIRM.

Chapter 7 (Project 5) introduces a new approach to the isolation of porcine primary muscle and tendon cells. This technique may be of value for future studies on muscle tendon junction regeneration using a porcine animal model.

Chapter 8 provides a summary of the findings of the written dissertation and points out research directions for the future.
CHAPTER 2

LITERATURE REVIEW

2.1 Overview

My study focuses on the application of current textile technologies to engineer 3D scaffolds that can be used for the regeneration of multiple tissue junctions. This review of the literature contains the following four major sections.

Section 1: Interface Tissue Engineering and Multiple Tissue Junctions

This review starts with an explanation of the concepts in tissue engineering (TE) in general, and continues with a discussion focused on interface tissue engineering (ITE) when it is necessary to regenerate two different tissue types that form a common junction. One of the more important types of tissue junction is the muscle-tendon junction (MTJ), which in this study is used as a model to design 3D textile scaffolds. The general challenges associated with developing a MTJ are delineated and discussed at the end of Section 1.

Section 2: Textile Scaffolds for Interface Tissue Engineering

Section 2 discusses general concerns in tissue engineering scaffolding and the current approach for designing textile scaffolds for interface tissue engineering (ITE) and their fabrication technologies. Because of the limited number of textile scaffolds that have been used for a muscle-tendon junction (MTJ) application; the wider experience in other interfacial tissue engineering (ITE) fields is reviewed. The materials, biological factors, culture environments and evaluation techniques used with such textile scaffolds are presented and compared with non-textile scaffolds used for similar applications.

Section 3: Warp Knitted Spacer Fabric Scaffolds

Advances in a wide range of different types of textile scaffolds have been summarized in Section 2. In order to find suitable textile structures for the scaffold for a muscle tendon junction (MTJ),
different textile fabrication technologies are presented and analyzed in Section 3. A 3D warp
knitted spacer fabric was considered as a promising candidate for an MTJ scaffold design, which
is discussed in terms of its multi-porous architecture, manufacturing processes, knitting
parameters, physical characteristics and mechanical proprieties. At the end of this section,
alternative knitting methods are proposed to create a series of heterogeneous integrated spacer
fabrics with engineered porosity gradients.

Section 4: Systematic Approach to Tissue Engineering a Muscle Tendon Junction

Based on reports from previous research studies, we propose a comprehensive list of criteria and
conditions that would be appropriate for tissue engineering a muscle tendon junction (MTJ). A
systematic approach is explored to develop and apply a MTJ scaffold including the development
of spacer fabric scaffold, the static in vitro culture environment, bioreactor dynamic culture
environment as well as the evaluation methodology. The potential for applying these criteria and
conditions to tissue regeneration at other multiple tissue junctions is also discussed.

2.2 Interfacial Tissue Engineering and Multiple Tissue Junctions

2.2.1 Interfacial Tissue Engineering (ITE)

The conventional treatments for patients who suffer tissue loss or end-stage organ failure include
autografting or allografting, which have limitations due to a shortage of donor tissue or
immunogenicity complications. In 1985, the concept of tissue engineering was mentioned by
Y.C. Fung for the first time, but it was not until 1993 that Robert Langer and Joseph P.
Vacanti defined tissue engineering as "an interdisciplinary field that applies the principles of
engineering and life sciences toward the development of biological substitutes that restore,
maintain, or improve tissue function".

In the past tissue engineering has used conventional biomaterials to engineer a range of
individual tissues such as skin, cartilage, bone and nerve. However, with the complexity of many
tissues and organs, our ability to engineer multiple tissue junctions especially at the soft-to-soft
and soft-to-hard tissue interfaces, is challenging traditional engineering approaches. There is a significant challenge in co-culturing different cell lines simultaneously and generating multiple types of contiguous tissues and assembling these tissues into functional complex organ systems.

Since the year 2000, the concept of interfacial tissue engineering has been developed. Interfacial tissue engineering (ITE) is an emerging field that aims to regenerate functional tissues in order to repair or regenerate diseased or damaged zones between different tissue types. The gradient of structural and mechanical properties at the tissue-to-tissue interface on one hand enables the interface to serve a number of functions, such as mediating load transfer and sustaining heterotypic cellular communications. On the other hand, this gradient adds to the risk of failure at the interface. Unfortunately, injuries at a tissue junction are not readily regenerated following standard surgical repair due to graft instability during long term healing in vivo.

The principles of interfacial tissue engineering are adapted from the general concepts of tissue engineering and include the use of advanced 3D scaffolds which can provide gradients in composition, structure and mechanical properties. This heterogeneous structure can also provide support for variations in a 3D co-culturing system (Figure 2.2.1).

Figure 2.2.1 Complex tissue interfaces in the shoulder and knee.
Therefore, in order to design an effective scaffold for interfacial tissue engineering, different types of tissue-tissue junctions need to be investigated in detail.\textsuperscript{11,12}

2.2.2 Muscle-tendon junctions (MTJ)

1) Definitions

Muscles are the engines or drivers of most skeleton movements, although they never connect directly to bone. Instead, they connect in most cases to tendon, except for some facial muscles, which connect to skin tissue. Muscles produce contractile forces and pull on tendons. The bones, muscles, and joints together form an integrated mechanical system called the musculoskeletal system, within which the combination of muscles and tendons is referred to as a muscle-tendon junction or myotendinous junction (MTJ).\textsuperscript{13}

Most muscles act together in groups. One end of the muscle attaches to a stationary bone via a tendon and the other end attaches at an insertion point to the movable bone also via a tendon. The characteristics of a MTJ will therefore vary in terms of its dimensions, shape, number of muscle ends (usually 2 to 4) and function.

The calcaneal tendon (better known as the Achilles tendon) is the strongest tendon of the human body. However, it often suffers from sports injuries and consequently has been the focus of attention among a number of orthopedic surgeons and researchers. As a result, the gastrocnemius muscle-achilles tendon junction will be focused in this review (Figure 2.2.2).\textsuperscript{14}
Figure 2.2.2: Muscle-tendon junctions in human lower limb. The Gastrocnemius muscle-Achilles tendon junction is marked.\textsuperscript{14}

2) Cells, Histology and Function

The tissue at a muscle-tendon junction (MTJ) is divided into three regions: the muscle region, the tendon region and the interface. The muscle region is composed of multiple components, and the major one is skeletal muscle cells.\textsuperscript{14} The tendon region is composed of tenocytes, an elongated fibroblast type cell; and the interface is composed of both skeletal muscle cells and tenocytes.
Figure 2.2.3: Light micrograph of MTJ tissue histology.  
(A) Human MTJ tissue consisting of skeletal muscle cells (yellow), collagen fibers and tenocytes (red)  
(B) Canine costal diaphragmatic muscle interface (CDI) with central tendon (CT). The pleural surface (PL) is attached by collagen strands (CS) to the muscle fibers (MF) and the central tendon. The peritoneum (P) is loosely attached to the abdominal surface of the diaphragm.

Muscle Region:

Skeletal muscle tissue is composed of a number of complex components, including muscle fiber (or muscle cells), deep fascia, perimysium, epimysium, endomysium, fascicles, motor neurons and blood capillaries.

Skeletal muscle fibers are elongated cylinders with multiple nuclei (Figure 2.2.3), often as many as 100 or more. They are very extensible. For example, a muscle fiber in the thigh muscle could measure 0.0004 inch (0.001 centimeter) in diameter and be 12 to 16 inches (30 to 40 centimeters) in length. The basic unit of the muscle fiber is the myofibril. The functional structures responsible for the typical striations of the myofibrils include: Z-discs, A bands, H zone, I band and sarcomere. The sarcomere can be viewed as a series of interlocking bands of thick and thin protein filaments and Z-discs. Contraction of the muscle myofibrils is caused by sliding of the proteins in the thick filaments past the thin filaments, which represents shrinkage and ultimately
the disappearance of the H zone and I band. When the muscle relaxes, the sliding of the thick and thin filaments is reversed and the H zone and I band are reformed (Figure 2.2.4). This deformation of the sarcomeres has similarities to the loop deformation of textile knitted structures, which will be discussed later in this chapter.

![Diagram of muscle cell and sarcomeres](image)

**Figure 2.2.4:** The muscle cell is composed of myofibrils. Skeletal muscle fibers contract and relax based on the striations of the myofibrils (Left). Changes in the structure of myofibrils during contraction and relaxation are illustrated (Right).

The various functions of muscle tissues include the creation of body movements, the stabilizing of body positions, regulating the volume of organs, moving materials within the body, such as the peristaltic movement of food in the GI tract and the production of heat.
**Tendon Region:**

As a connective tissue, a tendon is composed of fibroblastic cells, namely tenocytes that consist of long parallel fibrils of oriented Type 1 collagen which are interwoven with proteoglycans including decorin. Their glycosaminoglycan (GAG) side chains have multiple interactions with the fibril surface, showing that the proteoglycans are important structurally with the interconnection between fibrils. The major GAG components of the tendon are dermatan sulfate and chondroitin sulfate, which are associated with collagen and are involved in the fibril assembly process during tendon development. When decorin molecules are bound to a collagen fibril, their dermatan sulfate chains may extend and associate with other dermatan sulfate chains on decorin that is bound to separate neighboring fibrils, therefore creating interfibrillar bridges and eventually causing parallel alignment of the fibrils.

Because their fibrils contain oriented Type 1 collagen, tendon tissue is much stronger than muscle tissue in terms of its tensile properties when external forces are applied in the fiber axis direction. (Figure 2.2.3.A) The central tendon is essentially inextensible in the direction transverse to the fibers as well as along the fiber axis. Because of this, failure of tendon tissue usually starts at the interface between the fibrils.13,16,18,19

The contractile filaments in muscle tissue actively generate the contractile force which is passively carried by the collagen fibrils. The amount of elastic energy stored in muscles and tendons is roughly proportional to their relative length and is likely to be related to the cross-bridge elasticity in the muscle and the collagen-fibril elasticity in the tendon. Chondroitin sulfate occupies the volume between the collagen fibrils so as to keep them separated and help withstand deformation. It is therefore likely to play a significant functional role in transferring stresses between adjacent fibrils.5,20

**Interface Region:**

The interface is the combination of contiguous muscle and tendon tissues which constitute an integrated mechanical unit. The limits to the region are in the form of 3D cylindrical folds (Figure 2.2.3 B).
There is no distinct boundary, just a zone of transition between muscle and tendon. Individual tendon fibers penetrate the muscle region resulting in strong cohesion between the muscle and tendon. Nevertheless, the interface region is the weakest point in the muscle–tendon junction (MTJ), which makes it susceptible to strain injuries. Skeletal muscle cells transmit forces across their cell membranes to the extracellular matrix and ultimately to the adjoining tendons. The interface therefore has the following functions:

- To control the movement and position of each junction by acting as the prime mover, synergist, or antagonist;
- To stabilize the junction;
- To store and release elastic (strain) energy.

3) Tensile Properties

The tensile properties of muscle tendon junction (MTJ) tissues vary with the species of the animal as well as its age and sex. And in each case, the muscle, tendon and the interface regions have a different ultimate tensile strength and an ultimate tensile elongation. Generally the muscle is more compliant, while the tendon is stiffer and has a higher tensile modulus. This difference in stiffness between the muscle and tendon results in different strain profiles for the three regions of a MTJ tissue. In the muscle region, stiffness in the direction transverse to the fibers is much greater than that along the fibers, which is the opposite to what is found in the tendon region. In fact the muscle region has a more pronounced anisotropy than the tendon near the interface, and a gradient in muscle stiffness exists as one approaches the interface. At a given stress, the strain decreases from the muscle region to the tendon region, and naturally the interface lies in between. In effect, the interface serves as a buffer zone to reduce the level of stress-concentration, and so prevent failure.

Muscle Region:

The human skeletal muscle tissue from the rectus abdominis muscle has an average ultimate tensile strength of 11 g/mm² and ultimate percentage elongation of 61 %. The gastrocnemius has an ultimate tensile strength of 10 g/mm² and a remarkable ultimate tensile elongation of 105 %. The average for the ultimate tensile strength of different skeletal muscle tissues is 10-30 g/mm²,
while the ultimate percentage of elongation lies in the 60-105% range, regardless of any differences due to age and sex. From a practical and functional point of view, the initial working region corresponds to a yield stress of about 15-40% of the ultimate strength, and an initial yield strain of about 60-75% of the ultimate elongation. There is no clearly defined relationship between the ultimate strength of muscle tissue and the thickness of the muscle fiber.  

**Tendon Region:**

The tensile properties of human tendons are largely influenced by the person's age. People above 70 years old lose on average about 20% of the tensile strength of their tendons compared with people under 70 years old. Neither the sex of the person nor the anatomical location has a significant effect on the tensile properties of human tissue. In fact for the 1 - 79 year age group, the ultimate average tensile strength for human tendons is about 5.4 kg/mm², and the ultimate tensile elongation is about 83-90 %.  

In addition to human trials, cell culture evaluations have been studied using *in vivo* models, such as the horse, beef cattle, pig and guinea pig, dog, rabbit, rat and mice. The ultimate tensile strength of the calcaneal tendinous tissue in decreasing order of magnitude from highest to lowest has been found for the follows species: horses, beef cattle, rabbits (which are very close to humans), dogs, pigs, guinea piggery, rats and ultimately mice. The average thickness of the calcaneal tendon in decreasing order of magnitude from thickest to thinnest has been reported for the following species: horses, beef cattle, humans, pigs, dogs, rabbits, guinea pigs, rats and mice. The ultimate tensile strength is generally proportional to the thickness of the tendon. In a number of different animals the initial working region corresponds to a yield stress of about 10-20% of the ultimate strength of the calcaneal tendon, and an initial yield strain lies within about 20-33% of the ultimate elongation.

**4) Interface Region**

The tensile properties in the interface region lie in between those of the muscle and the tendon regions (Figure 2.2.5). In a study of a canine diaphragmatic MTJ, the following results have been reported. Muscle-tendon strips were harvested from 14 dogs and were tested under uniaxial
tension in the muscle fiber direction. The experimentally measured stress-strain curves are illustrated in Figure 2.2.5. The amount of strain in the muscle region was found to be greater than that in the interface region, which was greater than the tendon strain. The extensibility of the muscle ranged from about 33% at the interface to about 77% at about 1.5 cm away from the interface.\textsuperscript{16}

This study also found that: 1) the MTJ sheet was more compliant and considerably more extensible in the fiber direction compared to the direction at right angles to the fibers; 2) the central tendon was considerably stiffer than the adjacent muscle; 3) the central tendon near the MTJ was essentially isotropic, whereas the adjacent muscle was anisotropic; and 4) passive stiffness increased gradually as one approached the MTJ.

![Schematic of the Tested Diaphragmatic MTJ](image1)

![Experimental tensile stress strain curves](image2)

Figure 2.2.5: Sample preparation and results of tensile testing.\textsuperscript{16}

(A) Location of position markers placed on the MTJ tissue. (B) Experimental tensile stress strain curves for uniaxially loaded MTJ tissue in the direction of the muscle fibers using the samples prepared in (A).\textsuperscript{16}
5) Challenges in Engineering Muscle-Tendon Junction (MTJ) Tissue

Although many attempts have been made to engineer muscle and tendon tissues separately and independently, there is an increasing demand for methods that will facilitate the interface formation between muscle and tendon tissues. The challenges in developing an MTJ are:

1) To design a single scaffold system that is suitable for both tissue types,

2) To expand cell populations to form an *in vitro* interface and

3) To incorporate the use of a 3D dynamic bioreactor to introduce appropriate mechanical stimulation.

An integrated scaffold with gradients in both porosity and in mechanical performance is critical to the successful regeneration of a functional MTJ. The ideal design of the integrated MTJ scaffold should be based on the different biological structures and functions of the three regions. First of all the ideal muscle and tendon scaffolds share a number of common requirements in terms of porosity and aligned morphology and topography so as to support myofibril formation and alignment, the incorporation of growth factors, and the promotion of cell survival, attachment and proliferation. However, at the same time the two scaffolds need to differ according to their mechanical requirements. The ideal muscle scaffold should be compliant and elastic so as to generate contractile forces, while the tendon scaffold should be stiffer particularly in the axial direction. The properties at the interface should lie in between those of the muscle and tendon regions. The contiguous interface region of contact with adequate bonding between the muscle and tendon regions is critical to the integrity of the MTJ.

2.2.3 Textile Scaffolds for Interface Tissue Engineering

Section 2 discusses general concerns related to tissue engineering scaffolding and focuses on the current approach and the fabrication technologies for designing and producing textile scaffolds for interface tissue engineering (ITE). Because of the limited number of textile scaffolds that have been used for a muscle-tendon junction (MTJ) application; the wider experience in other interfacial tissue engineering (ITE) fields is reviewed. The materials, biological factors, culture environments and evaluation techniques used with such textile scaffolds are presented and compared with non-textile scaffolds used for similar applications.
1) General Concerns in Tissue Engineering Scaffolding

The main function of tissue engineering (TE) scaffolds is to maintain the mechanical integrity of the scaffold while promoting cell attachment, proliferation and differentiation within a porous biodegradable structure. The material properties and scaffold characteristics control communications at the biological level between the cells and the scaffold. In general, a tissue engineering scaffold should have the appropriate architecture and be capable of being formed into whatever the desired shape happens to be. It is necessary to have an internal interconnected pore network, adequate initial mechanical integrity, appropriately active surface chemistry and topography, as well as be comprised of materials that are bioresorbable. The rate of bioresorption or degradation should complement the generation and functioning of new host tissue. This means that the scaffold needs to be completely absorbed once the desired tissues have been regenerated and are functioning. In other words, the degradation rate of the TE scaffold should match the rate of tissue regeneration.

2) Scaffold should be biocompatible and bioresorbable

The biomaterials used to fabricate the scaffolds need to be compatible with the cellular components of the engineered tissues and endogenous cells in host tissue and demonstrate biocompatibility over the period they are present and interacting. A bioresorbable polymer is preferred rather than a biostable one because of the long-term biocompatibility issue and the need for a second surgery if one wishes to remove the permanent implant. The term “bioresorption” implies that the polymer and its degradation products, which are usually formed by simple hydrolysis, are removed from the body by cellular activity such as phagocytosis or the citric acid cycle in the biological environment. Bioresorbable scaffolds can be produced from natural polymers, such as collagen, glycosaminoglycans (GAG), chitosan, gelatin, starch, and alginate, or from synthetic biomaterials, such as aliphatic polyesters or poly(α-hydroxy acids) including poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and copolymers of poly(lactic-co-glycolic acid) (PLGA). The degradability of the bioresorbable material is mainly dependent on the selection of the polymer. The rate of degradation is dependent on the intrinsic properties of the polymer including the chemical structure, the presence of hydrolytically unstable bonds, the level of wettability, the degree of crystallinity, the copolymer ratio level if applicable and the
initial molecular weight. In addition, there are a number of physical factors that are also relevant, including the total porosity, the pore size distribution and the fiber diameter, as well as chemical factors such as the presence of enzymes and the pH at the in situ site.\textsuperscript{34,37,38} The degradation rate should match the rate of tissue proliferation so that there is sufficient scaffold support during tissue formation while at the same time enough space is created as the polymer degrades for new tissue ingrowth at the appropriate speed.\textsuperscript{34}

The advantage of natural biomaterials is that they closely simulate the native extracellular matrix. However they are also associated with serious limitations such as batch-to-batch variation, the introduction of toxic reagents during production, poor mechanical properties and the potential for a negative immune response after implantation.\textsuperscript{34,35,37,39} Synthetic biomaterials show advantages in flexibility to tailor mechanical properties and degradation rate, the capability to be fabricated into various shapes with the desired porosity and morphological features, and limited immune response compare to natural biomaterials. However, the greatest limitation of synthetic biomaterials is their lack of cell-recognition signaling and inferior biocompatibility.\textsuperscript{34, 35,37}

3) Scaffold architecture should mimic extracellular matrix (ECM)

A tissue engineering scaffold should have an appropriate architecture at the nanoscale ($10^{-6}$-$10^{-10}$ m) to mimic the features of natural extracellular matrix (ECM) and so encourage normal cell function as would occur in vivo (Table 2.2.1). The interaction between cells and ECM is a dynamic process, and each tissue has a unique ECM environment.\textsuperscript{35,37} For example, Type I collagen in a tendon is found in well-organized thick bundles so as to resist the force applied from the adjacent muscle. Therefore the degree of aligned porous morphology should be considered in tendon scaffold design in order to encourage the generation of Type I collagen stretching phenotype which will improve its tensile properties.\textsuperscript{31,40}
Table 2.2.1 Functions of extracellular matrix (ECM) in native tissues and in scaffolds for engineered tissues. 34,35

<table>
<thead>
<tr>
<th>Functions of native ECM</th>
<th>Analogous functions of scaffolds in tissue engineering</th>
<th>Characteristics of scaffolds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provides structural support for cells to reside.</td>
<td>Provide structural support for seeded cells to attach, proliferate, migrate, and differentiate.</td>
<td>Porous biomaterial with open, interconnected pores for cell migration, nutrient diffusion, and binding sites for cell attachment.</td>
</tr>
<tr>
<td>Supports mechanical properties of tissues.</td>
<td>Provide mechanical stability during tissue maturation and transmits mechanical cues.</td>
<td>Biomaterial with sufficient mechanical properties mimicking that of native tissue; tailored substrate stiffness/flexibility to support direct cell attachment and proliferation.</td>
</tr>
<tr>
<td>Provides bioactive cues for cells to respond to their microenvironment.</td>
<td>Interact with cells actively to facilitate proliferation or differentiation.</td>
<td>Biological cues such as cell-adhesive binding sites; physical cues such as surface topography, surface stiffness, and hardness.</td>
</tr>
<tr>
<td>Provides dynamic physical environment to allow tissue remodeling.</td>
<td>Provide void volume for angiogenesis, vascularization, and new tissue formation during remodeling.</td>
<td>Porous structure for nutrient supply and waste diffusion; engineered and controllable degradation rate.</td>
</tr>
</tbody>
</table>

4) **Scaffold should have appropriate porous internal structure.**

The three-dimensional (3D) architecture of tissue engineering (TE) scaffolds should facilitate efficient mass transportation to achieve tissue uniformity throughout the thickness and to avoid tissue formation only in the peripheral regions of the scaffold. Mass transportation associated with oxygen and nutrient delivery, waste removal, and cell migration and penetration in three dimensions is affected by the scaffold’s porous architecture, which is mainly characterized by its average pore size, total porosity, pore size distribution, pore interconnectivity and surface area. 34 For static culture without medium perfusion, the oxygen and nutrient diffusion is limited to within 100-200 µm from the external edges of the scaffold. 41 A 3D dynamic bioreactor system with medium perfusion is helpful to improve the mass transportation within the scaffold. The introduction of vascularization *in vivo* or *in vitro* is essential to retain a viable cell population within the engineered tissue construct, and the void space within a TE scaffold should make allowance for future vascularization. 34

Although the need for a porous architecture has drawn much attention in TE scaffold design, there has not been agreement on the optimal size or structure of the pores to mimic the ECM, due to the vague and subjective definition of a “pore” and the many different and conflicting
techniques for pore characterization. As a result, it is important to inspect the particular analytical technique and review the assumptions used when reporting and interpreting the experimental results for pore size reported in the literature. In general, the "pore" size or unit of void space within the scaffold should be big enough to allow the cell to penetrate, but not too big that results in the next closest surface being too far to make contact. Furthermore, if the pore size is too small, then the cells don't recognize it being there.

Numerous researchers have dedicated their time and energy to discovering the relationship between pore size and cell growth, differentiation and tissue formation. However, there is no general agreement on the optimal pore size. As for scaffolds for muscle and tendon tissue regeneration, it has been found that an optimal pore size should be 5µm for neovascularization and 5-15µm for fibroblast ingrowth. Zeltinger et al. found that vascular smooth muscle cells showed equivalent cell proliferation and extracellular matrix formation for pore sizes in the 38–150 µm range. Lee et al. studied the effect of scaffold pore size on smooth muscle cell growth across the surface as well as through the thickness. Three different pore size ranges (50–100, 100–150, and 150–200 µm) did not significantly affect the extent or direction of cell growth after 14 days of culture. After 7 days, the cell density in the scaffolds with pore sizes of 50–100 µm was significantly higher than scaffolds with larger pore sizes. After 14 days of culture, cell growth was observed only within the 100–200 µm outer layer of all the scaffolds, and very few cells were found in the interior of the scaffolds regardless of the pore size. The cell density continued to decrease after 14 days as cells near the edge of the scaffold continued to proliferate, but the extent of growth was limited by poor nutrient transport further into the scaffold. This suggests that pore size may be only influential during the initial period of cell culture and that diffusion limitation is still an issue within this pore size range.

Ma et al. have reported that human trophoblast ED27 cells (14 µm in diameter) could bridge the 15 µm distance or gap between adjacent fibers in a polyester (polyethylene terephthalate) nonwoven matrix. The cells spread and proliferated more rapidly than the cells grown on scaffolds with larger pores. In fact when the distance between neighboring fibers exceeded 20 µm, the cells were not able to bridge the gap between the fibers, and they tended to form large aggregates and became more differentiated. This indicates that the distance between fibers in a textile structure influences not only cell viability, but also cell phenotype and differentiation.
From a biotextile point of view, a “pore” can be considered as the space surrounded by fibers, which is usually an irregular shape that is open and well interconnected. The surface area/volume ratio and the “pore” size distribution of biotextiles can be controlled by yarn size and the type and frequency of the interweaving on the macroscale (10^{-4}-10^{-2}m) as well as the fiber diameter, cross-sectional shape and yarn crimp or texture on the microscale (10^{-6}-10^{-4}m).

In addition to pore size, an open, fully interconnected geometry, and a large surface area/volume ratio will allow cell ingrowth, uniform cell distribution, and facilitate the neovascularization of the construct. A higher level of total porosity enables better cell proliferation by providing more void space and faster oxygen and nutrient supply, but it can also limit the extent of differentiation.\(^{34}\) This finding appears to contradict the observation of Ma et al reported above that suggested that higher pore sizes led to aggregate formation and differentiation. However, note that the value for total porosity is not the same as the average pore size. A high total porosity in excess of 90% allows for adequate diffusion during cell culture. However, there is a conflict between increasing the total porosity and maximizing the mechanical properties. A highly porous structure can also contain closed pores that can reduce the path length for diffusion of gases into the core of the scaffold and prevent cell penetration and ingrowth. It has been reported that the degree of interconnectivity rather than the pore size has a greater influence on osteoconduction.\(^{34}\) Gao et al.\(^{47}\) and Taboas et al.\(^{48-50}\) have found that a wide distribution of both big pores (50-150 μm) and small pores (5-20 μm) within an integrated and interconnected scaffold can enhance nutrient delivery, cellular response and capillary ingrowth.

5) **Scaffold should provide physical support and mechanical functions.**

Scaffolds should provide support for either endogenous cells or those that have been purposely inoculated to attach, grow and differentiate during *in vivo* implantation and *in vitro* culture. The mechanical performance of the scaffold including both bulk and surface properties should resemble those of native tissues that are heterogeneous, viscoelastic, nonlinear, and anisotropic materials.\(^{20}\) In addition to being determined by the bulk properties of their constituent materials, these mechanical properties are also strongly influenced by their architecture and structural features, such as their pore size distribution, fiber diameter, and orientation.\(^{51,52}\)
Generally, scaffolds should have sufficient mechanical strength and resistance to deformation so that they can withstand typical hydrostatic and pulsatile pressures that can be found in the physiological environment. For instance, connective tissue cells and their mesenchymal stem cell progenitors experience contractile behavior. The stiffness of a tendon scaffold is important in resisting cell mediated contraction which can compress the scaffold, alter its shape and reduce the pore size. Scaffolds should also be able to maintain the space or pore volume required for cell ingrowth and matrix production. In addition, the bulk mechanical properties also directly control the surface mechanical behavior, such as surface hardness or stiffness, which is responsible for eliciting cellular interactions. Usually, the hardness or stiffness of a substrate's surface is characterized by atomic force microscopy.

Recent studies have demonstrated that different levels of substrate surface hardness or stiffness influence cell contractility, motility, adhesion, and spreading, as well as cell differentiation. For instance, it has been reported that myotubes optimally differentiate on materials with normal muscle stiffness. Myosin/actin striations emerge only on materials with stiffness values lying in the typical range of normal muscle, i.e. having a passive Young's modulus of about 12 kPa. Myocytes do not normally striate on materials that are either much stiffer or softer than this. For example, myocytes do not striate on a softer fibroblast layer, but do striate on the top myotubes regardless of whether the bottom myotubes striate or not. Furthermore, the stiffness or hardness of a substrate material is proportional to the adhesion strength. Engler et al. reported that matrix stiffness or hardness, which is the inverse of softness, directs stem cell lineage based on the observations that human mesenchymal stem cells display a phenotype of neurogenic lineage on the softest substrates (0.1–1 kPa), a myogenic phenotype on moderately hard matrices (8–17 kPa), and an osteogenic phenotype on the hardest surfaces (25–40 kPa).

6) Scaffold should sustain and support external mechanical stimulation

Mechanical stimulation is important for cell attachment, viability, differentiation and function. Mechanical forces promote cell differentiation, tissue maturation and function by controlling the maturation or disassembly of cell-matrix focal adhesions and cell-cell adhering junctions. This initiates the intracellular signaling cascades which ultimately direct most cellular
behaviors. In response to externally applied forces, cells actively rearrange the organization and contractile activity of the cytoskeleton and redistribute their intracellular forces. Many biological and cellular signaling mechanisms rely on mechanotransduction. In fact when engineering muscle and tendon tissues, numerous studies have shown that an appropriate level of mechanical stress and strain applied in a bioreactor promotes cell differentiation, tissue maturation and improved cellular function. The application of cyclic strain increases proliferation of smooth muscle cells and the expression of collagen and elastin, and significantly contributes to ECM formation. Sumanasinghe et al. have reported that collagen scaffolds under cyclic strain with bone marrow-derived mesenchymal stem cells can induce osteogenic differentiation without any osteogenic supplements.

Externally applied mechanical stimulation needs to mimic the specific mechanical conditions of the particular native tissue. Myers et al. studied the effect of strain rate on the dimensions and geometry of the tibialis anterior in the rabbit. The average isometric tetanic stress prior to elongation was 0.44 ± 0.15 MPa and the average stimulated modulus during elongation was 0.97 ± 0.34 MPa. Both the passive and stimulated stress-strain responses were sensitive to strain rates over the range of 1 to 25s⁻¹. It is suggested that engineered muscle tissue needs to be capable of providing isometric stress at the same level as normal tissue. And the scaffold needs to sustain the natural level of cyclic strain stimulation till the functional tissue is formed. The most appropriate architectural structure and material chemistry for tissue engineering scaffolds under conditions of cyclic strain have not yet been established. Several scaffolds have been reported to exhibit elastic properties, including poly(L-lactic acid) bonded to polyglycolide (PGA) fiber-based scaffolds, Type I collagen sponges, poly(glycolide-co-caprolactone), polyurethane, and poly(lactide-co-ε-caprolactone) (PLCL) constructs. Chung et al. have reported a PLCL electrospun scaffold that exhibits superior elastomeric properties with different sized fibers. The same biomaterial, PLCL, when exposed to radial distension and pressure by Jeong et al., was able to encourage cell proliferation and collagen deposition of rabbit smooth muscle cells as well as generate significant cell alignment in the radial direction compared with the static controls.

In short, designing and engineering a scaffold for tendon and muscle applications needs to focus on a number of important mechanical properties such as elasticity, dynamic fatigue and durability. The scaffold must be capable of withstanding cyclic mechanical strains over extended
periods of time without showing signs of damage, cracking or creep. So an appropriate combination of scaffold structural design and mechanical stimulation is critical to the successful generation of engineered tissue with appropriate mechanical and biological properties, particular for tissue with contractile functions such as tendon and muscle.\textsuperscript{31,40,66}

7) **Scaffold should be bioactive**

Scaffolds should be composed of biomaterials that include biological cues, such as cell-adhesive ligands to enhance attachment, or physical cues such as the appropriate topography to influence cell morphology and alignment. The scaffold may also serve as a reservoir and delivery vehicle for exogenous growth-stimulating signals such as growth factors to speed up regeneration. The level of bioactivity is dependent on the biomaterial surface properties, including surface chemistry, topography, softness/hardness, surface charge, polarity, and surface energy. In addition, the materials must possess requirements such as intrinsic biocompatibility and the appropriate surface chemistry to induce molecular biorecognition from cells.\textsuperscript{36} It has been reported that the rate of cell proliferation is relatively insensitive to surface chemistry, whereas cell attachment usually prefers hydrophilic surfaces.\textsuperscript{72} For the purpose of promoting cell adhesion, the surface of a scaffold can be modified either by immersion in culture media or competing proteins such as collagen and elastin,\textsuperscript{73} or by increasing the surface charge, or by introducing nanoscale and microscale (10-110 μm) texture\textsuperscript{74} or by introducing a combination of more than one of these approaches.

8) **Scaffolds with a three dimensional (3D) architecture for cell culture**

As discussed above, in order to exert mechanical stimulation on cells in a controlled manner, a dynamic bioreactor is required that can promote and develop cell culture in a planned and predictable procedure.\textsuperscript{17,31,75} Bioreactor systems, such as rotating wall vessels, direct perfusion systems, hollow fibers, spinner flasks and mechanical force systems, are designed to enhance the rate and quality of tissue generation \textit{in vitro}. Bioreactors facilitate the precise and reproducible control over many environmental conditions during cell culture including temperature, pH, medium flow rate, oxygen, nutrient supply, and waste metabolite removal.\textsuperscript{33,76}
Rotating wall vessels provide continuously moving culture conditions where cell constructs are grown under low shear stresses which enables high rates of mass transfer, allowing a high seeding density and enhanced cell ingrowth. Hollow fiber systems incorporate a matrix or scaffold contained within porous fibers so as to seed cells with a high metabolic rate. The medium that is perfused externally over the fibers increases mass transport. Spinner flasks can be used to seed cells onto scaffolds and then culture them over extended periods of time. Mass transfer and subsequent culture is maintained by the spinner mechanism. The mechanical force system can provide an applied cyclic force by stretching and contracting the scaffold and also measuring the forces generated by contractile tissue. Novel bioreactors involve non-invasive monitoring techniques such as 2-photon microscopy, magnetic resonance imaging (MRI) and/or computed tomography CT scanning to assess the extent of cellular growth and differentiation in situ.

*In vitro* culture of myoblasts have been successfully demonstrated on three dimensional (3D) constructs under both passive and isometric contractile stimulation. More recently an advanced 3D bioreactor has been developed to facilitate the co-culture of muscle and tendon cells, to measure the isometric responses, and to introduce mechanical stimulation during MTJ interface regeneration.

Dennis and Larkin cultured self-organized muscle-tendon constructs (Figure 2.2.6) in a “Developmental Emulator” bioreactor (Figure 2.2.7) that allowed the conditions present during growth and development of a muscle-tendon junction (MTJ) to be monitored and controlled. In addition, they proposed a compromise co-culture protocol for both tendon and muscle tissues so that the constructs would be generated with viable muscle-tendon interfaces and be able to maintain their integrity when exposed to pulsatile mechanical stimulation. In addition the researchers mounted the bioreactor on an optical microscope for non-invasive *in situ* monitoring so as to assess the morphology and mechanical properties during MTJ formation (Figure 2.2.8).

Seven to 10 days after the formation of the MTJ, the interface of the self-organized TE constructs was found to contain fibrils and myotubes oriented along the longitudinal axis. In addition, there was an increase of MTJ-specific protein expression, such as paxillin, similar to those found in fetal neonatal MTJs *in vivo*. All regenerated MTJs were robust and supported
tensile loading beyond the normal physiological strain range. No differences were observed between the groups with respect to diameter, maximum force, or specific force. The majority of the constructs failed in the muscle region.

The “Developmental Emulator” bioreactor was designed to measure the contractile force generated by the engineered constructs (Figure 2.2.7). The instrumentation in Figure 2.2.8 recorded the in situ changes in the thickness dimension or diameter of the constructs. The average diameter (mm), the maximum isometric force (mN), and the specific force (kPa) of the muscle-tendon constructs were measured continuously and non-invasively.

Dennis and Larkin study elucidated the following four conclusions. 1) By co-culturing skeletal muscle cells and tendon cells under in vitro static conditions it was possible to form a functional MTJ similar to those found in neonatal embryos developed in vivo. 2) The finger-like sarcolemma tissue folded at the interface between the muscle and tendon, demonstrated that the morphology of the MTJ could be formed without mechanical stimulation. 3) The protein paxillin was found at the focal adhesion between the muscle and tendon tissues and could be used to localize the MTJ interface. 4) A contractile mechanical stress was generated in the muscle portion of the construct and was found to signal the expression of paxillin.

Although it was possible to regenerate a muscle-tendon junction under static conditions without a scaffold, the dimensional and mechanical properties were not found to be adequate for clinical use. The average diameter of the engineered construct was about 200-400 μm, which is too small for surgical implantation. The average tangent modulus of the muscle constructs was found experimentally to be 37.2 kPa ± 10.3 kPa, about one quarter of the passive stiffness reported for a young adult rat soleus muscle.32 It is believed that these limitations could be explained by the absence of a biomaterial scaffold and the lack of cyclic mechanical loading during the co-culture.
Three different sources of tendon tissue, namely 1) self-organized tendon constructs, 2) segments of adult and 3) fetal rat-tail tendons, were co-cultured with skeletal muscle progenitor cells with dynamic mechanical stimulation. Two weeks after the initial plating of the muscle cells, the self-organized tendon constructs or the segments of adult or fetal rat tail tendons were pinned onto the muscle cell monolayer 15mm apart. Approximately 1 week later, the monolayer of muscle cells began to roll up around the tissue anchors and form a cylindrical construct. It took approximately 24 h from the initiation of delamination for a 3D construct to form.
Figure 2.2.7: The “Developmental Emulator” bioreactor.  
(A) is a modular unit that provides local control of the electromechanical stimulation by an embedded microprocessor. Visible to the extreme right is the culture plate containing the skeletal muscle construct (B), which is attached at the top by a linear servomotor, a stepper motor, planetary gear head and rack-and-pinion drive. At the bottom it is attached to a force transducer. The units can be maintained in organized stacks placed in a regular cell culture incubator and individually monitored and controlled by an external computer running LabView software.
Figure 2.2.8: Instrumentation developed for the measurement of the contractile (A) and tensile (B) properties of a 3-dimensional muscle-tendon construct. 32

For mechanical stimulation of the entire construct, platinum wire electrodes were positioned on either side of the construct, and a force transducer was attached to one end (A). After cell culture under direct field stimulation of the entire construct, the samples were transferred to a tensile tester to measure the stress-strain properties (B). 32
Du Geon Moon et al. developed an in vitro preconditioning protocol using a dynamic bioreactor that improves the contractility of engineered skeletal muscle after implantation in vivo. The primary human muscle precursor cells (MPCs) were seed onto a collagen based acellular tissue scaffold and showed a homogeneous muscle phenotype after 4 weeks implantation in a mouse model (Figure 2.2.9 A-D).

The linear motor-driven stimulation device used for applying cyclic unidirectional stretch and relaxation stresses consisted of an actuator mounted on a tissue culture container in which the cell-seeded scaffolds were placed (Figure 2.2.9 G). The linear actuator was calibrated, controlled and programmed by a computer. One end of the cell-seeded scaffold was tied to a stationary bar using sutures and the other end was secured to a movable bar that was attached to the linear motor and computer controller. The bioreactor container, which could be placed inside an incubator, could hold up to 10 tissue constructs at a time with a maximum distance between the 2 bars of 10 cm.

Once the bioreactor was in operation, viable muscle tissue constructs with unidirectional orientation were produced within 5 days. And after 3 weeks the constructs were able to generate a contractile response. Only the mechanically stimulated constructs generated tetanic and twitch contractile responses, while the statically cultured controls did not. Increasing the implantation time from 1 week to 4 weeks increased the contractile force from 1% to 10% of the value observed on native latissimus dorsi of mice.

This study revealed the importance of in vitro bioreactor preconditioning so as to accelerate skeletal muscle tissue organization, maturation, and function in vivo. The retrieved bioengineered constructs displayed measurable contractile responses induced by electrical field stimulation (EFS). The magnitude of the contractile force was dependent on the duration of the in vivo implantation. The dimensions of the scaffold that was used for surgical implantation was 1.5 cm x 1.5 cm. However, the maximum specific force was 10% of the native latissimus dorsi muscle of the mouse and the mechanical properties of an acellular tissue scaffold control were negligible.

The principle conclusion from the above discussion is that the most important aspect of bioreactor design for MTJ tissue engineering is to make sure that the conditions of the 3D culture
environment are reproducible and can controlled the regeneration of muscle and tendon cells simultaneously. The specific conditions address both biological and mechanical parameters, including the co-culture strategy, an effective culture medium that supplies sufficient biological cues (growth factors), the frequency and intensity of the mechanical strain/stress stimulation, as well as non-invasive methods to assess tissue growth during co-culture.

Figure 2.2.9: Cell culture and bioreactor system.  
Panels A–D depict the homogeneity of the muscle cell phenotype observed after dynamic cell culture of human skeletal muscle biopsies. Panels E and F illustrate the preparation of the decellularized bladder submucosa scaffold for cell seeding and bioreactor placement. Panel G illustrates the bioreactor chamber used in these studies, with the engineered skeletal muscle constructs in place.
2.2.4 Scaffold Fabrication Technologies

1) General Approaches to Scaffold Design

The different approaches to scaffold design are categorized into four main types (Figure 2.2.10) 34,36

1) Pre-made porous scaffolds
2) Decellularized extracellular matrix (ECM)
3) Cell sheets with secreted ECM
4) Cell encapsulation in self-assembled hydrogels

Figure 2.2.10: Schematic diagram showing four different scaffolding approaches in tissue engineering 36
All four approaches are illustrated in Figure 2.2.10 and a brief comparison of their raw materials, processing and fabrication technologies, strategies for combining with cells, host tissue transferring techniques, applications, advantages and disadvantages are listed in Table 2.2.2.

Table 2.2.2: Characteristics of different scaffolding approaches in tissue engineering

<table>
<thead>
<tr>
<th>Scaffolding approach</th>
<th>(1) Pre-made porous scaffolds for cell seeding</th>
<th>(2) Decellularized extracellular matrix for cell seeding</th>
<th>(3) Confluent cells with secreted extracellular matrix</th>
<th>(4) Cell encapsulated in self-assembled hydrogel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw materials</td>
<td>Synthetic or natural biomaterials</td>
<td>Allogenic or xenogenic tissues</td>
<td>Cells</td>
<td>Synthetic or natural biomaterials able to self-assemble into hydrogels</td>
</tr>
<tr>
<td>Processing or fabricating technology</td>
<td>Incorporation of porogens in solid materials; solid free-form fabrication technologies; techniques using woven or non-woven fibers</td>
<td>Decellularization technologies</td>
<td>Secretion of extracellular matrix by confluent cells</td>
<td>Initiation of self-assembly process by parameters such as pH and temperature</td>
</tr>
<tr>
<td>Strategy to combine with cells</td>
<td>Seeding</td>
<td>Seeding</td>
<td>Cells present before extracellular matrix secretion</td>
<td>Cells present before self-assembly</td>
</tr>
<tr>
<td>Strategy to transfer to host tissues</td>
<td>Implantation</td>
<td>Implantation</td>
<td>Implantation</td>
<td>Injection</td>
</tr>
<tr>
<td>Advantages</td>
<td>Most diversified choices for materials; precise design for microstructure and architecture</td>
<td>Most nature-simulating scaffolds in terms of composition and mechanical properties</td>
<td>Cell-secreted extracellular matrix is biocompatible</td>
<td>Injectable, fast and simple one-step procedure; intimate cell and material interactions</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>Time consuming cell seeding procedure; inhomogeneous distribution of cells</td>
<td>Inhomogeneous distribution of cells, difficulty in retaining all extracellular matrix, immunogenicity upon incomplete decellularization</td>
<td>Need multiple laminations</td>
<td>Soft structures</td>
</tr>
<tr>
<td>Preferred applications</td>
<td>Both soft and hard tissues; load-bearing tissues</td>
<td>Tissues with high ECM content; load-bearing tissues</td>
<td>Tissues with high cellularity, epithelial tissues, endothelial tissues, thin layer tissues</td>
<td>Soft tissues</td>
</tr>
</tbody>
</table>

The main advantage of the decellularized ECM approach is that the allogenic and xenogenic cellular antigens are removed from the tissues. The applications include heart valves, blood vessels, nerves, tendons and ligaments. Decellularized ECM can be used in both homologous and non-homologous applications. And while this approach has advantages because it benefits from the natural properties of the structure, there are also disadvantages due to inhomogeneous cell
seeding and the risk of immune reactions caused by incomplete removal of the cellular components.

An alternative approach is to use cell sheets that release self-secreted ECM on thermo-responsive polymer coated culture dishes until confluence is achieved. The detached confluent cell sheets are then laminated together to form thicker matrices. This approach is excellent for dense tissues that need close association between the cell layers, such as applications for the cornea and myocardium. The major advantages of this approach include an easy harvesting procedure, the possibility of sutureless transplantation, a homogeneous and uniform distribution of cells in the sheet without mass transfer problems and rapid neovascularization. However, this approach is limited by the thickness of engineered tissue, and the fabrication of rich ECM tissue for load bearing purposes.

The cell encapsulation approach uses a semi-permeable membrane or a homogenous solid mass to entrap living cells. This approach has been used in xenogenic pancreatic cell transplantation for diabetic therapy and liver failure. The main advantages of this approach include the simple procedure, homogenous cell distribution, good cell viability and the feasibility to use this approach in injectable applications. However, due to the inferior mechanical properties of the gel biomaterial, it is not usually used for engineering loading bearing tissues such as muscle and tendon.

The technique of seeding cells on pre-made bioresorbable scaffolds is the most commonly used and well-established scaffold fabrication strategy. This approach is flexible because a wide selection of biomaterials can be considered, and most of the materials that have been discussed in the previous section can be used. This approach can be readily applied to load bearing tissue engineering constructs if the appropriate biomaterials and fabrication methods are selected. Most the existing scaffold fabrication technologies fit within this approach.

2) Scaffold Fabrication Technologies

The majority of fabrication technologies can be categorized into three classes:

1) Physical methods and porogens are used to produce the pores within the polymeric biomaterial,
2) Rapid prototyping technologies, and
3) Fibers have been used to create a range of different porous textile structures.

**Physical methods**

Physical methods for the creation of conventional porous structures include phase separation, gas foaming (Figure 2.2.11), freeze-drying, solvent casting, particulate (such as salt or sugar) leaching (Figure 2.2.11), thermal processing and molding, as well as combinations of these technologies. Solvent casting and particulate leaching use porogens to achieve porous sponge-like structures. However the use of organic solvents introduces the risk in cytotoxicity. In the gas foaming process instead of using toxic organic solvents, high pressure CO$_2$ can be applied to form pores during high-temperature compression molding of polymers. However, with this approach, unconnected closed pores are often created within the scaffold. Physical methods have limitations in terms of their scope of application, pore interconnectivity and shape. The use of salt leaching and gas foaming methods can create structural gradients in porosity and mechanical properties. For example, Gao et al.$^{47}$ reported using salt fusion and leaching as well as polymer curing methods on poly(glycerol-sebacate) (PGS) to produce scaffolds with both macropores (70-150 μm) and micropores (5-20 μm). Taboas et al.$^{50}$ also created a bimodal distribution of global pores (50-100 μm) and micropores (5-10 μm) using an indirect solid freeform technique combined with phase separation and porogen leaching. In conclusion, the use of salt fusion and leaching has shown itself capable of creating tissue engineering scaffolds with porous interfaces.

**Rapid prototyping**

Rapid prototyping uses the approach in which selected materials are added layer by layer under the control of a computer to build up the 3D structure on top of a flat base. Unlike the physical methods this approach does not involve any removal of solid material. Rapid prototyping includes fused deposition modeling (FDM), three-dimensional printing (Figure 2.2.11), selective laser sintering (SLS), and stereolithography (SLA). Recently, rapid prototyping has been shown to include the introduction of cells during scaffold production by implementing novel robotic assembly and automated 3D cell encapsulation techniques. In addition to cell encapsulation,
rapid prototyping achieves detailed scaffold resolution, superior design flexibility, high pore interconnectivity as well as excellent reproducibility. Therefore, it is gaining popularity as a preferred tissue engineering scaffolding technique.\textsuperscript{33}

\textbf{Textile technologies}

There are a range of different textile technologies that can manipulate fibers and filaments such as monofilament sutures and multifilament yarns into various 2D ad 3D structures. They include flocked structures, hydroentangled fiber bonded webs, spunbonded nonwoven webs, electrospun membranes, as well as flat and tubular woven, knitted and braided fabrics (Figure 2.2.12). The raw material polymer resin that is used to fabricate textile structures is usually first of all made into fibers or yarns by a spinning method such as wet spinning, gel spinning, dry spinning, electrospinning or melt spinning. This last melt spinning option only applies to thermoplastic polymers. Electrospinning (Figure 2.2.11) is a widely used method to generate membranes with microscale and nanoscale fibers and pores. Electrospinning gains its popularity from its design flexibility, its applicability to degradable polymers, its simple operation and relatively small polymer requirements. In addition, electrospinning is able to resemble certain supra-molecular features of the extracellular matrix (ECM) in terms of its large surface area and nanofibrous architecture. It has been used as a carrier to attach and chemically bond proteins and other biomolecules to the surface in order to encourage, guide and control cell attachment and differentiation.\textsuperscript{34,35} Co-electrospinning collects two polymers simultaneously on the same collector to generate a scaffold architecture with a structural gradient. Therefore co-electrospun nanocomposite films have served well as interfacial tissue engineering scaffolds.\textsuperscript{31} However, the really small nanoscale pore size of electrospun membranes prevents cellular migration in the "z" direction, resulting in an effective 2D scaffold with no cell penetration through the thickness of the scaffold. Layering of electrospinning membranes that are pre-seeded with cells is one way to add the third dimension to the electrospun scaffold. In summary, the unique advantages of using textile scaffolds are listed in Table 2.2.3.
Table 2.2.3 Unique advantages of fiber-based biotextiles

<table>
<thead>
<tr>
<th>Fiber-based biotextiles can provide</th>
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<tbody>
<tr>
<td>• Thin, strong, flexible, lightweight, and porous structures</td>
</tr>
<tr>
<td>• Substrates with excellent fatigue properties</td>
</tr>
<tr>
<td>• A very large surface area desirable for drug delivery and cell attachment</td>
</tr>
<tr>
<td>• A structure that has the ability to be folded or compressed into a small volume for less invasive delivery through a catheter</td>
</tr>
<tr>
<td>• A structure that has the ability to tolerate repeated needle penetration and other types of iatrogenic damage (iatrogenic refers to any adverse condition induced in a patient by a physician’s activity, manner, or therapy)</td>
</tr>
<tr>
<td>• A structure that encourages the infiltration and proliferation of cells for improved tissue regeneration and biocompatibility</td>
</tr>
</tbody>
</table>

Among the main classes of fabrication technologies, the following six approaches have been used to create 3D tissue engineering scaffolds (Figure 2.2.11).

![Figure 2.2.11: Schematic of various 3D scaffold fabrication technologies.](image)
While implantable biotextiles have been used as permanent devices for interfacial tissue repair for decades, they have achieved limited long-term success due to poor host tissue integration.\textsuperscript{87-89} A clinical explant retrieval study based on 117 surgically excised anterior cruciate ligament (ACL) prostheses indicated that while the ACL prosthesis provided a satisfactory functional short term performance over 21-33 months, the long-term results beyond 3 years were invariably associated with failures. These prostheses were fabricated from non-degradable or permanent biomaterials, such as PET, PP, PTFE, PAA and UHMWPE, and fabricated into knitted, braided, woven, and twisted-cord structures. All of these structures had a higher strength than scaffolds made from bioresorbable polymers, but there clinical performance was limited by their poor tissue integration, premature abrasion and unacceptable flexural and rotational fatigue. In conclusion, the study proposed to apply tissue engineering techniques in the development of future devices that would generate an optimally oriented and integrated tissue composite that would contribute to the load bearing capacity of the device.\textsuperscript{90}

However the main limitation for all therapies involved in orthopedic tendon and ligament repair, whether implanting permanent prostheses or not, is the question of fixation to the neighboring tissues. To overcome this significant barrier it is proposed to develop an appropriate integrated interfacial tissue engineering scaffold that will mimic the morphology, mechanical properties and function of the multiple tissues at the interface simultaneously.

The native interface tissue is involved in a number of functions including homeostasis, load bearing, load transference, as well as maintaining cellular communications. The interfacial
scaffold should at least provide a seamless interface, have more than one internal structure and tissue specific morphology, and present a gradient of inhomogeneous characteristics and mechanical properties. Moreover, an interfacial tissue engineering scaffold should incorporate structures with different degradation rates to match and complement the different rates of tissue growth. It is emphasized that the integrity and porous architecture of the scaffold influence the interactions between the relevant cells and the scaffold at the interface. Thus it is critical to control the spatial distribution of the cell populations in the interfacial region. In recent years findings from retrieval studies have found that tissue engineered scaffolds have been more successful in achieving cell integration in vivo.10

A number of research studies have revealed details of the structure-function relationship of interfacial tissues7, and tissue engineers have developed different heterogeneous stratified and multi-phased scaffolds11,12,91,92 to recapture the multiple characteristics needed to represent junction tissue. The following review section focuses on interfacial tissue engineering scaffolds that involve a number of different textile fabrication technologies such as braiding91, weaving93 and electrospinning31.

1) Soft-to-hard interfacial textile scaffolds

Helen Lu's group has reported tissue engineering strategies using braiding and knitting technologies to regenerate soft tissue-to-bone interfaces such as ligament to bone, tendon-to-bone and cartilage-to-bone interfaces.7,11,92

Ligament-to-bone braided scaffolds

Cooper et al.91 fabricated a three-phase scaffold (Figure 2.2.13) combining a 3D braided structure of polylactide-co-glycolide (PLGA) fibers with a natural ligament and attaching them to two bony regions. The short term results were encouraging, with acceptable biocompatibility, healing and mechanical strength in a rabbit model. Forty eight yarns of polyglactin 10:90 (PLGA) (8 ply 52 denier) were braided into 3D circular braids with braiding angles from 26° to 31°, using a custom built 3D circular and rectangular braiding machines with a 3×16 carrier arrangement. As shown in Figure 2.2.13, the attachment sites had a high angle fiber orientation
at the bony attachment ends and a lower angle fiber orientation in the intraarticular zone. This pre-designed heterogeneity in the grafts was aimed to promote the eventual integration of the graft with bone tissue. The scaffold was composed of PLGA fibers with a diameter similar to that of Type I collagen fibers. By changing the braiding angle and the number of yarns, the scaffold had a range of properties such as a gradient in median pore size from 79-136 μm, in total porosity from 54-67%, in maximum load from 525-907 N and a range of ultimate tensile strengths from 212-439 MPa, similar to those of the native ligament. Primary rabbit ACL fibroblasts and BALB/C CL7 mouse fibroblasts were seeded separately for 1 and 8 days. The cell culture results revealed that the primary cells proliferated at a slower rate but showed better cell adhesion and growth on the scaffold in the direction of the fiber axis.

This study confirms that it is possible by 3D braiding to design and fabricate a heterogeneous customized scaffold with a continuous hierarchical multi-phase structure and control over the phases with a range of desired geometries, pore sizes, total porosities and mechanical properties. 3D braids allows for custom production of scaffolds with mechanical properties similar to those of natural ligament tissue in order to overcome issues of stress shielding during tissue ingrowth. The fabrication parameters, such as yarn diameter, number of yarns, braiding angle and braiding pattern can be manipulated on the braiding machine in order to alter the porosity and mechanical properties as well as cell behavior. In addition to changing the braiding parameters, the design flexibility can be extended by choosing different polymers or a combination of polymers. However, this study only used tendon cells and didn’t explore the tendon-bone interface, although the scaffold structure realized continuous multiple phases with appropriate mechanical properties.
Anterior cruciate ligament (ACL)-to-bone knitted scaffold

Altman et al.\textsuperscript{92} developed a multi-phase, porous knitted silk ACL graft which was evaluated in a goat model with promising results. However, the average pore size was not sufficient for cell infiltration. Spalazzi et al.\textsuperscript{10} pioneered the design of a tri-phasic scaffold (Figure 2.2.14) for the regeneration of the ACL-to-bone interface. The scaffold consisted of three distinct yet continuous and contiguous phases, each engineered for a specific tissue region found at the interface. Phase A was designed from a PLGA (10:90) knitted mesh for fibroblast culture and soft tissue formation. Phase B consisted of PLGA (85:15) microspheres and was the interface region intended for fibrochondrocyte culture. Phase C was composed of sintered PLGA (85:15) and 45S5 bioactive composite glass microspheres for bone formation. The results from the extended \textit{in vivo} tricell culture study showed that the chondrocytes, fibroblasts and osteoblasts were all able to make a viable contribution to the extensive collagen-rich matrix in all three phases of the scaffold. In fact, the construct resembled the cell shape and matrix morphology of a neonatal ACL–bone interface.\textsuperscript{10,94}

Their study revealed that: 1) a knitted scaffold successfully served as functional porous backbone for the multi-phase scaffold, 2) the biomimetic multi-phase hierarchical scaffold provided biological fixation for soft tissue to bone, 3) spatial control over the cell population distribution at the interface was critical to ensuring that the relevant cell phenotype and density were
regenerated, and 4) by using the appropriate co-culture or tri-culture techniques the hierarchical scaffold has the potential to regenerate the desired interface tissue both in vitro and in vivo.

Figure 2.2.14: Design of biomimetic triphasic scaffold. 95
(A) Posterior view of bovine anterior cruciate ligament (ACL) with the planned tibial ACL insertion outlined on the bone (red square). (B) Histological image of neonatal bovine tibial ACL insertion showing the three main tissue types found at the ACL-bone interface: ligament (ACL), fibrocartilage (FC), and bone (modified Goldner Masson’s trichrome stain). (C) Triphasic scaffold mimicking the three tissue regions found at the interface. 95

2) Soft-to-soft interfacial textile scaffolds

**Muscle-tendon junction (MTJ) co-electrospun scaffold**
Ladd et al. 31 co-electrospun mixtures of PCL/collagen and PLLA/collagen to produce a continuous multi-phase scaffold with dual porosity and dual mechanical properties that mimicked the native MTJ. The two components were electrospun simultaneously onto opposing ends of a rotating mandrel. The overlapping zone between the two components mimicked the MTJ interface. The dimensions of the integrated scaffold were about 10 cm x 12.9 cm, with a 3.5 cm length of PCL at one side and a 3.5 cm length of PLLA at the other side, leaving a 3 cm overlap zone in the middle representing the MTJ interface. The scaffold was found to be
cytocompatible in terms of cell adhesion and migration of fibroblasts and myoblasts as well as promoting myotube differentiation.

The PLLA/collagen side of the scaffold was stiffer and had a lower degree of strain than the PCL/collagen side, which was more compliant and had a higher strain. The middle interface zone had intermediate levels of stiffness and strain, which were analogous to the tendon, muscle, and junction respectively. However, none of the regions of the co-electrospun scaffold satisfied the requirements of the ultimate strength, Young’s modulus and strain at failure. The ultimate tensile strength of a native tendon (52-120 MPa) is 50-100 times the strength of the scaffold (1-4 MPa) in this study. The ratio of stiffness for the PLLA side compared to the PCL side was approximately 6 while the actual stiffness ratio of a natural tendon to muscle is 179-370,000. While the scaffold could sustain the cyclic stretching in the fiber direction for up to 100 cycles, nevertheless the structure suffered from creep and failure under tensile loading.

The presence of creep and the premature failure are not surprising because the electrospun film was thin and the fiber diameters were at the submicron level resulting in low tensile strength. Since the fibers in the electrospun web were stacked up on top of each other without any specific bonding agent, 3D entanglement, or cross-linking, the tensile strength of the electrospun layer was low. A limited amount of bonding may have occurred during solvent evaporation and between fibers in contact with each other. Therefore, the strain performance of the electrospun web would have depended mainly on the polymer properties such as the degree of polymerization, the level of crystallinity, and interchain hydrogen bonding.

The level of strain can be altered by increasing or decreasing the pore size and the number of cross-linked fibers. In short, the electrospun scaffold has limitations when it is applied to tissue engineering scaffolds requiring high strains and stresses.
Figure 2.2.15: Muscle-tendon junction (MTJ) co-electrospun scaffold.  
(A) SEM photomicrographs showing the three distinct regions of the scaffold, namely the PCL side, the interface zone and the PLLA side with methylene blue stain. (B-D) SEM photomicrographs from the different regions of the scaffold showing the different fiber morphologies and diameters. (B) PCL side (550±98 nm), (C) Interface central zone (504±93 nm), (D) PLLA side (452±40 nm). (E, H) Color maps of the strain profile of the scaffolds and
native tissues when undergoing uniaxial tensile testing. The maximum principal strain is displayed and the scale is from 0 to 0.25 mm/mm strain. The bar charts (F, I) show the differences in maximum strain for each region. The plots (G,J) show the maximum principal strain for the 3 regions of the scaffold at each overall strain interval for the whole scaffold. Analogous data is presented for native MTJ tissue tested in the same manner. From these figures, the scaffolds mimic the trends in mechanical properties observed in native tissue. Matching symbols indicate statistical difference from one another. Significance was defined as *p<0.05.\textsuperscript{31}

This study revealed that 1) a combination of co-cultured muscle and tendon cells with a multi-phase integrated scaffold is feasible to generate functional MTJ \textit{in vitro}, 2) the nanoscale texture of electrospun membranes is favorable for muscle and tendon cell attachment, 3) PLA and PCL are feasible polymers to support MTJ growth and their degradation rates are acceptable; however their biocompatibility needs to be improved using proteins such as collagen, 4) the electrospun membrane has disadvantages in terms of its thickness, the interconnected pores in the z-direction and the mechanical properties which make it unsuitable for generating a MTJ tissue junction, and 5) a bioreactor with cyclic stretching stimulation is feasible to guide muscle-tendon interface formation. However, this study didn’t employ any measurement of contractile force generated by the engineered tissue. It is suggested that future studies need to include a strong scaffold using other fabrication techniques, the use of primary cells \textit{in vitro} and large animal models such as dogs or pigs for the \textit{in vivo} study.

2.3 **Warp Knitted Spacer Fabric Scaffolds**

2.3.1 **Biotextile Fabrication Technologies**

Although biotextile scaffolds show unique advantages in engineering load-bearing interface tissues as discussed previously, the real challenge is during the design and fabrication phase. There are a number of questions that need to be kept in mind during concept development of a biotextile scaffold for an interface tissue engineering (ITE) application. What are the specific types of tissue that the scaffold needs to interact with? What is the prior function of the injured and/or damaged tissue that needs to be re-established by the biotextile scaffold? What alternative
fabric construction methods could be used alone or in combination with each other to mimic the prior function? Which textile technology would facilitate the fabrication of the desired fabric properties?

Given that the focus for this study is on the regeneration of a muscle-tendon junction (MTJ), there are three specific tissues that need to be targeted; they are the muscle, the tendon and their interface. Earlier in Section 1, their structure-function relationship was presented, and then in Section 2, alternative designs for a MTJ scaffold were discussed. The most important challenge now is to identify the functional requirements of the scaffold, which include a three-dimensional architecture with similar dimensions and shape as the human tissue, a combination of multiple phases, an integrated interface, adequate mechanical properties such as Young’s modulus, ultimate tensile strength and strain within each of the multiple phases and the ability to withstand dynamic mechanical fatigue.

The purpose of this section is to review and analyze different textile fabrication technologies and to assess their fabric constructions with respect of these functional requirements.

1) General Overview

Biotextile fabrication has inherited those mature technologies that have been used in the textile manufacturing industry for many centuries. They include weaving, braiding, knitting, nonwoven and fiber/yarn spinning techniques (Figure 2.2.12). These technologies offer superior control over design, scale-up for manufacturing, reproducibility and easy adaptation to clean room conditions for medical applications.  

Weaving is the interlacing of yarns at right angles to one another in an over-under pattern so as to form a textile structure that usually lies in one plane. The interaction between the yarns is what holds the 2D structure together and provides strength at the macro-level. More complex woven fabrics can be produced in a flat, tubular, bifurcated and tapered shape for different applications such as an abdominal aortic endovascular stent graft. For example, 3D woven fabrics incorporate a third feed yarn lying in the thickness plane or "z" direction so as to form a thick, dense and rigid scaffold, which is feasible for cartilage interface tissue engineering. However,
the high density of the yarns reduces its porosity and elasticity, resulting in the risk of poor vascularization and poor contractile/tensile performance. Therefore the 3D woven structure has been applied mainly to tissue engineering applications, such as creating a cartilage scaffold, where vascularization is less important.\textsuperscript{75,97-99} Therefore the use of a 3D woven structure has major limitations for muscle and tendon ITE applications.

Braiding is the interlacing of three or more yarns in a diagonally overlapping pattern. Braids can be manufactured over a central forming mandrel, which results in a 3D cylindrical hollow structure. Without the mandrel, the hollow braid becomes a solid double or multi-layer tape or soutache braid. By changing the braid angle, which is the angle between yarn direction and the braid axis, for each layer, the porosity can be tailored and customized for different applications. Recently Liang et al.\textsuperscript{100} reported the use of a double layer braid to serve as nerve conduit for peripheral nerve regeneration. However, the braided structure is limited in its extent of elastic deformation and contraction, and thus it does not fit the requirements of a muscle scaffold application well.

Nonwoven fabrics are bonded webs that are processed from either short, staple-length fibers or from continuous filaments that are spun directly by melt or melt-blown extrusion. Nonwovens have been widely used for surgical caps, gowns, drapes, and scrubs. These fabrics have a high absorption and filtration capacity. But they also have limitations such as low tensile strength and elongation, inferior fatigue resistance and the pores are not well interconnected through the thickness of the web.\textsuperscript{101} Nonwovens are usually thin fabrics so as to ensure they are flexible and easy to handle. Thicker structures are possible by laminating several layers together, but then they are stiffer, which makes them less attractive for muscle or tendon scaffold applications.

Knitted structures are formed through the interlacement of loops of yarn. Devices such as heart valves, hernia repair meshes, and products for incontinence treatment often employ knitted structures.\textsuperscript{102,103} Knitting technologies are categorized into weft and warp knitting. In weft knitted fabrics, a row of loops (a course) is made from one yarn moving in the weft direction, which is across the width of the fabric width. Warp knitted fabrics consist of many yarns moving in the warp or machine direction. Each yarn threaded to its own needle forms a column of loops (called a wale) simultaneously as the yarn moves in a zig-zag pattern down the length of the
fabric. Warp knits rather than weft knits have gained popularity in many medical applications due to their superior structural stability, the avoidance of yarn raveling and their higher suture retention strength. The total porosity, average pore size, pore size distribution and thickness of knits can be adjusted by changing the knitting variables such as loop length, without changing the material, resulting in a wide range of achievable mechanical properties such as stiffness, elasticity, ultimate strength and elongation and suture retention strength\textsuperscript{104-107}. With the right equipment, multiple phases of the same structure can be knitted simultaneously, and the interface between the contiguous zones can be integrated with the use of continuous yarns.

In addition, 3D knitting using a double-needle bed machine has facilitated the creation of a sandwich structure, called a spacer fabric, by including a third set of yarns to provide interlacement in the thickness direction between the two layers of knitted fabric\textsuperscript{108}. Anand et al\textsuperscript{87,106} has reported that spacer fabrics show advantages in compression bandage applications. They allow for consistent air circulation through the fabric to reduce heat build-up and increase moisture transfer as a result of the void space created by the spacer yarns.

He et al\textsuperscript{86} developed a poly(ethylene terephthalate) (PET) spacer fabric scaffold for tissue engineering applications and has evaluated its physical and mechanical properties as well as its cell attachment and penetration performance within the scaffold. It has been shown that the spacer fabric with multifilament yarns can not only support confluent cell proliferation on the surface, but the spacer yarns were able to guide cell infiltration in the "z" direction through the thickness. Moreover, the spacer fabric incorporated a distribution of multiple pore sizes through the thickness. The face and bottom knitted layers had a distribution of micropores between the knitted multifilament yarns, while the middle spacer layer had a distribution of macropores between the spacer yarns. It is believed that the larger void volume in the middle layer of the spacer fabric is more suitable for mass transportation and vascularization during tissue ingrowth. However, up until this time to the best of our knowledge, a multiphase structural design for a spacer fabric has not been reported. Therefore the fabrication methods to create a spacer fabric with a contiguous multiple phase design has still to be achieved.

In short, among the existing textile fabrication technologies, warp knitting technology shows the best potential for fabricating a multiphase integrated 3D scaffold with adequate mechanical
properties for a MTJ scaffold application. The rest of this section explores the potential methods for creating multiphase spacer fabric structures simultaneously during warp knitting, starting with a review of warp knitting fundamentals.

2) Warp Knitting Fundamentals

The basic elements of a warp knitting machine are illustrated in Figure 11. The basic principle of stitch formation together with the knitting elements in single and double needle bed warp knitting machines are illustrated in Figure 2.3.2.

Unlike weft knitting which uses the feed yarns directly from their individual packages, warp knitting requires pre-wound warp beams to be prepared (Figure 2.3.1), each with its precisely defined number and required length of warp yarns. Individual packages are mounted on a creel and placed parallel to each other to allow the required number of yarns to be threaded and wounded onto the beam. This process is called warp preparation, or warping. The proper tension and alignment of the yarns is critical to forming a good quality beam. Poor quality beams can lead to variable tensions across the warp, resulting in knitting faults, needle malfunctions and fabric defects. Variations in yarn thickness, elasticity, twist level, texture and other factors influence the beam quality. Therefore warping is an important and crucial step in the warp knitting manufacturing process.

Figure 2.3.1: Schematic diagram of a warp knitting machine109
From the beams, the yarns pass over several sets of rods and then through a series of small eyelets called guides that control the tension of the yarn entering the knitting elements (Figure 2.3.2). Guides are also mounted on movable bars called guide bars. The guide bars ensure that the individual yarns are separated and aligned parallel to each other, and their movements place the yarns around the needles to form loops. On a double needle bed warp knitting machine, the guided yarns are categorized into three groups: the face yarns, the spacer yarns, and the back yarns (Figure 2.3.2). The face yarns are fed to the front needle bed, the back yarns are fed to the back needle bed, and the spacer yarns are fed to both needle beds to connect the two layers. Since the movement of each guide bar is individually controlled it is usual to thread all the yarns from a single warp beam to a single and separate guide bar. That is why usually the number of guide bars equals the number of warp beams.

The number of needles per inch is defined as the “gauge”, which is often used as one way to describe the fabric density. The interlaced loop is defined as a “stitch”. The knitted fabric is pulled down by batch rollers at a controlled speed and then rolled up on a fabric take up roller at the front of the machine.

The feeding tension of the yarns from the beam is controlled by either spring loaded tensioners (passive feeding) or by a motor driven yarn deliver system (positive feeding). The tension of the yarn in the needles is mainly determined by the feeding tension, the knitting speed and the take-up speed, but it is also influenced by other factors such as the size of the yarn, the distance between the needle beds on a double needle bed machine and the design pattern which is referred to as the lapping sequence.\(^{109}\)

The knitted design is controlled by the vertical movement of the needles as well as the “lapping” sequence which describes the movement of the yarn in between each course or row of stitches. Lapping for the spacer yarns is controlled by the swing and shogging movements of the guide bars (Figure 2.3.3). The cam shaft drives the guide bars to continuously swing back and forwards in a horizontal motion between the needle beds, and move the yarns to the back or the front needle bed. At the same time the pattern drum pushes and pulls the guide bar sideways in the needle bed direction, producing underlaps or overlaps between successive courses. Traditionally the pattern drum consists of chain links with different heights that direct the degree of shogging
of the guide bar. The design pattern for the fabric construction on each needle bar is transferred by the height sequence of the chain links to direct the zig-zag motion of the guidebar and control the sideways displacement of the yarns.\textsuperscript{109} However the pattern drum limits the knitting capability. The length of a repeat unit of the lapping sequence is influenced by the size of the drum. If the height difference between neighboring chain links is too great, this can cause failure of the shogging motion and lead to dropped stitches. Also in order to change the lapping sequence and/or the needle gauge one needs to reset or replace the links, resulting in down time, increased cost of operation and the need to store supplies. To answer this challenge, the latest warp knitting machines\textsuperscript{110} use a digital stepper motor instead of chain links.

![Diagram of stitch formation](image_url)

Figure 2.3.2: Schematic diagram of the basic principle of stitch formation showing the knitting elements in a single and double needle bed warp knitting machine \textsuperscript{109}
3) **Warp knitted spacer fabrics**

A 2D warp knitted fabric consists of columns of stitches called "wales" and rows of stitches called "courses" as illustrated in Figure 2.3.4A. The “wales” which are represented as two columns of red loops in Figure 2.3.4A are formed by a single feed yarn. A spacer fabric consists of two distinctive surface layers linked together by the spacer yarn in the middle (Figure 2.3.4B). The face and back surface layers and the spacer layer can each be designed individually. The spacer fabric is heterogeneous with different properties in the weft and warp "x" and "y" directions, in addition to the "z" direction through the thickness.
Figure 2.3.4: Simulation of a 2D knitted fabric (A) and a 3D warp knitted spacer fabric (B).

The mechanical properties of a spacer fabric are dependent on the structural characteristics as well as the selection of the biomaterial. The structural characteristics such as the wales per inch (WPI), courses per inch (CPI), thickness, total porosity and porous size distribution, can be determined by the particular yarns selected for each beam, and the design variables, such as the lapping sequence and knitting parameters. The lapping sequence is the main driving factor for controlling the fabric construction. A monofilament yarn usually has higher strength and bending rigidity than a multifilament yarn, and the spacer fabric with a monofilament spacer yarn can provide superior compression resistance. Multifilament yarns have a larger surface area, which facilitates cell attachment. The distance between the yarns, also known as the void volume or "total porosity", can be altered by the level of twist and texture of the yarns as well as the cross-sectional shape of the individual fibers. The spacer fabric construction can be changed significantly by modifying various knitting variables such as gauge, number of guide bars, yarn feed tension, take-up speed, threading method and the distance between the needle beds, without any change in the lapping sequence.
Since any spacer fabric has anisotropic properties, there are two strategies for creating a continuous multiphase warp knitted spacer fabric. One is to create a warpwise gradient in the machine direction and the other is to create a weftwise gradient across the fabric width. For each strategy the table below proposes modifying several processing variables and knitting parameters (Table 2.3.1).

In order to create the gradient in the machine direction, it is possible to change the lapping sequence and/or the fabric take-up speed. Reducing the take-up speed leads to a larger loop length and more yarn being held on the needle hence knitting a denser fabric. Alternatively, by increasing the fabric take-up speed, the loop length will be reduced and a more porous open knitted structure will result.

Table 2.3.1 Possible variables to create multiphase warp knitted spacer fabrics in two different directions.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Warpwise gradient</th>
<th>Weftwise gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lapping sequence</td>
<td>Selection of different yarns</td>
</tr>
<tr>
<td></td>
<td>Take-up speed</td>
<td>Number of guide bars</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Threading sequence</td>
</tr>
</tbody>
</table>

Regarding the direction across the fabric width, one approach is to change the number of guide bars (GB) that are threaded. So by threading 4GB for half the width on the left hand side and then changing to 6GB for the other half on the right hand side, the fabric density on the right hand side will be much higher which will significantly change the mechanical properties between the two sides of the fabric sample. Alternatively, one can select different threading procedures between the two sides of the fabric. For example, half the width on one side could be all-threaded and the other half could be partially threaded. This would provide another strategy for fabricating a single continuous multiphase knitted structure with different mechanical properties on the two sides.
2.4 Systematic Approach to Tissue Engineering a Muscle Tendon Junction (MTJ)

2.4.1 Developmental Strategy and Methods

Based on reports from previous studies, we have summarized the challenges in developing a muscle tendon junction (MTJ):

1) to design a single scaffold system that is suitable for both tissue types,
2) to expand cell populations to form an in vitro interface and
3) to incorporate the use of a 3D dynamic bioreactor to introduce appropriate mechanical stimulation.

This section reviews previous procedures and methodologies in developing and evaluating a tissue engineered MTJ. A systematic approach is explored including the development of a spacer fabric scaffold, the static in vitro culture environment, the dynamic culture environment in a bioreactor as well as the evaluation methodology. The potential for applying these criteria and conditions to tissue regeneration at other multiple tissue junctions is also discussed.

Figure 2.4.1: Systematic approach of developing MTJ scaffold using warp knitted spacer fabric.

1) Development of MTJ Spacer Fabric Scaffold

This section reviews how previous research studies have divided the approach of developing an interfacial tissue engineered (ITE) biomaterial scaffold into a sequence of steps including polymer selection, yarn preparation, fabric fabrication and stabilization, modification of the fabric's surface and lastly sterilization. According to these experiences, the development of an MTJ scaffold using knitted spacer fabric technology is proposed as follow (Figure 2.4.2).
Figure 2.4.2: Steps in the Development of an MTJ Spacer Fabric Scaffold

The yarn properties, warp beams preparation and warp knitting variables have been discussed in Section 3. Obviously a bioresorbable polymer is recommended for the final sample. However, in the interim a permanent or biostable polymer can be used in the prototyping phase to optimize the structural properties. During the process of warping and warp knitting, the yarn is exposed to the manufacturing environment and may well be contaminated by dirt, grease, lubricant and other particles. It is necessary to wash or “scour” the fabric using biocompatible detergent that will not cause premature degradation and/or resorption of the polymer. The stability of the structure can be improved by heat setting which involves heating the fabric above the polymer's glass transition temperature so that the yarns are set to the desired loop length and loop shape within the fabric. The most commonly used polymeric materials include thermoplastic melt spun synthetic polymers or natural polymers such as silk fibroin. Usually the surface chemistry of synthetic spun yarns needs to be modified so as to improve the hydrophilicity and biocompatibility. The different surface activation methods that can be applied to various
biomaterials have previously been discussed in Section 3. Among the most commonly used methods, coating or immobilization with collagen and immersion in serum or culture media is easy to operate and is effective.\textsuperscript{34,34} The coated scaffold needs to be sterilized before being exposed to the cell culture environment. The selection of an appropriate sterilization method, such as the use of an autoclave, ethylene oxide, gamma radiation or UV exposure, is dependent on the properties of the biomaterial.\textsuperscript{112} In view of the thickness and density of the spacer fabric, the choice of UV exposure is not recommended.

2) **Static Culture**

Cell culture refers to the removal and isolation of cells from an animal or plant and their subsequent growth in a favorable artificial \textit{in vitro} environment. Although the use of dynamic 3D culture conditions in a bioreactor has several key advantages, an initial static 2D culture is still recommended for the following several reasons.\textsuperscript{31,33,76,113}

1) Static expansion is required to generate a larger enough cell population. Intracellular communication is crucial for cell survival and function. It requires a particularly elevated starting cell density for a given unit volume. The starting cell concentration should be high for a spacer fabric scaffold because of the high surface area, the thickness and the relatively large size (several millimeters in diameter) of the construct.

2) Static culture ensures that the cells do attach themselves to the scaffold. Cells may experience poor or limited attachment when they are seeded directly onto the biotextile scaffold with shear forces and a dynamic flow environment. Initially cells prefer a static environment at the initiation of the proliferation stage. The high void volume of biotextiles also increases the difficulty for cells to locate. Therefore a small volume with high cell density is recommended for cell seeding.

3) The interaction between muscle and tendon cells could be challenging depending on the cell sources and the culture techniques. Some cell culture recipes using compromised medium for the co-culture of muscle and tendon cells need to be developed first under static conditions.

4) The evaluation of cell viability can be done more readily and reliably in static culture.
The steps and parameters for undertaking the static co-culture of muscle and tendon cells are listed in Figure 2.4.3. However, there are still questions that need to be answered for the successful use of static culture of muscle and tendon cells, such as the origin, phenotype and source of the cells.

Stem cells and progenitor cells have been used to engineered tissues because of their potential to develop into different cell phenotypes in a similar way as occurs in natural tissue. Stem cells have three general properties that can benefit tissue engineering.\textsuperscript{40,113}

1) They are capable of dividing and renewing themselves for long periods of time.
2) They are not specialized cells with limited specific functions, and
3) They can differentiate into specialized cells.

However culturing stem cells involves the development of efficient protocols for directing cells to differentiate under controlled conditions. And this demands more complex and interdisciplinary cooperation between engineers and cell and molecular biologists than is planned for this study\textsuperscript{27,114,115}.

The cells used for \textit{in vitro} cell culture can also be categorized into cell lines and primary cells. For example, skeletal muscle can be engineered from primary cells from neonatal, adult or aged mammals or from myogenic cell lines. Primary cells refer to the cells that can be harvested directly from the tissue and disaggregated by enzymatic and/or mechanical means before isolation, purification and expansion. Primary cells have a limited life span. They have a slow proliferation speed, and as they are passaged, the cells with the fastest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population. A primary cell culture may be composed of mixtures of cell types. Therefore primary cell harvesting always incorporates some cell isolation and purification. The primary cells derived from different animals of the same species can behave differently in culture depending on the genetics, the sex and the age of the individuals.\textsuperscript{27,116}

The major challenge for primary cell culture is the development of efficient isolation and culture protocols. An \textit{in vitro} study is designed to represent the \textit{in vivo} conditions inside the living animal so the primary cells need to adapt to their different \textit{in vitro} culture conditions. Regarding an \textit{in vivo} study, a large animal model such as pig is more representative of the human condition.
Generally, primary cells from large animals such as the pig are more difficult to isolate than those from smaller animals such as mice and rats. This is because the tissue from larger animals contains more connective collagen, which requires harsher conditions to digest and isolate\textsuperscript{115}. There is therefore a fine balance between disaggregating cells and maintaining their cell viability because the digestion process can causes some cell damage. Also cells from larger animals can be more complex, expensive and difficult to harvest.

Cell lines have at least one passage after the first subculture. With each subsequent subculture, the cell population becomes more homogenous as the faster growing cells predominate. Immortal cell lines have long been used for \textit{in vitro} studies due to their genotypic and phenotypic uniformity. This leads to convenient maintenance, standard analysis and ease of comparison of

*Figure 2.4.3: Steps and parameters of static co-culture of muscle and tendon cells*
results between studies. However, these immortal cell lines are also criticized because they don’t represent what occurs \textit{in vivo}.\textsuperscript{117}

After the desired primary cells have been obtained, the compromised co-culture conditions for muscle and tendon cells needs to be established on the scaffolds before dynamic bioreactor culture can be attempted.

3) Dynamic Culture in a Bioreactor

As previously mentioned in Section 2, the dynamic culture conditions involved in a 3D bioreactor need to be optimized in terms of the specific conditions for mechanical stimulation and contractile force detection.\textsuperscript{31,40,61,118} The development of a dynamic culture system includes an understanding of the bioreactor design and function as well as the protocol for mechanical and electrical stimulation. The design of the bioreactor requires an evaluation of the size and shape of the culture chamber, the mechanism to supply the electrical and/or mechanical stimulation, the materials, the fabrication as well as the method of assembly (Figure 18). In particular, the protocol for mechanical stimulation during co-culture needs an assessment of the frequency, applied force and level of strain to be supplied by the bioreactor.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{bioreactor_diagram.png}
\caption{Steps and parameters for developing the dynamic bioreactor co-culture protocol.}
\end{figure}
4) Evaluation Methodology

Every day new perspectives are being reported in the literature describing new approaches and methodologies to evaluate the performance of tissue engineering scaffolds. However, at this time, there has been no agreement on a list of standard methods to be applied to biotextile tissue engineering scaffolds. Researchers have in the past usually borrowed testing standards from the fields of textiles and medical devices as well as previously established methods from other related research. Some important test standards for implantable spacer fabrics should include either medical device or textile related standards such as ASTM F2150-10, "Characterization and testing of biomaterial scaffolds used in tissue-engineered medical products, ASTM D6571-01 "Compression resistance and recovery properties", ASTM D5035-11 "Breaking force and elongation of textile fabrics" and EN 29073-3:1992 "Tensile strength in dry and wet conditions", ASTM D1388-08 "Fabric stiffness", ASTM D3787 "Bursting strength" and ISO 10993 "Biological evaluation of medical devices". Some of the more commonly used evaluation methods for testing the functional properties of MTJ spacer fabric scaffolds are listed below (Figure 2.4.4). \(^{119,120}\)

There are three main categories of functional properties for an MTJ spacer fabric scaffold. They are the physical characteristics, the mechanical properties and the biological properties.
Figure 2.4.4: Summary of properties and test methods for evaluating MTJ spacer fabric scaffolds

The physical characteristics of spacer fabric scaffolds include the thickness, fabric density, stitch count (wales per inch (WPI) and courses per inch (CPI)) and the total porosity of the porous structure. The thickness, fabric density and stitch account can be measured from image analysis following the standard methods BS 1051 and ASTM D 3887-91. There is no standard method to measure the porosity of the scaffold. However, as previously discussed in Section 2, the porous structure has been described using terms such as “total porosity”, “average pore size”, “pore size distribution” and “air permeability”. These porous features can be tested using theoretical
calculations, microscopic analysis, mercury porosimetry, and micro-computed tomographic (μ-CT) analysis.\textsuperscript{34}

The ultimate tensile strength, elongation at break and tensile modulus of a spacer fabric can be tested using ASTM D 5035-11 "Breaking force and elongation of textile fabrics" and EN 29073-3:1992 "Tensile strength in dry and wet conditions". The cyclic tensile and contractile properties can be measured using a video strain gauge which records the changes in the shape, dimensions and structure at the same time as an external tensile force is applied (Figures 3 and 10) or as the contractile force is generated from the engineered tissue (Figure 3).\textsuperscript{31,121}

Biological properties such as cytocompatibility, cytotoxicity, cell viability and cell distribution within the scaffold, cell morphology and protein expression are usually tested routinely during \textit{in vitro} culture. Cell viability is a determination of the ratio of living to dead cells, based on the total cell sample and can be used to correlate cell behavior with viable cell numbers. Sometimes the cell viability assay is used to exam the cytotoxicity and cytocompatibility. Cell viability assay usually involves observing a sample cell population and staining the cells to distinguish the living from the dead population. There are wide arrays of cell viability methods which range from the most routine trypan blue dye exclusion assay to measuring mitochondrial activity or caspase assays such as MTT, MTS, XTT and alamarBlue.\textsuperscript{122,123} Cell morphology can be observed histologically by analyzing the optical microscopic images of ultra-thin sectioned slices of stained tissue. It is critical that representative stained sections are selected for histological analysis so as to distinguish cells of different colors. For example, Mason’s Trichrome staining has been found suitable for viewing MTJ histological images (Figure 2.4.5A). The distribution of cells within the scaffold can be analyzed using optical microscopic images. The microscopy used at the cellular level for a number of the tests is more demanding in terms of high resolution. In that case one should use transmission electron microscopy (TEM), scanning electron microscopy (SEM) for viewing the surface of the scaffolds and laser scanning confocal microscopy (LSCM) for viewing sections through the whole thickness of the scaffolds.\textsuperscript{33}
Figure 2.4.5: Frozen sections (A) of a normal adult muscle tendon junction (MTJ) with Mason’s Trichrome staining for collagen (blue), skeletal muscle fibers (red), and cell nuclei (black). Immunostaining of Paxillin (red) that is clustered at the MTJ in (B) neonatal and (C) adult rat muscle. 

Analysis of specific protein expression can identify the formation of certain tissues. With respect to MTJ tissue, the specific markers one is looking for include desmin, M-cadherin, myogenin, MyoD and myosin heavy chain in the muscle; scieraxis, Type 1 collagen and decorin in the tendon; as well as vinculin, paxillin (Figure 2.4.5B and 26C), talin, focal adhesion kinase and integrins in the MTJ interface. Specific markers can be stained and shown in immunohistochemical (IHC) and immunocytochemical (ICC) images, but the process requires special protocols for appropriate fixing, embedding, sectioning, antibody staining and microscopic examination. At the gene level, the use of the polymerase chain reaction (PCR) is able to identify the DNA sequence of the specific markers, and therefore it can verify the regeneration of muscle tissue, tendon tissue and the muscle-tendon interface.

2.5 Summary

This review has given an overview to the challenges and approaches to tissue engineering interfacial tissue junctions taking the muscle tendon junction as an example. After an evaluation and comparison of the different fabrication technologies currently in use for preparing different types of tissue engineered scaffolds, the warp knitted spacer fabric was nominated as a promising
candidate for an MTJ scaffold. This is primarily due to its design flexibility, mechanical properties and ability to integrate a range of different multiphase structures contiguously into one single architecture. A systematic approach to exploring the requirements of a tissue engineering MTJ scaffold was undertaken, and the use of a spacer fabric was evaluated in terms of the number of steps to reach production scale-up, the static in vitro culture environment, the dynamic bioreactor cultural environment as well as the evaluation methodologies. This systematic approach to designing an MTJ scaffold illustrates the potential for a spacer fabric to serve as an interfacial tissue engineering scaffold. By altering the knitting design variables, an appropriate continuous multiphase spacer fabric can be obtained with a variety of architectures and mechanical properties. From this exercise, it is readily feasible to use a spacer fabric to engineer other interfacial tissue junction scaffolds such as a soft-tissue-to-bone junction scaffold. Bone tissue engineering scaffolds require superior stiffness and a high degree of total porosity with sufficient interconnected pores. High porosity warp knitted spacer fabrics demonstrate their ability to provide a remarkably high interconnected void volume for vascularization that is crucial for the initiation of bone regeneration. In this case, other fabrication methods may be incorporated so as to introduce calcification and mimic the regeneration of rigid bone tissue.

In summary, a warp knitted spacer fabric meets all the major requirements to provide a continuous integrated multiphase scaffold for interface tissue engineering applications.
CHAPTER 3

DEVELOPMENT OF MUSCLE-TENDON JUNCTION SPACER FABRIC SCAFFOLD

3.1 Introduction

A three dimensional (3D) scaffold with structural gradient is essential to tissue engineer a multiple tissue construct such as a muscle-tendon junction (MTJ). A warp knitted spacer fabric is a promising candidate for such a 3D scaffold. However, the knitting technology to create a structural gradient within a spacer fabric has yet to be available on the market. This study initiates the design and fabrication of a 3D muscle-tendon junction spacer fabric scaffold from first principles.

The specific aims are:

5) to design multi-phase warp knitted spacer fabrics with contiguous structural and mechanical gradients that can be used as scaffolds for muscle-tendon junction tissue engineering.

6) to develop and optimize the knitting methods to prototype the proposed designs using a non-degradable surrogate polymer.

7) to identify the optimal knitting methods based on the efficiency of knitting production and the properties of the non-degradable prototype structures.

8) to use the optimal knitting methods to fabricate and evaluate degradable MTJ spacer fabric scaffolds.

In order to achieve the specific aims, this thesis project is divided into the following stages.

![Diagram of development process](image)

Figure 3.1.1: Steps to develop a MTJ spacer fabric scaffold.
3.2 Design of Multiple Phase Spacer Fabric

3.2.1 Muscle-tendon Junction (MTJ) Tissue Model

A multiple phase spacer fabric has been designed to mimic the structural and mechanical properties of native muscle-tendon tissue.

Figure 3.2.1: Design strategy of the MTJ spacer fabric scaffold

The Wake Forest Institute of Regenerative Medicine (WFIRM) has developed a muscle-tendon junction scaffold for small animal models. However, their scaffold is not suitable for a large animal model, and so it has limited long term clinical value. This study aims to develop a construct for a large animal model such as a pig. A porcine muscle-tendon junction was tested and so it is used as the target. The tensile properties for a human muscle-tendon junction are referenced below in Table 3.2.1, Figure 3.2.2 and Figure 3.2.3.

Table 3.2.1 Normal Human and Porcine Tissue Tensile Properties from the Literature

<table>
<thead>
<tr>
<th></th>
<th>Human 30</th>
<th></th>
<th>Porcine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tendon</td>
<td>Muscle</td>
<td>Tendon</td>
</tr>
<tr>
<td>Tensile stress (MPa)</td>
<td>52-120</td>
<td>0.1-0.3</td>
<td>2.4 ± 0.8</td>
</tr>
<tr>
<td>Tensile strain (%)</td>
<td>5-16</td>
<td>60-105</td>
<td>30.1% ± 12.7%</td>
</tr>
<tr>
<td>Young’s Modulus (MPa)</td>
<td>500-1850</td>
<td>0.005-2.8</td>
<td>7.2 ± 3.8</td>
</tr>
</tbody>
</table>
Figure 3.2.2: Human muscle-tendon junction tensile properties.\textsuperscript{30}

Figure 3.2.3: Comparison of human and porcine muscle-tendon junction properties
Porcine tissue was harvested from the tibialis anterior together with the tibialis tendon from the hind legs of two 1-year old female pigs. The muscle, tendon, and interfacial junction were tested on a CellScale Mechanical BioTester (CellScale, Waterloo, ON, Canada). The experimentally measured tensile strength and Young's modulus of the porcine tissue was much lower than the values for human tissue quoted from the literature. The muscle-tendon junction in large animals experiences less than 20% strain during daily activities, and therefore, in addition to measuring the ultimate tensile stress and failure strain, the stress level and Young's modulus at 20% strain were also monitored.

Porcine tissue has been reported to have similar mechanical properties as human tissue. However, the test results from different research centers may vary due to the use of different testing equipment, sample preparation and test method.

The ideal multiple phase muscle-tendon scaffold should have distinct mechanical profiles at the two ends. Each end should mimic natural porcine or human tissue at strain levels of up to 20%, while at the same time, supporting stresses higher than those normally experienced by natural tissue.

In order to create this mechanical gradient, a multi-phase scaffold should be customized using a combination of unique knitted structures. The following sections in Chapter 3 will discuss how to create this structural gradient and provide a systematic approach to the evaluation of such a multi-phase spacer fabric scaffold.

### 3.3 Development of Non-Degradable Surrogate Prototype

#### 3.3.1 Materials

Non-degradable poly (ethylene terephthalate) (PET) in Figure 3.3.1 was melt spun into 150 denier/48 filament yarn (Unifi Inc., Yadkinville, NC, USA). The non-degradable polymer is resistant to moisture, lubricants and variations in temperature in the knitting production environment, which facilitated the knitting and evaluation steps for this preliminary trial. PET is
suitable surrogate polymer because the medical products made from PET have been approved for medical use by the FDA.\textsuperscript{126} The technical details of the PET yarns are listed in Table 3.3.1.

Table 3.3.1 Measured Unifi PET Yarn Properties

<table>
<thead>
<tr>
<th>Properties</th>
<th>Cross-section</th>
<th>Tenacity (gpd)</th>
<th>Elongation at Break</th>
<th>Shrinkage (180F)</th>
<th>Density (g/cm(^3))</th>
<th>Twist Direction</th>
<th>Torque Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET yarn</td>
<td>Round</td>
<td>4.7</td>
<td>20.4%</td>
<td>14.8%</td>
<td>1.38</td>
<td>S</td>
<td>Z</td>
</tr>
</tbody>
</table>

Figure 3.3.1 Left: chemical formula of poly(ethylene terephthalate). Right: optical microscopic image of PET yarn.

3.3.2 Knitting Method

There are two possible directions in which a structural gradient can be created in a knitted fabric, namely in the machine (warp) direction, or in the width (weft) direction. The multiple structures can be created using different knitting variables in the knitting elements (Figure 3.3.2), such as the threading method, the number of guide bars (GB), the stitch notation, the take-up speed and a combination of these variables.
Four knitting methods were proposed to create a contiguous interface in the spacer fabric both in the machine direction and across the width. The 3D loop structure of the spacer fabric scaffold can be pre-viewed according to the simulation images from the ProCAD warpknit software (TEXION Software Solutions, Germany) (Table 3.3.2).
Table 3.3.2 ProCAD warpknit software simulations of two directions to create a gradient.

Gradient Changed in Machine Direction (Warp Direction)
Knitting methods: 1. change the take-up speed and 2. change the stitch notation

Gradient Changed across the Width (Weft Direction)
Knitting methods: 3. Change the number of guide bars and 4. Change the threading method
**Knitting Method 1 (KM1)** creates a structural gradient in the warp direction by changing the take-up roll speed of the fabric after the knitting elements. One can vary the take up speed in proportion to the main motor of the knitting bed. For example, 1000% take up speed means that the fabric take-up roll spins 10 times faster than the needle bed motor speed. The take-up speed is usually higher than 100% in order to apply a tension and pull the fabric off the knitting elements. The take-up speed range of 200%-1000% is commonly used on the RIUS® Mini-Tronic Machine. Reducing the take-up speed allows more time for the guide bars to jog between needles and lay in yarns between the two needle beds. By releasing the tension applied to the fabric roll, the density and thickness of the spacer fabric scaffold will increase.

Table 3.3.3 Knitting parameters of Knitting Method 1

<table>
<thead>
<tr>
<th></th>
<th># of guide bars</th>
<th>Take-up speed</th>
<th>Gauge</th>
<th>Stitch notation</th>
<th>Threading method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle region</td>
<td>6</td>
<td>250%</td>
<td>12</td>
<td>King 1</td>
<td>Full</td>
</tr>
<tr>
<td>Tendon region</td>
<td>6</td>
<td>1000%</td>
<td>12</td>
<td>King 1</td>
<td>Full</td>
</tr>
</tbody>
</table>

**Knitting Method 2 (KM2)** combines two stitch notations to create a structural gradient in the warp direction. This is also known as a change in design pattern or lapping sequence. The stitch notation determines the movement of individual guide bars. The open pore structure and yarn orientation in the spacer layer can be manipulated by the stitch notation.

Table 3.3.4 Knitting parameters of Knitting Method 2

<table>
<thead>
<tr>
<th></th>
<th># of guide bars</th>
<th>Take-up speed</th>
<th>Gauge</th>
<th>Stitch notation</th>
<th>Threading method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle region</td>
<td>6</td>
<td>500%</td>
<td>12</td>
<td>C1, D1</td>
<td>Full</td>
</tr>
<tr>
<td>Interface</td>
<td>6</td>
<td>500%</td>
<td>12</td>
<td>C2, D2</td>
<td>Full</td>
</tr>
<tr>
<td>Tendon region</td>
<td>6</td>
<td>500%</td>
<td>12</td>
<td>C3, D3</td>
<td>Full</td>
</tr>
</tbody>
</table>
Knitting Method 3 (KM3) creates a structural gradient in the weft direction by changing the number of threaded guide bars and hence the density of the yarns across the width. That means, 6 guide bars are used in half of the width, while 4 guide bars are used in the other half.

Table 3.3.5 Knitting parameters of Knitting Method 3

<table>
<thead>
<tr>
<th></th>
<th># of guide bars</th>
<th>Take-up speed</th>
<th>Gauge</th>
<th>Stitch notation</th>
<th>Threading method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle region</td>
<td>4</td>
<td>1000%, 750%, 500%, 250%</td>
<td>12</td>
<td>King 1</td>
<td>Full</td>
</tr>
<tr>
<td>Tendon region</td>
<td>6</td>
<td>1000%, 750%, 500%, 250%</td>
<td>12</td>
<td>King 1</td>
<td>Full</td>
</tr>
</tbody>
</table>

Knitting Method 4 (KM4) creates a structural gradient in the weft direction by changing the threading method on the guide bar. For half the width the 6 guide bars are fully threaded, and for the other half they are half threaded.

Table 3.3.6 Knitting parameters of Knitting Method 4

<table>
<thead>
<tr>
<th></th>
<th># of guide bars</th>
<th>Take up speed</th>
<th>Gauge</th>
<th>Stitch notation</th>
<th>Threading method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle region</td>
<td>6</td>
<td>1000%, 750%, 500%, 250%</td>
<td>12</td>
<td>King 1</td>
<td>Half</td>
</tr>
<tr>
<td>Tendon region</td>
<td>6</td>
<td>1000%, 750%, 500%, 250%</td>
<td>12</td>
<td>King 1</td>
<td>Full</td>
</tr>
</tbody>
</table>

A total of eleven PET prototype samples were knitted using these 4 knitting methods. Their sample specifications are listed in Table 3.3.7.
Table 3.3.7  Specifications of 11 PET spacer fabric prototypes

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Knitting Method #</th>
<th>Stitch Notation</th>
<th># of Guide Bars</th>
<th>Take-up Speed</th>
<th>Threading Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>KM1</td>
<td>1</td>
<td>K1</td>
<td>6</td>
<td>1000% &amp; 250%</td>
<td>Full</td>
</tr>
<tr>
<td>KM2-C</td>
<td>2</td>
<td>C1, C2, C3</td>
<td>6</td>
<td>500%</td>
<td>Full</td>
</tr>
<tr>
<td>KM2-D</td>
<td>2</td>
<td>D1, D2, D3</td>
<td>6</td>
<td>500%</td>
<td>Full</td>
</tr>
<tr>
<td>KM3-1000%</td>
<td>3</td>
<td>K1</td>
<td>4 &amp; 6</td>
<td>1000%</td>
<td>Full</td>
</tr>
<tr>
<td>KM3-750%</td>
<td>3</td>
<td>K1</td>
<td>4 &amp; 6</td>
<td>750%</td>
<td>Full</td>
</tr>
<tr>
<td>KM3-500%</td>
<td>3</td>
<td>K1</td>
<td>4 &amp; 6</td>
<td>500%</td>
<td>Full</td>
</tr>
<tr>
<td>KM3-250%</td>
<td>3</td>
<td>K1</td>
<td>4 &amp; 6</td>
<td>250%</td>
<td>Full</td>
</tr>
<tr>
<td>KM4-1000%</td>
<td>4</td>
<td>K1</td>
<td>6</td>
<td>1000%</td>
<td>Half &amp; Full</td>
</tr>
<tr>
<td>KM4-750%</td>
<td>4</td>
<td>K1</td>
<td>6</td>
<td>750%</td>
<td>Half &amp; Full</td>
</tr>
<tr>
<td>KM4-500%</td>
<td>4</td>
<td>K1</td>
<td>6</td>
<td>500%</td>
<td>Half &amp; Full</td>
</tr>
<tr>
<td>KM4-250%</td>
<td>4</td>
<td>K1</td>
<td>6</td>
<td>250%</td>
<td>Half &amp; Full</td>
</tr>
</tbody>
</table>

3.3.3  Fabrication

The fabrication includes the following steps:

Yarn preparation  ➔ Warping  ➔ **Warp knitting**  ➔ Scouring  ➔ Heat setting

Figure 3.3.3  Steps in the fabrication of an MTJ Spacer Fabric Scaffold

The 150D/48F fully-oriented PET yarns were warped onto six 288 end beams. The RIUS® Mini-Tronic narrow width double needle-bed warp knitting machine (RIUS, Spain) was used to knit the spacer fabric scaffold under ambient conditions. After knitting, the PET fabric scaffolds were washed in 1% Triton X-100 aqueous solution 3 times for 10 minutes each time at room temperature. Then the fabric scaffolds were rinsed by DI water 3 times for 10 minutes each time at room temperature. After rinsing, the PET fabric scaffolds were mounted onto frames and passed through a hot air oven (CH-8155 Niederhasli/Zurich) at a temperature of 130 °C and a constant speed of 1 m/min for 90 seconds.
3.4 Evaluation of Non-degradable Polymer

3.4.1 Methods

The heat set fabrics were conditioned overnight at 65±3 % RH and a temperature of 20±2 °C. Then they were analyzed by the following methods.

Fabric Surface Morphology

The fabric surface morphology was analyzed using a Nikon SMZ 1000 optical microscope. Five images were taken of each region of the sample including the face of the muscle and tendon regions, and the cross-section of the interface.

Fabric Stitch Count

The fabric stitch count was determined by counting the number of wales per inch (WPI) in the weft direction and courses per inch (CPI) in the warp direction according to the standard test method ASTM D 3887–9 Standard Specification for Tolerances for Knitted Fabrics. The specimens were chosen at random from different locations all at least 1 inch from the edges of the samples. Five specimens of each sample were counted four times each and the mean values and standard deviations were calculated.

Fabric thickness

The thickness of the spacer fabrics was determined using an SDL 94 thickness gauge (Shirley Developments Ltd, Stockport, England) (Figure 3.4.1). The area of the presser foot was 412mm², and the gauge measured thickness to a precision of 0.01 mm. For thickness testing in this study the pressure applied to the presser foot was 20 g/cm² for all specimens. Five specimens of each sample were counted four times each and the values averaged. The test method followed standard ASTM test method D 1777 – 96 Standard Test Method for Thickness of Textile Materials (2007).
Total Porosity

According to ASTM F2150-10, the porosity is defined as the “property of a solid which contains an inherent or induced network of channels and open spaces. Porosity can be measured by the ratio of pore (void) volume to the apparent (total) volume of a porous material and is commonly expressed as a percentage.” However, since it is difficult to measure the total porosity directly, it was calculated using the Equation 3.1 which relies on the density of the 3D fabric scaffold and the density of the polymer as provided in the ASTM standard F2450 – 10 Standard Guide for Assessing Microstructure of Polymeric Scaffolds for Use in Tissue-Engineered Medical Products.

\[
p = \left(1 - \frac{d_s}{d_p}\right) \times 100 = \left(1 - \left\{\frac{m_s}{v_s}\right\}/d_p\right) \times 100 = \left(1 - \left\{\frac{m_s}{w \times l \times h}\right\}/d_p\right) \times 100 \quad (3.1)
\]

Where,

- \( p \) = total porosity
- \( d_s \) = the density of the scaffold in \((g/cm^3)\),
- \( d_p \) = the density of the polymer in \((g/cm^3)\),
m_s = the mass of the scaffold (g),
vs = the volume of the scaffold (cm³),
w = the width of the scaffold (cm),
l = the length of the scaffold (cm),
h = the thickness of the scaffold (cm)

The density of the polyester (d_p) was assumed to be 1.38 (g/cm³) [2]. Each sample was cut into sixteen 4.00 cm by 5.00 cm specimens and conditioned overnight at 65±3 % RH and 20±2 ºC. The width and length were measured using vernier calipers, while the thickness was measured using the SDL 94 Thickness Gauge as described above. The mass of the specimen was measured on a Mettler weighing balance (Model AG 245) with a precision of 0.1 mg, and the total porosity, p, was calculated as a mean value.

Tensile Stress, Strain and Young’s Modulus

In order to study the maximum tensile strength in the gradient direction, samples were tested on a Model # 5544 Bluehill Instron Universal Tester (Instron, Norwood, MA, USA) following the principles of the standard ASTM D 5035-11 test method ASTM D5035-11(2015) Standard Test Method for Breaking Force and Elongation of Textile Fabrics (Strip Method). A strip test specimen measuring 2.0 cm wide was mounted between flat pneumatic clamps, which were at an initial gauge length of 2.5 cm and a force was applied to the specimen (Figure 3.4.2). The test used a constant crosshead speed of 50 mm/min until the specimen failed. Values for the breaking force and elongation at break were recorded. The maximum tensile stress, strain and Young’s modulus were calculated from the experimental stress-strain curves. The maximum tensile stress is defined as the maximum load divided by the initial cross-sectional area.

Young's modulus, E, is determined as the slope of the stress/strain curve in the initial linear portion of the curve. The Young's modulus is reported as the steepest slope between the lower and upper bounds, as shown in the following equation (3.2):

\[ E = \frac{\text{tensile stress}}{\text{tensile strain}} = \frac{\sigma}{\varepsilon} = \frac{F/A}{\Delta L/L} = \frac{F \times L}{A \times \Delta L} \]  
(3.2)
Where,
\[ E = \text{the Young's modulus (MPa)}, \]
\[ F = \text{the absolute force applied to the fabric (N)}, \]
\[ A = \text{the original cross-sectional area through which the force is applied (mm}^2\text{)}, \]
\[ \Delta L = \text{the amount by which the length of the object changes (mm)}, \]
\[ L = \text{the original length of the object or gauge length (mm)}. \]

In this test, 8 specimens were cut into 2 cm x 8 cm strips from each sample. The maximum tensile stress (MPa), tensile strain (%) and Young’s modulus (MPa) were calculated and averaged.

Figure 3.4.2 Left: Bluehill Intron Model No. 5544 Universal Tester. Right: Test specimen clamped between flat pneumatic clamps.
3.4.2 Results and Discussion

Fabric Surface Morphology

The samples were observed and analyzed using optical microscopic images at x8 magnification. The optical images of KM1, KM2, KM3 and KM4 samples are listed below. The reported WPI and CPI results are based on these optical microscopic images.

All four knitting methods are able to create distinct knitted structures according to our microscopic observations. The interface is contiguous and integrated in every sample. KM3 and KM4 provided the most difference between the two ends in terms of the appearance on the face and though the cross-section. Note that the interface region for the KM1 and KM2 samples is a longer and smoother transition than for the other designs.

Knitting Method 1

Figure 3.4.3 Left: Overview of Knitting Method 1 Sample. Center: Cross-sectional view. Right: Face of the interface region.
Table 3.4.1 Optical microscopic images of KM1 Samples

<table>
<thead>
<tr>
<th>Regions</th>
<th>250%</th>
<th>Middle</th>
<th>1000%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Warp-wise cross-sectional view</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Weft-wise cross-sectional view</td>
<td><img src="image7.png" alt="Image" /></td>
<td>NA</td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Knitting Method 2

Figure 3.4.4  Left: Appearance of Knitting Method 2-C (KM2-C) Sample. Right: Appearance of Knitting Method 2-D (KM2-D) Sample.

Table 3.4.2  Optical microscopic images of KM2.

<table>
<thead>
<tr>
<th>Region</th>
<th>C3 (Tendon)</th>
<th>C2 (Interface)</th>
<th>C1 (Muscle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td></td>
<td>D3 (Tendon)</td>
<td>D2 (Interface)</td>
<td>D1 (Muscle)</td>
</tr>
<tr>
<td>Face</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
Knitting Method 3

Note: 4GB = Four guide bars, 6GB = Six guide bars.

Table 3.4.3 Optical microscopic images of KM3-1000%

<table>
<thead>
<tr>
<th>Regions</th>
<th>4GB</th>
<th>Middle</th>
<th>6GB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warp-wise cross-sectional view</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weft-wise cross-sectional view</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4.4 Optical microscopic images of KM3-750%

<table>
<thead>
<tr>
<th>Regions</th>
<th>4GB</th>
<th>Middle</th>
<th>6GB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warp-wise cross-sectional view</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weft-wise cross-sectional view</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.4.5 Optical microscopic images of KM3-500%

<table>
<thead>
<tr>
<th>Regions</th>
<th>4GB</th>
<th>Middle</th>
<th>6GB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Warp-wise cross-sectional view</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Weft-wise cross-sectional view</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Table 3.4.6 Optical microscopic images of KM3-250%

<table>
<thead>
<tr>
<th>Regions</th>
<th>4GB</th>
<th>Middle</th>
<th>6GB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Warp-wise cross-sectional view</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td>NA</td>
</tr>
<tr>
<td>Weft-wise cross-sectional view</td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Knitting Method 4

Figure 3.4.5 Appearance of full width of KM4-1000% fabric.

Table 3.4.7 Optical microscopic images of KM4-1000%

<table>
<thead>
<tr>
<th>Region</th>
<th>Fully Thread</th>
<th>Middle</th>
<th>Half Thread</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weft-wise cross-sectional view</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4.6 Appearance of full width KM4-750% fabric

Table 3.4.8 Optical microscopic images of KM4-750%

<table>
<thead>
<tr>
<th>Region</th>
<th>Fully Thread</th>
<th>Middle</th>
<th>Half Thread</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Face</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>Weft-wise cross-sectional view</strong></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 3.4.7  Appearance of full width KM4-500% fabric

Table 3.4.9  Optical microscopic images of KM4-500%

<table>
<thead>
<tr>
<th>Region</th>
<th>Fully Thread</th>
<th>Middle</th>
<th>Half Thread</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Weft-wise cross-sectional view</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
By observing the optical microscopic images, the defects of each sample were identified and examined. The defects are caused by missed stitches during knitting, which happens when the yarn feeding tension is too low. Generally, the KM1 and KM2 samples showed fewer defects than KM3 and KM4 samples.
3.5 Evaluation and Refinement of Knitting Methods

Warp knitting technology has been widely used for commercial fabric production for decades. However, its application to the production of tissue engineered (TE) scaffolds has not been studied. This section compares the four knitting methods in terms of their production speed and efficiency and addresses issues related to knitting production.

**Knitting Method 1 (KM1)** creates a structural gradient in the warp direction by changing the take-up speed during knitting. However, it may require stopping the machine to change the take-up speed, depending on the type of knitting machine. Frequently stopping the machine limits the production speed and the continuity of the knitted scaffold structure. Also, stopping the machine may cause changes in yarn tension from the feeding bars. This can be a cause for dropped stitch defects in the fabric. Thus, this method is not recommended for commercial production.

**Knitting Method 2 (KM2)** combines three stitch patterns to create a structural gradient in the warp direction. However, the nature of warp knits makes the wales resistant to deformation in the warp direction. There is a limited type of stitch notation to create an elastic structure in the warp direction. Therefore, the KM2 structure may well be suited for a stiffer tissue scaffold, such as for ligament or bone, but is limited when applied to an elastic tissue scaffold such as muscle. The advantage of this method is that it provides a continuous and contiguous knitting approach. The production speed is high and the yarn tension across and between each guide bar is evenly distributed. There are fewer fabric defect concerns compared with the other three knitting methods.

**Knitting Method 3 (KM3)** creates a structural gradient in the weft direction by changing the number of threaded guide bars across the width. However, the yarns are not uniformly distributed between the needles, because the 6 guide bars region consumes more yarns than the 4 guide bars region. If the difference of yarn consumption is too great, there will be fabric defects. The needles may miss stitches at the low tension end, and be broken by the tensed yarns at the high tension end. Certain stitch notations, such as King 1, can tolerate the difference in yarn consumption between the 4 guide bar and 6 guide bar regions. Slower production speeds can also reduce the difference in yarn consumption.
In addition, not all the stitch pattern designs are suitable for this method. Some more open porous structures need all the guide bars to be threaded to complete a full course of stitches, and for those structures the fabric cannot be knitted in the zone where there are fewer threaded guide bars. For example, the King1 is suitable for this method, but C1, C2, C3, D1, D2, and D3 are not suitable for use with this approach.

Although knitting production is continuous, operating at a slower speed is recommended. There could be yarn tension issues from the feeding bar because of the non-uniform yarn consumption in the knitting area.

**Knitting Method 4(KM4).** In principle, KM4 can create a structural gradient in the weft direction by changing the threading sequence. This means that half the needle bed is all-threaded and the other half is partially threaded. This method shows the most defects associated with missed stitches. Like KM3, the yarn consumption across the width is not uniform. And the difference in yarn consumption is even greater than for the KM3 sample. The uneven tension leads to dropped stitches at faster yarn feeding rates. Alternatively, needle breakage is likely to occur at low yarn feeding rates. The non-uniform yarn consumption requires a more tolerance structure and low production speeds.

Table 3.5.1 Comparison of four knitting methods

<table>
<thead>
<tr>
<th></th>
<th>KM1</th>
<th>KM2</th>
<th>KM3</th>
<th>KM4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Production Speed</strong></td>
<td>Low (&lt;20 rpm)</td>
<td>High (20-100 rpm)</td>
<td>Medium (25-50 rpm)</td>
<td>Low (&lt;20 rpm)</td>
</tr>
<tr>
<td><strong>Yarn Tension</strong></td>
<td>Even</td>
<td>Even</td>
<td>Uneven</td>
<td>Uneven</td>
</tr>
<tr>
<td><strong>Risk of Defects</strong></td>
<td>High</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td><strong>Workable Stitch Notation</strong></td>
<td>No limits</td>
<td>No limits</td>
<td>Restricted to certain stitch patterns</td>
<td>Restricted to certain stitch patterns</td>
</tr>
</tbody>
</table>

Table 3.5.2 Structural stability of three of the knitting methods.

Here, structural stability refers to the amount of dimensional and structural change that occurs across the transition region before and after fabric relaxation. KM1 shows the lowest structural stability, while KM2 shows the highest. Although the structure for each region can be fixed by
heat setting, there is a tendency for relaxation to occur naturally before heat-setting, which can introduce variability between batches.

Table 3.5.2 Comparison of fabric structural stability between the four knitting methods

<table>
<thead>
<tr>
<th>KM</th>
<th>KM2</th>
<th>KM3</th>
<th>KM4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural stability (Stitch count change through the transition region %)</td>
<td>Low (30-50%)</td>
<td>High (3-5%)</td>
<td>Medium (5-10%)</td>
</tr>
</tbody>
</table>

One of the most important performance properties for multiple tissue engineered junction scaffolds is the difference in tensile properties between the two ends.

In summary, KM1 and KM4 are the least preferred structures. KM2 shows more advantages in terms of knitting production and dimensional and structural stability, but it has limited heterogeneity in mechanical performance between the different regions. KM3 shows advantages in terms of its mechanical properties, but there are limitations in terms of knitting production.

For the next step of knitting with PLA yarns, we select Knitting Method 3 and Knitting Method 2 with the following modifications:

1. Two stitch notations (King 1 and D1) were selected to increase the heterogeneity in mechanical properties between the muscle and tendon regions. The interface between these two regions was designed to serve as the junction's structural and porosity gradient.
2. The optimum take-up speed and number of guide bars were selected to maximize the difference in mechanical properties between the two regions.

**Fabric Thickness, Fabric Stitch Count and Total Porosity**

Among the knitting parameters, take-up speed is the most versatile. By changing the take-up speed, each knitting method can create a variety of structures. Altering the take-up speed can manipulate the fabric thickness, total porosity, WPI and CPI, and the difference in these properties between the muscle and tendon regions can be precisely tuned.
Knitting Method 3 samples were tested in terms of fabric thickness, fabric stitch count, and total porosity. The results are illustrated in Figure 3.5.1.

Figure 3.5.1 Fabric structural characteristics of PET-KM3 prototypes

The use of more guide bars resulted in higher WPI. Higher take-up speed resulted in lower WPI and CPI, making a more porous structure. The CPI is more sensitive to take-up speed in comparison to WPI. 1000% take-up speed creates a structure with the thinnest and most porous
structure. As a result, the thickness and total porosity in the tendon region are significantly higher than that in muscle region.

**Tensile Properties**

The ultimate tensile strength of the KM3 samples is related to the number of guide bars (GB) and the take-up speed. According to Figure 3.5.2, by increasing the take-up speed from 250% to 1000%, the ultimate tensile strength decreased significantly for the 4GB samples. On the other hand, by changing the take-up speed from 250% to 750% for the 6GB samples, only a marginal change was observed for the tensile strength of the 6GB samples. The 500% and 750% samples gave similar trends in ultimate tensile strength between the 4GB and 6GB regions.

The number of guide bars shows a positive influence on the ultimate tensile strength in the take-up speed range of 500% to 1000%. As the take-up speed falls to 250%, no significant difference in ultimate tensile strength was observed between the 4GB and 6GB samples. The 500% and 750% take-up speed samples showed the greatest difference in ultimate tensile strength between the 4GB and 6GB regions of the fabric.

![Tensile Stress](image_url)

Figure 3.5.2 Ultimate tensile stress of PET-KM3 prototypes
According to Figure 3.5.3, the number of guide bars influences the ultimate tensile strain of the sample. The largest difference in tensile strain between the muscle (4GB) and tendon (6GB) regions was observed for the 1000% take-up speed sample, followed by the 750% take-up speed sample.

For those samples knitted from the same number of guide bars, increasing the take-up speed significantly increased the ultimate tensile strain. The lowest ultimate tensile strain was about 150% for the 4GB 250% sample, while the highest ultimate tensile strain was about 400% for the 6GB 750% and 6GB 1000% samples.

The 750% and 1000% take-up speed samples showed similar ultimate tensile strain values for the 4GB and 6GB regions. In terms of production efficiency, the 1000% samples resulted in higher standard deviations than for the 750% samples.

Figure 3.5.3 Ultimate tensile strain of PET-KM3 prototypes

Regarding the stiffness or initial tensile modulus, the highest Young’s Modulus was obtained by using 4 guide bars and a 250% take-up speed as shown in Figure 3.5.4. In general the take-up
speed showed a negative relationship with the initial Young’s modulus. The more guide bars, the lower the initial Young’s modulus in the weft direction.

![Young's Modulus](image)

**Figure 3.5.4** Young's modulus of PET-KM3 prototypes

When viewing the tensile properties in summary, a higher take-up speed ratio resulted in a weaker fabric when only 4 guide bars were used. With more layers of yarn, i.e. with 6 guide bars knitted together, the higher take-up speed ratio resulted in the ability to achieve larger strains in the weft direction. So by increasing the take-up speed and increasing the number of guide bars from 4 to 6, one can knit a fabric with lower initial Young's modulus and higher fabric extensibility in the weft direction.

**Conclusion**

Eleven PET prototypes have been successfully created. KM2 and KM3 are recommended for further study using resorbable PLA yarns. A take-up speed ratio of 1000% is recommended because it creates the highest total porosity, and the largest difference in strain and Young's modulus between muscle and tendon regions.
3.6 Fabricate Degradable Fabric Scaffold

Materials and Sample Specification

Poly(lactic acid) yarns were spun into a 2 ply/70 denier/48 filament fully oriented yarns (Xinxiang Sunshine Textiles Co., Ltd, Xinxiang, Henan Province, China) The original molecular weight (Mw) was $140,909 \pm 2,921$ with $1.57 \pm 0.01$ PDI.

![Figure 3.6.1 Scanning electron micrograph showing the air-entanglement two-ply PLA filaments in a longitudinal view (left, Magnification: x385) and cross-sectional view (right, Magnification x1620)](image)

The PLA degradable yarns were knitted on the same Ruis knitting machine using the following knitting parameters (Table 3.6.1)
Table 3.6.1 Specification for the PLA degradable scaffolds

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Knitting Method</th>
<th>Stitch Notation</th>
<th>Take-up Speed</th>
<th>Gauge</th>
<th>Number of Guide Bars</th>
<th>Threading method</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA-KM2</td>
<td>2</td>
<td>King1 &amp; D1</td>
<td>1000%</td>
<td>12</td>
<td>6</td>
<td>Full</td>
</tr>
<tr>
<td>PLA-KM3</td>
<td>3</td>
<td>King1</td>
<td>1000%</td>
<td>12</td>
<td>4 &amp; 6</td>
<td>Full</td>
</tr>
</tbody>
</table>

Scouring and Heat Setting

The PLA fabrics were washed by 1% Triton-X100 aqueous solution at 40 ºC for 1 minute, and magnetic stirring bars were used to improve the washing efficiency. Washed PLA fabrics were rinsed twice using DI water stirring for 10 minutes each at 50 ºC. The final wash was with DI water at room temperature. Then the PLA fabrics were air dry at room temperature for 24 hours. Triton-X100 was selected because it is nonionic and is recommended for cleaning PLA resin products by the manufacturer NatureWorks LLC.

The PLA fabric was cut into 10 inches strips and the two ends were fixed onto a 10 inch long frame so the fabric was free of tension at the edges. The fixed PLA fabrics were passed through a hot-air oven at 65 ºC for 60 seconds and then cool down in the air at the room temperature.

Although NatureWorks LLC suggested a heat-setting temperature between 120º C-125º C, this range of elevated temperatures has been found to make the fabric rigid. So in order to keep the softness, flexibility and low Young’s modulus, the heat-setting temperature was performed for a longer period between 65 ºC-75 ºC.

Knitting

The fabrics were taken off the knitting machine and relaxed in a standard atmosphere overnight. The fabric width on the knitting machine and after relaxation has been measured and reported in Appendix I and II.

After relaxation, the following images were taken of the PLA-KM2 and PLA-KM3 degradable fabrics (Figure 3.6.2, Figure 3.6.3, Figure 3.6.4 and Figure 3.6.5).
Figure 3.6.2 Appearance of full width of PLA-KM2 degradable scaffold.
Figure 3.6.3 Appearance of full width of PLA-KM2 D1 Region (tendon region).

The yellow line indicates a zigzag pattern of the loops.

Figure 3.6.4 Appearance of full width of PLA-KM2 King 1 Region (muscle region).

The yellow line indicates a zigzag pattern of the loops.
3.7 Evaluation of Degradable Fabric Scaffold

3.7.1 Methods

The same methods were used as for the non-degradable scaffolds described in Section 3.4.1.

3.7.2 Results

Fabric Morphology

The PLA-KM2 and KM3 microscopic images are listed in Table 3.2.1 and Table 3.7.2. The interface is contiguous in both degradable scaffolds. For the PLA-KM2 sample the interface includes 3-5 courses with continuous yarns from the muscle to the tendon region. In order to mark the interface, a blue yarn was inserted in the spacer layer at the interface. In comparison, the PLA-KM3 sample has bigger pores in the muscle region compared to Sample PLA-KM2. In fact the difference in structure and total porosity between the muscle and tendon region is more pronounced for the PLA-KM3 scaffold. Again, the PLA-KM3 interface includes 3-5 wales and provides a clearly visual demarcation at the interface.
Table 3.7.1 Optical microscopic images of PLA-KM2 degradable scaffold

<table>
<thead>
<tr>
<th></th>
<th>D1 (Tendon Region)</th>
<th>Interface (MTJ)</th>
<th>King I (Muscle Region)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face (8x)</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>Face (20x)</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
</tbody>
</table>

Table 3.7.2 Optical microscopic images of PLA-KM3 degradable scaffold.

<table>
<thead>
<tr>
<th></th>
<th>6GB (Tendon Region)</th>
<th>Interface (MTJ)</th>
<th>4GB (Muscle Region)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face (8x)</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>Face (20x)</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
</tbody>
</table>
Fabric Thickness, Fabric Stitch Count and Total Porosity

PLA-KM2

The tendon region in the PLA-KM2 scaffold is significantly thinner and has a lower WPI than the muscle region. There is no significant difference between the muscle and tendon regions in terms of their total porosity and CPI (Figure 3.7.1). The porosity values are similar for the two regions in PLA-KM2 samples (Table 3.7.3), but different for the two regions in the PLA_KM3 sample (Table 3.7.4).

![Graphs showing total porosity, thickness, WPI, and CPI for muscle and tendon regions in PLA-KM2 degradable scaffold](image_url)

Figure 3.7.1 Total porosity, thickness, WPI and CPI of the PLA-KM2 degradable scaffold
PLA-KM3

The total porosity of the PLA-KM3 sample is listed in Table 3.7.4 and Figure 3.7.2. The tendon region in PLA-KM3 is significantly thicker than the muscle region. The tendon region has a tighter construction with 19% more yarns (WPI) than the muscle side. The muscle region has 5% more total porosity.

Figure 3.7.2 Total porosity, thickness, WPI and CPI of the PLA-KM3 degradable scaffold
Table 3.7.3  Total porosity of the PLA- KM2 degradable scaffold

<table>
<thead>
<tr>
<th>Total Porosity</th>
<th>Specimen 1</th>
<th>Specimen 2</th>
<th>Specimen 3</th>
<th>Specimen 4</th>
<th>Average</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>King 1 (Muscle Region)</td>
<td>74.89%</td>
<td>76.88%</td>
<td>78.16%</td>
<td>80.54%</td>
<td>77.6%</td>
<td>2.4%</td>
</tr>
<tr>
<td>D1 (Tendon Region)</td>
<td>81.48%</td>
<td>81.48%</td>
<td>78.23%</td>
<td>82.68%</td>
<td>81.0%</td>
<td>1.9%</td>
</tr>
</tbody>
</table>

Table 3.7.4  Total porosity of the PLA- KM3 degradable scaffold.

<table>
<thead>
<tr>
<th>Total Porosity</th>
<th>Specimen 1</th>
<th>Specimen 2</th>
<th>Specimen 3</th>
<th>Specimen 4</th>
<th>Specimen 5</th>
<th>Average</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4GB (Muscle Region)</td>
<td>84.9%</td>
<td>84.80%</td>
<td>85.40%</td>
<td>83.70%</td>
<td>85.40%</td>
<td>84.9%</td>
<td>0.7%</td>
</tr>
<tr>
<td>6GB (Tendon Region)</td>
<td>77.30%</td>
<td>79.50%</td>
<td>79.10%</td>
<td>79.20%</td>
<td>80.70%</td>
<td>79.1%</td>
<td>1.2%</td>
</tr>
</tbody>
</table>

Table 3.7.5 compares the fabric thickness of the degradable scaffolds PLA-KM2 and PLA-KM3. The literature provides limited discussion of any change in thickness across a muscle-tendon junction scaffold. The muscle region for the PLA-KM2 scaffold is 11% thicker than its tendon region. In the contrast, the tendon region for the PLA-KM3 scaffold is 41% thicker than its muscle region.

Table 3.7.6 and Table 3.7.7 compare the WPI and CPI of the PLA-KM2 and PLA-KM3 scaffolds. For the PLA-KM2 scaffold, the WPI of the muscle region is 15% higher than for the tendon region. On the other hand, the CPI's are similar for the two regions. For the PLA-KM3 scaffold, the WPI of the tendon region is 17% higher than for the muscle region. Again, the CPI is similar for both regions.
<table>
<thead>
<tr>
<th>King 1 (Muscle Region)</th>
<th>Specimen Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>1</td>
</tr>
<tr>
<td>Specimen 1</td>
<td>2.21</td>
</tr>
<tr>
<td>Specimen 2</td>
<td>2.22</td>
</tr>
<tr>
<td>Specimen 3</td>
<td>2.11</td>
</tr>
<tr>
<td>Specimen 4</td>
<td>2.29</td>
</tr>
<tr>
<td>Specimen 5</td>
<td>2.04</td>
</tr>
<tr>
<td>King 1 average thickness</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D1 (Tendon Region)</th>
<th>Specimen Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading (mm)</td>
<td>1</td>
</tr>
<tr>
<td>Specimen 1</td>
<td>1.83</td>
</tr>
<tr>
<td>Specimen 2</td>
<td>1.94</td>
</tr>
<tr>
<td>Specimen 3</td>
<td>1.74</td>
</tr>
<tr>
<td>Specimen 4</td>
<td>1.81</td>
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<tr>
<td>Specimen 5</td>
<td>1.87</td>
</tr>
<tr>
<td>D1 average thickness</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4GB (Muscle Region)</th>
<th>Specimen Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>1</td>
</tr>
<tr>
<td>Specimen 1</td>
<td>1.72</td>
</tr>
<tr>
<td>Specimen 2</td>
<td>1.86</td>
</tr>
<tr>
<td>Specimen 3</td>
<td>1.75</td>
</tr>
<tr>
<td>Specimen 4</td>
<td>1.62</td>
</tr>
<tr>
<td>Specimen 5</td>
<td>1.77</td>
</tr>
<tr>
<td>4GB average thickness</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>6GB (Tendon Region)</th>
<th>Specimen Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading (mm)</td>
<td>1</td>
</tr>
<tr>
<td>Specimen 1</td>
<td>2.29</td>
</tr>
<tr>
<td>Specimen 2</td>
<td>2.41</td>
</tr>
<tr>
<td>Specimen 3</td>
<td>2.28</td>
</tr>
<tr>
<td>Specimen 4</td>
<td>2.50</td>
</tr>
<tr>
<td>Specimen 5</td>
<td>2.63</td>
</tr>
<tr>
<td>6GB average thickness</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.7.6 Wales per inch (WPI) and courses per inch (CPI) for the PLA-KM2 scaffolds

<table>
<thead>
<tr>
<th>Specimen</th>
<th>King1 Average WPI</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>King1 (Muscle Region)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reading</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Specimen 1</td>
<td>18.5</td>
<td>17</td>
</tr>
<tr>
<td>Specimen 2</td>
<td>19</td>
<td>18.5</td>
</tr>
<tr>
<td>Specimen 3</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Specimen 4</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Specimen 5</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>King1 average WPI</td>
<td>18.1</td>
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Table 3.7.7 Wales per inch (WPI) and courses per inch (CPI) for the PLA-KM3 scaffolds

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<td>16.5</td>
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<td>17</td>
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<tr>
<td>4GB average WPI</td>
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<tr>
<td>Standard deviation</td>
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</table>

<table>
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</thead>
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<td>19</td>
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<tr>
<td>Specimen 2</td>
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<td>Specimen 3</td>
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<tr>
<td>Specimen 4</td>
<td>18.5</td>
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<tr>
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<td>18.5</td>
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<td>6GB average WPI</td>
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<tr>
<td>Standard deviation</td>
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<table>
<thead>
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<th>4GB (Muscle Region)</th>
<th>Specimen Average</th>
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</thead>
<tbody>
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<td>13</td>
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<tr>
<td>4GB average CPI</td>
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<tr>
<td>Standard deviation</td>
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<table>
<thead>
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<th>6GB (Tendon Region)</th>
<th>Specimen Average</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>Specimen 5</td>
<td>14.5</td>
</tr>
<tr>
<td>6GB average CPI</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
</tr>
</tbody>
</table>
Ultimate Tensile Properties

The ultimate tensile properties of the PLA-KM2 and PLA-KM3 scaffolds were significantly different. The test method used was a uniaxial tensile test oriented in the direction of the gradient. It is expected that warp knit fabrics show a much higher stress in the warp direction where the yarns are aligned, and a higher strain level in the weft direction.

The PLA-KM2 scaffold that had the gradient in the warp direction exhibited an ultimate tensile stress over 100 times the value for the PLA-KM3 scaffold in the warp direction. In the contact, the ultimate strain for the PLA-KM3 scaffold was triple the value for the PLA-KM2 sample in the muscle region and 5 times in the tendon region.

The PLA-KM2 scaffold had a higher ultimate tensile stress than human tissue. And the PLA-KM3’s ultimate tensile stress value was close to that of porcine tissue.

Table 3.7.8 Ultimate tensile properties of the PLA-KM2 degradable scaffold

<table>
<thead>
<tr>
<th></th>
<th>King 1 (Muscle Region)</th>
<th>D1 (Tendon Region)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Std Dev</td>
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<tr>
<td>Tensile Modulus (MPa)</td>
<td>181.04</td>
<td>22.24</td>
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<td>Stress (maximum load) (MPa)</td>
<td>44.14</td>
<td>3.94</td>
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<tr>
<td>Strain (maximum load) (%)</td>
<td>58.56</td>
<td>4.60</td>
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</table>

Table 3.7.9 Ultimate tensile properties of the PLA-KM3 degradable scaffold

<table>
<thead>
<tr>
<th></th>
<th>4GB (Muscle Region)</th>
<th>6GB (Tendon Region)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Std Dev</td>
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<tr>
<td>Tensile Modulus (MPa)</td>
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<td>0.59</td>
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<td>Stress (maximum load) (MPa)</td>
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<tr>
<td>Strain (maximum load) (%)</td>
<td>172.7</td>
<td>10.51</td>
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</table>
Figure 3.7.3  Stress-strain curves of PLA-KM2 degradable scaffolds.

The yellow-red groups are the King 1 muscle region specimens. The blue-purple groups are the D1 tendon regions. The two different regions of the PLA-KM2 scaffolds show distinctive stress-strain curves.
Figure 3.7.4 Stress-strain curves of the PLA-KM2 degradable scaffolds in comparison with human tissue.

The human tissues are identified with a black solid line. The stress-strain curves of the muscle and tendon regions of PLA-KM2 scaffolds lie in between the human tissue curves.
Figure 3.7.5  Stress-strain curves of the PLA-KM2 muscle region scaffold in comparison to natural porcine muscle tissue.
Top: stress scale bar is 0-10MPa; Bottom: stress scale bar is 0-0.2 MPa. The PLA-KM2 muscle region is stiffer than porcine muscle tissue.
Figure 3.7.6 Stress-strain curves of the PLA-KM2 tendon region scaffold in comparison to normal porcine tendon tissue. The PLA-KM2 tendon region is stiffer than porcine tendon tissue.
Figure 3.7.7 Stress-strain curves of the PLA-KM3 degradable scaffolds. The muscle region (4GB) and the tendon region (6GB) of the PLA-KM3 fabrics show distinctive stress-strain curves.
Figure 3.7.8  Stress-strain curves of the PLA-KM3 scaffold compared with porcine muscle and tendon tissue. The PLA-KM3 tendon region is stiffer than the porcine tendon tissue. The muscle region has a similar stress-strain curve to that of porcine muscle tissue.
In conclusion, the ultimate tensile properties of the scaffold PLA-KM2 are close to human muscle-tendon junction tissue, while the properties of the PLA-KM3 scaffold are closer to porcine muscle-tendon junction tissue. The different range of tensile properties between the two ends is determined by the knitting method, and can be tuned by the take-up speed.

**Conclusions for Chapter 3**

1) Designed warp knitted spacer fabrics that have a continuous multi-phase structure and mechanical gradients so as to serve as a tissue engineering scaffold for human muscle-tendon junction regeneration.

2) Designed and evaluated four different knitting methods to produce a continuous multi-phase design having distinct muscle and tendon regions on a commercial production machine.

3) Successfully prototyped 3D spacer fabric multi-phase structures from surrogate PET yarns using four different knitting methods. Evaluated the PET prototypes in terms of their WPI/CPI, thickness, total porosity and tensile properties include ultimate tensile stress, tensile strain and Young’s Modulus.

4) Selected the preferred knitting methods according to the production efficiency and prototype PET fabric properties.

5) Selected two of the PET knitting methods to successfully fabricate multiphase 3D spacer fabrics from PLA resorbable yarns having distinct muscle and tendon regions. Evaluated the PLA resorbable scaffolds in terms of their WPI/CPI, thickness, total porosity and tensile properties include ultimate tensile stress, tensile strain and Young’s Modulus in each region.

6) Made direct comparisons between the opposite ends of the PLA scaffolds with porcine muscle and tendon tissues to demonstrate similarities with fresh native tissues.
CHAPTER 4

REGENERATE MUSCLE-TENDON JUNCTION

4.1 Introduction

The specific aims for this research project are:

3) to study the cytocompatibility and cell proliferation of the two optimal scaffolds from Project 1 (Chapter 3) by culturing commercially available murine (mice) muscle and tendon cell lines separately on the different regions of the scaffold.

4) to study the formation of a muscle-tendon interface zone by co-culturing commercially available murine (mice) muscle and tendon cell lines in a cyclic stretching bioreactor developed at the Wake Forest Institute of Regenerative Medicine (WFIRM).

4.2 Materials

In order to develop the co-culture technology, prototype scaffolds were first knitted from surrogate PET yarns. After establishing the cell culture laboratory procedure for knitted textile scaffolds, then PLA degradable scaffolds were cultured. A PET-KM3 750% prototype and a PLA-KM3 1000% degradable scaffold were used for the initial two co-culture experiments. Both fabrics were sterilized in ethylene oxide prior to co-culturing, but they used different surface coating methods. PET used a collagen coating, while PLA used a hydrogel coating in order to prevent cell leakage during cell seeding.

Figure 4.2.1 Dimensions of PET and PLA spacer fabric samples in the dynamic bioreactor.
**Sterilization:** Ethylene oxide gas will be used as the sterilizing agent. It was released from a 20 cc ampoule inside the sterilizer bag, slowly diffusing out over a 12 hour sterilization period under ambient temperatures (approximately 70 °F).

### 4.3 PET Co-Culture

**Cell Expansion**

C2C12 myoblasts (muscle cells) + NIH 3T3 fibroblasts (tendon cells)

**Static Co-Culture**

Both cells were seeded on the scaffolds side by side for 2-4 weeks

**Dynamic Co-Culture**

The scaffolds were kept in the cyclic stretching bioreactor for 2 weeks of co-culture (1Hz, 10% Strain)

---

Figure 4.3.1 Steps in the dynamic co-culture on 3D spacer fabrics

**Cell lines:** Murine C2C12 myoblasts and NIH 3T3 fibroblasts (ATCC, Manassas, VA, USA).

**Collagen Coating:** Type 1 collagen from calf skin (Sigma-Aldrich, USA) was dissolved in a 0.1% (w/v) solution of acetic acid. The spacer fabric scaffolds were immersed in the collagen solution overnight and then dried at room temperature in a vented hood for 48 hours.

**Dynamic Co-culture:**

The co-culture process is illustrated in Figure 4.3.1. Initially murine myoblasts (C2C12) and fibroblasts (3T3) were co-cultured on the KM3 PET prototype scaffold (PET-KM3 750% 4/6GB) using static 6-well plates for 4 weeks. The seeding density was 2 million cells per well per sample. The medium was changed every other day.
Then the cultured samples were transferred to the WFIRM dynamic cyclic stretching bioreactor (Figure 4.3.2) for 2 weeks of dynamic culture. The gauge length between the clamps was 2.5 cm, and the medium was changed every other day.

The cyclic stretching bioreactor was kept under 95% air and 5% CO₂ at 37°C inside the incubator. The compromised differentiation medium was changed every other day. The cocultured scaffolds were then subjected to a 10% stretch and relaxation regime (2.5 mm) at 1 Hz.

Figure 4.3.2 The bioreactor consisting of an actuator mounted in a tissue culture biocompatible matchbox which is designed to provide controlled cyclic strain to the attached scaffolds. ⁶²

The constructs were supported between the two holders A and B. There was a removable lid, C, a single pass-through drive (D) consisting of a water resistant bearing and mounting hardware. The bioreactor contained vertical adjustments to the actuator between the mounting clamps E and F. The linear actuator, or stator (G) was controlled by a linear motor controller system (H) and a computer used for programming and subsequent monitoring of the actuator. The shaft (J) provided motion to the connector (E), which moved the horizontal piece A, and caused cyclic deformation of the constructs. The media surface line shown by K and the volume occupied by the seeded scaffolds is represented by the cylindrical spaces L. ⁶²
At the completion of the static and dynamic cell culture trial, biological tests involving q-PCR, routine histological analysis and scanning electron microscopy (SEM) were performed on the samples in order to examine their gene expression, the cell attachment and proliferation into the 3D PET scaffold. The sample specification and testing methods are listed in Table 4.3.1 and Table 4.3.2.

Table 4.3.1 Experimental design for PET sample co-culture

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
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<tbody>
<tr>
<td>Muscle fabric</td>
<td>Tendon fabric</td>
<td>Muscle Tendon</td>
</tr>
<tr>
<td>muscle region only</td>
<td>tendon region only</td>
<td>three-region scaffold</td>
</tr>
<tr>
<td>4GB</td>
<td>6GB</td>
<td>4GB + 6GB</td>
</tr>
<tr>
<td>KM3-750% 4GB</td>
<td>KM3-750% 6GB</td>
<td>KM3 750%</td>
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Table 4.3.2 Test methods and sample size used for the PET co-culture experiment

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<th>SEM</th>
<th>Histology</th>
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<tr>
<td>Day 42 (End of Dynamic Culture)</td>
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<td>2</td>
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</tbody>
</table>

4.4 **Testing Results of PET Co-culture**

**PCR Test:** After 28 days of static co-culture followed by 14 days of dynamic co-culture in the bioreactor, the scaffolds and their proliferating cells were exposed to a polymerase chain reaction (PCR), which identified the DNA sequence of the cells on the scaffolds. This was a key test
method to identify the differentiation of the interface cells that would provide a unique gene expression different from the tendon cells and the smooth muscle cells.

The confocal samples were preserved in 2.5% gluteraldehyde, the IHC samples were preserved in 4% paraformaldehyde, and the PCR samples were frozen at -80 degrees.

### 4.4.1 SEM Results of PET Co-culture

Figures 4.4.1, 4.4.2 and 4.4.3 show the scanning electron micrographs of the co-cultured fabric scaffolds after 4 weeks of static culture (control group) and 4 weeks of static plus 2 weeks of dynamic culture (test groups).

At the tendon end of the scaffolds Figure 4.4.1 illustrates the fibroblasts have successfully attached to the PET fibers under both static and dynamic conditions. The crossing points of the yarns and filaments attracted lots of cells that form confluent cell sheets. The cells in the dynamic group appear to be more aligned and have formed flat sheets between the yarns.

In the muscle region of the scaffolds (Figure 4.4.2), the myoblasts show more confluent attachment than did the fibroblasts. The fabric facilitated myoblast attachment and proliferation under both static and dynamic conditions. Myoblasts showed significant alignment in the direction of the external stimulus after 2 weeks of dynamic culture with cyclic stretching. The yarn loops guided cell proliferation and alignment. In the static group, the cells show a star-shaped morphology and aggregated into groups at the crossing points of yarns and fibers. In comparison, in the dynamic group, the cells extended along the yarn loops with a more uniform distribution and smoother sheet morphology.

At the interface between the muscle and tendon regions (Figure 4.4.3), the myoblasts and fibroblasts interacted with each other, and formed an integrated interface with inter-digitation, which followed the wavy shape of the warp loops. The PET scaffold was able to support the formation of a 3D cell interface structure under both static and dynamic culture conditions. Similar to the muscle region, the interface region showed a smooth and confluent cell morphology as well as significant alignment in the dynamic group.
Tendon region under static conditions          Tendon region under dynamic conditions

Figure 4.4.1 SEM micrographs of the 3T3 fibroblast morphology in the tendon region

Muscle region under static conditions          Muscle region under dynamic conditions

Figure 4.4.2 SEM micrographs of the C2C12 myoblast morphology in the muscle region
Figure 4.4.3 SEM micrographs showing the morphology of the fibroblasts and myoblasts at the interface region.
4.4.2 Histological Analysis of PET Co-culture

The cultured fabric samples were embedded in a paraffin block and then sectioned into 5µm thick slices using a diamond blade (Figure 4.4.4). The PET scaffold samples were sliced in the lengthwise or weftwise direction so that all three regions could be viewed in the same section. Images of the cross-sections of the fibers and cells were taken by a Nikon LABPHOT-POL optical microscope. After extracting the paraffin, the slides were stained by hematoxylin and eosin (H&E). (Figure 4.4.5)

![Figure 4.4.4 Sectional view of the cell cultured PET-KM3 prototype scaffold.](image)
Figure 4.4.5 Microscopic sectional views following H&E staining. The PET fibers appear clear and contain titanium dioxide particles. They are surrounded by cells, which are stained purple with dark purple nuclei. (Magnification 400X)

- **Dynamic Group**

Figure 4.4.6 Microscopic sectional views following H&E staining.

The PET scaffold in the dynamic group showed confluent cell sheets at the surface (red arrow). The cell density at the surface was higher than inside the 3D spacer fabric. The cells inside the fabric were isolated and evenly distributed throughout the thickness (Left image, x100 magnification). The cells at the surface were extended and appeared to connect the fibers. The
cell sheets were aligned in the direction of mechanical stimulation (Right image, x400 magnification).

- **Static Group**

![Image](image.png)

**Figure 4.4.7 Microscopic sectional views following H&E staining.**

The cells (red arrow) in the static group did not show a confluent sheet at the scaffold surface. The cells were evenly distributed between the surface and the inside layers. Collagen (blue arrows) was observed in the spaces between the fibers. Some cells aggregated into small groups where the fibers crossed over each other.

In both the dynamic and static groups, the cells proliferated through the entire thickness of the fabric scaffolds during their 6 weeks of culture (Figure 4.4.6 and Figure 4.4.7). In random areas the polyester fibers were observed to be associated with collagen (light purple) (Figure 4.4.8). The connected open pores of the fabric scaffold ensured that the cells penetrated through the thickness. The porosity or space inside the 3D scaffolds encouraged cell proliferation and migration in all three directions.
• Comparison

Figure 4.4.8 Microscopic sectional views following H&E staining. Confluent cell sheets (red arrows) can be seen along the entire length and on both sides of the scaffold surface in the dynamic group sample. Few cells were observed on the surface of the static group sample.

Compared to the inner spacer fabric layer, the surface structure was denser with smaller pores, which appear to promote cell attachment and confluence at the surface under dynamic culture conditions (Figure 4.4.8 and Figure 4.4.9). In Figure 4.4.9, the dynamic culture conditions enhanced cell proliferation at the surface of the scaffolds. The cells in the dynamic group show aligned morphology at the surface. Both static and dynamic groups showed isolated cells inside the fabric. But in the dynamic group, the presence of collagen (light purple) was observed together with more densely packed cells having an aligned appearance. The aligned yarn loops of the spacer fabric helped the cells to attach, stretch and pack together in a regular pattern, while the stimulus of dynamic stretching appeared to provide the mechanical signal to direct the cell alignment.
Dynamic Group

Static Group

Surface x100

Surface x100

Inside x100

Inside x100

Figure 4.4.9 Microscopic sectional views following H&E staining.

4.4.3 qPCR Results of PET Co-culture

Quantitative PCR analysis (q-PCR) was used to test for the presence of specific markers that are associated with the muscle-tendon junction. Each co-cultured specimen was cut into three parts: the muscle, tendon, and junction region. Four components of the RNA that are known to be specific markers for a muscle tendon junction (MTJ) (Vinculin, Talin1, Talin 2 and Paxillin) were isolated from the cells in each region.

Relative Expression: The junction marker concentration was normalized by the muscle region (Figure 4.4.10) and by the tendon region (Figure 4.4.11). The values of relative expression were
averaged from 6 separate specimens. If the MTJ specific marker had a higher expression in the junction region, then the relative expression was larger than 1 (the red dashed line in Figure 4.4.10 and Figure 4.4.11). Native mouse tissue was used as the positive control.

From the results in Figures 4.4.10 and 4.4.11, the four markers in the dynamic group samples showed higher levels of gene expression in comparison to the static group samples. In addition, the dynamic group values were closer to the level to native mouse tissue,

In conclusion, the use of in vitro dynamic cell culture demonstrated that viable muscle-tendon junction tissue was generated on the multiphase PET prototype scaffolds, as confirmed by gene expression levels close to those of natural murine muscle-tendon junction tissue.

![Graphs showing relative expression of MTJ markers](image1)

*Native: mouse MTJ tissue

Figure 4.4.10 MTJ specific marker expression normalized by muscle region (C2C12 cells).

![Graphs showing relative expression of MTJ markers](image2)

*Native: mouse MTJ tissue

Figure 4.4.11 MTJ specific marker expression normalized by tendon region (3T3 cells).
4.4.4 Conclusion of Dynamic Co-Culture Study on PET Fabric Scaffolds

The experimental results indicate that the polyester (PET) tri-phase spacer fabric prototype structure could serve as a tissue engineering scaffold for muscle-tendon junction regeneration. It could ensure that muscle and tendon cells proliferated and penetrated throughout the 3D spacer fabric as well as interacted at the muscle/tendon interface to form a viable and genetically appropriate junction tissue.

4.5 PLA Culture

Based on this PET experience, the co-culture experiment was modified to accommodate PLA scaffold material:

The laser scanning confocal microscope (LSCM) was introduced to exam the two different cell lines on the 3D scaffold.

The static culture period was shortened to 2 weeks.

In order to increase the cell density and prevent leakage at the time of cell seeding, the initial seeding density was increased to 4 million cells per sample. And a gelatin methacrylate GelMA hydrogel was introduced to encourage the cells to stay inside the scaffolds.

Table 4.5.1 Experimental plan for PLA co-culture samples

<table>
<thead>
<tr>
<th>Fluorescent Labeling</th>
<th>qPCR</th>
<th>SEM, Histology &amp; LSCM</th>
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</thead>
<tbody>
<tr>
<td>Dynamic culture</td>
<td>4 specimens x 3</td>
<td>3 specimens x 3</td>
</tr>
<tr>
<td>Static co-culture</td>
<td>4 specimens x 3</td>
<td>4 specimens x 3</td>
</tr>
<tr>
<td>Static muscle culture</td>
<td>4 specimens x 3</td>
<td></td>
</tr>
<tr>
<td>Static tendon culture</td>
<td>4 specimens x 3</td>
<td>3 specimens x 3</td>
</tr>
</tbody>
</table>
Samples of a 2D nonwoven spunbond PLA membrane were introduced in the PLA co-culture experiment so as to study the difference in cell morphology between a 2D and 3D environment. However, because the 2D spunbond PLA membrane was not elastic enough, it could not sustain the dynamic cyclic stretching in the WFIRM bioreactor.

Samples:

3D HBD: 3D hybrid structure (PLA-KM3 1000% 4/6GB) dynamic group
3D HBS: 3D hybrid structure (PLA-KM3 1000% 4/6GB) static group
3D PD: 3D pure structure (PLA-KM3 1000% 4GB) dynamic group
3D PS: 3D pure structure (PLA-KM3 1000% 4GB) static group
2D S: 2D structure spunbond nonwoven PLA fabric, (30 g/mm²) static group

Table 4.5.2 Test methods and sample size used for the PLA co-culture experiment

<table>
<thead>
<tr>
<th>Tests</th>
<th>qPCR</th>
<th>SEM</th>
<th>Histology</th>
<th>LSCM</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Specimens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14 (End of Static Culture)</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Day 28 (End of Dynamic Culture)</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Cell expansion:

Murine C2C12 myoblasts and NIH 3T3 fibroblasts were expanded using cell factory for 4 weeks in order to reach the desired cell population. Both cell lines used the same compromised growth medium (GM) as used previously in the static culture experiment.

Synthesis of gelatin methacrylate (GelMA):

Gelatin (Type A, bloom 300g, Sigma) was functionalized by methacrylic anhydride. Briefly, gelatin was completely dissolved in PBS buffer (pH 7.4, 1X) at 60°C. At 50°C, methacrylic
anhydride (Sigma, USA) was added to the gelatin solution with vigorous stirring for 1 h. The reaction mixture was diluted 5 times by PBS buffer (pH 7.4, 1X), following by dialysis for 3 days in distilled water at 40°C. After dialysis, the GelMA was freeze-dried and stored at 4°C until used.

**Hydrogel preparation:**

The GelMA was dissolved in DMEM medium (3-10 % w/v) containing 0.1-0.2% (w/v) of photoinitiator 2959 (Sigma, USA) at 40°C. The mixture was then cross-linked by exposing it to UV light for 120 s so as to form the hydrogel.

**Cell Seeding and co-culture:**

The co-culture process is illustrated in Figure 4.3.1 Steps in the dynamic co-culture on The cell seeding density was increased to 4 million cells/cell type/sample. The cells were mixed with the GelMA medium mixture, seeded onto the PLA scaffolds and sealed in the petri dishes without adding extra medium. Then the samples in the petri dishes were exposed to UV light for 120 s to form the hydrogel. After the transparent hydrogel was formed, growth medium was added into the petri dishes. All the samples were transferred to the incubator for 14 days of static co-culture followed by 14 days of dynamic co-culture. At the end of the static and dynamic cell culture periods (Day 14 and Day 28), biological tests (q-PCR), routine histological analysis, scanning electron microscopy (SEM) and laser scanning confocal microscope (LSCM) were performed on the samples. It was necessary to examine the samples for gene expression, cell proliferation and cell penetration into the 3D PLA degradable scaffold. The planned test methods and sample size are listed in Table 4.5.2.

### 4.6 Test Results of PLA Co-culture

#### 4.6.1 SEM Results

Table 4.6.1 shows the SEM micrograph images of the 2D nonwoven membrane at Day 28. The nonwoven structure is flat and isotropic. The surface is smoother than the 3D spacer fabric, and
the average pore size is much smaller. The small pores promote cell attachment after seeding, but limit the extent of cell penetration through the thickness of the membrane later. The isotropic surface structure did not provide any direct guidance for cell alignment. As a result, the cells on the 2D nonwoven membrane were randomly distributed without any orientation.

In the contrast, the 3D PLA spacer fabric scaffold showed cell alignment without dynamic cyclic stretching stimulation. The 3D pure structure (Table 4.6.2) and hybrid structure (Table 4.6.3) took advantage of the yarn orientation to guide the cell alignment in both the tendon and muscle sections. Since the pure structure only contained the muscle region fabric structure which was more porous than the tendon region fabric, the cell density and morphology of the tendon cells appeared to be different for the pure structure under static culture. The tendon region of the hybrid structure showed more rapid cell confluency and the cells were elongated.

Table 4.6.1  SEM micrograph images of 2D nonwoven membrane after static culture for 28 days

<table>
<thead>
<tr>
<th>2D S</th>
<th>Muscle</th>
<th>MTJ</th>
<th>Tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>110 x</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>550 x</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Table 4.6.2 SEM micrograph images of 3D hybrid structure (PLA-KM3 1000% 4/6GB) after static culture for 28 days

<table>
<thead>
<tr>
<th>3D HBS</th>
<th>Muscle</th>
<th>MTJ</th>
<th>Tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>110x</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>550x</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
</tbody>
</table>

Table 4.6.3 SEM micrograph images of 3D pure structure (PLA-KM3 1000% 4GB) after static culture for 28 days

<table>
<thead>
<tr>
<th>3D PS</th>
<th>Muscle</th>
<th>MTJ</th>
<th>Tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>110 x</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>550 x</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
</tbody>
</table>
Table 4.6.4 and Table 4.6.5 list the 3D pure and hybrid structure scaffolds after dynamic co-culture. Both the muscle and tendon cells form three dimensional tissues within the knitted spacer fabric under conventional static culture conditions. However, note that the cells alone are not able to support any mechanical stimulation.

With the application of dynamic stimulation, the fabric loops guide cell alignment in the direction of the applied external force. The knitted loop structure at the muscle-tendon junction interface helps the muscle and tendon cells to interact in three dimensions and generate the unique interwoven morphology of the load-bearing junction tissue. The yarn loops provide an elastic substrate so that the attached cells can experience the stretching and contraction under cyclic mechanical stimulus. The loops are able to maintain the volume of the tissue and prevent the tissue from collapsing under tensile and compressive forces.

Both muscle and tendon cells show alignment in the direction of mechanical loading, which indicates that in vitro cyclic mechanical stimulation successfully trains the cells to develop a more natural morphology.
Table 4.6.4  SEM micrograph images of 3D pure structure (PLA-KM3 1000% 4GB) after
dynamic culture at Day 28

<table>
<thead>
<tr>
<th>3D PD</th>
<th>Muscle</th>
<th>MTJ</th>
<th>Tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>110x</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>550x</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Table 4.6.5  SEM micrograph images of 3D hybrid structure (PLA-KM3 1000% 4/6GB) after
dynamic culture at Day 28

<table>
<thead>
<tr>
<th>3D HBD</th>
<th>Muscle</th>
<th>MTJ</th>
<th>Tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>110x</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>550x</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Table 4.6.6  Comparison SEM micrograph images of 2D nonwoven membrane after static culture for 28 days, with the 3D hybrid structure (PLA-KM3 1000% 4/6GB) after dynamic culture also after 28 days

Table 4.6.6 compares the cell morphology generated on the 2D and the 3D scaffolds, and shows that the cell alignment is significantly different. There was no preferred orientation of the cells on the 2D nonwoven membrane. However, the cells on the 3D knitted fabric were oriented along the yarns, which meant that the loop structure was able to manipulate the cell alignment and generate tissue texture.

Table 4.6.7  SEM micrograph images of the 3D hybrid structure (PLA-KM3 1000% 4/6GB) and 3D pure structure (PLA-KM3 1000% 4GB) after dynamic culture for 28 days
In Table 4.6.7 both the pure and hybrid 3D spacer fabric scaffolds are able to form three dimensional tissues within the knitted spacer fabric under dynamic culture conditions. The hybrid structure shows denser cell growth inside the yarn bundles, whereas the cell orientation and migration was more obvious in the hybrid structure. The distinct tri-phase scaffold structure was able to distinguish between the level of local strain in the muscle and tendon regions. This may have contributed to the interlocked 3D tissue interface in the junction region.

Table 4.6.8 SEM micrograph images of the 3D hybrid structure (PLA-KM3 1000% 4/6GB) after static culture and the 3D pure structure (PLA-KM3 1000% 4GB) after dynamic culture for 28 days

<table>
<thead>
<tr>
<th>3D HBS MTJ</th>
<th>3D HBD MTJ</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="SEM Image" /></td>
<td><img src="image2.png" alt="SEM Image" /></td>
</tr>
</tbody>
</table>

Table 4.6.8 compares the same hybrid structure after being co-cultured under different conditions. The cells migrated along the yarns and formed a 3D network for both samples. But any preferred cell alignment is found in the stretching direction after dynamic cyclic stimulation. More cells appeared to proliferate towards confluence in the dynamic group. Whereas the degree of confluence was limited among the cells in the static group where they tended to aggregate in circular clusters.
4.6.2 Laser Scanning Confocal Microscopy (LSCM) Results

Because of the very narrow depth of field it is difficult to view the distribution of cells on non-transparent 3D textile structures using an optical microscope. An alternative microscopic approach is SEM, which has a greater depth of field, but the limitation is that it only scans the surface structure. In order to examine the cell penetration through the thickness of the scaffold, routine histology has been widely used. However, the sample must be exposed to dehydration, embedding and sectioning, which can alter the cell morphology. Therefore, LSCM was introduced to apply a laser scan to the inside of the 3D non-transparent scaffold and generate a series of compact images and 3D movies through the thickness of the construct.

Introducing one or more synthetic polymers and scanning them on a large scale (20 mm$^3$), adds to the complication of LSCM technology. In addition, the synthetic polymers must be distinguishable from the two different cell types (C2C12 and 3T3), which makes the situation unique and more complex. Chapter 5 explains how my innovation in LSCM technology and multiple fluorescent labeling adds to the versatility of the technique.

A Zeiss LSM 710 (Zeiss, Germany) laser scanning confocal microscope was used to scan the images. In order to distinguish between the two cell lines, the cells were labelled with Vybrant® Multicolor Cell-Labeling Kit DiI and DiD Solutions (Invitrogen™, ThermoFisher Scientific, USA) during cell expansion. The C2C12 myoblasts were labeled with a fluorescent nucleus stain DiD solution and 3T3 fibroblasts were labeled with Dil solution. PLA yarns are not fluorescent so they appear transparent under the laser microscope. However, due to reflection, the PLA yarns did show the color of the neighboring fluorescent components.

Figure 4.6.1 shows the 3D hybrid structure (PLA-KM3-1000% 4/6GB) after dynamic culture for 28 days. The 3T3 tendon cells are shown in pink and yellow, marked by the pink and yellow arrows. The C2C12 muscle cells are in green, marked with green arrows. The PET yarns were knitted as indicator yarns at the interface region because the PET is auto-fluorescent, showing a greenish color, a similar color to the C2C12 cells. The PLA yarns were transparent but reflected the pink color of the 3T3 cells because 3T3 cells grew inside the yarn bundle in high density.
There are several imaging processing modes that are provided by the ZEN software, which was supplied with the Zeiss LSM 710 confocal microscope. The 3D Maximum mode and 3D Surface mode are both used to generate combined 3D images from the series of 2D scans. The 3D Maximum mode allowed an inside view to be shown while the 3D Surface mode provided surface illustration similar to SEM, but with colors. Since the PLA yarns were transparent, both modes were used to provide more information.

Figure 4.6.1 An example of multiple fluorescent labelling. The sample is 3D PLA-KM3-1000% 4/6GB with a PET interface indicator yarn, cultured with Dil labeled 3T3 cells and DiD labeled C2C12 cells.
The 2D nonwoven membrane did not support cell penetration through the thickness as shown in Table 4.6.9 both muscle C2C12 cells and 3T3 cells showed an isolated circular shape, meaning that the 3D network between the cells was weak. There was a dark line shown at the interface region, suggesting the junction tissue growth inside the membrane was limited.

Table 4.6.10 makes a comparison between the different 3D structures which show cell growth inside the yarns. 3T3 tendon cells tend to stay at the surface and form cell clusters (marked with red circles) in the static group. The cell distribution is more uniform in the dynamic group. The hybrid structure had the least number of cell clusters, which could be due to the localized staining. The pure structure did not have the tendon region; and the muscle region tensile data did not support the 3T3 cells to elongate.

Table 4.6.9  LSCM images of 2D nonwoven membrane after static co-culture for 28 days.

<table>
<thead>
<tr>
<th>2D S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image processing mode: 3D Maximum</td>
</tr>
<tr>
<td>Image processing mode: 3D Surface</td>
</tr>
</tbody>
</table>

![LSCM images of 2D nonwoven membrane after static co-culture for 28 days.](image)
Table 4.6.10 LSCM images of 3D spacer fabric scaffolds after co-culturing for 28 days.

<table>
<thead>
<tr>
<th>3D HBD</th>
<th>3D HBS</th>
<th>3D PD</th>
<th>3DPS</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image processing mode: 3D Maximum" /></td>
<td><img src="image2.png" alt="Image processing mode: 3D Maximum" /></td>
<td><img src="image3.png" alt="Image processing mode: 3D Maximum" /></td>
<td><img src="image4.png" alt="Image processing mode: 3D Maximum" /></td>
</tr>
<tr>
<td><img src="image5.png" alt="Image processing mode: 3D Surface" /></td>
<td><img src="image6.png" alt="Image processing mode: 3D Surface" /></td>
<td><img src="image7.png" alt="Image processing mode: 3D Surface" /></td>
<td><img src="image8.png" alt="Image processing mode: 3D Surface" /></td>
</tr>
</tbody>
</table>
4.6.3 qPCR Results

The qPCR analysis method has been described in Section 4.4.3. The junction marker concentration was normalized separately either by the muscle region (Figure 4.6.2) or by the tendon region (Figure 4.6.3). The relative expression values were averaged from 2 separate specimens. If the MTJ specific marker had a higher expression in the junction region, then the relative expression was larger than 1, which is represented by the red dashed line in Figure 4.6.2 and Figure 4.6.3. Native mouse tissue was used as the positive control.

In Figure 4.6.2, the 3D PLA degradable scaffold showed a higher expression of the MTJ specific markers compared to natural mice MTJ tissue, regardless of whether it was under static or dynamic co-culture conditions. The dynamic group (3D Dynamic Hybrid and 3D Dynamic Pure) also showed a relative expression higher than 1, and in fact showed the highest expression of Paxillin and Vinculin. The 3D Dynamic hybrid gave the highest expression of all four MTJ specific markers, while the 3D Pure structure gave the second highest expression of Talin 1 and 2 for both static and dynamic conditions. The 2D nonwoven structure showed the lowest gene expression markers, which was close to or lower than the mice MTJ tissue.

In Figure 4.6.3, the mice MTJ tissue gave the highest MTJ specific marker expression, while the 2D nonwoven membrane gave the lowest value which was less than the relative expression of 1.

In conclusion, the use of in vitro dynamic cell culture has demonstrated that muscle tendon junction tissue was generated on the multiphase PLA degradable scaffold. The PLA scaffold improved the gene expression of the co-culture in 3D compared to the PET prototypes. The 2D nonwoven membrane was not able to form a true viable MTJ junction under static conditions. The 3D PLA degradable scaffold improved the junction formation in comparison with the 2D nonwoven structure. But the maximum amount of relative MTJ specific marker expression was not achieved until the 3D scaffold experienced stimulation with cyclic stretching conditions.
Figure 4.6.2 MTJ specific marker expression normalized by the muscle region (C2C12 cells)

Figure 4.6.3 MTJ specific marker expression normalized by the tendon region (3T3 cells)
4.7 Conclusions

Based on the two co-culture experiments with the PET surrogate prototype and the PLA degradable scaffolds, it has been demonstrated that the 3D multiple phase spacer fabric structure is suitable for muscle tendon junction regeneration. The co-culture approach developed for the 3D multiple phase spacer fabric is efficient in encouraging cell proliferation, migration and penetration as well as generating viable junction tissue formation in the interface region.

Particularly:

1) **The tri-phase spacer fabric satisfied the structural requirements of an MTJ scaffold.**

   The tri-phase muscle and tendon prototype structure supports muscle and tendon cell proliferation and the formation of a confluent tissue layer. The orientated spacer yarns facilitate cell attachment and penetration through the thickness. The open porosity between the yarns enables cell connections in three dimensions, and promotes the uniform distribution of cells throughout the spacer fabric. With the application of dynamic stimulation, the fabric loops guided cell alignment in the direction of the applied external force. Oriented and aligned cell morphology is essential for muscle and tendon tissues to function mechanically. It is also critical for the regeneration of an engineered muscle-tendon junction. The knitted loop structure at the muscle-tendon junction interface helped the muscle and tendon cells to interact in three dimensions and generate the unique interwoven morphology of the load-bearing junction tissue.

   Moreover, the distinct pore size distribution between the two outer surfaces and the middle layer of the spacer knitted structure results in different levels of cell attachment and proliferation. The pores on the surface of the fabric are smaller than the spacer layer, which promotes cell confluence at the fabric surface. In another words, by creating a distinct pore size gradient within the thickness of the fabric, the rate of cell proliferation and cell confluence, as well as the location of cell clusters can be manipulated.

2) **The elastic spacer fabric provides mechanical support for MTJ regeneration.**

   Both the muscle and tendon cells were able to form three dimensional tissues within the knitted spacer fabric under conventional static culture conditions. However the cells alone
were not able to support the mechanical stimulus. The yarn loops provided an elastic substrate so that the attached cells could experience the stretching and contraction under typical cyclic mechanical stimulation. Also, the loops were able to maintain the volume of the tissue and prevent the tissue from collapsing under tensile and compressive forces. Moreover, the unique multiple phase scaffold structure was able to distinguish between the level of local strain within the muscle and tendon regions. Muscle cells showed better alignment than tendon cells. This supports our hypothesis that regional variations in tensile properties within the scaffold results in different cell morphologies when cultured under mechanical stimulation.

3) **The PET and PLA fabrics are non-cytotoxic.**

Both muscle and tendon cells have been found to proliferate on the knitted polyester spacer prototype fabrics after 4-6 weeks of *in vitro* culture conditions. New and unique junction tissue has been created on both the PET and PLA scaffolds at the muscle tendon interface, confirming that both the muscle and tendon cells were viable and functioning.

4) **A cyclic stretching stimulus promotes MTJ development on spacer fabric scaffolds.**

The cyclic dynamic stretching stimulus (frequency 1Hz, strain 10%) that was applied in this MTJ developmental study has proven to be effective. The gene expression of the cells at the MTJ interface was enhanced and found to be closer to that of natural tissue compared to the MTJ tissue cultured under static conditions. Both muscle and tendon cells showed preferred alignment in the direction of mechanical loading, which indicates that an *in vitro* cyclic mechanical stimulus successfully trained the cells to develop a more natural morphology.

In summary, the regeneration of an MTJ using PET and PLA spacer fabric scaffolds has been successfully achieved with 3D multiphase warp knitted spacer fabric constructs. The scaffold was purposely designed to have regional variations in structural and mechanical properties, and has proven to be an attractive candidate to serve as a tissue engineering scaffold for a muscle tendon junction. The cyclic stretching bioreactor system provided the essential dynamic mechanical stimulation for the regeneration of a functional muscle tendon junction using the tri-phase prototype scaffolds.
5.1 Introduction

Various types of microscopic technology are essential for observing cell morphology during tissue engineering studies. For tissue engineering applications involving 3D textile scaffolds, it is challenging to view non-transparent fibrous scaffolds containing cells.

Figure 5.1.1 Compound optical microscopic images of cell cultured 3D spacer fabric.

The optical compound microscope is not a good option, because it uses visible light which is blocked by the reflective fibers in the scaffold and creates a black field (Figure 5.1.1). In Figure 5.1.1, the compound optical microscopic cannot create clear images of cellular structure within 3D spacer fabric scaffold. Because the limited view depth can only focus on portion of the structure (left) and the non-transparent structure will block the light, and create a black field (right).

Another commonly used microscopy is scanning electron microscopy (SEM), also used for this study, but despite its increased depth of field, the major limitation with SEM is that it only scans the surface structure. In order to examine the cell penetration through the thickness, histology has been widely used. However, the sample must go through dehydration, embedding and sectioning
which may alter the cell morphology. As a result laser scanning confocal microscopy (LSCM) has been introduced so as to scan a series of thin sections inside of the 3D non-transparent sample and generate compact images and movies in 3D.

However, LSCM alone is not enough to identify, distinguish and measure multiple types of cells on a 3D scaffold. It involves multiple color fluorescent stains in order to label and distinguish more than one cell type using difference fluorescent spectra. However, the existence of PLA yarns makes the process difficult. First, the PLA yarns are wavy in 3D loops which increase the scanning depth and field area for the laser. However, increasing the view depth will decrease the laser energy, which leads to a weak signal. Second, the PLA is not fluorescent. It has to be either dyed by fluorophores or mixed with other auto-fluorescent polymers to be detectable by the laser. Third, the precision and resolution of the scanning should be at the cell scale (10-100µm), while the scanning area and laser penetration depth should fit within the fabric scale (at least 1mm width x 10 length mm x 1 mm height). Fourth, the combination of fluorophores for the cells and the PLA yarns need to have minimal overlap in absorption and emission spectra. If the wavelength overlaps too much, the cross excitation and emission bleed into each other, masking several components within the same spectra. In other words, the peaks cannot be distinguished.

In order to answer the above challenges, the specific aim of this chapter was to develop an analytical approach to use multiple fluorescent labeling kits, auto-fluorescent fibers and a laser confocal microscope to view and measure the cellular activities of multiple cell types on and in a 3D subtract.

The key to distinguishing the different components under a confocal microscope is 1) to label each component with fluorescence that gives a separate emission and excitation spectra and 2) to collect spectra separately by laser tracking. In order to visualize moving structures with multiple stains simultaneously, it is necessary to choose fluorophores with minimal overlap in their absorption and emission spectra. In addition, sequential imaging can be used in order to reduce cross excitation and emission bleed.
5.2 3D imaging using LSCM

**Microscopy settings:** A Zeiss LSM 710 Confocal Microscope (with UV laser and 458, 488, 514, 561, 633nm laser units) is shown in Figure 5.1.2. The software for image capture and processing used ZEN 2011 software (Zeiss, Germany). The scanning set up was one track using DAPI (415-553 nm), Dil (562-631 nm) and DiD (637-725 nm) emission detectors. An example of the scanning setting on ZEN software is illustrated in Figure 5.1.3.

![Zeiss LSM 710 Confocal Microscopy](image)

**Figure 5.2.1** Zeiss LSM 710 Confocal Microscopy

![An example of scanning setting on ZEN 2011 software](image)

**Figure 5.2.2** An example of scanning setting on ZEN 2011 software
Since the spacer fabric scaffold was a 3D structure, Z-stack scanning method (Figure 5.2.3) was used to combine a series of 2D scanned images into a 3D image and video. The scanning area under the the maximum view was about 1mm x 1mm. Therefore Tile scanning method (Figure 5.2.4) was used to add several scanning areas together to create an overview of the spacer fabric dimensions of 2mm x 10mm of dimension. The combination of the Z-stack and the Tile scanning method (Figure 5.2.5) was able to create a 3D overview of the scaffold.
Figure 5.2.6 Sixty nine images of 2D scans through the thickness direction (Z-stack) of PET-KM3 500% 4GB spacer fabric.

It was cultured with C2C12 myoblasts (380,000 seeding density) for 3 days. Before microscopic imaging, the sample was fixed and dyed with 4',6-diamidino-2-phenylindole (DAPI) fluorescent DNA stain. The emission channel is DAPI (415-553 nm).

In order to create 3D images of scaffold, a series of 2D scans through the thickness direction (Z-stack scanning) were combined together. Figure 5.2.6 shows an example of a series of Z-stack scanned images. The thickness interval between each section is adjustable. The viewing depth in each thickness direction is up to 500 µm. The color in the image is selected by the user. Different emission spectra can be coded by different colors.

The series of Z-stack scanned images was combined into a 3D image (Figure 5.2.7). The 3D image can be rotate at different angle. The “Depth Code” function (Figure 5.2.8) is useful to mark the 3D morphology for the scaffold. Different colors were used to label the depth of view: red is close to readers and blue is further away. The view depth (Z-direction) was 400µm, about
half the width of the wale, and the Y-axis was 850 µm, about a quarter of the fabric thickness. With the depth color coding, the cell penetration was confirmed by the uniform distribution of cells in three dimensions.

Figure 5.2.7. 3D combined image with 69 Z-stack sections from Figure 5.2.6 using Zeiss 2011 software.

Figure 5.2.8. 3D combined image of spacer yarns with depth coding colors. The XY plane is the cross-section of the fabric. The Unit is micron meter. The rainbow color along the Z-axis indicates the depth of view, red for closed to and blue for farther away.
5.3 **Design labeling methods for cell co-cultured PLA scaffolds.**

PLA is transparent but not fluorescent. On the other hand, PET is auto fluorescent with emission and excitation spectra similar to DAPI stain (4’,6-diamidino-2-phenylindole). Therefore, one thread of PET yarn (UNIFI, USA) was knitted as the indicator between the muscle and tendon zones.

There are commercial available cell labeling kits which can individually label the protein/antibody or nuclei of different cells. A nucleus stain kit: Vybrant® Multicolor Cell-Labeling Kit (Invitrogen™, ThermoFisher Scientific) has been reported to stain C2C12 myoblasts and 3T3 fibroblasts. In this study, the C2C12 myoblasts were labeled by DiD solution and the 3T3 fibroblasts by Dil solution.

![Spectral wavelength distributions](image)

Figure 5.3.1 The emission (dotted line) and excitation (solid line) spectral wavelength distributions of auto-fluorescent PET polymer, Dil and DiD stain.

In Figure 5.3.1, the auto- fluorescent PET yarns and cell labelling stain Dil (for fibroblasts 3T3) and DiD (for myoblasts C2C12). The area color is not the reflecting color on the scanning images. The confocal microscope software ZEN has the function to freely code any wavelength in a color selected by the user.
Figure 5.2.7 shows PET and DAPI stained C2C12 cells. This image gives an example of the scaffold and the stained cells being excited at the same spectral wavelength. The cells and fibers that are present in the same channel cannot be analyzed separately.

Figure 5.3.2 shows the 3D combined images with multiple fluorescent colors. The 3T3 fibroblasts (pink and yellow) can be distinguished with C2C12 myoblasts (green). Figure 5.3.3 uses two 3D image processing modes 3D Maximum and 3D Surface to provide a 3D visualization of the PET and PLA yarns. For the non-fluorescent polymer PLA, the 3D Surface mode makes it “visible”, while the 3D Maximum mode (Figure 5.3.2, right) provides more information about the surrounding cell morphology.

Figure 5.3.2 The 3D combined image of C2C12 and 3T3 co-cultured spacer fabric scaffolds using Vybrant® Multicolor Cell-Labeling Kit.

The PET polymer is auto-fluorescent (left) and the PLA is not fluorescent and reflect the cells’ color (right).
Figure 5.3.3 Three dimensional combined image of PLA-KM3 1000% dynamic cultured Day 28 scaffold in 3D Maximum mode (left) and 3D Surface mode (right). The non-fluorescent PLA yarns (pink) show more clearly visible in 3D Surface mode.

5.4 Steps to generate a 3D image of multiple cell co-cultured spacer fabric scaffold

As mentioned above, a unique scanning confocal microscopic methodology has been developed that combines multiple cell labeling, a fluorescent polymer indicator (auto-fluorescent PET) and a 3D scanning method using Tile and Z-stacking.

This 3D scanning method involves the following steps:

Step 1, one channel of 2D scanning

Step 2, three channels of 2D scanning
Step 3, three channels of 2D Tile Scanning

Step 4, Z-stack of 2D Tiles.
5.5 Conclusion

A new 3D microscopic visualization methodology has been developed that uses a multiple fluorescent labeling kit, auto-fluorescent fibers and laser confocal microscopic Tile and Z-stack scanning methods to view and measure the cellular activities of multiple cell types on and throughout 3D spacer fabrics. It can be applied to any polymeric, fibrous and textile scaffold in 3D. By changing the labelling kit, different cell functions can be monitored. For example, by using fluorescent antibodies, the specific cell activity, such as primary cell differentiation, can be observed within a 3D scaffold. This methodology avoids fixation, embedding and sectioning of cultured samples and preserves the original cell morphology to the greatest extent. By using non-toxic fluorescent labeling, such as the alamarBlue® assay, viable cell activity can be captured in situ in 3D. This combined methodology makes it easier to observe interactions at cell-to-cell and cell-to-scaffold interfaces.
CHAPTER 6

DEVELOPMENT OF A 3D DYNAMIC BIOREACTOR

6.1 Introduction

The bioreactor that developed by Wake Forest Institute of Regenerative Medicine (WFIRM) was used in Project 2 for developing a composite multi-phase muscle tendon tissue junction (MTJ). And during that project there were issues, such as an overheating motor, unreliable operation, insufficient energy output and contamination, that made it unsuitable for applications involving textile based scaffolds. Therefore, it was necessary to develop a new dynamic bioreactor that was small, convenient to operate and functional to provide cyclic stretching stimulation to a textile based scaffold structure.

Figure 6.1.1 Left: the WFIRM bioreactor. Right: the NCSU MTJ bioreactor

The specific aims were:

1) to develop a 3D dynamic bioreactor with cyclic stretching and recovery mechanical stimulation for muscle-tendon junction regeneration

2) to evaluate bioreactor function by co-culturing muscle and tendon cells on the 3D scaffold and evaluating the engineered junction tissue.
6.2 Review of WFIRM Bioreactor:

The WFIRM bioreactor was used in Project 2 to provide cyclic stretching and recovery force to the scaffolds during cell culture. However, since the bioreactor was initially designed for dynamic muscle cell culture on a thin electrospun web there were several disadvantages when trying to apply it to serve for MTJ dynamic culture using a spacer fabric scaffold.

The following section will go through the components, materials and assembly methods of the WFIRM bioreactor, and will then discuss its advantages and disadvantages.

6.2.1 Components of WFIRM bioreactor

![Block diagram of WFIRM’s cyclic stretching bioreactor circuit](image)

Figure 6.2.1 Block diagram of WFIRM’s cyclic stretching bioreactor circuit

The bioreactor system (Figure 6.2.1) consists of a controller that connects to a computer (Figure 6.2.2 C-E), a power supply and switch (Figure 6.2.2 A and B), a bioreactor (Figure 6.2.3 A and B) and a linear motor actuator called a “Stator” (Figure 6.2.3 C-E).

In Figure 6.2.2 the WFIRM’s bioreactor usually works inside a sealed incubator. Due to its large size, the LinMot controller is left outside the incubator, while the actuator is attached to the
bioreactor and kept inside the incubator. The linear actuator (referred to as a “Stator”) is controlled by a linear motor controller system and computer which is used for programming and subsequent monitoring of the actuator. One controller can control two actuators having different protocols. The power supply of the controller is controlled by an on-off switch.

Figure 2 shows the parts of the control system.

![Figure 2: Parts of the control system](image)

Figure 6.2.2 System controls: A) power switch, B) power supply for motor, C) LinMot controller, D) RS 232 computer connector and E) serial connectors to LinMot stator actuators.

The bioreactor includes an actuator mounted on a tissue culture-compatible box, which is designed to provide controlled cyclic strain to the attached scaffolds or other tissue constructs.

Figure 6.2.3 shows the bioreactor box with a removable lid and the actuator. The bioreactor box is white and made from a single block of Teflon® (polytetrafluoroethylene) resin. The box lid is transparent and made from acrylic sheet material (e.g. Plexiglass). The reciprocating stretching function is implemented by the actuator, which pushes and pulls the slider. The slider provides the motion to the support connector inside the box, which by moving one sample clamp transfers the deformation to the clamped scaffolds. There are two strip clamps with holes inside the box. One is attached to the slider, and the other is fixed on the opposite wall. The strip clamps contain 8 holes along their length, which means that the bioreactor can theoretically hold and stretch up to 8 scaffolds simultaneously.
Method of Assembly of WFIRM Bioreactor

Step 1: Slide the magnetic slider through the hole in the bioreactor box. Attach the holder for the magnetic slider to the bioreactor box.
Step 2: Apply LinMot lubricant to the holder so as to reduce friction. Fasten the holder with the O-rings, washer and nut in the specified order.

Step 3: Attach the removable clamp (B) to the magnetic slider with the Allen screw (A). Adjust the height of the screw in the vertical slot on the removable clamp to match the other clamp that is fixed to the inside wall of the bioreactor box.

Step 4: Sterilize the box and lid using ethylene oxide gas followed by aeration.

Step 5: Clamp the cell seeded scaffolds inside the bioreactor box and cover with the lid.
Step 6: Place the actuator holding bracket and bioreactor box inside the incubator. In order to maintain a sealed environment inside the incubator, the power cords to the actuator should pass through the back of the incubator, not through the front door. Attach the actuator holding bracket to the box, and attach the actuator to this holding bracket.

Step 7: Set the computer software to start the actuator and generate reciprocating (cyclic) motion in the bioreactor.

6.2.3 Advantage and Disadvantage of WFIRM Bioreactor

Advantages:

1) The motion can be programmed in the computer connector. The program file could be modified according to application.

2) Bioreactor box can hold 500ml medium, which can provide the efficient amount of medium for large cell population on multiple samples.

Disadvantage:

1) Sealing

The sealing between the acrylic cap and PTFE box is loose, and operation is likely to introduce non-sterilized air inside the chamber and lead to contamination.

The magnetic slider that stretches the sample cyclically is able to move inside and out of the bioreactor box. The sealing of the holder relies on a rubber O-ring around the hole. The ageing of the rubber is observed to cause sealing problems and contamination issues. Introduction of lubricant into the bioreactor box

2) Introduction of lubricant into the bioreactor box

The application of a lubricant to the LinMot actuator and rod is helpful in reducing friction. However, the pushing motion is likely to introduce lubricant into the box, which will contaminate the cell culturing environment.

3) “Non-linear” movement of the linear actuator
The cyclic stretching and recovery movement is determined by the linear actuator. However, the movement is also influenced by the lubricant, the strength and rigidity of the scaffold sample and the way in which the magnet holder is fastened. Non-linear motion occurs when the lubricant runs out or the magnet holder is held too tightly.

4) Large size needs bench space

The control system takes up a lot of bench space next to the incubator. Also, the total length for a pair of linear actuators inside the bioreactor box is 40-50 cm. This leaves limited space to manoeuvre the bioreactor box and the stator inside the incubator.

5) Poor durability after sterilization.

The box was created from a single block of white PTFE. This ensured that there were no leaks or sealing problems along the sides and edges during the extended periods of cell culture. In order to operate easily during handling and sealing, an acrylic resin lid was used as the transparent cover. However, the acrylic sheet became brittle after sterilization in ethylene oxide or UV radiation. In fact the cracking of the cover reduced the durability of the box.

6.3 Development of MTJ Bioreactor System

6.3.1 Components of MTJ Bioreactor System:

In order to improve the bioreactor performance, a more compacted bioreactor system has been designed. The NCSU MTJ bioreactor system (Figure 6.3.1) consists of multiple bioreactor plates with a single motor and a power cord with a branched connector.
Unlike the WFIRM bioreactor, the MTJ bioreactor doesn’t require any space outside the incubator. The majority of the parts are to be installed inside the incubator, leaving only the power control system outside.

Thanks to the small size of the MTJ bioreactor plates, it is easy to put 6-8 plates on top of each other on one shelf in the incubator. In addition, the MTJ bioreactor leaves screw holes at the four corners (Figure 6.3.2), which allow the plates to be stacked on top of each other. Stacking plates can also save shelf space.
Figure 6.3.2 The NCSU MTJ bioreactor plate consists of a plate and the main disc in a 100 ml petri dish and an adjustable arm with a spin motor.

As the motor spins, the attached cylinder magnet drives another magnet fixed in the main disc.
Figure 6.3.3 A C-Shaped piece is half glued on the bottom of the main disc. The free end of the C-Shaped piece being wider than the main disc acts like a spring to hold the main disc at the bottom of the 100 ml petri dish.

The NCSU MTJ bioreactor consists of a plate and a main disc in a 100 ml petri dish. There is also an adjustable arm with a spin motor as shown in Figure 6.3.2. The stretching motion is driven by the two cylinder Neodymium magnets, one of which is fixed to the spin motor, "the motor magnet", and the other is glued to the main disc, "the plate magnet". The main disc is held firmly at the bottom of 100 ml petri dish by the C-shaped piece (Figure 6.3.3). The side of plate magnet on the main disc is carved and hollowed-out and has two slender arms which swing from side to side (Figure 6.3.4). The motor magnet is covered by an acrylic case, with only the two ends exposed. When the motor spins, the motor magnet moves to a place on the main disc where the motor magnet end is facing the opposite end of the plate magnet, the plate magnet is driven to the motor magnet and the arms under the plate magnet swing to the side (Figure 6.3.4).

The driving force on the fabric scaffolds is controlled by the initial distance between the two magnets, which are adjusted by the knobs on the adjustable arms. The initial distance for the muscle-tendon junction co-culture was set at 3mm, which provides 0.84 lb pullout force.
Figure 6.3.4 Swinging of slender arms provides linear displacement of the fabric scaffold. The maximum displacement is 4mm, which is 40% strain of a 10 mm gauge length.

Figure 6.3.5 The initial distance between the two magnets is controlled by the adjustable arm.
### 6.3.2 Parts and Assembly of the NCSU MTJ Bioreactor System

![Image of bioreactor components](image.png)

**Figure 6.3.6** The parts and assembly of the NCSU MTJ bioreactor components

**Parts:**
The parts of the bioreactor are illustrated in Figure 6.3.6.

**Bioreactor:** The plate, main disc and C-shaped piece are laser cut from low density polyethylene (LDPE) sheet. The bioreactor plate is 15 cm in length, 12.5 cm in width and 5 cm high, which can easily fit into 100 ml petri dish.

**The power cord** has branches so as to link the connector to the motor on the bioreactor plate. When the motor is connected to the power cord, the bioreactor is on. There is no separate on-off switch. The connector branches are left inside the incubator while the power cord is left outside. Each bioreactor can be connected or disconnected individually when in a stacked configuration.

**The glue** that fixes the magnet to the main disc is EP30MED (Master Bond Inc., NJ, USA). EP30MED is able to securely seal metal to hard plastic surfaces. EP30MED has a two part low viscosity resin and hardener to provide an optically clear epoxy resin. When cured it is rigid and provides superior dimensional stability and ethylene oxide (ETO) resistance. In addition, it meets USP Class VI rating for medical applications. It can be cured at room temperature or with heat. The optimum curing conditions involves a slow overnight cure at room temperature, followed by thermal curing at 65°C for 5 hours.
**The magnet** is cylindrical Neodymium magnet, Grade N52 (K&J Magnetics, Inc., PA, USA) as shown in Figure 6.3.7. It is made of NdFeB, Grade N52 with plating of Ni-Cu-Ni (Nickel). The magnetization direction is along the axis. The dimensions are 0.25 inch diameter by 0.375 inch in thickness. The weight is 0.0798 oz. (2.26 g), and the maximum pullout force is 4.42 lb.

![Figure 6.3.7 The cylindrical Neodymium Magnet, Grade N52 and its fallout force with distance (magnet to magnet repulsion)](image)

The stainless steel **screw and hexagon head nuts** are used to hold fabric scaffolds onto the bioreactor's main disc. The Pan Head Phillips Machine Screw (Cat # 91772A059) is an 18-8 stainless steel, 0-80, #0 Drive, and 1/2 inch length screw. The hexagon nut is Catalog number 91841A115, 18-8 stainless steel, thread size 0-80 Width 5/32” and height 3/64”. Both were purchased from McMaster-Carr, USA (Figure 6.3.8).

![Figure 6.3.8 Stainless steel screw and hexagon head nut were used to fix the fabric scaffolds onto the bioreactor main disc](image)

**The Motor** is a standard DC hobby motors, 60rpm.
6.3.3 Advantages and Disadvantages of the NCSU MTJ Bioreactor System

Advantages:

1) The operation is easy and time saving. When changing the medium, the plate can be detached from the connector and moved into a sterile biosafety cabinet. The compact size of the plate makes it easy to handle and operate in one hand.

2) The sealing of the culture environment depends on the petri dish and its lid. The petri dish is a commercially available product. Its ability to seal and its durability are dependent on the quality of the petri dishes purchased.

3) The bioreactor system provides linear movement for cyclic stretching and recovery without sudden jerkiness that was experienced with the WFIRM's bioreactor drive system. It is also easy to monitor the motion during cell culture since the entire bioreactor is transparent.

Disadvantages:

1) The design of the motion is limited by the hardware. The cyclic stretching motion is regulated by the motor. In order to change the frequency of the cyclic motion, one needs to replace the motor with the appropriate spin frequency. The cyclic strain is determined by the initial distance between the two magnets. And the maximum strain is 4 mm which is equivalent to 10% strain.

2) The length of the test sample scaffold is limited to 2 cm. In future, the size of the bioreactor could be enlarged by using bigger petri dishes and stronger magnets and motors.

6.3.4 Co-culture using MTJ bioreactor

Co-culture of Murine Muscle and Tendon Cells: The co-culture procedure that will be adapted from the one used in Project 2. Three co-seeded scaffold samples PET-KM3 750% were placed in 100ml petri dishes in the bioreactor and subjected to constant stretching stimulation while kept with 5% CO₂ at 37°C in the incubator (Figure 6.3.9). The compromised grow medium was changed every other day. Routine histology, laser scanning confocal microscopy
(LSCM) and an Alamar Blue® cell viability assay were used to evaluate the cell response after 1, 5, 10 and 19 days of the dynamic co-culture.

Figure 6.3.9 Murine muscle and tendon cells are co-cultured on PET-KM3-750% samples inside the MTJ bioreactor.

Routine histology (Figure 6.3.10) and confocal microscopy images (Figure 6.3.11) both confirm good cell penetration through the thickness at the two ends of 3D multi-phase scaffold. The MTJ bioreactor encouraged cell proliferation and 3D connections in comparison to the 2D static condition (Figure 6.3.11). Cell viability results are shown in Figure 6.3.12.

Figure 6.3.12 shows the fluorescence readings from the Alamar Blue® assay. The figure includes the results from i) cultured muscle C2C12 cells on the 750% 4GB scaffold in a static 2D control culture plate, ii) cultured muscle C2C12 cells on the 750% 4GB scaffold and iii) cultured NIH 3T3 tendon cells on the 750% 6GB scaffold in a dynamic culture environment. There was significantly higher cell density on the 2 dynamic culture groups at Day 5. This demonstrates that the MTJ bioreactor can improve the extent of cell proliferation during a limited in vitro trial.
Figure 6.3.10 Murine muscle and tendon cells were co-cultured on PET KM3-750% scaffold sample inside the NCSU MTJ bioreactor for 19 days.
Dynamic MTJ bioreactor

Figure 6.3.11 Laser scanning confocal microscopy (LSCM) image of PET-KM3 750% at 19 days of dynamic co-culture.

Static Plate culture
Figure 6.3.12 Alamar Blue results showing cell viability after 1 and 5 days of dynamic co-culture.

6.4 Conclusions:

An MTJ bioreactor system has been developed at NCSU to provide 1Hz 10% strain mechanical stimulation to multiple fabric scaffolds simultaneously. It shows advantages in linear motion, ease of operation and environmental sealing over the previous dynamic bioreactor that was developed at WFIRM. The dynamic muscle-tendon co-culture trial has demonstrated its functional ability to encourage cell proliferation and penetration at tissue junction regions. Future work will be needed to continue to test for the genetic expression of the junction tissue. This dynamic bioreactor system is believed to be capable of serving other tissue engineering applications, which could be the direction for future research.
CHAPTER 7

HARVESTING AND ISOLATION OF PORCINE PRIMARY MUSCLE AND TENDON CELLS

7.1 Introduction

Chapter 2 described earlier how I used purchased murine cell lines from a standard cell bank and supplier to regenerate an in vitro muscle-tendon junction through co-culturing the two cell lines. However, in the future, after completing in vitro cell studies on standard cell lines of small animals, such as the mouse or rat, it is essential to use larger animals after the rodent model. A porcine model is an ideal candidate because its mechanical tissue properties are generally similar to those of a human and they are more readily accessible than with smaller animals. However, porcine skeletal muscle cells and viable tendentious cells are not available on the market. So the specific aim of Chapter 7 is to develop the methodology for harvesting and isolating primary skeletal muscle cells and tendentious cells from pigs.

Harvesting Pig Tissue

Pig tissue was harvested from the tibialis anterior together with the tibialis tendon in the hind legs of two 1-year old female pigs. The muscle and tendon tissues were surgically removed under aseptic conditions from the shoulder and forelimb of euthanized pigs within 2 hours of the sacrifice. The tissue was weighed, washed in 70% ethanol and preserved in cooled PBS solution for up to 30 mins in an icebox.

7.1.1 Primary Cell Isolation

A single-edged razor blade and a pair of No. 5 forceps (Fine Science Tools, USA) will be used to slice the muscle and tendon tissues into small pieces measuring less than 1mm in size (Figure 7.1.1).
**Primary porcine muscle cell digestion**

The minced muscle tissue was digested in 10ml/gram tissue PBS solution with 15 unit/mL Type I collagenase and 15 unit/ml Type IV collagenase and 2% antibiotic-antimycotic agent as shown in Figure 7.1.2. Increasing the digestion time assisted in the degradation of the collagen bonds between the myotubes. Based on our experimental findings, 3-4 hours was found to be the optimal time for digesting primary muscle tissue.

**Primary porcine tendon cell digestion**

The minced tendon tissue was dissociated in 10ml/gram tissue of PBS solution with 125 unit/mL Type I collagenase and 140 unit/ml Type IV collagenase and 2% antibiotic-antimycotic agent for up to 8 hours. The differences in digestion are shown in
Figure 7.1.3. The tendon tissue was more difficult to digest since it contained almost entirely collagen. The optimal digestion time was found to be about 6 hours.

![Digestion of primary porcine tendon tissue over time](image)

**7.1.2 Culture of primary digested cells**

After digestion the isolated primary cells were mixed together with muscle and tendon cell fragments and myotube debris. A cell strain was used to filter the cells from the broken tissue mixture. The isolated cells were then seeded on cell culture plates coated in horse serum for 14 days. The culture medium recipes for primary muscle and tendon cells are listed in Appendix III. The medium was changed every other day. Optical microscopic images were taken every day to monitor cell attachment and proliferation (Figure 7.1.4 and Figure 7.1.5).

![Porcine muscle cells proliferate after isolation.](image)
In Figure 7.1.4, porcine muscle cells becoming attached to the horse serum coated culture plates. After 24 hours the muscle cells stretched from the myotubes and began to proliferate on the culture plates. The proliferation rate increased after 7 days.

Figure 7.1.5 Porcine tendon cells proliferate after isolation.

In Figure 7.1.5, porcine tendon cells becoming attached to the horse serum coated culture plates. After 24-48 hours, the fibroblast cells started to stretch and proliferate on the culture plates. The proliferation rate increased after 7 days.

**Conclusion and Future Plan**

Porcine primary muscle and tendon cells have been isolated and cultured under 2D static conditions. Future studies involving the genetic engineering of primary muscle and tendon cells are recommended. Porcine primary cell *in vitro* studies are essential before undertaking *in vivo* animal studies. However it is an interdisciplinary field which requires the expertise of primary cell biologists, tissue engineers and genetic engineers. Compared to the availability of mice and rabbit cell lines and related biomolecular supplies, there are few, if any, porcine tissue engineering supplies and regents available on the market. For example, it is difficult to find
commercial porcine skeletal muscle cells, customized medium to control differentiation and the proliferation of porcine myoblasts, as well as the primers for MTJ specific markers for q-PCR testing. To achieve these primary supplies, close collaboration will be needed in the future between cell biologists, genetic engineers, material scientists as well as textile and tissue engineers.
CHAPTER 8

CONCLUSION AND FUTURE PLANS

The primary goal of this study focused on interfacial tissue engineering of a muscle-tendon junction, and how to move the concept of a multiple heterogeneous scaffold from the laboratory bench to a practical clinical application at the patient's bedside. A number of innovative steps have been studied including:

1) the design and fabrication of a multiple phase 3D warp knitted spacer fabric scaffold that has a contiguous structure and an appropriate gradient of physical and mechanical properties so as to mimic a muscle tendon junction.
2) the use of a dynamic co-culture method to regenerate muscle tendon junction tissue \textit{in vitro} using a cyclic stretching bioreactor.
3) the design and assembly of an innovative compact bioreactor which is customized for muscle tendon junction regeneration and has specific advantages over existing bioreactor systems.
4) the development of a nondestructive imaging methodology to identify and monitor the regeneration of muscle tendon junction tissue cultured on 3D textile scaffolds,
5) a new approach to harvesting and isolating primary porcine muscle and tendon cells for the regeneration of multiple tissue interfaces using a large animal (e.g. pig) model in the future.

The 3D warp knitted spacer fabric scaffold with multiple phases designed and fabricated in this study is the first ever reported multiple phase warp knitted structure used for a biomedical application. This 3D textile structure provides an improvement in tissue engineering applications compared to conventional MTJ scaffolds, which have been made by electrospinning a 2D membrane. The unique structural feature of this multiphase spacer fabric scaffold is its integrated interface. The 3D multiple phase scaffold also shows potential for use in other connective tissue applications. When one end of the multiphase spacer scaffold is calcified, the scaffold would be
used for hard-to-soft junction applications, such as tendon-bone and ligament-bone junctions. Also, the compression resistance of the spacer fabric makes it a potential candidate for cartilage regeneration.

As shown in Chapter 3, the spacer fabric scaffold can be fabricated from either permanent or degradable yarns. And without altering the type of polymer, the structural, physical and mechanical properties of each region can be tuned by changing the knitting parameters. The flexibility in the structural design makes the knitted scaffold competitive among tissue engineering products because the available material has been approved in the USA by the FDA. A lot of different natural and synthetic fiber-base materials can be fabricated using this warp knitting approach.

The dynamic co-culture method developed in this study has been successfully demonstrated to generate viable oriented confluent tissue on synthetic 3D textile scaffolds. The compacted MTJ bioreactor developed in Chapter 6 improved the operation, linear motion and sterile sealing of the previous cyclic stretching bioreactor designed and assembled at WFIRM. In addition, this compacted MTJ bioreactor has potential for use in other tissue engineering applications, which require a tensile stimulus, such as in tendon, muscle, ligament, and skin regeneration. Also, it can be used for non-biological applications, such as providing a tensile fatigue test method for films, fabrics, membranes and other textile structures.

The laser scanning confocal microscope technique together with multiple fluorescent labelling can be applied to other functional tissue engineering applications. It is especially beneficial when applied to large scale 3D scaffolds for big animal models.

The co-culture experiment in this study was originally designed to use primary porcine muscle and tendon cells. However, this approach met with several technical difficulties including the identification of reliable genetic markers of porcine MTJ's being analyzed by qPCR analysis. This means that the presence of a muscle tendon junction cannot be genetically detected and confirmed. Which means that tissue engineering research is currently placing high demands on the interdisciplinary collaboration of biologists, geneticists, material scientists and engineers. And there are still lots of challenges for a team of interdisciplinary researchers to conquer. The
primary future goal continues to be the regeneration of functional complex tissue in order to provide the patient with viable and functioning tissues and organs.
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APPENDIX

Cell culture media protocols

1. Growth medium for the primary porcine muscle cells
   HG-DMEM + 20% FBS + 1 vial Chick Embryo Extract/500 mL + 1% Antibiotic/antimycotic agent
   * For 1 L of media, use 1 L of HG-DMEM, 200 mL of FBS, 2 vials of CEE, 10 mL abx

2. Growth medium for the isolated porcine tendentious cells
   HG-DMEM + 10% FBS + 1% Antibiotic/antimycotic agent
   * For 1 L of media, use 1 L of HG-DMEM, 100 mL of FBS, 10 mL abx

3. Differentiation medium for culturing primary cells seeded on a scaffold:
   HG-DMEM + 2% HS + 1 vial insulin-transferrin-selenium (“ITS” from Lonza) + 250 nM dexamethasone + 1% Antibiotic/antimycotic agent
   * For 1 L of media, use 1 L of HG-DMEM, 20 mL HS, 2 vials ITS, the dilution of the stock dexamethasone, and 10 mL abx

Reagents:
HG-DMEM: high-glucose Dulbecco’s Modified Eagle Medium (Thermo Fisher Scientific, USA)
FBS: fetal bovine serum (Thermo Fisher Scientific, USA)
CEE: the chick embryo extract (Sera Laboratories International)
Abx: Antibiotic/antimycotic agent (Thermo Fisher Scientific, USA)
ITS: insulin-transferrin-selenium (Thermo Fisher Scientific, USA)
Dexamethasone (Thermo Fisher Scientific, USA)
HS: horse serum (Thermo Fisher Scientific, USA)