ABSTRACT

TUIN, STEPHEN ALEXANDER. Enhanced Mass Transport Nonwovens as Industrial Scale Tissue Engineering Scaffolds Using Human Adipose Derived Stem Cells. (Under the direction of Dr. Elizabeth Loboa).

Many tissue engineering strategies seek to replace damaged or diseased tissue through controlled proliferation, differentiation, and extracellular matrix (ECM) production via stem cells seeded on a biodegradable support scaffold. However, many scaffold fabrication techniques are time consuming, expensive, and require specialized techniques not suitable for industrial scale production. In order to translate tissue engineering strategies from the laboratory to clinical practice, a high throughput, repeatable, scalable, and economical manufacturing method is needed. We hypothesized that nonwoven industry standard high throughput manufacturing techniques (spunbond, meltblowing, and carding) can meet this need. An additional challenge to the successful generation of relatively thick tissue engineering constructs is the formation of necrotic cores. As cells populate the scaffold and lay down ECM, nutrient and gas exchange becomes a limiting factor for viable cell growth in the interior of the scaffold. We further hypothesized that porous and hollow porous fibers may result in enhanced mass transport of nutrients and gases throughout the construct, limiting the formation of necrotic cores.

Many stem cell sources have been implemented for a variety of tissue engineering applications. Recently, adipose derived stem cells (hASC) have gained momentum as an abundant stem cell source for a variety of tissue engineering strategies, particularly for mesodermal lineages. Human ASC are relatively easy to harvest compared to other mesodermal stem cell sources, such as those derived from bone marrow. The goal of this research was to implement industry standard, scalable nonwoven manufacturing fabrication
methods for the generation of full thickness tissue engineering scaffolds composed of fibers with enhanced mass transport properties, and validated using hASC.

The three most common industry standard nonwoven manufacturing techniques (spunbond, meltblowing, and carding) were validated as tissue engineering scaffolds using hASC. We demonstrated that solid fiber scaffolds manufactured via these techniques support viable cellular proliferation and adipogenic and osteogenic differentiation of hASC. This work was extended to fabricate scaffolds composed of porous fiber scaffolds with enhanced mass transport properties fabricated via the Spunblown process, a specialized version of meltblowing. We demonstrated the successful fabrication of Spunblown scaffolds composed of porous fibers via inclusion of a sacrificial component (AQ55S) in the primary polymer backbone, composed of poly(lactic acid) (PLA). Subsequent washing in deionized water resulted in the formation of porous fibers. We showed that porous fibers led to increased adipogenic and osteogenic differentiation of hASC, and promoted better cellular attachment throughout the scaffold thickness. Building on this work, we describe the successful fabrication of hollow porous multifilaments, and conversion to carded nonwoven scaffolds composed of hollow porous fibers using the PLA/AQ55S polymer system. Similar to porous fibers we showed that hollow porous fibers led to enhanced adipogenic and osteogenic differentiation of hASC.

Lastly, this work was extended to alternative fiber cross sections with enhanced mass transport properties. We described for the first time fabrication of novel mushroom gilled fibers, exhibiting a hollow central channel with multiple fin like extensions extending to the solid fiber surface, and subsequent conversion to gilled fiber carded scaffolds. We demonstrated that gilled fiber scaffolds led to increased proliferation of hASC and increased
expression of the early osteogenic gene marker, runt related transcription factor 2 (RUNX2) when exposed to one hour of pulsatile fluid flow in the absence of soluble osteogenic induction factors. As a whole, this work demonstrates the feasibility of standard commercial nonwoven manufacturing methods as scalable, high throughput, economical fabrication techniques for clinical translation of enhanced mass transport tissue engineering scaffolds using hASC.
Enhanced Mass Transport Nonwovens as Industrial Scale Tissue Engineering Scaffolds
Using Human Adipose Derived Stem Cells

by
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DEDICATION

This dissertation is dedicated to my parents, Van and Trish Tuin, for their unwavering support and guidance through my collegiate career and life in general. To my wife, best friend, and Editor-In-Chief Jill Blecha – RAWR! And to my son Miles Tuin, who is my reason for getting up every morning, figuratively and literally.
**BIOGRAPHY**

Stephen Tuin was born in Brighton, Colorado on July 7th, 1983 to parents Van and Trish Tuin. Stephen attended high school in the small mountain town of Paonia, CO and graduated as class valedictorian in May, 2002. The following fall he entered the College of Engineering at the University of Colorado at Boulder in the Department of Chemical Engineering with an emphasis on bioengineering. During his senior year he completed a senior thesis under the direction of Dr. Melissa Mahoney developing drug delivery vehicles for the treatment of chronic pain, inspiring him to further his education and focus on biomedical research. He graduated with a Bachelor of Science in chemical engineering in the fall of 2006 and entered the Department of Chemical Engineering at Auburn University in Auburn Alabama, where he received a Master of Engineering degree in Chemical Engineering under the direction of Dr. Steve Duke. In the fall of 2011 he joined the Cell Mechanics Laboratory in the Joint Department of Biomedical Engineering at North Carolina State University and the University of North Carolina under the direction of Dr. Elizabeth Loboa. Dr. Behnam Pourdeyhimi of the Nonwovens Institute also mentored Stephen during his tenure as a Ph.D. student.
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CHAPTER 1 Interconnected, Microporous Hollow Fibers for Tissue Engineering: Commercially Relevant, Industry Standard Scale-Up Manufacturing

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1.1. Introduction

Significant progress has been achieved in the field of tissue engineering to create functional tissue using biomimetic three-dimensional scaffolds that support cell growth, proliferation, and extracellular matrix production. However, many of these constructs are severely limited by poor nutrient diffusion throughout the tissue-engineered construct, resulting in cell death and tissue necrosis at the core. Nutrient transport can be improved by creation and use of scaffolds with hollow and microporous fibers, significantly improving permeability and nutrient diffusion. The purpose of this review is to highlight current technological advances in the fabrication of hollow fibers with interconnected pores throughout the fiber walls, with specific emphasis on developing hollow porous nonwoven fabrics for use as tissue engineering constructs via industry standard processing technologies: Spunbond processing and polymer melt extrusion. We outline current methodologies to create hollow and microporous scaffolds with the aim of translating that knowledge to the production of such fibers into nonwoven tissue engineering scaffolds via spunbond technology, a commercially relevant and viable melt extrusion manufacturing approach that allows for facile scale-up.
Hollow fibers are lightweight with superior surface area to volume ratio [1], [2], thermal [3], [4] and acoustic insulation [5], and water retention properties [6] compared to solid fibers. Given their advantages over traditional solid fibers, hollow fibers have been used in a variety of applications over the past several decades. Applications range from membrane separation [7], [8], liquid and gas filtration [8], desalination [9], and biomedical separation processes [10]. Fabrication of hollow fibers has been described in the literature [11–17], including processing parameters and resultant effects on physical and mechanical properties [11–13]. Specific to the fabrication method and processing parameters, both physical and mechanical properties can be tailored to meet the requirements of a specific application.

In addition to their use in these more traditional applications, hollow and microporous fibers are a promising material in the field of tissue engineering. Traditional nonwoven round fiber materials have been shown to support cell growth and proliferation while providing structural integrity of the tissue engineered construct/scaffold [17], [18]. Biodegradable scaffolds add further benefit by providing initial structural integrity required for early cellular growth and proliferation. As the cells proliferate within the scaffold, they generate their own extracellular matrix (ECM) to replace the degrading scaffold, leaving behind only newly formed tissue [18].

However, a major challenge to current scaffold fabrication approaches is the formation of necrotic cores within thick scaffolds that are needed for repair of large tissue defects. Cells seeded on the periphery of such scaffolds deposit ECM proteins that can quickly block mass transport of vital nutrients to cells in the interior of the scaffolds [19–22]. Total porosity of the fabric can also affect cellular viability and proliferation. Previous
investigators have shown that many cell lines require pores in a specific size range to allow
cellular infiltration into the center of the scaffold structure [20], [23]. Optimal pore sizes for
a variety of cell types, ranging from 5 µm for neo-vascularization, to 200-350 µm for
osteoconduction were documented in a recent review by Guarino and Ambrosio [20]. By
incorporating both hollow channels within fibers, and a microporous fiber wall, mass
transport of nutrients to the core of a cell-seeded scaffold can be enhanced, allowing for
cellular growth throughout the scaffold. Further, cellular waste products and polymer
degradation products can be removed from the core.

Current limitations of engineered tissues lie in the inability to supply nutrients via a
biological or engineered vasculature system [22], [24–34]. In order to successfully translate
tissue constructs created in vitro for implantation in vivo, a sufficient vasculature system
must be in place. Jain et al. have shown that cells further than 200 µm from the nearest
blood vessel are limited by diffusional limitations of oxygen and nutrients[24], [31], [34].

Historically there are two approaches for generating vascularized tissue in order to overcome
these limitations: cellular based strategies and scaffold based strategies [24]. These
strategies are briefly described here. For a thorough review of vascularization approaches,
we refer the reader to Novosel et al [24].

Cellular based strategies aim to generate new vessels de novo via vasculogenesis.
Appropriate cellular linages capable of vasculogenesis such as endothelial progenitor cells
can be seeded within a scaffold along with appropriate growth factors such as vascular
endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth
factor (HGF), platelet-derived growth factor (PDGF), and transforming growth factor beta
(TGFβ) in order to promote prevascularization in vitro [24], [26], [35]. Upon implantation,
the prevascularized tissue may connect to the host vasculature via anastomose [24]. While powerful, this strategy may require the use of complex co-culture systems, expensive and potentially unstable growth factors, and leads to slower perfusion due to lack of direct microsurgical connections to the host vasculature [24].

Alternatively, in vivo cellular based strategies may be employed to promote prevascularization [24], [25], [28]. Avascular scaffolds implanted within healthy host tissue may undergo vascularization, taking advantage of endogenous cells and growth factors to generate a vascularized network within the scaffold [24]. The newly vascularized scaffold is then explanted and re-implanted to the injured site. This approach takes advantage of the entire physiologic system of the host to generate a well-connected vasculature system within the scaffold, however three surgeries are required [24], [25].

In contrast to cellular based approaches, scaffold based vascularization strategies have also shown promise [22], [24], [33]. In this method, existing decellularized capillary networks or synthetically fabricated artificial vessel like structures provide nutrient and oxygen diffusion throughout a tissue engineered construct [22], [24], [33]. Recently, Bettahalli et al. have developed an artificial vasculature perfusion bioreactor by incorporating four hollow porous fibers perfused with nutrient media into a porous scaffold seeded with murine pre-myoblasts [22]. Incorporation of hollow porous fibers led to improved cellular distribution throughout the entirety of the scaffold, whereas cellular infiltration was limited to the scaffold boundaries in control scaffolds [24]. In this review, we outline commercially relevant fabrication methodologies for the fabrication of hollow porous fibrous scaffolds as a synthetic vascular network for use as tissue engineering constructs.
The four most common fabrication methods for producing hollow fibers are electrospinning, wet spinning, dry spinning, and melt spinning (Figure 1.1) [36–38]. Wet and dry spinning methods are used for polymers that must be dissolved in a solvent to spin. Melt spinning is preferred for polymers that can be melted. Higher spinning speeds are generally achieved, thus increasing throughput [39], [40]. Forcing the dissolved or molten polymer through an annular spinneret creates a hollow fiber. Hollow fibers can also be fabricated via co-electrospinning in which a sheath core configuration is generated utilizing two spinning syringes. Subsequent removal of the core component yields a hollow fiber [41]. This review will focus primarily on melt spinning, particularly spunbond processing (direct to web melt spinning), for hollow fiber formation as these techniques are the most attractive methods from a high throughput, commercially relevant standpoint with attainable spinning speeds of 3000-6000 m/min [39].
In addition to hollow fibers, the presence of micropores in the fiber walls can lead to improved mass transport. Micropores allow a diffusion path for nutrients into the scaffold and removal of cellular waste products. Micropores can be created after the spinning process via cold stretching [42–45], thermally induced phase separation (TIPS) [11], [46–50], or by
introducing a secondary sacrificial component into the base polymer [16], [20], [51]. Subsequent removal of the sacrificial component leaves a microporous network throughout the fiber wall generated by the primary polymer. The aim of this paper is to provide a comprehensive review of fabrication methods to generate hollow and microporous fibers. We focus on melt spinning and spunbond technologies for an emphasis on creating or utilizing commercially viable, high throughput, fabrication processes.

1.2. Hollow Microporous Fibers for Tissue Engineering Applications

Nonwovens have been used extensively as tissue engineering scaffolds for a variety of cell types and tissue applications [20], [22], [52–57], however many of these constructs have inherent limitations. For example, electrospun nonwovens have been extensively investigated as tissue engineering constructs. Our group and many others have shown that the size scale of electrospun fibers closely mimic the size scale and structure of native ECM structures, particularly collagen fibers, and promotes attachment, proliferation, and differentiation of a variety of cell types [36], [58–65]. For a detailed review of the electrospinning process in the context of tissue engineering, we direct the reader to a review by Pham et al [66]. However, the fabrication of large three-dimensional scaffolds via electrospinning for critical defect repair is challenging and time consuming; and, scaffolds created using this technique generally result in mechanical properties much lower than the tissue they are being used to replace. Further, cell growth and migration is often limited to the scaffold surface. Cells within the interior of the scaffolds are limited by mass transport of nutrients via diffusion and consequent cell death can result in necrotic cores with relatively thick constructs [19–22]. Scaffolds consisting of hollow fibers with microporous walls could
effectively act as an artificial vasculature system, enhancing nutrient diffusion and circumventing these issues.

Many researchers have successfully fabricated hollow and porous nonwovens for tissue engineering applications using wet, dry, and melt spinning methods (Table 1.1) [20], [22], [52–57]. While the size scale of fibers produced via these methods are typically larger than electrospun fibers, and thus larger than native ECM structures, nonetheless several groups have demonstrated their potential utility as tissue engineering scaffolds [20], [22], [52–57]. Higher spinning speeds and thicker scaffolds are attainable compared to electrospinning [39], [40].
Table 1.1. Approach and results of major studies utilizing hollow porous and nonwoven fiber scaffolds for tissue engineering applications.

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PEOT/PBT, poly(ethylene oxide terephthalate)/poly(butylene terephthalate); PES, polyethersulfone; PLACL, poly(lactic acid-co-caprolactone); PLGA, poly(lactic-co-glycolic acid); PET, polyethylene terephthalate; PLA, poly(lactic acid); CO₂, carbon dioxide; TIPS, thermally induced phase separation; NaOH, sodium hydroxide; hMSC, human mesenchymal stem cells; SCID, severe combined immunodeficiency; hASC, human adipose derived stem cells.
Although the size scale of ECM structures are on the order of hundreds of nanometers, Unger et al. have cultured a variety of human cells on large (about 2.2 mm diameter) hollow porous polyethersulfone (PES) fibers fabricated without the use of solvents by supercritical CO$_2$ phase inversion [52]. PES fibers were prepared for cell culture by cutting thin ring sections 0.2-0.5 mm thick, as well as longer 2-4 mm sections subsequently cut in half lengthwise. Human glial, epithelial, keratinocyte, osteoblast, fibroblast, and glioblastoma cell lines were then cultured on the cut fiber sections. They reported that all PES hollow porous fibers supported cell growth on the inner and outer surfaces, as well as cut surfaces [52]. Hollow porous fibers composed of poly(lactic acid)/polycaprolactone (PLACL) copolymer fabricated via the TIPS process and manufactured into nonwoven fabrics have also shown promise as tissue engineering constructs [53]. Pertici et al. have shown via Alamar blue$^\text{TM}$ assays and scanning electron microscopy (SEM) analysis that murine fibroblasts, human osteosarcoma cells, and bovine chondrocytes attach and proliferate on hollow porous PLACL fibers to a greater extent compared to tissue culture plate controls [53]. Similarly, Ellis and Chaudhuri have successfully fabricated hollow poly(lactic-co-glycolic acid) (PLGA) hollow fibers via wet and dry spinning using 1-methyl-2-pyrrolidinone (NMP) as the solvent and water as the nonsolvent [54]. The spinning temperature was found to have a significant effect on fiber morphology leading to a more open central channel at 40 °C compared to 20 °C. Immortalized osteogenic cells (pZIP) were then cultured on hollow fibers in a flow bioreactor and exhibited similar attachment and proliferation as those cultured on traditional tissue culture polystyrene [54].

In addition to these in vitro studies, hollow porous fibers have also shown promise as tissue engineering scaffolds in vivo. Morgan et al. examined the in vivo utility of hollow
porous scaffolds for skeletal tissue engineering [55]. Hollow porous poly(DL-lactide-co-glycolide) (PDLG) fibers were prepared via the TIPS process and seeded with human bone marrow stromal cells (hMSC). hMSC were cultured on hollow PDLG fibers for 9 or 42 days prior to being impacted in a perforated acrylic graft chamber to secure the implant in the flanks of severe combined immunodeficiency (SCID) mice. Control grafts of PDLG without cells and experimental grafts with hMSC plus bone allograft chips loaded into the graft chamber were also analyzed. Animals were sacrificed after 42 days and the grafts were evaluated for new osteoid formation. Immunohistological staining indicated that grafts containing hMSC cultured on hollow porous PDLG fibers exhibited increased expression of type I collagen and led to osteoid formation and organized new woven bone tissue [55]. These in vitro and in vivo studies demonstrate the potential utility of hollow porous fibers for tissue engineering scaffolds. However in order to be successfully translated for the treatment of critical size defects, these scaffolds must be able to support cell growth, migration, and proliferation throughout a relatively thick construct.

In order to create a full thickness scaffold for the treatment of critical size defects for soft tissue, Bettahalli et al. recently reported incorporation of hollow fibers into a large (4 mm²) poly(ethylene oxide terephthalate)/poly(butylene terephthalate) (PEOT/PBT) copolymer scaffold representing such a defect [22]. Aligned layered PEOT/PBT fiber mats were sequentially stacked at 90 degrees using a bioplotter to construct the scaffolds. Three commercially available porous hollow fibers (PP, Membrana GmbH; modified polyethersulfone, Gambro GmbH; modified polysulfone, Asahi Kasei Medical Co. Ltd.) used for plasmapheresis were inserted into the constructs in order to simulate artificial vasculature. Scaffolds were seeded with mouse pre-myoblast C2C12 cells and cultured under static and
flow perfusion conditions. Scaffolds cultured under static conditions showed a high concentration of cells and ECM on the scaffold surface with few cells in the interior. However, results indicated that the incorporation of 4 hollow porous fibers into the scaffold construct led to improved cellular distribution and ECM deposition throughout the entirety of the scaffold [22]. Surprisingly, scaffolds with 8 hollow porous fibers exhibited a decrease in cellular proliferation compared to 4 hollow fibers, attributed to a decrease in the scaffold porosity, indicating that careful consideration of scaffold pore structure is also of critical importance when designing porous tissue engineering constructs. Edwards et al. examined the effects of pore size and pore geometry on mouse NR6 fibroblast infiltration and proliferation into carded, through-air thermally bonded sheath core poly(ethylene terephthalate) (PET) nonwovens [56]. They observed that tissue growth was initiated at fiber crossover points, with decreasing tissue growth at increasing distance from these fiber crossovers. Over 6 days in culture, circumferential pore infiltration occurred, with few cell aggregates observed, indicating that pore sizes studied were suitable for fiber-fiber bridging [56]. Similar studies on human trophoblast ED27 cells cultured on NaOH treated PET carded nonwovens with larger pore sizes indicated significant cell aggregation on fiber surfaces as the inter fiber distance was too large for cellular bridging [57]. These studies show that careful scaffold morphology must be considered depending on the type of cell lines cultured [22], [56], [57], [67].

Not only must the scaffold morphology be considered in the context of cellular interactions, ideally, the degradation rate of the scaffold material should match the rate of new tissue formation. Depending on the chosen polymer, the degradation rate of the scaffold can be affected by: Temperature, pH, crystallinity, molecular weight, additives, solvents,
morphology, and porosity [68–72]. Further, the degradation rate of implantable devices can also be affected by sterilization methods [73], [74] and exposure to the in vivo environment via cellular and enzymatic interactions[68–72], [75]. In the case of hollow porous materials, the unique structural morphology must also be considered from a degradation standpoint.

Wen and Tresco have analyzed the in vitro degradation of hollow porous PLGA fiber membranes fabricated via the TIPS process [75]. Hollow porous membranes were exposed to 0.1 M phosphate buffered saline (PBS) at 37 °C and pH 7.4 for eight weeks and compared to solid PLGA pellets. Counter intuitively, it was found that the solid polymer pellets completely dissolved after six weeks, however the hollow fiber membranes did not completely dissolve until week eight of the study. Reduction in weight average molecular weight for the pellets was greater than that of hollow porous membranes, supporting the total weight loss results. The disparity in the degradation rate was attributed to increased autocatalysis in the pellet form. Carboxyl end groups exposed by hydrolytic ester bond cleavage in the interior of the pellets cannot readily diffuse to the surface and results in a local acidic environment, accelerating degradation. The increased surface area of the hollow fiber membranes allows acidic degradation products to be removed from the surface of the structure, reducing the effect of autocatalysis[75]. This phenomena was also investigated computationally by Chao et al [70]. A cellular automaton analysis was employed to study the effect of porosity on the degradation rate of porous PLA scaffolds. Bulk erosion of scaffolds in media with initial porosities of 80, 90, and 95% were considered. Simulation results indicated that increasing initial scaffold porosity leads to a slower degradation rate [70]. These results were consistent with experimental results by Wen and Tresco and others
[71], [72] and were attributed to enhanced diffusion of acidic breakdown products out of more porous scaffolds, leading to a reduction of the autocatalysis effect [70].

1.3. Spunbond Process

Unlike carding or laydown processes that use preformed staple fibers to fabricate a nonwoven fabric, the hallmark of the spunbond process (a specialized melt spinning technique) is that the web is formed directly from polymer pellets or powder in one unit operation, followed by an in line bonding step [76]. Therefore, due to the nature of the process the raw material must be a thermoplastic material in order to facilitate polymer extrusion and subsequent solidification into fibers.

Several spunbond line configurations are in use today, including commercially available configurations as well as proprietary systems [77]. Each of these configurations fabricate nonwoven fabrics via fiber spinning, web laydown, and bonding in a single continuous operation (Figure 1.2). The properties of the resultant fabrics depend greatly on the processing conditions. The spunbond process and variables affecting the performance of the fabrics produced have been described elsewhere [76]. The spunbond process is briefly described again here, but for a comprehensive review of the processing parameters affecting the spunbond process we refer the reader to Bhat and Malkan [76].
Figure 1.2 [78] outlines the spunbond process with an emphasis on forming hollow and microporous fibers, highlighting processing parameters in each step that will influence the final performance of the fabric produced. Polymer chips are fed to an extruder and molten polymer is forced through a spinbeam to form filaments. In order to increase fiber coverage and web uniformity, several spinbeams are often used together [76]. As the filaments exit the spinbeam, cool air solidifies the fibers before pneumatic drawing, producing fibers with typical diameters of 10-20 μm [79], while simultaneously increasing molecular orientation, improving mechanical properties [76]. Filaments are then collected on
a moving perforated belt as an unbonded web. The web is then bonded to lock the structure in place and provide mechanical integrity to the fabric. If required, post processing and finishing is applied after bonding and before winding the fabric into a rolled good.

**Figure 1.3.** Overview of the spunbond process highlighting processing parameters that will affect fabric properties with an emphasis on the formation of hollow microporous fibers via incorporation of a sacrificial secondary polymer component, adapted from [78].
1.4. Selection of Polymers

Initiation of the design process should begin with careful consideration of the final application of the nonwoven fabric. Desired mechanical and physical properties that dictate the performance of the nonwoven start with the raw polymer materials used to fabricate the individual fibers. Polymer selection is therefore a key step in the design process. Selection of the secondary, sacrificial polymer component used to create the hollow and porous structure in the fibers and the method of the sacrificial polymer’s removal must also be taken into consideration early in the design phase. Thermal and rheological properties of the sacrificial component must be sufficiently similar to the primary polymer to ensure good spinnability [80], but at the same time must be soluble in a solvent in which the primary polymer has limited solubility.

Typically, materials suitable for spunbond processing have melt flow indices (MFI) in the range of 5-40 dg/min [81]. MFI is a relatively easy measurement and a good indicator of processability. However, MFI does not take into account discreet contributions of molecular weight distribution, tacticity, and degree of branching. Polypropylene (PP), followed by polyethylene (PE), are the most well studied materials used for the spunbond processes. PP continues to be the most widely used raw material today due to its good strength, low melting temperature, chemical resistance, strength, and cost [82]. Polyesters and polyamides have also found commercial application when superior physical properties are required, although cost of the raw materials and processing conditions are higher than that of PP [82].

In theory, hollow fibers can be fabricated from any polymer suitable for melt spinning. In the case of multicomponent spinning systems, the selection of the secondary sacrificial polymer will depend greatly on the properties of the primary polymer. Close
matching of rheological properties is critical to processability and subsequent fiber formation [80], [83]. If thermal and rheological properties differ greatly, spinning may not be feasible.

Tsebrenko et al. investigated the fiber forming properties of polymer blends consisting of polypropylene/co-polyamide (PP/CPA), polyoxymethylene/co-polyamide (POM/CPA), polyoxymethylene/co-ethylene vinyl acetate (POM/EVAC), PP/EVAC, EVAC/CPA, and polypropylene/polyvinyl alcohol (PP/PVA) via capillary viscometer measurements [84]. Their findings indicate that the fiber forming properties of the resultant polymer blends depend strongly on the miscibility of the polymers and interactions between the polymers [84]. In a related study poly(lactic acid)/PVA (PLA/PVA) blends were prepared via melt blending at varying PLA:PVA blend ratios and molded into thin plates [85]. Results indicated that blending with PLA decreased the melt viscosity of PVA. Blends were miscible as indicated by the observance of a single glass transition temperature (Tg) and a melting temperature (Tm) between the Tm of the pure polymers. Interestingly, the strongest interaction was observed for 80:20 PLA:PVA blends, determined by maximum blending torque. This was attributed to maximal interactions between the α-hydroxyl group of PVA and the carbonyl group of PLA [85]. These studies show that the rheological properties of polymer blends must be considered carefully when determining a polymer mixture for fiber formation.
1.5. Formation of micropores

Several methodologies to form micropores are described in published literature and patents. These include the addition of soluble additives [86], thermally induced phase separation [11], [46–50], and melt spinning cold stretching [42–45].

Mizutani described a process to fabricate molded porous polyolefins by including silica, polysiloxane, or crosslinked vinyl polymer in the polymer melt. Pore sizes of 0.005 to 0.1 µm were reported. The diameter of the additives ranged from 0.01 to 0.1 µm and had a significant effect on the diameter of the resultant pores. Decreasing the diameter of the additive resulted in smaller pore sizes. However, as the size of the additives was decreased the cohesion between particles increased and agglomerates formed, resulting in poor dispersion throughout the molten polymer phase. Such agglomerates led to larger pore diameters and a broader pore size distribution [86].

Micropores can also be introduced by incorporation of a secondary polymer into the fiber wall. After the fibers have been extruded, removal of the secondary polymer results in the formation of a porous structure. In order to form an interconnected pore structure, a co-continuous blend of the two polymers is desirable. Subsequent removal of either component leads to an interconnected porous structure. Studies have shown that the ratio of the viscosities of the two polymers, blend weight ratio of the two components, the interfacial tension between the polymers, and the processing conditions all affect the formation of co-continuous blends [87], [88]. A viscosity ratio of one is desirable to promote formation of a co-continuous blend [87], [88], however blends of PLA and other co-polyesters have been shown to form co-continuous blends at viscosity ratios varying from 0.1-10 [87]. Li et al. fabricated molded interconnected porous structures from 50:50 weight percent
PLA/polystyrene (PS) blends [88]. The viscosity ratio was kept near one by controlling the screw speed in the extruder. Upon extraction of the PS phase, an interconnected porous structure was observed [88]. We have previously investigated PLA/EastONE blends as a method to form porous fibers. EastONE (Eastman Chemical Company, Kingsport, TN) is a sulphonated co-polyester that can be dissolved in water. Blend ratios of 1, 3, 5, and 10% EastONE by weight were compounded with PLA in a Haake MiniLab and extruded into fibers. Fibers were washed in de-ionized water, followed by sonication to remove the EastONE component. Fourier transform infrared spectroscopy (FTIR) mapping was employed to determine the distribution of EastONE throughout the fibers. Results indicated that 10% EastONE led to fibers with uniform distribution throughout the fibers, however lower blend ratios resulted in regions of concentrated EastONE near the fiber surface [51].

In addition to the incorporation of a soluble additive, thermally induced phase separation (TIPS) is also routinely employed to fabricate porous structures. In the TIPS process, a homogenous blend of polymer and diluent undergoes liquid-liquid or solid-liquid phase separation into a polymer rich phase and diluent rich phase upon cooling [11], [46–50]. Removal of the diluent then results in a porous network. Different porous morphologies can be achieved based on materials selected and the cooling conditions employed [45], [47], [89]. In a study by Kim et al., porous hollow fibers were fabricated via a modified wet spinning process using soybean oil as the diluent [11]. Hollow PP fibers were achieved via a tube-in-orifice spinneret with nitrogen forming the lumen of the fibers. Phase separation was achieved using Freon 133 as a coagulant and diluent extractant. The effects of diluent concentration, spinning temperature, draw ratio, and cold stretching were explored. Decreasing the concentration of diluent led to a decrease in the porosity of the fibers.
Further, a pore size gradient was observed across the fiber cross section that was attributed to differences in cooling rate between the outer surface exposed to air and the inner lumen exposed to nitrogen [11]. In a more recent study, Li et al. investigated the effect of take-up speed (subsequently referred to as spinning speed) on polyvinylidene fluoride porous hollow fibers fabricated via TIPS [49]. At spinning speeds less than 41 m/min an asymmetric gradient structure was observed[49] similar to that reported by Kim et al.,[11] due to differences in the cooling rate across the fiber walls. However when spinning speed was increased to 72 m/min, the cross-section became uniform and an apparent decrease in porosity was observed. As the spinning speed increased, both the inner and outer diameters of the fibers decreased resulting in an increase in the cooling rate. An increase in elongational stress was suggested as a possible explanation for this phenomenon [49].

The final method of micropore formation that will be examined in this review is the melt spinning cold stretching (MSCS) process. Unlike TIPS or the inclusion of dissolvable additives, the MSCS process introduces pore structure by mechanically stretching fibers or films that are fabricated from a pure polymer. MSCS can be advantageous due to elimination of waste products and solvents and ease of handling, however control of pore size is relatively difficult. Pores are formed during stretching as microcrystalline lamellae oriented normal to the fiber direction are spread apart [45], [89]. Kim and Jang investigated the effect of draw ratio, spinning temperature, and annealing temperature on MSCS PP hollow membranes [89]. As draw ratio was increased, molecular orientation also increased, resulting in more segregated amorphous and crystalline phases. It was proposed that this phase segregation led to the formation of larger pores upon cold stretching [89]. More recently, Liu et al. investigated the effect of draw ratio and annealing time on the air flux of
microporous hollow PP membranes fabricated via MSCS. A nearly linear increase in air flux was observed with increasing draw ratio from 1000 to 2700 [44]. Shen et al. reported similar results during the formation of porous PE membranes [45]. Increased draw ratio resulted in the formation of more row-nucleated crystals, which upon annealing and cold stretching, led to the formation of larger micropores, increasing the flux of nitrogen [45].

Although these studies have shown promise as a strategy to create micropores via the MSCS process, several groups have attempted to fabricate highly porous fibers by combining the TIPS and MSCS processes, taking advantage of the unique aspects of each process [11]. Kim et al. proposed that microcrystalline lamellae also align during the TIPS process despite the presence of a diluent [11]. As such, further application of MSCS could result in changes in the pore structure across the fiber cross-section. They found that PP membranes fabricated via TIPS with soybean oil as the diluent subjected to 50 or 100% MSCS extension had a lower bubble point and density. It was estimated that the combined effect of doubling the draw ratio and MSCS extension led to a global porosity increase of 100%, however the authors were careful to point out that this did not coincide with an increase in the maximum pore size [11].

1.6. Formation of the Hollow Channel

Forcing a molten polymer through a hollow fiber spinneret produces hollow fibers. The geometry and arrangement of the spinneret governs the resultant cross section of the extruded fibers. Typical spinneret geometries include dies with either open or closed slits [90]. In the case of open slits, the molten polymer exits the spinneret and fuses just beyond the die to form the hollow channel [12–14]. Closed slit designs typically allow for better
control of fiber diameter as well as elimination of grooves on the surface where the polymer streams converge [14]. These designs also allow for the inclusion of either a gas stream in the center lumen to form the hollow channel, or a secondary sacrificial polymer that can be subsequently removed to form the hollow channel [11], [15–17].

Historically, wet and dry spinning have been the most common methodologies used to fabricate hollow fibers [11]. However, such strategies are limited due to high cost associated with low spinning speeds and the need for coagulation baths and solvent removal systems [40]. Advances in melt spinning and spunbond technologies offer a possible solution to make the formation of hollow fibers more economical as spinning speeds of up to 6000 m/min are possible for spunbond lines [76]. By combining principles of hollow fiber formation and spunbond processing, the fabrication of hollow fibers with interconnected microporous walls may be feasible.

Some groups have investigated the effects of melt spinning processing parameters on the formation of hollow fibers with various die configurations [12], [13]. Based on two-dimensional finite element analyses and experimental validation, Oh et al. reported that spinning temperature is the most important factor governing the formation of the hollow channel, followed by mass throughput rate [12]. A three-segmented arc die was employed to produce hollow PP fibers for model validation. Unlike solid cylindrical fibers, as the spinning temperature is increased, both the overall fiber radius and the ratio of the hollow cross-sectional area to the total cross-sectional area ($A_h/A_t$) (a measure of the hollowness of a fiber) decrease. The $A_h/A_t$ ratio decreased nearly 10% as spinning temperature was increased from 190 °C to 250 °C. This behavior was attributed to faster cooling of the molten polymer at lower temperature leading to a shorter zone of deformation. Mass throughput rate was
reported to have a slightly weaker effect on the hollowness of the fibers, with take-up velocity (spinning speed) having little effect [12]. In a more recent follow up study, Oh reported similar results for hollow PET fibers [13]. Spinning temperature had a strong effect on the cross-sectional area ratio \( A_i/A_t \), followed by mass throughput rate and spinning speed [13].

1.7. Islands in the Sea Multicomponent Fibers

Bicomponent fibers, such as island in the sea (INS), offer an alternative method for the fabrication of hollow fibers. In this method, specialized spinnerets allow simultaneous spinning of two polymers forming a sheath (sea component) around the second polymer (island component) (Figure 1.4). Typical island counts are 1 (sheath core), 7, 12, 19, 37, 108, 216, and 360 [79], [91–94]. Hills Inc. (West Melbourne, FL) has reported fabrication of filaments containing up to 1200 islands, with the potential of achieving over 4000. The island components are liberated from the sea component, leaving behind the islands that form individual filaments. In this manner, micro-denier fabrics can be achieved via the spunbond process, circumventing limitations of processes such as electrospinning and meltblowing that are traditionally used to fabricate micro- and nanofibers. Fedorova and Pourdeyhimi fabricated spunbond INS fabrics with Nylon 6 (N6) and PLA as the islands and sea polymers, respectively [91]. The PLA sea component was subsequently removed using a winch beck machine in 3% caustic soda at 100 °C for 10 minutes. The island count was varied from 0 to 360 and the percentage of island to sea polymer was set at 25% island, 75% sea; and, 75% island, 25% sea. By increasing the number of islands from 36 to 360, the final fiber diameters decreased from 1.3 to 0.36 \( \mu \text{m} \) for the 25% N6 samples [91].
Liberation of the island component results in the formation of smaller fiber diameters [93]. However, the washing process often requires solvents and thus can pose an environmental concern. Island components can also be liberated from the sea matrix through mechanical fibrillation, typically achieved via high-pressure water jets (hydroentangling or spunlace), carding, or drawing [91]. Durany et al. studied the effects of island count and sea polymer composition on the mechanical properties of INS spunbond webs fibrillated via hydroentangling [93]. The island and sea polymer combinations investigated included N6/PE, PET/N6, and N6/PET. The island to sea polymer ratio was set at 75% island and 25% sea for all samples analyzed. As expected, mechanical properties including tensile grab, tongue tear, and trap tear increased with increasing hydroentangling energy up to a level of 67,452 kJ/kg, followed by a slight decline. This decline was attributed to excess energy imparted to the fabric causing fiber damage. More energy was required to fibrillate the samples due to increased island/sea interfacial area, compared to other bicomponent
configurations such as segmented pie. They also reported that as the number of islands increased, the burst strength remained comparable to the sheath core control, indicating that increasing the number of islands did not result in a loss of mechanical properties. Further, mechanical properties were found to be superior to commercially available Evolon® samples of the same basis weight and did not require use of any solvents during processing [93].

Spunbond INS is a versatile process that can be implemented for a variety of applications using a variety of polymer combinations [83]. The selection of the island and sea components will depend on the desired application or functionality of the resultant fabric. Polyamide/polyolefin and polyamide/polyester are often used to form the island and sea components [79], [91–95]. Spunbond INS studies of N6 islands and PE sea by Anantharamaiah et al. suggest that fibrillation can be improved by limiting the molecular orientation of the sea component relative to the islands [92]. A modified cross section was employed that resulted in the sea forming a sheath around the islands. They reported that this enhanced spinnability and resulted in reduced orientation of the PE sea because the islands solidified first [92]. Fedorova and Pourdeyhimi examined the crystallinity of the island and sea components in N6/PLA spunbond fabrics as a function of the island count via wide angle X-ray scattering [91]. Overall, the crystallinity of both phases decreased compared to the pure polymers. However, as the number of islands increased from 36 to 360 the crystallinity of the N6 islands decreased slightly, while the crystallinity of the PLA phase was highest for the 360 island fibers. These results were attributed to changes in the solidification kinetics as the island count was varied. Increasing islands led to faster solidification of the N6 islands, resulting in higher stresses and ultimately increased molecular orientation. Further, the N6
phase reached final spinning speed faster than the PLA sea leading to further drawing of the sea component and thus improved orientation [91].

Alternatively, the island component can instead be removed, retaining the sea component and thus forming fibers with a varying number of hollow channels. Haslauer fabricated hollow porous INS filaments composed of 12 EastONE islands in a 20% EastONE/80% PLA sea matrix [17]. The EastONE islands were removed by subjecting the filaments to mechanical agitation in de-ionized water and subsequently sonicating for two hours at 69 ºC [17]. Wong et al. verified that the EastONE component was removed via focused ion beam characterization, resulting in filaments with 12 hollow channels and microporous fiber walls [16] (Figure 1.5).

![Figure 1.5. Induced secondary electron images of an EastONE/PLA fiber after removal of the EastONE component. Island and sea components were 100% EastONE and 80%PLA/20%EastONE, respectively. Fiber cross section (A). Increased magnification (B,C) denoted by rectangular box. Reproduced with permission from [16].](image)

1.8. Conclusions

We have attempted to describe in depth current advances in polymer extrusion and nonwovens processing in the context of hollow fiber manufacture with interconnected microporous walls with the goal of producing such structures via spunbond technology for
use as tissue engineering scaffolds. Hollow and porous fibrous scaffolds can serve as an artificial vascular network, increasing nutrient transport throughout the scaffold as well as elimination of cellular waste and degradation products. Spunbond processing is an advantageous melt extrusion technology as commercially viable spinning speeds can be obtained compared to traditional hollow fiber membrane technologies such as dry and wet spinning or electrospinning. Further, spunbond processing does not require any solvents as the molten polymer is extruded directly into filaments. Environmental impacts and energy consumption are thereby reduced, resulting in reduction in cost. Although this review is focused on these structures as tissue engineering constructs, through careful selection of the raw materials and tailoring of the fabric properties, hollow porous fibers could be useful for a variety of other applications such as thermal and acoustic insulation, odor absorption, liquid and gas separation, technical textiles, and medical separation membranes.

Understanding the complex interaction between the raw materials, processing parameters, and the resulting fabric structure is key to designing highly engineered nonwoven fabrics comprised of hollow fibers with interconnected micropores. Not only will the raw materials affect the properties of the final fabric, processing parameters that affect the various attributes such as fiber orientation distribution, fiber diameter and distribution, basis weight, pore size and distribution, and bonding methodology will affect the final product and must be considered. By combining established hollow membrane technologies and spunbond nonwovens processing, fabrication of spunbond nonwovens composed of hollow fibers with interconnected micropores may be realized.
CHAPTER 2 Scalable Nonwoven Manufacturing Techniques for Clinical Translation of Tissue Engineering Strategies Using Human Adipose Derived Stem Cells

This chapter has been submitted for publication to Tissue Engineering Part C: Methods.

2.1. Introduction

Electrospun nonwovens have been used extensively for tissue engineering applications due to their inherent similarities with respect to fiber size and morphology to that of native extracellular matrix (ECM). However, fabrication of large scaffold constructs is time consuming, may require harsh organic solvents, and often results in mechanical properties inferior to the tissue being treated. In order to translate nonwoven based tissue engineering scaffold strategies to clinical use, a high throughput, repeatable, scalable, and economic manufacturing process is needed. We suggest that nonwoven industry standard high throughput manufacturing techniques (meltblowing, spunbond, and carding) can meet this need. In this study, meltblown, spunbond and carded poly(lactic acid) (PLA) nonwovens were evaluated as tissue engineering scaffolds using human adipose derived stem cells (hASC) and compared to electrospun nonwovens. Scaffolds were seeded with hASC and viability, proliferation, and differentiation were evaluated over the course of three weeks. We found that nonwovens manufactured via these industry standard, commercially relevant manufacturing techniques were capable of supporting hASC attachment, proliferation, and both adipogenic and osteogenic differentiation of hASC, making them promising candidates.
for commercialization and translation of nonwoven scaffold based tissue engineering strategies.

Tissue engineering strategies aim to repair or replace damaged tissues using a stem cell source seeded on a biological or artificial scaffold structure. As the cells proliferate and differentiate creating new tissue, the original scaffolding material is gradually replaced, leaving behind the desired replacement tissue. A wide range of scaffolding materials have been investigated as tissue engineering constructs with varying degrees of success [96]. Nonwoven fabrics are one such example that show great promise as scaffolding for tissue engineering [17], [18], [20], [22], [52–57], [97]. In basic terms, a nonwoven is a fabric or web composed of fibers. The orientation and properties of the fibers and fabric can be controlled to mimic native cellular structures, such as collagen fibers of the extracellular matrix (ECM). Electrospinning is the most common nonwoven manufacturing method used in the field of tissue engineering. The size scale of electrospun fibers mimics that of native ECM and provides an ideal environment for cellular attachment, growth, and differentiation into the target tissue [36], [59–61], [64–66], [98–100]. However one of the many hurdles facing translation of tissue engineering strategies to clinical practice lies in scalable and repeatable fabrication of scaffold material on an industrial scale. Fabrication of large scale electrospun scaffolds is time consuming, may require harsh organic solvents, difficult to generate sufficient three-dimensional structures, and typically results in mechanical properties inferior to the target tissue to be replaced. In order to translate nonwoven tissue engineering from the lab to the clinic, a scalable, repeatable, and economical scaffold fabrication method is needed. The goal of this study was to test our hypothesis that other industry standard nonwoven manufacturing technologies may meet this need.
Although there are many nonwoven fabrication processes, three of the most common in use are meltblowing, spunbond, and carding. These processes and the processing parameters affecting fabrication and properties of the resulting nonwovens are described in detail elsewhere [101], but are briefly described here to orient the reader. Meltblowing and spunbond nonwovens are composed of continuous fiber filaments fabricated by forcing molten thermoplastic polymer through very fine orifices arranged in a spinbeam. As the filaments are formed, they are drawn and accelerated, and collected on a moving screen or belt to form the nonwoven web. The main difference in the meltblowing and spunbond processes lies in the drawing step. In the meltblowing process, the filaments are drawn and accelerated toward the collector screen via hot air knives, keeping the filaments in a molten state, allowing for fine fiber attenuation. Collected fibers are still in a tacky state, allowing self-bonding between fibers [101]. Conversely, in the spunbond process, as the filaments exit the spinbeam they are rapidly solidified by cool air before being drawn pneumatically. Drawing of filaments in the solid state increases molecular orientation and leads to improved mechanical properties compared to meltblowing, typically at the cost of greater fiber diameter [76], [79]. In the carding process, short fibers of a few inches in length (staple fibers) are separated and entangled by a series of specialized combed rollers to form an unbonded web. Carded webs are typically layered to increase the fabric basis weight and improve fiber orientation (crossslapping) before being bonded to lock the structure in place [101]. Typical processing capabilities and fiber and fabric properties achievable via the methods described here are presented in Table 2.1. The high speed, repeatable, economical production capabilities of these techniques make them attractive candidates for the commercial production of nonwoven based tissue engineering scaffolding materials.
Interest in human adipose derived stem cells (hASC) for tissue engineering applications has increased recently given their relative ease of harvest, accessibility, and multipotent differentiation capacity, in particular for lineages derived from the mesoderm [102–107]. Ease of harvest from excessive adipose tissue makes them particularly attractive compared to other mesodermal cells such as mesenchymal stem cells that require invasive collection procedures and yield smaller quantities. The goal of this study was to compare industry standard nonwovens fabricated via meltblowing, spunbond, and carding to electrospun materials as tissue engineering scaffolds with respect to their effects on viability, proliferation, and differentiation of hASC.

Table 2.1. Estimated typical processing capabilities and cost of commercial nonwoven manufacturing techniques.

<table>
<thead>
<tr>
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<th>Electrospinning</th>
<th>Meltblowing</th>
<th>Spunbond</th>
<th>Carding</th>
</tr>
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<tbody>
<tr>
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<td>20</td>
<td>300</td>
<td>600</td>
<td>200</td>
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<td>Max Line Width (m)</td>
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<td>5.5</td>
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<tr>
<td>Fiber Diameter Range (µm)</td>
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<td>0.5-10</td>
<td>10-50</td>
<td>10-50</td>
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<tr>
<td>Basis Weight Range (g/m²)</td>
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<td>5-500</td>
<td>10-800</td>
<td>10-2000 (with crosslap)</td>
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<tr>
<td>Typical Cost for PLA ($/m²)</td>
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<td>1-2</td>
<td>0.3-3</td>
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2.2. Materials and Methods

*Scaffold Fabrication*

All nonwoven scaffolds used in this study were composed of poly(lactic acid) (PLA) 6202D (NatureWorks LLC, Minnetonka, MN), with a melt index (g/10 min at 210 °C) of 15-
30, engineered for staple fiber and spunbond production. Spunbond, carded, and meltblown nonwovens were fabricated at the Nonwovens Institute pilot facilities (NWI, Raleigh, NC). Meltblown processing of this PLA grade was achieved via a 5-inch Biax Fiber-Film (Greenville, WI) co-concentric spinnerette mounted on a custom meltblowing unit. Electrospun scaffolds were produced by dissolving PLA in chloroform and dimethylformamide at a ratio of 3:1 (Sigma, St Louis, MO) at a concentration of 11 wt% for four hours at 80 °C. Electrospinning was performed at room temperature at a feed rate of 0.7 µL/hr, 15 kV, and spinning distance of 15 cm on a static grounded collector for 3 hours on a custom electrospinning system.

**Scaffold Characterization**

Mechanical and physical properties of nonwovens used in this study were characterized prior to cell seeding. Fabric surface and fiber morphology was imaged on a Phenom G2 Pro Scanning Electron Microscope (SEM) (Eindhoven, Netherlands). Fiber diameter was calculated using ImageJ software (NIH, Bethesda, MD) with a minimum of 100 fibers measured. Fabric thickness was measured using a Hanatek FT3 Precision Thickness Gauge (East Sussex, UK). Thickness measurements were taken from random locations across fabric surfaces, at least two inches from the fabric edge (n=10) in accordance with ASTM D5729. Mean flow pore size was analyzed using a Porous Materials INC advanced capillary flow porometer (Ithaca, NY) using GalWick as the wetting agent in a wet up/dry up, no wait at dry configuration. Basis weight samples of each fabric type were cut and weighed in accordance with IST 130.2. The solid volume fraction was calculated from thickness and basis weight measurements and fiber density (1.24 g/cm³) according to
Equation 2.1. Fabric porosity is reported as the difference of 100% and the solid volume fraction (SVF).

Equation 2.1. \[ \mu = \left( \frac{B}{z} \right) / \rho \]

where: \( \mu = \) solid volume fraction
\( B = \) fabric basis weight
\( z = \) fabric thickness
\( \rho = \) fiber mass density

A strip test in the machine direction (MD) and cross direction (CD) was used to evaluate mechanical properties measured on an Instron 4400R (Norwood, MA) in accordance with ASTM D5035. Fabric width was insufficient for strip test in the CD for meltblown fabrics (less than 6 inches) and both MD and CD for the electrospun fabrics.

hASC Isolation

A pre-menopausal hASC superlot that we have previously described, consisting of five gender and age matched donors, was used for cellular growth and differentiation studies [107]. Use of a superlot allows streamlining of the experimental process, reducing time and reagents required to test individual donor cells, while capturing cellular proliferation and differentiation behaviors of the individual cell lines [107]. hASC were obtained from excess waste tissue from liposuction procedures performed at the University of North Carolina at Chapel Hill under an IRB exempt protocol (10-0201).

Scaffold Seeding

Fabrics were punched into 9/16 inch diameter circular scaffolds and placed in 24 well non-tissue culture treated polystyrene plates and sterilized in 70% ethanol for four hours and subsequently washed with phosphate buffered saline (PBS) three times. Scaffolds were then soaked in complete growth medium (CGM; Eagle α-Minimum Essential Medium, 10% fetal
bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin) for 12 hours prior to cell seeding. hASC superlot (second passage) cells were seeded in 75 cm² tissue culture treated flasks and expanded to 75% confluency in CGM. hASC were trypsinized and resuspended in CGM at a concentration of 200,000 cells/mL. Scaffolds (n=3/condition) were then seeded with 20,000 cells/cm² and allowed to attach for one hour in a humidified incubator at 37 °C and 5% CO₂ prior to flooding the well with CGM. Cells were allowed to attach for an additional 12 hours prior to turning the scaffolds over and seeding the other side with an additional 20,000 cells/cm². After an additional 12 hours, scaffolds were transferred to new tissue culture plates, and the remaining cells on the plate surfaces were trypsinized and counted on a hemocytometer to determine cell seeding efficiency. Scaffolds were then cultured in CGM for seven days, with media changes performed every three days. On day seven, culture medium was changed to osteogenic differentiation medium (ODM; Eagle α-Minimum Essential Medium, 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 50 µM ascorbic acid, 0.1 µM dexamethasone, and 10 mM β-glycerophosphate), or adipogenic differentiation medium (ADM; Eagle α-Minimum Essential Medium, 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 1 µM dexamethasone, 10 µg/mL human insulin, 100 µM indomethacin, and 500 µM isobutylmethylxanthine), or continued in CGM for an additional 14 days with media changes every three days.

**Analysis of Scaffold Performance**

On days 1, 3, 5, 7, and 21 cellular viability was evaluated with a mammalian cell Live/Dead viability/cytotoxicity kit (Invitrogen Molecular Probes, Eugene, OR) and cellular
proliferation using the AlamarBlue™ assay (BioRad AbD Serotec, Oxford, UK). Live/Dead scaffolds were washed twice in PBS and dead cells stained with 4 μM ethidium homodimer-1 and live cells with 2 μM calcein AM in PBS for 15 minutes in a humidified incubator at 37 °C and 5% CO₂. Stained scaffolds were then mounted on glass microscope slides and imaged under 10x magnification on a Leica DM5500B fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Calcein AM produces a strong green fluorescence in the cytoplasm of live cells while ethidium homodimer-1 produces a strong red fluorescence in the nuclei of dead cells. At each experimental time point, AlamarBlue™ scaffolds (n=3/condition) were washed twice with PBS and wells flooded with media containing 10% (v/v) of AlamarBlue™. Scaffolds were incubated for 3 hours in a humidified incubator at 37 °C and 5% CO₂, at which time 200 μL of treated media was removed and absorbance read at 570 and 600 nm on a GENios microplate reader (Tecan, Männedorf, Switzerland). Scaffolds were then washed three times with PBS and wells flooded with media. Metabolic activity results in the reduction of AlamarBlue™, resulting in a detectable color change. The percent reduction is related to metabolic activity and thus an indicator of cellular proliferation. Because AlamarBlue™ is nontoxic to cells, the same scaffolds can be evaluated for cellular proliferation for the duration of the experiment.

Cell mediated calcium and lipid accretion was evaluated on day 21 qualitatively using Alizarin Red S and Oil Red O stains, respectively (both reagents from Acros Organics, Geel, Belgium), and quantitatively using total calcium (StanBio Laboratory, Boerne, TX) and adipogenesis colorimetric assay kits (BioVision, Milpitas, CA). Scaffolds for staining were washed three times in PBS and fixed in 10% buffered formalin for 30 minutes (Thermo Scientific, Waltham, MA). Scaffolds were subsequently rinsed three times in PBS and
stained with 40 mM Alizarin Red S or 0.4 mM Oil Red O for 5 minutes. Scaffolds were rinsed several times with distilled water to remove excess stain and imaged on an Olympus CKX41 inverted microscope at 10x magnification (Olympus, Center Valley, PA). Scaffolds for quantitative analysis (n=3/condition) were rinsed twice in PBS and subsequently carefully cut in half with a razor. For calcium quantification one half was transferred to 1 mL of 0.5N HCl and placed on an orbital shaker overnight at 4 °C. The remaining half was transferred to 200 µL of RIPA buffer (Thermo Scientific, Waltham, MA) and incubated on ice for 10 minutes, centrifuged at 15,000 rpm and the supernatant collected. Similarly, scaffolds for triglyceride quantification were cut in half and one half transferred to 200 µL of adipogenesis assay buffer and the remaining half in RIPA buffer. Calcium and triglyceride samples were assayed according to manufacturer’s instructions and absorbance read on a GENios microplate reader at 550 and 570 nm, respectively. Protein samples for normalization were analyzed using a BCA total protein assay (Thermo Scientific, Waltham, MA) according to manufacturer’s instructions and absorbance read at 562 nm.

Statistical Analysis was performed using Microsoft Excel for Mac OS X, Version 10.10.2 (Microsoft, Redmond, WA). An unpaired Student’s t-test was used to analyze statistical difference and to distinguish statistical significance between test conditions. For each quantitative assay n=3 biological replicates and n=3 technical replicates were utilized (n=9 per condition). Error bars represent standard error of the mean. Statistical significance is indicated as p < 0.05.
2.3. Results

Scaffold Characterization

The nonwoven fabrics employed in this study as tissue engineering scaffolds were chosen if they were the most representative “typical” fabric and fiber properties of their respective manufacturing methods. Scaffolds exhibited a wide range of fiber and fabric properties (Table 2.2). SEM micrographs of scaffold surfaces at 500x magnification are presented in Figure 2.1. Scaffolds varied from thin fabrics with fine micron sized fibers and small pore size to very open structures with very high porosity and larger fibers. Fiber diameter varied from 1.1 – 26.9 µm, while thickness and pore size ranged from 160.8 – 965.7 µm and 2.6 – 655 µm, respectively. Similarly, mechanical properties, measured as peak load, varied from 1.0 – 102.2 N. As expected, sample size of electrospun scaffolds was insufficient for mechanical testing in the machine and cross directions (MD and CD), and the width of meltblown samples was insufficient for CD testing.
Table 2.2. Fiber and fabric properties of nonwovens evaluated as tissue engineering scaffolds using hASC. MD and CD refer to machine and cross directions respectively. Fabric size was insufficient for MD and CD peak load testing of electrospun scaffolds and fabric width was insufficient for meltblown CD peak load testing.

<table>
<thead>
<tr>
<th></th>
<th>Electrospun</th>
<th>Meltblown</th>
<th>Spunbond</th>
<th>Carded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber Diameter (µm)</td>
<td>1.1 ± 0.3</td>
<td>4.8 ± 4.6</td>
<td>12.8 ± 2.4</td>
<td>26.9 ± 3.0</td>
</tr>
<tr>
<td>Fabric Thickness (µm)</td>
<td>160.8 ± 1.4</td>
<td>343.2 ± 12.3</td>
<td>562.3 ± 21.2</td>
<td>965.7 ± 52.2</td>
</tr>
<tr>
<td>Mean Flow Pore Size (µm)</td>
<td>2.6 ± 0.7</td>
<td>27.2 ± 12.5</td>
<td>24.0 ± 14.9</td>
<td>655.0 ± 373.4</td>
</tr>
<tr>
<td>Basis Weight (g/m²)</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>Solid Volume Fraction</td>
<td>0.125</td>
<td>0.117</td>
<td>0.143</td>
<td>0.046</td>
</tr>
<tr>
<td>Porosity (%)</td>
<td>87.5</td>
<td>88.3</td>
<td>85.7</td>
<td>95.4</td>
</tr>
<tr>
<td>MD Strip Test Peak Load (N)</td>
<td>-</td>
<td>25.5 ± 7.4</td>
<td>102.2 ± 12.8</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>CD Strip Test Peak Load (N)</td>
<td>-</td>
<td>-</td>
<td>55.7 ± 5.5</td>
<td>1.0 ± 0.6</td>
</tr>
</tbody>
</table>
Figure 2.1. Scanning electron micrographs of nonwoven fabric surfaces evaluated as tissue engineering scaffolds for hASC at 500x magnification. (a) Electrospun, (b) meltblown, (c) spunbond, and (d) carded. Fiber diameters ranged from 1.1, 4.8, 12.8, and 26.9 µm while average pore size ranged from 2.6, 27.2, 24.0, and 655 µm for electrospun, meltblown, spunbond, and carded fabrics respectively. Scale bars represent 100 µm.

**Seeding Efficiency**

hASC were able to adhere to all scaffolds examined with seeding efficiencies exceeding 60% for all nonwoven fabric types (Figure 2.2). Scaffolds exhibited seeding
efficiencies of 84, 82, 78, and 64% for electrospun, meltblown, spunbond, and carded fabrics, respectively. There was no significant difference in seeding efficiency between electrospun, meltblown, and spunbond scaffolds. However, carded nonwovens exhibited a significant decrease in seeding efficiency compared to the other scaffold types (Figure 2.2).

**Figure 2.2.** Seeding efficiency nonwoven scaffolds evaluated as tissue engineering scaffolds for hASC. After seeding procedure, scaffolds were transferred to new tissue culture plates and remaining cells were trypsinized and counted on a hemocytometer (n=3). No statistical differences were observed among electrospun, meltblown, and spunbond scaffolds, while carded scaffolds exhibited a significant reduction in seeding efficiency. This was attributed to much larger fabric pore sizes in carded scaffolds, reducing overall surface area and probability of cells interacting with scaffold fibers. Error bars represent standard error of the mean. Different letters represent statistical significance (p<0.05).

**Proliferation and Viability**

hASC proliferation and viability was analyzed qualitatively over the course of the experiment using a Live/Dead staining assay. All scaffolds exhibited an increase in the number of viable cells over the first seven days of culture in CGM (Figure 2.3). hASC on
electrospun, meltblown, and spunbond scaffolds exhibited a typical hASC morphology and attached across multiple PLA fibers. However, hASC cultured on carded samples initially attached to single PLA fibers and exhibited a more elongated morphology along the fiber direction (Figure 2.3, d₁ and d₂). After five days, cells appeared to be concentrated in regions where fibers overlap, with far fewer cells in adjacent open pore volumes (Figure 2.3, d₃ and d₄). At the end of the 21 day experiment, electrospun, meltblown, and carded scaffolds were uniformly covered in viable cells for all culture media (CGM, ODM, and ADM), whereas carded samples showed regions of concentrated cells in regions of fiber overlap, with significant ingrowth into adjacent pore volumes (Figure 2.4). Further, hASC seeded on scaffolds and cultured in ADM exhibited fewer viable cells compared to those cultured in CGM and ODM on all scaffold types (Figure 2.4, a₃, b₃, c₃, d₃). Electrospun and carded scaffolds exhibited relatively few dead cells over the first seven days, whereas meltblown and spunbond scaffolds exhibited a relative increase in the number of dead cells.
Figure 2.3. Live/Dead staining of hASC over time for CGM treated scaffolds at 10x magnification. All fabric types supported viable cell growth over the first seven days of the experiment. Meltblown and spunbond samples exhibited a greater number of viable cells, attributed to cell attachment and spreading across multiple fibers throughout the thickness of the scaffolds. Electrospun scaffolds exhibited cell growth in a monolayer on the fabric surface. Cells initially attached to single fibers on carded scaffolds and gradually filled in fiber-fiber crossover points. Meltblown and spunbond scaffolds also exhibited a greater number of dead cells compared to electrospun and carded fabrics. This is attributed to mass transport limitations in cells that have infiltrated the fabric thickness. Scale bars represent 100 µm.
Figure 2.4. Live/Dead staining of hASC at the end of the 21 day experiment in CGM, ODM, and ADM at 10x magnification. hASC remained viable on all fabric types for the duration of the experiment. By day 21, electrospun, meltblown, and spunbond fabrics exhibited similar cell spreading, covering the entire surface of the fabrics. hASC on carded scaffolds showed significant ingrowth into pore volumes, originating from fiber-fiber crossover points, but did not entirely occupy scaffold pores. hASC treated with CGM or ODM exhibited typical spread hASC morphology while ADM treated scaffolds assumed a rounded shape. Electrospun, meltblown, and spunbond scaffolds exhibited a similar number of dead cells, while carded scaffolds exhibited relatively few dead cells. Scale bars represent 100 µm.
Proliferation of hASC was quantified using the AlamarBlue™ assay. All scaffolds exhibited an increase in hASC proliferation over time, however the meltblown and spunbond scaffolds led to a significant increase in cellular proliferation compared to electrospun and carded scaffolds over the first seven days in culture (Figure 2.5a). These results were in agreement with the qualitative Live/Dead imaging. At the end of the 21 day experiment in each culture media evaluated, there were no significant differences in proliferation between scaffold types (Figure 2.5b).
Figure 2.5. AlamarBlue™ cellular proliferation of hASC across different nonwoven fabric types (n=3). (a) hASC proliferated across all fabric types over the first seven days of the experiment, however meltblown and spunbond fabrics showed a significant increase compared to electrospun and carded fabrics, confirming Live/Dead staining. (b) All fabric types showed similar proliferation at the end of the 21 day experiment across each growth media type evaluated. Different letters represent statistical significance (p<0.05).
Differentiation Capacity

In order to examine the effect of scaffold type on the adipogenic and osteogenic differentiation capacity of hASC, lipid and calcium accretion was visualized and quantified on day 21. Oil Red O staining revealed the presence of lipid vacuoles in hASC on all scaffold types when cultured in ADM (Figure 2.6, a3, b3, c3, d3). Similarly, Alizarin Red S staining indicated the presence of calcium accretion for all scaffolds cultured in ODM (Figure 2.7, a2, b2, c2, d2). These results were confirmed quantitatively and indicated a significant increase in lipid and calcium accretion for all scaffolds compared to controls (Figure 2.8). Electrospun, meltblown, spunbond, and carded scaffolds resulted in normalized triglyceride concentrations of $0.048 \pm 0.007$, $0.074 \pm 0.013$, $0.069 \pm 0.010$, and $0.077 \pm 0.005$ nmol lipids/µg protein respectively, and calcium mass of $0.0095 \pm 0.0006$, $0.0099 \pm 0.0009$, $0.0074 \pm 0.0006$, and $0.0108 \pm 0.0006$ µg calcium/µg protein respectively. As expected, lipid accretion was significantly lower for CGM and ODM treated scaffolds, and calcium accretion was significantly lower for CGM and ADM treated scaffolds.
**Figure 2.6.** Oil Red O lipid staining for all nonwoven fabric types evaluated as tissue engineering scaffolds for hASC at 10x magnification. All fabric types exhibited the presence of lipid vacuoles when treated with ADM (lipid vacuoles appear stained as cherry red spheres). Electrospun scaffolds exhibited a monolayer of lipid vacuoles in a single focal plane, whereas lipid vacuoles were observed in multiple planes throughout the fabric thickness for meltblown, spunbond, and carded scaffolds. CGM controls and ODM treated scaffolds did not exhibit any lipid vacuoles, indicating cells are not undergoing random differentiation. Scale bars represent 100 µm.
Figure 2.7. Alizarin Red S calcium staining for all nonwoven fabrics types evaluated as tissue engineering scaffolds for hASC at 10x magnification. Calcium deposits appear dark red after staining. All fabric types exhibited the presence of intense calcium staining when treated with ODM. Calcium staining was concentrated in regions of greatest fiber density for meltblown, spunbond, and carded scaffolds, while electrospun scaffolds exhibited uniform staining over the entire fabric surface. CGM controls did not exhibit any calcium staining, however relatively small amounts of calcium were observed for ADM treated scaffolds. Scale bars represent 100 µm.
Figure 2.8. End product expression of hASC after 21 days in culture on all nonwoven fabric types evaluated (n=3). (a) Total triglyceride content normalized to total protein across all media types evaluated. All fabric types exhibited a significant increase in triglyceride content when treated with ADM, indicating the presence of an adipogenic cell phenotype. No statistical differences were observed amongst ADM treated scaffolds across all fabric types. (b) Calcium content normalized to total protein. All fabric types exhibited a significant increase in calcium content when treated with ODM, indicating the presence of an osteogenic cell phenotype. No statistical differences were observed amongst ODM treated scaffolds between electrospun, meltblown, and carded scaffolds, however a small but significant decrease was observed on the spunbond scaffolds. Different letters represent statistical significance (p<0.05).
2.4. Discussion

The goal of this study was to evaluate commercially relevant, repeatable, and economical high throughput nonwoven manufacturing techniques as tissue engineering scaffolds. Specifically, we examined the viability, proliferation, and adipogenic or osteogenic differentiation of hASC on meltblown, spunbond, and carded PLA nonwovens compared to the nonwoven gold standard of electrospinning for tissue engineering. Our group and many others have demonstrated the potential utility of electrospun nonwovens as tissue engineering scaffolds for a wide range of cell sources and target tissues [36], [59–61], [64–66], [98]. Electrospun fibers are similar in size scale to native ECM and provide an ideal environment for cellular attachment, proliferation, and differentiation. However, electrospinning large quantities is time consuming, may require harsh organic solvents, and typically results in structures with inferior mechanical properties to the tissue being treated. We hypothesized that industry standard nonwoven manufacturing techniques such as meltblowing, spunbond, and carding may provide economical, repeatable, and scalable methods for the fabrication of large quantities of nonwovens for use as tissue engineering scaffolds with precise control of fabric and fiber properties.

All scaffolds used in this study were able to support attachment, proliferation, and differentiation of hASC. Seeding efficiency data indicated there was no significant difference between seeding efficiency for electrospun, meltblown, and spunbond scaffolds, however a significant decrease was observed on the carded scaffolds (Figure 2.2). This is likely due to differences in fabric properties, namely pore size and fiber size. The carded scaffold had a very open and porous structure; with an average pore size of 655 µm, compared to pore sizes in the range of 1-25 µm for the other fabric types. The fiber diameter
of 26.9 µm was also significantly larger for the carded scaffolds, compared to fiber diameters ranging from 1.1 – 12.8 µm for the other scaffolds. This open structure and larger fiber size may have decreased the opportunity for cellular attachment to the scaffold fibers during the seeding process as hASC seeded in static culture could instead collect and adhere to the culture plate.

Live/Dead staining revealed that cells remained viable on all nonwoven scaffold types over the course of three weeks (Figures 2.3 and 2.4). Further, by the end of the experiment all scaffolds across all media types appeared to exhibit similar hASC coverage on all nonwoven types (Figure 2.4). However, at early time points, meltblown and spunbond samples appeared to have more viable cells compared to electrospun and carded fabrics (Figure 2.3, b₁–b₄, c₁–c₄). This was consistent with AlamarBlue™ cellular proliferation assays, which showed a statistically significant increase in hASC proliferation on meltblown and spunbond scaffolds for days 1 – 7 compared to electrospun and carded nonwovens (Figure 2.5a). We speculate that this increase is likely attributed to cellular infiltration into the thickness of meltblown and spunbond scaffolds. Cellular growth is restricted to the surface of electrospun fabrics due to very small pore sizes, effectively leading to less total growth area for cells. Carded samples also indicated cell spreading throughout the thickness of the scaffolds, however cellular attachment was focused on the surface of single fibers, whereas cells appeared to attach across multiple fibers on the other fabric types. Cell growth on carded samples began with attachment of individual cells to single fibers, and over time cells populated fiber-fiber cross over points (Figure 2.3, d₁–d₄, Figure 2.4, d₁–d₃). These findings are consistent with work by Edwards et al. examining tissue growth on carded PET nonwovens using mouse fibroblasts [56]. Meltblown and spunbond scaffolds also exhibited
a greater number of dead cells over the course of the experiment compared to electrospun and carded fabrics (Figure 2.3, b1–b4, c1–c4. Figure 2.4, b1–b3, c1–c3). This was likely attributed to mass transport limitations of nutrients throughout the thickness of the scaffold constructs. Several groups have shown that cell seeded scaffolds quickly become covered in a dense layer of ECM and cells on the periphery, effectively blocking mass transport of nutrients to cells in the interior of the scaffold and removal of cellular waste products [19–22]. In the case of electrospun scaffolds, cell growth is restricted to the surface of the fabric due to the small pore size restricting migration through the fabric thickness, however at the end of the 21 day experiment electrospun scaffolds showed an increase in the number of dead cells compared to earlier time points. This effect was not observed with the carded scaffolds and is likely a result of the very porous nature of the material. hASC are not observed to occupy the entirety of the pore volume, likely resulting in less ECM deposition on the surface, effectively retaining a degree of porosity and route of mass transport.

AlamarBlue™ proliferation data at day 21 indicated minimal differences in the number of cells across all fabric and media types (Figure 2.5b). Although the initial proliferation rate of the meltblown and spunbond samples was higher compared to those of the electrospun and carded samples (Figure 2.5a), by the end of the experiment cell growth equalized across the different scaffold types (Figure 2.5b). Taken together, this data suggests that there is an important relationship between fiber size, pore size, and fabric thickness that governs the ability of cells to attach and proliferate throughout the scaffold construct over time.

Analysis of the differentiation capacity of hASC cultured on high throughput nonwoven fabrics indicated that all fabrics were capable of supporting adipogenesis and
osteogenesis when treated with appropriate growth factors (Figures 2.6–2.8). Oil Red O staining revealed the presence of lipid vacuoles for hASC seeded on all scaffold types and cultured in ADM (Figure 2.6, a₃, b₃, c₃). Visual inspection of Oil Red O micrographs would suggest that electrospun scaffolds exhibit the largest number of lipids compared to the other scaffold types. However, all of the lipid vacuoles on the electrospun scaffolds were observed in a single plane, suggesting cell growth was restricted to the surface of the scaffold with little to no ingrowth throughout the fabric thickness (Figure 2.6, a₃). Conversely, all other scaffolds exhibited the presence of lipid vacuoles in multiple planes, throughout the thickness of the fabrics. Although not a significant measure of cellular infiltration, this suggests that cells are capable of penetrating the thickness of meltblown, spunbond, and carded scaffolds to some extent. Additional experiments would be required to verify cellular infiltration and distribution throughout the thickness of the scaffolds. Additionally, lipid vacuoles on the carded samples appeared concentrated in the space between fiber-fiber crossover points (Figure 2.6, d₃), further suggesting cells are able to bridge these points after three weeks, similar to work by Edwards et al. on carded PET [56]. Oil Red O staining results were confirmed with quantitative evaluation of total triglycerides present on ADM treated scaffolds. Figure 2.8a revealed that all hASC seeded scaffolds cultured in ADM show a striking increase in total triglycerides compared to CGM controls and ODM treated scaffolds, indicating the presence of an adipogenic phenotype. Further, there was no statistical difference in normalized triglyceride content for ADM treated scaffolds between nonwoven fabrication method, indicating all three high throughput fabrication methods are suitable for hASC adipogenesis.
Similar to Oil Red O results, Alizarin Red S staining revealed the presence of intense red calcium staining across all nonwoven scaffold types seeded with hASC and cultured in ODM (Figure 2.8, a₂, b₂, c₂, d₂). Calcium staining was not observed for CGM controls; however there was a small amount of staining for ADM treated scaffolds (Figure 2.8, a₃, b₃, c₃, d₃). Microscopic evaluation revealed that calcium staining was concentrated in areas of greatest fiber density, i.e. staining was not present in porous regions. This suggests that cells are also concentrated in these regions and do not fully occupy pore volume. This was particularly striking for the carded samples, where red staining was observed near fiber cross over points, leaving no stain in the large open pores. Small regions of light staining were also observed in ADM treated scaffolds with no apparent pattern discernable.

Alizarin Red S staining results were confirmed with quantitative evaluation of total calcium present on each scaffold type. Figure 2.8b revealed that all ODM treated fabric types showed an increase in total calcium compared to CGM controls and ADM treated scaffolds, indicating the presence of an osteogenic phenotype. ADM treated scaffolds showed an increase in calcium content relative to CGM controls, however to a much lesser extent than ODM treated scaffolds. While there was no difference in scaffold type for adipogenesis, osteogenesis results indicated that there was no difference in normalized calcium accretion across electrospun, meltblown, and carded samples, however there was a small but significant decrease in calcium accretion on spunbond scaffolds.

We have demonstrated that meltblown, spunbond, and carded high throughput nonwoven manufacturing methods are suitable as tissue engineering scaffolds for hASC. hASC viability, proliferation, adipogenesis, and osteogenesis were similar to electrospun gold standard nonwoven tissue engineering scaffolds. These results are promising in the
effort to move tissue engineering strategies out of the lab and into commercial production and clinical use as they allow large quantities of material to be produced quickly, economically, and with a wide range of controlled fabric properties. However, it is important to note that further studies are needed to understand the interplay of fabric properties and their effects on cellular growth and differentiation. In order to directly state that one method is superior to another, studies normalizing specific fabric properties such as fiber size, solid volume fraction, or pore size are needed. This becomes difficult, as each method has unique benefits and disadvantages. For example, it would be nearly impossible to create electrospun and carded scaffolds with the same fiber diameter and porosity. However these results do indicate that these methods are suitable for cell growth and differentiation and merit additional study as commercially viable, high throughput tissue engineering scaffolding materials. Understanding the interplay between fiber and fabric properties on the behavior of hASC is crucial for successful tissue engineering strategies and will vary depending on the target tissue and cell source. In future studies, we will examine the effects of processing parameters as well as fiber and fabric properties on cellular growth and differentiation across different nonwoven manufacturing methods with the goal of better understanding the relationship between scaffold properties and hASC behavior.
CHAPTER 3 Industry Standard Scalable Fabrication of Biax Spunblown™ Porous Fiber Poly(Lactic Acid) Melblown Nonwovens for Tissue Engineering Applications Using Human Adipose Derived Stem Cells

This chapter has been submitted for publication to Advanced Healthcare Materials.

3.1. Introduction

Due to their similar fibrillar structure to native extracellular matrix, nonwoven fibrous materials are promising candidates as tissue engineering scaffolds. However, for relatively thick, three dimensional scaffolds, enhanced mass transport strategies to eliminate or reduce the formation of necrotic cores are required. The Biax Spunblown™ process is capable of producing meltblown nonwovens at industrial speeds in a repeatable, economical, and scalable fashion using a variety of polymers, including higher viscosity polymers that cannot be easily meltblown in conventional meltblowing processes. In this study, fabrication and characterization of porous poly(lactic acid) (PLA) meltblown nonwovens is reported. The inclusion of a water dispersible polymer, AQ55S, into the PLA backbone and subsequent removal in hot water, results in a porous internal fiber morphology. Further processing with sodium hydroxide exposes the internal pore structure. The economical, scalable, high-speed Spunblown™ fibers are evaluated as tissue engineering constructs using human adipose derived stem cells (hASC). The effects of fiber morphology on viability, proliferation, adipogenic and osteogenic differentiation, and scaffold infiltration are discussed. Porous
fibers promote adipogenic and osteogenic differentiation as evidenced by significantly increased triglyceride and cell mediated calcium accretion levels compared to control fibers. Further, porous fibers exhibit increased cellular attachment throughout the scaffold thickness.

Many tissue engineering strategies seek to replace damaged or diseased tissues via controlled proliferation, differentiation, and matrix production by stem cells seeded on a scaffolding support structure. Nonwoven fabrics have shown promise as tissue engineering scaffolds for these applications [18], [20], [22], [52], [56], [57], [97]. Electrospinning is the most common nonwoven manufacturing technique used for creation of tissue engineering scaffolds, due to its simplicity of set up and low cost for a table top system. Although useful in applications requiring utilization of thin constructs consisting of micro and nanofibers, clinical translation of large scale three dimensional electrospun scaffolds on an industrial scale is challenging, time consuming, and generally results in materials with weak mechanical properties. Further, electrospun structures tend to consolidate greatly, resulting in dense structures with small pore sizes. Among many nonwoven manufacturing techniques, we propose that meltblown (single row) as well as Spunblown™ (multi-row, Figure 3.1) technologies may be well suited for commercial fabrication of three dimensional tissue engineering constructs, capable of producing fine fiber diameter scaffolds in scalable, high throughput, economical, and repeatable fashion.
In the meltblowing process, polymeric filaments in the liquid state are attenuated via a heated, high velocity gas stream [108–110]. Attenuation in the liquid state results in the formation of fine fibers, on the order of 1–5 µm (for most low viscosity polymers of melt flow rate (MFR) 400 decigrams/min and higher) and in some cases on the nanometer scale.
depending on polymer viscosity, mass throughput, and air velocity employed [109]. The
subsequent bending instabilities as the fibers slow down when approaching a collector
system, result in the formation of self-bonded webs when fibers come into contact with one
another in the molten state. Depending on the die to collector distance (DCD), the degree on
bonding in these meltblown fabrics can be controlled. Low DCD results in membrane-like
structures while higher DCD results in bulkier structures with lower solidity. Fiber
attenuation in the molten state allows formation of finer diameter fibers at commercially
relevant throughputs, not possible with other nonwoven continuous filament processes such
as spunbond fabrication (continuous filament extrusion followed by rapid fiber cooling via
cool air prior to pneumatic fiber attenuation [76]), although typically at the expense of
reduced mechanical properties [76], [111]. Unlike traditional meltblown systems which use
two air knives on each side of a single row of orifices for fiber attenuation [112], [113], the
hallmark of the Biax-Fiberfilm Spunblown™ process lies in the incorporation of an
attenuating air stream for each individual fiber (Figure 3.1). Surrounding each capillary is a
co-concentric orifice that delivers high velocity heated air such that each fiber is completely
enveloped in an attenuating air stream [114–116]. Partly because the process can use higher
viscosity, higher molecular weight polymers, the Spunblown™ process is currently capable
of producing nonwoven fabrics and textiles with fiber diameters ranging from 1–25 μm,
using multi-row spinnerettes that result in significant throughput.

The Spunblown™ process is the first meltblowing configuration to utilize a co-
concentric air stream around each filament for fiber attenuation. This unique design allows
for multiple rows of capillaries, thus increasing the system’s throughput and exceeding those
possible with current standard meltblowing technology such as those manufactured by
Reicofil [114–116]. However, there exists a tradeoff as capillary density is reduced because each orifice must be separated by at least 1.3 times the radius of the orifice in order to minimize interference in the neighboring airstream [115]. The extruder design allows for decreased polymer residence time, limiting degradation and producing stronger fabrics and textiles [114].

The Spunblown™ system has demonstrated successful spinning of high quality webs from relatively low MFR polymers, down to 70 decigrams/min [115], as opposed to a typical lower limit of 1000 decigrams/min for traditional meltblown nonwovens [117]. The ability to spin a wide range of MFR polymers results in the potential to achieve meltblown fabrics using polymers not previously possible on standard geometries in typical meltblowing machines. Interestingly, Biax-Fiberfilm and Reicofil recently entered an agreement that enables the use of the Biax co-concentric spinnerette on a Reicofil meltblown unit. Combining the ability to spin lower MFR polymers and its modular design to fit a variety of line configurations, the Spunblown™ process is uniquely positioned to address a variety of applications serviced by meltblown fabrication, such as medical barriers, filtration, and separation processes [113]. We propose it holds great potential for high throughput, low cost, scaffold creation for tissue engineering applications.

A major challenge to utilization of large, three dimensional scaffolds for tissue engineering applications is the formation of necrotic cores due to the scaffold’s inability to supply nutrients and remove cellular waste products throughout the scaffold construct. It is known that cells can only tolerate a maximum distance of approximately 200 µm from the nearest blood vessel before diffusion of oxygen and nutrients becomes limited [34]. Incorporation of porous scaffolding materials has been proposed to alleviate this issue. In
particular, it has been proposed that microporous scaffolds may enhance mass transport of nutrients and oxygen to the internal scaffold structure [22], [24], [33]. Microporous fiber nonwovens are ideally suited for meltblown fabrication, particularly with the Spunblown™ system. Introducing a secondary sacrificial component into the primary polymer, and subsequent removal leaves behind a microporous network throughout the fiber wall.

Careful consideration of the primary fiber forming polymer must also be taken into account. Thermal properties of the primary and sacrificial polymers must be sufficiently similar to ensure spinnability, however solubility of the primary polymer in the dissolution solvent for the sacrificial component must be very low. Poly(lactic acid) (PLA) is an FDA-approved polymer that we and others have used extensively as a scaffold material in a variety of tissue engineering strategies [51], [61], [98], [118–120]. We have found the rheological properties of PLA are suitably compatible with AQ55S for fiber spinning. AQ55S is readily dispersible in water while PLA has little to no solubility in water. Further, the rate of degradation can be controlled for specific applications by varying the polymer properties, such as crystallinity and molecular weight [120].

Lastly, a variety of stem cell sources have been investigated for tissue engineering applications. We and others have reported extensively on the potential of human adipose derived stem cells (hASC) for a variety of tissue engineering applications, particularly for mesodermal lineages [63], [98], [102], [104–107], [121–124]. Human ASC are relatively easy to harvest from excess adipose tissue as opposed to other cell sources such as mesenchymal stem cells that require more invasive procedures, and are capable of multipotent differentiation; particularly with respect to adipogenesis, osteogenesis, and chondrogenesis [102], [104–107], [121].
The goal of this study was to fabricate and characterize both solid fiber and porous fiber PLA nonwovens using the economical, scalable, high speed fabrication Spunblown™ process and to evaluate their use as tissue engineering scaffolds using hASC. We hypothesized that both solid and porous fiber PLA nonwoven scaffolds could be created in a high throughput fashion using a Spunblown™ fabrication process and that porous fiber scaffolds would lead to enhanced hASC viability, proliferation, differentiation, and infiltration relative to solid fiber scaffolds.

3.2. Experimental Section

Polymer Characterization

PLA grade 6202D was supplied by NatureWorks LLC (Minnetonka, MN) and AQ55S was supplied by Eastman Chemical Company (Kingsport, TN). PLA/AQ55S was dried overnight at 40 °C and was compounded at a ratio of 80:20 PLA:AQ55S by weight (w/w). Rheological characterization of the dried compounded blend as well as the dry pure polymers was performed on a Rosand RH7 capillary rheometer (Malvern Instruments, Malvern, UK) fitted with a long die (16 mm length, 1 mm diameter). Samples were subjected to a shear rate sweep from 20-10,000 1/s at constant temperature (225, 235, 245, and 255 °C).

Scaffold Fabrication

Nonwoven scaffolds were fabricated using a Biax Spunblown™ unit fitted with a 38 cm die composed of 368 active spinning orifices (0.508 mm diameter) arranged in two rows. Melt temperature was set at 282 and 270 °C for pure PLA and PLA/AQ55S, respectively.
Air temperature for both polymer systems was set at 230 °C. A Box-Behnken surface response design [125] was employed in order to investigate the effects of processing parameters on the formation and properties of the resultant scaffolds (basis weight, throughput, DCD, and air pressure). Preliminary trials were undertaken to determine the feasible maximum and minimum values for processing parameters for each polymer system. Levels for basis weight, throughput, DCD, and air pressure for pure PLA were set at 20, 30, 40 and 80 g/m² (gsm), 0.14, 0.22, and 0.29 g/hole/min (ghm), 15, 25 and 35 cm, and 55, 76, and 96 kPa, respectively, resulting in the fabrication of 52 distinct scaffolds. Levels for basis weight, DCD, and air pressure for PLA/AQ55S were set according to pure PLA conditions, however throughput was limited to 0.32 ghm, resulting in the fabrication of 36 distinct scaffolds.

Additional bonding was employed in order to provide sufficient mechanical integrity during the washing step to remove the AQ55S component. Scaffold fibers were bonded with a heated point bond calendar (125 °C, 2.07 MPa) prior to washing. Scaffolds were washed in agitated DI water with 250 µL surfactant per 1 L DI water at 85 °C for 15 minutes, followed by sodium hydroxide (2% w/w) at 85 °C for one minute and immediately rinsed in DI water for several minutes. Residual sodium hydroxide was neutralized in an acetic acid bath (1% v/v) for 15 minutes followed by rinsing in DI water for several minutes. Scaffolds were dried for 24 hours at 40 °C before being weighed to determine weight loss from the washing procedure.


**Scaffold Thickness and Solid Volume Fraction**

The scaffold thickness and fiber density (1.24 and 1.268 g/cm³ for PLA and PLA/AQ55S, respectively) of each scaffold type was used to calculate the solid volume fraction (SVF) for each condition according to Equation 3.1. The SVF is a useful measure of the fraction of space occupied by fibers that takes into account not only scaffold weight, but also scaffold density. Dent has shown the SVF can be correlated with air permeability of nonwovens, allowing visualization of a large data set that would otherwise be grouped into weight specific categories [126]. By using SVF, our entire scaffold data set can be visualized for a variety of scaffold properties in a single plot. Fiber diameter and SVF for pure PLA and PLA/AQ55S scaffolds as well as design of experiment parameters studied are presented in Table 3.1 and Table 3.2, respectively.

**Equation 3.1.**

\[
\mu = \frac{\left(\frac{w}{xyz}\right)}{\rho} = \frac{\left(\frac{BW}{z}\right)}{\rho}
\]

Where:

- \(\mu\) = solid volume fraction
- \(w\) = scaffold weight
- \(x\) = scaffold length
- \(y\) = scaffold width
- \(z\) = scaffold thickness
- \(BW\) = scaffold basis weight
- \(\rho\) = scaffold mass density
Table 3.1. Nonwoven fabrication design of experiments based on Box-Behnken surface response design [125] for pure PLA scaffolds with processing parameters in bold. First number represents fiber diameter (µm), and second number represents solid volume fraction.

<table>
<thead>
<tr>
<th>Throughput [ghm](^a)</th>
<th>0.14</th>
<th>0.22</th>
<th>0.29</th>
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<tbody>
<tr>
<td>DCD [cm]</td>
<td>15</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>Basis Weight [gsm](^b)</td>
<td>55</td>
<td>20</td>
<td>96</td>
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<tr>
<td>Air Pressure [kPa]</td>
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<td>96</td>
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<td>55</td>
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<td>80</td>
<td>76</td>
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<td>10.1 ± 2.8</td>
<td>10.8 ± 2.8</td>
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<tr>
<td>Throughput [ghm]</td>
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<td>0.026</td>
<td>0.033</td>
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<tr>
<td></td>
<td>16.4 ± 1.9</td>
<td>13.9 ± 2.3</td>
<td>14.7 ± 2.0</td>
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<td>0.035</td>
<td>0.023</td>
<td>0.030</td>
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<tr>
<td></td>
<td>20.7 ± 1.9</td>
<td>17.2 ± 2.2</td>
<td>15.9 ± 2.5</td>
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<tr>
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<td>0.036</td>
<td>0.023</td>
<td>0.030</td>
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<tr>
<td></td>
<td>9.1 ± 3.7</td>
<td>8.5 ± 2.5</td>
<td>9.9 ± 2.9</td>
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<tr>
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<td>0.033</td>
<td>0.036</td>
<td>0.045</td>
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<tr>
<td></td>
<td>13.1 ± 1.7</td>
<td>12.0 ± 1.9</td>
<td>12.6 ± 2.0</td>
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<td>0.025</td>
<td>0.023</td>
<td>0.033</td>
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<tr>
<td></td>
<td>17.4 ± 2.1</td>
<td>14.9 ± 1.6</td>
<td>13.9 ± 1.7</td>
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<tr>
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<td>0.027</td>
<td>0.022</td>
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<td>8.4 ± 3.8</td>
<td>7.3 ± 2.9</td>
<td>6.7 ± 3.0</td>
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<td>0.029</td>
<td>0.032</td>
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<td>9.9 ± 2.1</td>
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<td>0.025</td>
<td>0.026</td>
<td>0.047</td>
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<td>14.2 ± 1.8</td>
<td>13.0 ± 1.8</td>
<td>12.3 ± 1.9</td>
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<td>0.025</td>
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<td></td>
<td>13.7 ± 2.0</td>
<td>12.5 ± 2.3</td>
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<td>0.034</td>
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<td>0.077</td>
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<tr>
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<td>16.6 ± 2.1</td>
<td>12.0 ± 2.3</td>
<td>12.2 ± 1.7</td>
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<td>0.079</td>
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<td>0.081</td>
<td>0.115</td>
<td>0.133</td>
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<td>14.7 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>0.102</td>
<td>0.095</td>
<td>0.115</td>
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| a) g/hole/min; b) g/m².
Table 3.2. Nonwoven fabrication design of experiments for PLA/AQ55S scaffolds with processing parameters in bold. First number represents fiber diameter (µm), and second number represents solid volume fraction.

<table>
<thead>
<tr>
<th>Throughput [ghm](^a)</th>
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<td>DCD [cm]</td>
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<tr>
<td>Basis Weight [gsm](^b)</td>
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<tr>
<td>Air Pressure [kPa]</td>
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<tr>
<td>55</td>
<td>28.0 ± 3.4</td>
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<td></td>
<td>0.067</td>
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<td>26.3 ± 3.7</td>
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<tr>
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<td>23.0 ± 3.3</td>
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<td>28.3 ± 3.8</td>
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<td>25.8 ± 3.3</td>
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<td>25.9 ± 3.3</td>
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<td>24.9 ± 3.5</td>
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<td>24.5 ± 3.2</td>
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<td>0.131</td>
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\(a\) g/hole/min; \(b\) g/m²

**Scaffold Characterization**

Surface and cross sectional morphology of scaffolds produced were imaged using a Phenom G2 Pro desktop scanning electron microscope. Acellular scaffolds were dried overnight at 40 °C. Cell seeded scaffolds were chemically dried in a series of ethanol and
hexamethyldisilazane (HMDS) for 10 minutes per dilution (50, 70, 95, and 100% ethanol followed by 1:3, 1:1, and 3:1 HMDS:ethanol followed by pure HMDS and allowed to air dry in a chemical fume hood) (Thermo Scientific, Waltham, MA) to prevent damaging the cellular and ECM structures. Scaffolds were then fractured in liquid nitrogen to expose the scaffold cross section. Average fiber diameter was determined using ImageJ software (NIH, Bethesda, MD) with a minimum of 100 fibers measured per scaffold. A Hanatek FT3 Precision Thickness Gauge (East Sussex, UK) was used to determine average scaffold thickness. An advanced capillary flow porometer (Porous Materials INC, Ithaca, NY) was used to determine mean flow pore size of as fabricated and calendared scaffolds. Burst strength of scaffolds fabricated was measured using a TruBurst (Halifax, UK) for pure PLA scaffolds and on an Instron 4400R (Norwood, MA) with a 250 lb load cell fitted with a burst ball attachment for PLA/AQ55S scaffolds. Burst strength was evaluated at random points across the fabric width and length (n=5), at least 5 cm from the fabric edge. Scaffolds selected for use in cell culture analysis were also subjected to a tensile strip test to failure in the machine direction according to the ASTM D5035 using an Instron 4400R. Surface areas of scaffolds selected for cell culture experiments were determined using the Brunauer-Emmett-Teller method[127] with a Quantachrome ASiQwin Automated Gas Sorption chamber (Quantachrome Instruments, Boynton Beach, FL) with Krypton as the analysis gas and an outgassing temperature of 40 °C.

**AQ55S Cytotoxicity**

Cytotoxicity of AQ55S was evaluated prior to cell seeding on PLA/AQ55S scaffolds. Second passage hASC were seeded in 24 well tissue culture treated plates at 2500 cells per
well and allowed to proliferate in complete growth medium (CGM: Eagle α-Minimum Essential Medium, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin) for three days. CGM was then replaced with medium containing dissolved AQ55S from 0 to 10 mg/mL (maximum solubility in CGM). Cells were incubated for an additional three days and cellular proliferation was evaluated using the AlamarBlue™ assay (BioRad AbD Serotec, Oxford, UK) at a concentration of 10% by volume (v/v) AlamarBlue™ in CGM for three hours and absorbance subsequently read on a GENios microplate reader (Tecan, Männedorf, Switzerland) at 570 and 600 nm, and viability using a Live/Dead viability/cytotoxicity kit (Invitrogen Molecular Probes, Eugene, OR) and imaged on a Leica DM5500B fluorescence microscope under 10x magnification (Leica Microsystems, Wetzlar, Germany).

**Scaffold Seeding**

Select PLA/AQ55S nonwovens were chosen for analysis as tissue engineering scaffolds. Scaffolds analyzed consisted of calendared nonwovens of basis weight 80 gsm, 25 cm DCD, and 76 kPa air pressure. Pure PLA controls and experimental PLA/AQ55S scaffolds were fabricated at 0.29 and 0.32 ghm, respectively. PLA/AQ55S scaffolds were evaluated unwashed, after DI water wash only, and after washing in sodium hydroxide and neutralized in acetic acid. Additionally, a series of pure PLA Spunblown™ scaffolds without any additional bonding were selected to examine the effects of total scaffold porosity on the behavior of hASC (referred to as PLA pore size series). Pore size series scaffolds were fabricated with constant throughput (0.22 ghm) and DCD (25 cm), with varied air pressure (55, 76, and 96 kPa) to fabricate scaffolds of varied porosity (50, 73, and 90 µm).
Human ASC were harvested from excess waste tissue from liposuction procedures performed at the University of North Carolina at Chapel Hill under an IRB exempt protocol (10-0201) and pooled to create a superlot of five age and gender matched pre-menopausal donors that we have previously described [107]. Scaffolds were seeded with second passage hASC at a density of 20,000 cells/cm² at a concentration of 200,000 cells/mL and allowed to attach for one hour in a humidified incubator at 37°C and 5% CO₂ prior to flooding the well with CGM. After 12 hours, the scaffolds were turned and the remaining side was seeded with an additional 20,000 cells/cm² and allowed to attach for one hour before flooding the well with CGM. Scaffolds were cultured in CGM for seven days with medium changed every 3–4 days. Culture medium was then replaced with osteogenic differentiation medium (ODM: Eagle α-Minimum Essential Medium, 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 50 µM ascorbic acid, 0.1 µM dexamethasone, and 10 mM β-glycerophosphate), adipogenic differentiation medium (ADM: Eagle α-Minimum Essential Medium, 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 1 µM dexamethasone, 10 µg/mL human insulin, 100 µM indomethacin, and 500 µM isobutylmethylxanthine), or continued in CGM for an additional 14 days with medium changes every 3–4 days.

Analysis of Scaffold Performance

Seeding efficiency for each scaffold type was calculated by trypsinizing the cells attached to the tissue culture plates after the seeding procedure to determine the percentage of seeded cells that attached to each scaffold. Cellular viability and proliferation was analyzed on days 1, 3, 5, 7, and 21 using a mammalian cell Live/Dead viability/cytotoxicity kit
(Invitrogen Molecular Probes, Eugene, OR) and imaged on a Leica DM5500B fluorescence microscope under 10x magnification and the AlamarBlue™ assay (10% AlamarBlue™ v/v in medium, absorbance read at 570 and 600 nm), respectively. On day 21, cell mediated lipid and calcium staining was evaluated with Oil Red O and Alizarin Red S stains (both from Acros Organics, Geel, Belgium), respectively and imaged under 10x magnification on a Leica DM5500B microscope. Quantitative measurements of total lipids and calcium were determined using an adipogenesis colorimetric assay kit (BioVision, Milpitas, CA) and a total calcium assay kit (StanBio Laboratory, Boerne, TX) according to manufacturer’s instructions. Lipid and calcium accretion were normalized to total DNA present in each scaffold by carefully cutting each scaffold in half with a razor. One half of each scaffold was assayed for total lipids or calcium and the other half was assayed for total DNA using Hoechst 33258 fluorescent dye (Fisher Life Technologies, Waltham, MA). SEM images of scaffold surfaces and cross section were captured on days 7 and 21 across each media type evaluated to visualize cellular migration and ECM deposition on each scaffold type. Infiltration of pure PLA pore size series scaffolds was also analyzed using the fluorescent nuclear stain DAPI. Scaffolds were fixed in 10% formalin for 15 minutes, followed by washing in PBS three times and allowed to air dry before fracturing under liquid nitrogen with a razor. Scaffolds were then incubated in PBS containing DAPI at a concentration of 1:1000, DAPI:PBS for 15 minutes and subsequently rinsed several times in PBS to remove excess stain. Scaffolds were mounted on glass slides and imaged under 10x magnification on a Leica DM5500B fluorescence microscope (Leica Microsystems, Wetzlar, Germany). DAPI imaging could not be assessed on AQ55S containing scaffolds due to autofluorescence of scaffold fibers.
3.3. Results and Discussion

3.3.1. Fabrication and Characterization of Nonwovens

*Fabrication and Characterization of Polymer Blend*

We have previously demonstrated that EastONE, a water dispersible amorphous linear sulphonated copolyester, leads to the formation of a porous network when blended with PLA and subsequently removed via washing in water [51], [119]. AQ55S, a related polymer in the same family, was chosen as the sacrificial component for this study due to its commercial availability. The water dispersible nature of the polymer is attributed to sodiosulpho groups on the polymer backbone. Our previous studies with EastONE and AQ55S have shown that PLA:sacrificial polymer ratios (sacrificial polymer being either EastONE or AQ55S) of 80:20 w/w result in the most consistent pore morphology and polymer distribution [51], [119]. A PLA/AQ55S blend was prepared by compounding (blending in the melt state) at a ratio of 80:20 w/w in a twin screw extruder. PLA and AQ55S were physically mixed and dried at 40 °C for 24 hours prior to compounding.

The shear rate profile of the compounded blend was analyzed on a capillary rheometer and compared to that of pure PLA over the typical operating window for filament spinning of PLA (225–255 °C). Shear rate profiles of PLA/AQ55S are provided in Figure 3.2. Previous experiments have shown that stable filament spinning of pure PLA is feasible in this operating window with respect to temperature, however the best results were achieved at 245 °C resulting in the fastest spinning speeds attainable and absence of instabilities in fiber formation or doglegging (filament bending at the die exit). We found that the viscosity profile of the PLA/AQ55S compounded blend was three times less than that of pure PLA at 245 °C, indicating that stable filament spinning of this system needed to be carried out at
lower temperatures compared to pure PLA (Figure 3.2). Such a reduction in viscosity is desirable from a meltblowing fabrication standpoint, as lower viscosity polymers are typically required for meltblown processing.

![Figure 3.2. Apparent shear viscosity (Pa s) versus shear rate (1/s) for compounded PLA/AQ55S over the operating window for pure PLA filament spinning (225–255 °C), compared to pure PLA at optimal filament spinning temperature (245 °C). A three fold decrease in viscosity was observed for the PLA/AQ55S polymer compared to pure PLA at 245 °C, indicating stable filament spinning should be carried out at a reduced temperature.](image)

**Figure 3.2.** Apparent shear viscosity (Pa s) versus shear rate (1/s) for compounded PLA/AQ55S over the operating window for pure PLA filament spinning (225–255 °C), compared to pure PLA at optimal filament spinning temperature (245 °C). A three fold decrease in viscosity was observed for the PLA/AQ55S polymer compared to pure PLA at 245 °C, indicating stable filament spinning should be carried out at a reduced temperature.

**Fabrication and Characterization of Biax Spunblown™ Nonwovens**

A 38 cm wide Biax die with the outermost rows delivering air only was used to fabricate compounded PLA/AQ55S scaffolds and pure PLA control scaffolds. The die orifice of 0.508 mm was suitable from a rheological standpoint for both polymer systems, resulting in sufficient polymer melt pressure at the die opening for fiber spinning. An initial trial to determine the optimal spinning conditions was conducted prior to completion of the
full design of experiments (DOE) as well as determination of the practical operating limits for throughput, DCD, and air pressure. Spinning under the same temperature profile was not possible due to the reduction in viscosity of the compounded blend compared to pure PLA. A higher temperature profile was required for stable spinning of pure PLA with uniform scaffold formation. Spinning of the compounded blend at these temperatures led to fiber sputtering, and scaffolds were unable to be collected. Final melt temperature for the full DOE was set to 282 °C for pure PLA and 270 °C for compounded PLA/AQ55S.

After determining the optimal spinning conditions for pure PLA and the compounded blend, the range of operating parameters for throughput, DCD, and air pressure were determined. In the case of pure PLA under the temperature profile determined, a minimum throughput of 0.14 grams/hole/min (ghm) was required for the production of a stable scaffold. Below this value, sputtering and fiber breakage occurred leading to poor quality scaffolds. The maximum throughput was limited to 0.29 ghm due to melt pressure restrictions. At this throughput the melt pressure reached the maximum safe pressure for the extruder. High quality, consistent fiber morphology scaffolds were achieved over the operating range of the machine for air pressure with the minimum and maximum values of 55 and 96 kPa, representing the machine limits. It may be of interest to note that a six-row Biax die has as many as 3,300 capillaries. A throughput of 0.14 ghm would translate to 27.7 kg/m/hr, and 0.29 ghm would yield as much as 57.4 kg/m/hr. The operating window for DCD was determined to be 15–35 cm. Below 15 cm, scaffolds were highly consolidated and became membrane like structures. At DCD values above 35 cm, fiber bonding was very poor and led to weak scaffolds that could not be easily collected. For the compounded blend under the temperature profile determined, the throughput was extruder speed limited due to
the lower viscosity of the blend compared to pure PLA. Stable scaffold formation was possible only at the maximum extruder speed, below this level sputtering and fiber breakage occurred and scaffolds could not be collected. In future experiments, using smaller orifice dies (resulting in increased melt pressure) would extend the operating range of throughput for the compounded blend system. Therefore, one throughput was used in the final DOE for the compounded blend. In future studies, it is suggested that smaller orifice dies (resulting in increased melt pressure at the die exit) also be investigated to potentially extend the operating ranges of throughput for the compounded blend system. However, with the throughput utilized in this study, similar to pure PLA, high quality scaffolds comprised of the compounded PLA/AQ55S blend were achieved over the operating range of the machine for air pressure and DCD levels of 15–35 cm.

After determination of the optimal temperature profiles and operating parameters, a full DOE based on a Box-Behnken surface response design [125] was completed. Stable scaffold formation was achieved across all DOE settings for both pure PLA and the compounded PLA/AQ55S blend, resulting in the fabrication of 52 pure PLA scaffolds and 36 PLA/AQ55S scaffolds spanning the operating window for throughput, DCD, and air pressure for a series of basis weights (scaffold weight per area (g/m²)). Fiber diameter for pure PLA scaffolds ranged from 6.7–20.7 µm, while PLA/AQ55S scaffolds resulted in fiber diameters of 18.0–28.5 µm. The larger diameter of the PLA/AQ55S scaffolds was expected, given that the throughput was necessarily set higher than that of pure PLA. Scaffold thickness was consistent between polymer systems and ranged from 270–798 µm for pure PLA and 227–729 µm for PLA/AQ55S, respectively. Fiber diameter and solid volume fraction (SVF), as
well as DOE parameters are available in Table 3.1 and Table 3.2 for pure PLA and PLA/AQ55S, respectively.

Capillary flow analysis was implemented to determine the pore size range of PLA and PLA/AQ55S scaffolds. Not all scaffolds could be analyzed using this method, particularly, the low weight 20 gsm samples, as they were too open and porous for accurate analysis. Pore sizes measurements for these samples would require alternative methods, such as direct geometric analysis for sample imaging. Therefore, 27 of the 88 samples were unable to be measured accurately for pore size. Pore size results of the remaining 61 samples are provided in Figure 3.3. In general and as expected, pore size decreased with increased SVF. Further, the compounded PLA/AQ55S blend resulted in larger pore sizes than that of pure PLA; this was expected given the larger fiber size with these scaffolds. Pore sizes ranged from 39–185 µm for pure PLA and 128–424 µm for PLA/AQ55S. These pore size ranges represent an ideal range for cell growth and differentiation of a variety of cell types as documented by Guarino and Ambrosio, in particular with respect to osteoconduction [20].
Figure 3.3. Average pore size for pure PLA and compounded PLA/AQ55S scaffolds. A reduction in pore size was observed with increased solid volume fraction. Larger fiber diameters in the PLA/AQ55S scaffolds led to an increase in the average pore size compared to pure PLA scaffolds.

Burst strength (pressure required to rupture scaffold through application of multiaxial force via a ball attachment on an Instron machine or spherical membrane apparatus) was measured in order to determine the effects of processing parameters on the mechanical properties of the resultant scaffolds. Burst strength was significantly lower for the PLA/AQ55S scaffolds compared to pure PLA controls. Burst strength data is provided in Figure 3.4. There was a strong correlation of increased burst strength with increased SVF for both polymer systems. From a tissue engineering perspective, the burst strength of PLA/AQ55S scaffolds is suitable for static culture. However, application of mechanical loading modalities such as tensile strain, shear stress, and hydrostatic pressure may be limited to low magnitudes and could prove a hindrance to the development of differentiated tissues.
Figure 3.4. Average burst strength of pure PLA and compounded PLA/AQ55S scaffolds. Burst strength increased in a linear fashion with increased solid volume fraction. (a) Pure PLA and PLA/AQ55S scaffolds. PLA/AQ55S scaffolds exhibited significantly reduced burst strength compared to pure PLA scaffolds. (b) Expanded view of PLA/AQ55S burst strength. Error bars represent standard error of the mean.
**Removal of Sacrificial Component**

Washout trials were performed in order to determine the feasibility of removing the AQ55S component from the PLA/AQ55S scaffolds. Scaffolds were dried overnight at 40 °C to remove any absorbed water before being weighed prior to washing. Samples were washed in deionized water (DI) at 85 °C under gentle mechanical agitation with 250 µL surfactant per 1 L DI water to reduce surface tension (hereinafter referred to as AQ55S water washed). Timed wash trials indicated that a maximum washing efficiency of 40% was observed after four minutes, with additional wash time having no additional effect on washing efficiency (Figure 3.5a). Subsequent wash trials to determine the effects of processing conditions on washing efficiency were fixed at 10 minutes to allow adequate time for maximum removal of the AQ55S component.
Figure 3.5. Effect of fiber and scaffold properties on removal of AQ55S component after DI water wash procedure. Washing efficiency of 100% indicates complete removal of AQ55S component. (a) Washing efficiency versus time. Maximum removal of AQ55S was observed after four minutes with no additional removal with increased wash time. (b) Effect of scaffold basis weight. Increased basis weight led to a reduction in washing efficiency. (c) Effect of die to collector distance (DCD). Increased DCD led to increased washing efficiency. Large error bars for 35 DCD are attributed to fiber loss with loosely bonded samples. (d) Effect of fiber diameter. Fiber diameter range was too narrow to adequately determine the effect of fiber diameter on washing efficiency. Error bars represent standard error of the mean; gsm = g/m\(^2\).
The effects of basis weight, DCD, and fiber diameter on washing efficiency were also investigated. DCD, basis weight, and fiber diameter series were examined, with all other processing conditions fixed within the series. There were no significant differences in washing efficiency for 20 and 30 gsm scaffolds, however there was a slight decrease with increasing basis weight up to 80 gsm (Figure 3.5b). This was attributed to hindrance of water penetration into thicker scaffolds and subsequent removal of the AQ55S component. An opposite trend was observed for DCD. There was a significant increase in the washing efficiency with increased DCD (Figure 3.5c). As the scaffold structure is more open and porous with increasing DCD, water can more readily penetrate the entire structure and disperse a greater amount of AQ55S. However at 35 cm DCD, there was substantial fiber loss due to the loosely bonded nature of the scaffold, which is reflected in the increase in standard error of the mean (Figure 3.5c). There was no significant difference in washing efficiency with respect to fiber size (Figure 3.5d). This was attributed to the very narrow fiber diameter range available for analysis with all other conditions constant (5 µm). We expect that a larger fiber diameter range would lead to an increase in washing efficiency with a decrease in fiber diameter.

Scanning electron microscopy (SEM) imaging revealed a porous internal morphology after washing in DI, however the presence of a thin PLA skin was observed on the surface of the fibers (Figure 3.6c). Unwashed samples clearly show phase separation of the PLA and AQ55S components (Figure 3.6b). Separation at the polymer interfaces was observed and likely due to drying and freeze fracturing. The washed samples led to a pseudo “islands in the sea” [128] morphology with the PLA forming the polymer ”sea” and AQ55S leaving behind hollow interconnected “island” channels. This morphology, while oriented in the
fiber direction, was expected to enhance mass transport properties in relatively thick tissue engineered constructs.

Figure 3.6. Scanning electron microscopy images of PLA/AQ55S fiber cross sections within Spunblown™ scaffolds at 4000x magnification. PLA control (a), PLA/AQ unwashed (b), PLA/AQ water washed (c), PLA/AQ caustic washed (d). Unwashed samples (b) revealed phase separation of the PLA and AQ55S components. Upon washing in DI water a pseudo “islands in the sea” (multiple discreet polymer filaments or channels “islands” oriented in the fiber direction surrounded by a second polymer “sea”) interconnected porous morphology with a thin PLA outer skin was observed (c). The PLA skin was removed with further washing in caustic (d). Scale bars represent 10 μm.
However, in order to expose the internal pore structure, additional samples were subjected to a brief sodium hydroxide wash to remove the PLA skin (hereinafter referred to as AQ55S caustic washed). Previous experiments to remove the PLA skin via sodium hydroxide resulted in dissolution of fiber bond points, resulting in rapid scaffold degradation before the PLA skin was removed. Scaffolds were subjected to additional bonding via a point bond thermal calendar (heated roller patterned with raised pegs that selectively bonds fibers together via melting) to provide sufficient mechanical integrity to withstand the washing procedure. After the initial DI wash, scaffolds were then subjected to a 2 or 6% w/w sodium hydroxide wash for 60 seconds at 55 or 85 °C and rinsed in DI water followed by neutralization in 1% acetic acid by volume (v/v). SEM surface images of scaffolds subjected to the caustic washes are provided in **Figure 3.7**. Scaffold samples were able to withstand all washing conditions except 6% caustic at 85 °C. As expected, PLA skin removal increased with increased sodium hydroxide concentration or wash temperature. A caustic treatment of 2% at 85 °C led to the greatest degree of skin removal while maintaining scaffold integrity and was chosen for cell culture experiments (**Figure 3.6d**). Brunauer-Emmett-Teller (BET) surface area analysis [127] results indicated pure PLA control, unwashed AQ55S, water washed AQ55S, and caustic washed AQ55S scaffolds exhibited specific surface areas of 0.178, 0.169, 1.109, and 1.423 m$^2$/g, respectively. Water washed scaffolds led to a five fold increase in specific surface area compared to unwashed controls, indicating the porous channels were interconnected throughout the fiber cross section. Further, removal of the PLA skin increased the surface area compared to water washed scaffolds by 0.314 m$^2$/g. Pure PLA pore size series scaffolds exhibited specific surface areas...
of 0.333, 0.236, and 0.340 m$^2$/g, respectively. Graphical representation of BET surface area is provided in Figure 3.8.

**Figure 3.7.** Optimization of caustic washing procedure; scanning electron microscopy images of fiber surfaces after caustic wash treatment at 4000x magnification. All scaffolds were imaged regardless of final scaffold integrity. Increased temperature and or increased caustic concentration resulted in a greater degree of PLA removal from the surface of fibers. All scaffolds remained intact except under the harshest condition (6% caustic at 85 °C). 2% caustic at 85 °C led to the best fiber surface morphology while maintaining scaffold integrity, and was chosen for cell culture experiments. Scale bars represent 10 µm.
Figure 3.8. Surface area of acellular scaffolds selected for cell culture evaluation measured using the Brunauer-Emmett-Teller (BET) method [127]. a) PLA/AQ55S scaffolds: Water washed scaffolds exhibited a five fold increase compared to unwashed controls, indicating the porous channels are interconnected throughout the fiber cross section. Removal of the PLA skin via caustic treatment further increased surface area compared to water washed scaffolds. b) Pure PLA pore size series: Scaffold pore size had little effect on surface area.
**Scaffold Selection**

After characterization, a series of calendared Spunblown\textsuperscript{TM} nonwoven scaffolds were selected to examine the effects of fiber morphology on the behavior of hASC. Processing parameters and properties of PLA/AQ55S scaffolds selected for cell culture studies is provided in Table 3.3. The effect of fiber morphology on proliferation, viability, differentiation, and scaffold infiltration of hASC was investigated. In subsequent sections, the following terms are used to refer to the different fiber morphologies studied: PLA control (pure PLA round fibers), AQ unwashed (unwashed PLA/AQ55S scaffolds), AQ water washed (porous fibers with PLA skin), and AQ caustic washed (porous fibers with PLA skin removed). Additionally, pure PLA scaffolds were selected for cell culture to determine the effects of scaffold porosity on the behavior of hASC. Processing parameters and properties of pure PLA scaffolds selected for cell culture studies is provided in Table 3.4. Mechanical properties of scaffolds selected for cell culture were evaluated using a tensile strip test to failure in the machine direction according to the American Society for Testing and Materials (ASTM) D5035 and are provided in Figure 3.9 for PLA/AQ55S scaffolds and in Figure 3.10 for pure PLA pore size scaffolds.
Table 3.3. Properties and fabrication parameters of scaffolds selected for cell culture evaluation. PLA/AQ55S scaffolds were chosen to investigate the effect of fiber morphology on hASC behavior.

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<td>25</td>
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<td>21.3</td>
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<tr>
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<td>25</td>
<td>76</td>
<td>21.3</td>
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a) g/m²; b) g/hole/min

Table 3.4. Properties and fabrication parameters of scaffolds selected for cell culture evaluation. Pure PLA scaffolds were chosen to investigate the effect of scaffold pore size on hASC behavior.

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<td>55</td>
<td>14.4</td>
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a) g/m²; b) g/hole/min
Figure 3.9. Mechanical properties of PLA/AQ55S acellular scaffolds selected for cell culture evaluation. (a) Representative engineering stress strain curves for each scaffold type. (b) Peak load for each scaffold type. Water wash treatment decreased peak load for PLA/AQ55S scaffolds. A further reduction was observed for caustic treated scaffolds. Error bars represent standard error of the mean.
Figure 3.10. Mechanical properties of pure PLA pore size series acellular scaffolds selected for cell culture evaluation. (a) Representative engineering stress strain curves for each scaffold type. (b) Peak load for each scaffold type. Increased scaffold pore size led to decreased peak load. Error bars represent standard error of the mean.
3.3.2. Cytotoxicity of AQ55S

The water and caustic wash procedures developed for removal of AQ55S, while exposing the desired pore morphology, did not lead to the complete removal of the AQ55S component. In order to determine if residual AQ55S in the washed scaffolds affected hASC behavior, prior to conducting cell culture experiments, the cytotoxicity of AQ55S to hASC was evaluated. Human ASC seeded on 24 well tissue culture plates were exposed to increased concentrations of AQ55S dissolved in complete growth medium (CGM) from zero to maximum solubility (10 mg/mL) for three days. Cellular proliferation was evaluated with the AlamarBlue™ assay and viability via a mammalian cell Live/Dead cytotoxicity kit. The median lethal dose (LD₅₀) was determined for AQ55S by taking the ratio of percent reduction for AQ55S treated cells to pure CGM controls (Figure 3.11a). A significant statistical difference in proliferation was only observed at the maximum dose of AQ55S (10 mg/mL). Further no obvious differences were observed for all AQ55S concentrations analyzed with respect to the number of live and dead cells (Figure 3.11b). Taken together, AlamarBlue™ and Live/Dead analyses suggested that AQ55S has little to no negative effect on hASC proliferation and viability. Even at the maximum solubility of AQ55S, cells remained viable and proliferation was only slightly less (although significant) than that of untreated controls. Scaffolds used in subsequent experiments contained 1.6 mg of AQ55S before washing. Hypothetically, unwashed scaffolds would lead to an AQ55S concentration of 3.2 mg/mL if all AQ55S dissolved (before the first media change). Even at this concentration, no significant inhibition of hASC proliferation or viability was observed.
Figure 3.11. (a) AQ55S median lethal dose (LD$_{50}$) for hASC as determined by AlamarBlue$^\text{TM}$ reduction. A slight reduction in proliferation was observed with increased AQ55S concentration, however the LD$_{50}$ (50% growth of control) was never reached even at maximum AQ55S solubility (10 mg/mL). A significant statistical difference in proliferation relative to 0 mg/mL was only observed at the maximum dose of AQ55S (10 mg/mL). The shaded inset represents total possible range of AQ55S concentration that could be released from scaffolds, from 0 (100% washing efficiency) to 3.2 mg/mL (0% washing efficiency). (b) Live/Dead images of hASC treated with media containing dissolved AQ55S at 10x magnification. No difference was observed for all concentrations with respect to the number of live (green) and dead (red) cells. Scale bars represent 100 µm.
3.3.3. Porous Fiber Nonwovens as Tissue Engineering Scaffolds

**Cell Seeding Efficiency**

After scaffolds had been seeded on both sides with hASC, they were transferred to new tissue culture plates for the duration of the experiment and remaining cells that had not adhered to the scaffolds were counted using a hemocytometer in order to determine the percentage of cells that were able to adhere to the scaffolds. Human ASC were able to adhere to all scaffolds investigated with scaffolds exhibiting seeding efficiencies of 73.3 ± 6.9, 57.9 ± 13.4, 61.7 ± 11.6, and 51.3 ± 5.7% for PLA control, AQ unwashed, AQ water washed, and AQ caustic washed scaffolds, respectively. Seeding efficiency data is provided graphically in Figure 3.12. Seeding efficiency data does not account for any cellular division that may have occurred throughout the scaffold seeding process. Although there was no significant difference between seeding efficiencies of PLA control, AQ unwashed, and AQ water washed scaffolds, there was a significant reduction for AQ caustic washed scaffolds compared to pure PLA controls. These results contradicted our hypothesis that additional surface area on the caustic treated fibers would lead to enhanced cellular attachment. We speculate that this may be due to reactive species or changes in pH on the surface of the fibers as a result of the caustic treatment. Although the scaffolds were neutralized after caustic treatment with acetic acid, residual reactive species may have been present that negatively affected cellular attachment. We suggest that future studies investigate additional approaches to optimize the neutralization step, followed by molecular characterization of the fiber surface as a potential methodology to address this limitation.

Although not significantly different, the pure PLA scaffold pore size series exhibited a reduction in seeding efficiency with an increase in scaffold pore size, resulting in seeding
efficiencies of $79.2 \pm 8.1$, $65.8 \pm 13.7$, and $55.4 \pm 12.5\%$ for scaffold pore sizes of 50, 73, and 93 µm, respectively. This was expected as increased pore area likely led to a smaller probability of hASC coming into contact with scaffold fibers and adhering before settling through the scaffold onto the plate surface.
Figure 3.12. Seeding efficiency on Spunblown™ nonwoven scaffolds. a) PLA/AQ55S scaffolds. No significant differences were observed between PLA control, AQ unwashed, and AQ water washed scaffolds. There was no difference between AQ unwashed, AQ water washed, and AQ caustic washed scaffolds, however caustic washed scaffolds exhibited a significant decrease in seeding efficiency compared to pure PLA controls. b) Pure PLA pore size series scaffolds. Although not significant, increased pore size led to a decrease in seeding efficiency. Seeding efficiency does not account for cell division during the seeding process. Different letter represent statistical significance (p<0.05); error bars represent standard error of the mean.
AlamarBlue™ data revealed that cells proliferated on all scaffolds investigated with little difference between PLA controls, AQ unwashed, and AQ water washed scaffolds over the first seven days (Figure 3.13a). However AQ caustic treated scaffolds exhibited a lag in proliferation at day five compared to the other scaffold types. By day seven, all scaffold types exhibited no significant differences in rates of proliferation. These results are consistent with Live/Dead imaging over the first seven days of the experiment, which revealed similar cellular viability on PLA control, AQ unwashed, and AQ water washed scaffolds and fewer cells on AQ caustic treated scaffolds (Figure 3.14). At the end of the three week experiment, all scaffolds analyzed exhibited no significant difference in rates of proliferation in each media type evaluated (Figure 3.13b). Similarly, Live/Dead imaging revealed similar numbers of live cells across all scaffolds tested in each media type (Figure 3.15). These results indicated that unwashed and AQ water washed (porous with skin) scaffolds do not increase (or negatively affect) viability and proliferation of hASC compared to PLA controls. We speculated that AQ water washed scaffolds did not exhibit increased cellular attachment and mass transport due to the presence of the PLA skin on the surface of the fibers, effectively blocking the internal pore structure and presenting like a solid fiber to attached cells. We hypothesized that AQ caustic treated scaffolds would lead to an increase in viability and proliferation by exposing the internal porous structure, increasing available surface area for cellular attachment and a conduit for nutrient transfer. However, results indicated an early reduction in viability and proliferation, followed by comparable performance to the other scaffold types for the remainder of the culture period. We believe that this initial decrease is a result of the caustic treatment, as there was no evidence of
AQ55S cytotoxicity on hASC. Changes in pH and the formation of reactive species not fully neutralized in the washing procedure might have negatively affected initial cellular attachment and proliferation. We hypothesized that these species could be depleted through interactions with cells and medium and this effect thus be diminished.
Figure 3.13. AlamarBlue™ cellular proliferation on PLA/AQ5S Spunblown™ scaffolds for day one to seven in complete growth medium (a) and on day 21 across all media types evaluated (b). Cells proliferated across all scaffolds over the first seven days, however a lag on day five was observed for caustic treated scaffolds, attributed to residual reactive species generated during the caustic washing procedure (a). By day 21, proliferation equalized across all scaffold types (b). Different letters represent statistical significance (p<0.05); error bars represent standard error of the mean.
Figure 3.14. Live/Dead staining of hASC over the first seven days for hASC-seeded PLA/AQ55S scaffolds cultured in complete growth medium at 10x magnification. All scaffolds supported viable cell growth (stained green); although caustic treated scaffolds (d1–d4) appeared to exhibit fewer viable cells compared to other scaffold types, although viable cells were seen at all time points. AQ water washed (c1–c4) and AQ caustic washed (d1–d4) scaffolds exhibited the fewest number of dead cells (stained red) compared to PLA (a1–a4) and AQ unwashed (b1–b4) controls. Scale bars represent 100 µm.
Figure 3.15. Live/Dead staining of hASC at the end of the 21 day experiment across all media types evaluated on PLA/AQ55S scaffolds at 10x magnification. All scaffolds supported viable cell growth (stained green) over the course of the experiment; although caustic treated scaffolds (d₁–d₃) appeared to exhibit fewer viable cells compared to other scaffold types. AQ water washed (c₁–c₃) and AQ caustic washed (d₁–d₃) scaffolds exhibited the fewest number of dead cells (stained red) compared to pure PLA (a₁–a₃) and AQ unwashed (b₁–b₃) controls. Calendar bond points (regions of compressed and partially melted fibers) appear as dark ellipses (indicated by arrows) and, as expected, exhibited fewer viable cells compared to fibrous regions, particularly for caustic treated scaffolds (d₁–d₃). Scale bars represent 100 µm.
In order to test this hypothesis, additional scaffolds were created and treated with caustic, but the acid neutralization step was omitted. Proliferation was evaluated for one week and compared to acid neutralized scaffolds (Figure 3.16). Results indicated that acid neutralized scaffolds exhibited a statistically significant increase in cellular proliferation over one week, compared to caustic treated only scaffolds. These results support our hypothesis that the caustic treatment negatively affects early cellular attachment. Future studies to optimize the caustic treatment to reduce or eliminate this effect are needed to overcome this limitation. However, our results indicated that neutralization in caustic reduces this negative effect, significantly increasing proliferation compared to caustic only treated scaffolds.

Figure 3.16. AlamarBlue™ cellular proliferation on caustic treated scaffolds with and without additional acid neutralization step. Neutralization in acetic acid after caustic treatment resulted in a significant increase in proliferation on all days analyzed, with greatly enhanced proliferation observed on day seven. (*) Indicates p<0.05 and (#) indicates P<0.01. Error bars represent standard error of the mean.
AlamarBlue™ results indicated that decreased scaffold pore size led to an increase in cellular proliferation. The smallest scaffold pore size (50 µm) led to the highest levels of cellular proliferation over the first seven days of the experiment (Figure 3.17a). This effect may partly be due to the observed increased seeding efficiency with decreased scaffold pore size. A greater number of hASC attached during the seeding process to scaffolds with 50 µm pores compared to 73, and 90 µm, leading to a higher initial seeding density (Figure 3.12b). Live/Dead imaging was consistent with AlamarBlue™ data over the first seven days, and revealed a greater number of live cells on scaffolds of smaller pore size (Figure 3.18). However, an increase in the number of dead cells was also observed with decreased scaffold pore size. This effect may be attributed to decreased mass transport of nutrients and oxygen throughout the less porous scaffolds. At the end of the three week experiment, there were no statistical differences observed in cellular proliferation across all scaffold pore sizes analyzed (Figure 3.17b). However, similar to early time points, more dead cells were observed for the smallest scaffold pore size (Figure 3.19). These results indicated that there is a trade off between cellular viability and proliferation with respect to scaffold porosity over the range of pore sizes analyzed. Smaller pore sizes led to greater proliferation, at the expense of cellular viability. Further, we hypothesize that that there is likely a minimum in the optimal pore size with respect to cellular proliferation that is not captured in the data. As the scaffold pore size is decreased, we expect that cellular infiltration will become limited and cellular attachment will only occur at the scaffold surface, similar to typical hASC attachment on small pore size electrospun scaffolds.
Figure 3.17. AlamarBlue™ cellular proliferation on pure PLA Spunblown™ scaffolds for day one to seven in complete growth medium (a) and on day 21 across all media types evaluated (b). Cells proliferated across all scaffold types over the first seven days, however increased scaffold pore size led to decreased proliferation (a). No statistical differences were observed on day 21 across all scaffold pore sizes and media types evaluated (b). Different letters represent statistical significance (p<0.05); error bars represent standard error of the mean.
Figure 3.18. Live/Dead staining of hASC over the first seven days for hASC-seeded pure PLA pore size scaffolds cultured in complete growth medium at 10x magnification. All scaffolds supported viable cell growth (stained green); however, a greater number of dead cells (stained red) were observed for smaller scaffold pore sizes ($a_1$–$a_4$). Scale bars represent 100 µm.
Figure 3.19. Live/Dead staining of hASC at the end of the 21 day experiment across all media types evaluated for hASC-seeded pure PLA pore size scaffolds at 10x magnification. All scaffolds supported viable cell growth (stained green) over the course of the experiment; however a greater number of dead cells (stained red) were observed for smaller scaffold pore sizes (a₁–a₃). Scale bars represent 100 µm.

Differentiation of hASC

After allowing for three weeks of growth and proliferation throughout the scaffolds, the effect of fiber morphology on hASC adipogenic and osteogenic differentiation was evaluated. PLA control, AQ unwashed, AQ water washed, and AQ caustic washed scaffolds resulted in total triglycerides normalized to total DNA of 1.72 ± 0.10, 2.46 ± 0.33, 1.73 ± 0.32, and 4.47 ± 0.62 nmol triglycerides per µg DNA, respectively, when treated with
adipogenic differentiation medium (ADM) (Figure 3.20a). As expected, hASC-seeded scaffolds cultured in complete growth medium (CGM) or osteogenic differentiation medium (ODM) led to significantly lower levels of normalized triglycerides compared to hASC-seeded scaffolds cultured in adipogenic differentiation medium (ADM). Adipogenesis results were confirmed with Oil Red O staining, provided in Figure 3.21. Oil Red O staining revealed the presence of lipid vacuoles across all scaffold types treated with ADM, while lipid vacuoles were not observed for CGM controls of scaffolds cultured in ODM.
Quantification of hASC adipogenic and osteogenic end product differentiation normalized to total DNA content for PLA/AQ55S scaffolds. (a) Total triglycerides and (b) cell mediated calcium accretion. All hASC-seeded scaffolds cultured in adipogenic differentiation medium (ADM) exhibited increased levels of triglycerides compared to those cultured in complete growth medium (CGM) or osteogenic differentiation medium (ODM). Caustic treated scaffolds seeded with hASC and cultured in ADM exhibited a significant increase in triglycerides relative to other fiber types (a). All hASC-seeded scaffolds cultured in ODM exhibited increased levels of cell-mediated calcium accretion compared to those cultured in CGM or ADM. No difference was observed between PLA controls, AQ unwashed, and AQ water washed scaffolds, however a significant increase in cell mediated calcium accretion was observed for hASC seeded on caustic treated scaffolds (b). Different letters represent statistical significance (p<0.05); error bars represent standard error of the mean.
Figure 3.21. Oil Red O lipid staining on PLA/AQ55S Spunblown™ scaffolds after 21 days in culture at 10x magnification. All scaffold types exhibited the presence of lipid vacuoles (stained cherry red) when treated with adipogenic differentiation medium (ADM). Lipid vacuoles were not observed for scaffolds treated with complete growth medium (CGM) or osteogenic differentiation medium (ODM). Calendar bond points appear as bright ellipses beyond the focal plane. Scale bars represent 100 µm.

Similarly, for osteogenesis, total cellular accreted calcium normalized to total DNA was evaluated on day 21 for all scaffolds. PLA control, AQ unwashed, AQ water washed, and AQ caustic washed scaffolds resulted in total cell mediated calcium accretion normalized to total DNA of 0.249 ± 0.011, 0.300 ± 0.047, 0.240 ± 0.001, and 0.457 ± 0.026 µg calcium per µg DNA, respectively when treated with ODM (Figure 3.20b). Cell-seeded scaffolds cultured in CGM and ADM exhibited significantly less cell mediated calcium accretion.
compared to those cultured in ODM. Osteogenesis results were confirmed with Alizarin Red S staining, provided in **Figure 3.22**. Similar to Oil Red O results, Alizarin Red S staining resulted in the presence of intense red calcium staining across all scaffold types cultured in ODM. In agreement with quantitative measurements, calcium staining was not observed for CGM controls; however there was a slight amount of staining for cell-seeded scaffolds cultured in ADM.

**Figure 3.22.** Alizarin Red S calcium staining on PLA/AQ55S Spunblown™ scaffolds after 21 days in culture at 10x magnification. All scaffold types exhibited the presence of intense red calcium staining when treated with osteogenic differentiation medium (ODM). A small amount of staining was observed for scaffolds treated with complete growth medium (CGM) and adipogenic differentiation medium (ADM). Calendar bond points appear as bright ellipses beyond the focal plane. Scale bars represent 100 µm.
These results suggest that surface morphology is an important scaffold parameter, capable of regulating the extent of hASC differentiation. Adipogenesis and osteogenesis were increased on caustic treated scaffolds; while no significant differences were observed between AQ water washed and control scaffolds, suggesting that the open porous, rough surface morphology is conducive for hASC differentiation. Our results are consistent with other investigators who have reported similar findings for a variety of polymer systems and cell sources with regard to surface morphology [23], [129–131]. Woo et al. have shown that neonatal murine osteoblastic cells cultured on nanofibrous PLA scaffolds exhibited enhanced osteoblastic differentiation and biomineralization compared to solid walled scaffolds. The interconnected nanofibrous porous surface led to increased von Kossa staining as well as increased levels of runt related transcription factor two and bone sialoprotein mRNA [129]. Our results suggest that increased surface roughness and porosity on the caustic treated scaffolds lead to similar results, promoting differentiation of hASC.

Similar to AQ55S containing scaffolds, pure PLA pore size series hASC-seeded scaffolds cultured in CGM or ODM led to significantly lower levels of normalized triglycerides compared to hASC-seeded scaffolds cultured in ADM (Figure 3.23a). Further, there were no significant differences total triglycerides observed across all scaffold pore sizes cultured in ADM. Adipogenesis results were confirmed with Oil Red O staining, provided in Figure 3.24. Oil Red O staining revealed the presence of lipid vacuoles across all scaffold types cultured in ADM, while lipid vacuoles were not observed for CGM controls of scaffolds cultured in ODM. Similarly for osteogenesis, pure PLA pore size series scaffolds cultured in CGM and ADM exhibited significantly less cell mediated calcium accretion compared to those cultured in ODM (Figure 3.23b). Further, there were no significant
differences in cell mediated calcium accretion observed across all scaffold pore sizes cultured in ODM. Osteogenesis results were confirmed with Alizarin Red S staining, provided Figure 3.25. Similar to Oil Red O results, Alizarin Red S staining resulted in the presence of intense red calcium staining across all scaffold types cultured in ODM. These results suggest that scaffold pore size does not significantly affect adipogenic and osteogenic differentiation of hASC within the scaffold porosity range studied.
Figure 3.23. Quantification of hASC adipogenic and osteogenic end product differentiation normalized to total DNA content for hASC-seeded pure PLA pore size scaffolds. (a) Total triglycerides and (b) cell mediated calcium accretion. All hASC-seeded scaffolds cultured in adipogenic differentiation medium (ADM) exhibited increased levels of triglycerides compared to those cultured in complete growth medium (CGM) or osteogenic differentiation medium (ODM). There were no significant differences in total triglycerides for all pore size scaffolds cultured in ADM (a). All hASC-seeded scaffolds cultured in ODM exhibited increased levels of cell-mediated calcium accretion compared to those cultured in CGM or ADM. There were no significant differences in cell mediated calcium accretion for all pore size scaffolds cultured in ODM (b). Different letters represent statistical significance (p<0.05); error bars represent standard error of the mean.
Figure 3.24. Oil Red O lipid staining on pure PLA Spunblown™ scaffolds after 21 days in culture at 10x magnification. All scaffold types exhibited the presence of lipid vacuoles (stained cherry red) when cultured in adipogenic differentiation medium (ADM). Lipid vacuoles were not observed for scaffolds treated with complete growth medium (CGM) or osteogenic differentiation medium (ODM). Scale bars represent 100 µm.
Figure 3.25. Alizarin Red S calcium staining on pure PLA Spunblown™ scaffolds after 21 days in culture at 10x magnification. All scaffold types exhibited the presence of intense red calcium staining when treated with osteogenic differentiation medium (ODM). A small amount of staining was observed for scaffolds treated with complete growth medium (CGM) and adipogenic differentiation medium (ADM). Scale bars represent 100 µm.

Attachment and Infiltration of hASC

In order to evaluate the extent of cellular migration over the surface and throughout the thickness of the scaffolds, SEM surface and cross section images were captured after one week in CGM and at the end of the three week experiment across all media types studied. Scaffold surface images (Figure 3.26 PLA/AQ55S scaffolds, Figure 3.27 pure PLA pore size scaffolds) provided dramatic evidence of the need for enhanced mass transport strategies throughout the thickness of three dimensional tissue engineered constructs. After one week in culture hASC were observed on the surface of all scaffolds analyzed and covered a significant portion of the surface with extracellular matrix (ECM). By three weeks, the surface of all scaffold types was completely covered with cells and ECM, blocking the
internal structure of the scaffold. Cross sectional images revealed the presence of hASC in the interior of all scaffolds analyzed, with an increase in the number of cells and extent of ECM observed across one and three weeks in culture (Figure 3.28 PLA/AQ55S scaffolds, Figure 3.29 pure PLA pore size scaffolds). Very fine fibrillar ECM structures were observed originating from attached cells, spanning multiple polymer fibers creating a three dimensional ECM network. As expected, the presence of cellular generated ECM fibers was decreased for hASC-seeded scaffolds cultured in ADM compared to CGM and ODM, as adipocytes generate ECM to a lesser extent than osteocytes.
Figure 3.26. Scanning electron microscopy surface images at 2000x magnification of PLA/AQ55S Spunblown™ hASC-seeded scaffolds after one week of culture in complete growth medium (CGM) and at the end of the 21 day experiment in CGM, adipogenic differentiation medium (ADM), and osteogenic differentiation medium (ODM). Extensive deposition of extracellular matrix and hASC was observed after one week, and nearly covered the entire scaffold surfaces after three weeks. Scale bars represent 50 µm.
Figure 3.27. Scanning electron microscopy surface images at 2000x magnification of pure PLA Spunblown™ hASC-seeded scaffolds after one week of culture in complete growth medium (CGM) and at the end of the 21 day experiment in CGM, adipogenic differentiation medium (ADM), and osteogenic differentiation medium (ODM). Extensive deposition of extracellular matrix and hASC was observed after one week, and nearly covered the entire scaffold surfaces after three weeks. Scale bars represent 50 µm.
Figure 3.28. Scanning electron microscopy cross section images at 2000x magnification of PLA/AQ55S Spunblown™ hASC-seeded scaffolds after one week of culture complete growth medium (CGM) and at the end of the 21 day experiment in CGM, adipogenic differentiation medium (ADM), and osteogenic differentiation medium (ODM). hASC were observed throughout the interior of all scaffold types, with an increase in the number of cells and extent of extracellular matrix (ECM) deposition observed between one and three weeks in culture. Fine fibrillar ECM structures were observed originating from attached cells, spanning multiple polymer fibers creating a three dimensional ECM network. hASC seeded on caustic treated scaffolds (d₁–d₅) appeared to have a greater number of cellular attachment sites per cell compared to other scaffolds analyzed. Scale bars represent 50 µm.
Scanning electron microscopy cross section images at 2000x magnification of pure PLA Spunblown™ hASC-seeded scaffolds after one week of culture complete growth medium (CGM) and at the end of the 21 day experiment in CGM, adipogenic differentiation medium (ADM), and osteogenic differentiation medium (ODM). hASC were observed throughout the interior of all scaffold types, with an increase in the number of cells and extent of extracellular matrix (ECM) deposition observed between one and three weeks in culture. Fine fibrillar ECM structures were observed originating from attached cells, spanning multiple polymer fibers creating a three dimensional ECM network. Scale bars represent 50 µm.

DAPI imaging of pure PLA pore size series scaffolds revealed a similar number of hASC nuclei on the surface of all pore size scaffolds (Figure 3.30). However, an increased number of nuclei were observed throughout the scaffold cross section for 90 µm scaffolds, compared to 73, and 50 µm. These results indicated that hASC spreading and infiltration throughout the scaffold thickness increased with increased scaffold pore size.
Figure 3.30. DAPI surface (a₁–c₁) and cross section (a₂–c₂) images at 20x magnification of pure PLA Spunblown™ hASC-seeded scaffolds after 21 days of culture in complete growth medium (CGM). Little difference in the number of hASC nuclei were observed on scaffold surfaces (a₁–c₁), however increased scaffold pore size led to increased numbers of hASC throughout the scaffold thickness (a₂–c₂). Scale bars represent 50 µm.
Qualitatively, hASC seeded on caustic treated scaffolds appeared to have a greater number of cellular attachment sites per cell compared to the other scaffolds analyzed (Figure 3.28, d_2–d_5). Further, cellular morphology appeared more conserved, with whole cells visible, compared to cell fragments observed for other scaffold types suggesting improved cell adhesion on the caustic treated scaffolds. We believe this is a result of the surface morphology of the caustic treated fibers. The increased surface area, increased roughness, and small surface features provide an ideal surface for cellular attachment compared to the relatively smooth fiber surface of other scaffold types analyzed [129–131]. Our findings are consistent with those of other investigators. Papenburg et al. have shown that porous nano-fibrous scaffolds fabricated via phase inversion resulted in improved adhesion and proliferation of murine pre-myoblasts compared to controls [130]. Similarly, Diban et al. have demonstrated that surface morphology of flat poly(ε-caprolactone) scaffolds prepared via phase inversion affects hASC adhesion and proliferation. Scaffolds exhibiting the highest level of surface roughness led to the best cell attachment and spreading of hASC [131]. While difficult to draw definitive conclusions regarding the extent of migration throughout each scaffold type, SEM imaging revealed that hASC attach differently to fibers with surface roughness and porosity, and were able to populate the entirety of each scaffold and generate ECM spanning multiple polymer fibers.

**Statistical Analysis**

An unpaired student’s t-test was used to analyze statistical difference for scaffold characterization techniques. A one-way ANOVA was used for statistical analysis of
biological assays. Error bars represent standard error of the mean. Statistical significance is indicated as $p<0.05$.

3.4. Conclusions

We have demonstrated successful production and characterization of solid and porous fiber PLA meltblown nonwoven scaffolds fabricated via the Biax Spunblown™ process, an economical, scalable, repeatable, high-speed fabrication approach. Inclusion of water dispersible AQ55S and subsequent removal via washing in DI water led to the formation of an interconnected porous internal fiber morphology. The porous interior was exposed by additional treatment with caustic, opening the structure for cellular interactions. Further, the effect of fiber morphology on the behavior of hASC was evaluated. Porous fibers led to similar levels of cellular proliferation and viability compared to control fibers after three weeks in culture. However, an initial decrease in proliferation on caustic treated fibers was observed, and attributed to residual reactive species created during the caustic treatment. Porous fibers led to an increase in the differentiation capacity of hASC with respect to adipogenesis and osteogenesis, attributed to surface pores and increased roughness compared to control fibers. Lastly, hASC attachment was improved for caustic treated scaffolds, resulting in more cellular attachments across multiple scaffolding fibers and the production of a cell generated fibrillar ECM network. To the best of our knowledge, this is the first study to report the fabrication of porous fibers at commercially relevant speeds using the Biax Spunblown™ process for the production of repeatable, scalable, and economic tissue engineering scaffolds with increased mass transport properties.
CHAPTER 4 Hollow Porous Fiber Carded Nonwovens for Full Thickness Tissue Engineering Applications Using Human Adipose Derived Stem Cells

This chapter is in preparation for submission to Acta Biomaterialia.

4.1. Introduction

In order to translate scaffold based tissue engineering strategies to clinical practice, a repeatable, economical, high throughput scaffold manufacturing method is needed. Further, for the treatment of full thickness three dimensional critical defects, enhanced mass transport strategies to reduce or eliminate the formation of necrotic cores are needed. In this study, the fabrication and characterization of thick (2–3 mm) nonwoven tissue engineering scaffolds composed of porous or hollow porous fibers with enhanced mass transport properties created using nonwoven industry standard commercially relevant carding technology is described. Scaffolds are validated as tissue engineering constructs using human adipose derived stem cells (hASC), and the effects of fiber morphology on hASC proliferation, viability, adipogenic and osteogenic differentiation, and cell spreading are described. Porous and hollow porous fiber scaffolds were found to promote osteogenesis as evidenced by significantly increased levels of cell mediated calcium accretion compared to pure PLA controls. Further, hollow porous fibers led to increased levels of total triglycerides, indicating enhanced adipogenesis, compared to porous and control fiber morphologies.
Scaffold-based tissue engineering strategies often aim to replace damaged tissues via controlled proliferation, differentiation, and extracellular matrix (ECM) production by stem cells seeded on a biodegradable scaffold. Chemical and mechanical cues guide controlled proliferation and differentiation of cells into the desired phenotype, producing new extracellular matrix (ECM), and gradually replacing the scaffolding material with newly formed tissue. Many materials and fabrication techniques have shown great promise as tissue engineering scaffolds on the lab scale, however a large challenge remaining to be addressed is implementation of an economical, repeatable, scalable scaffold manufacturing method to move tissue engineering out of the lab and into clinical practice. In addition, cell seeding of three-dimensional, full-thickness tissue engineering scaffolds often result in necrotic cores due to mass transport limitations of nutrients and oxygen throughout the scaffold thickness [22], [24], [26], [31], [32], [34]. Utilization of hollow and porous scaffolds to improve mass transport throughout tissue engineering scaffolds has been proposed to reduce or eliminate the formation of necrosis in the core [20], [22], [24], [33], [119]. We propose that carded nonwovens composed of hollow and porous fibers may be implemented for the industrial scale production of full-thickness tissue engineering scaffolds with enhanced mass transport properties throughout the full thickness of the construct.

Carding technology is one of the oldest nonwoven fabrication methods (Figure 4.1). In the carding process, short fibers of a few inches (staple fibers) are separated into individual fibers from compact bundles or tuft and subsequently entangled and intermixed via specialized combed rollers to fabricate a loose unbonded web of fibers (Figure 4.1c) [101], [132–134]. High loft, relatively thick fabrics can be produced by layering the unbonded web at a specified angle upon itself (crosslapping) until the desired fabric weight
per area (basis weight) is achieved [101]. In this manner, carded webs of up to 2000 g/m² may be produced at industrial relevant speeds. The web structure is then locked into place via bonding. Many bonding techniques are available and in use today including but not limited to mechanical bonding (needle punching (Figure 4.1d) and hydroentangling), chemical bonding (adhesive), and thermal bonding (partial melting of fibers (through air or calendared) or specialized binder fibers to each other (Figure 4.1d)) [101].
Figure 4.1. Idealized schematic of hollow porous fiber carded scaffold fabrication. a) Multifilament spinning: Polymer pellets are fed to an extruder, melted, and forced through the spinnerette/die. Individual filaments are formed at each orifice and collected as a fiber bundle or spinline and wound around the collector godet roll via a vacuum gun (lurgi gun). Multifilaments are wound and collected on a bobbin or transferred via additional godet rolls for subsequent processing. b) Crimping and cutting: Fibers are crimped into a zig-zag pattern to facilitate transfer to carding rolls and cut into short (about 5 cm) individual staple fibers. c) Carding: Staple fibers are converted to an unbonded fiberweb via a series of specialized combed rollers. d) Bonding: Web structure is locked into place via needle punching. Barbed needles snare and entangle fibers through the scaffold thickness. Partial melting sets PET binder fibers as the scaffold is transferred on a conveyor through an oven. e) AQ55S component is removed via washing in deionized water, followed by washing in caustic (sodium hydroxide) to remove outer PLA skin. Excess caustic is rinsed, followed by neutralization of residual caustic via an acetic acid bath. Finally, excess acetic acid is rinsed and scaffold is dried in an oven followed by winding on a collector roll. Fabrication is depicted as a continuous process, scaffolds fabricated in this study were processed in batch operations (filament spinning, crimping and cutting, carding, bonding, and washing).

Carding is suitable for the production of nonwoven webs composed of many fiber types, including natural fibers such as cotton as well as synthetic fibers composed of
thermoplastic and thermoset polymers, and even glass and metal fibers. In the context of tissue engineering scaffolds, biodegradable polymeric fibers such as those composed of poly(lactic acid) (PLA), an FDA-approved polymer, are particularly attractive. The fiber construct provides initial support for early cellular growth, and is gradually replaced by newly formed ECM and cells [18]. Our group and many others have reported on the use of PLA as a scaffolding material in a variety of tissue engineering strategies [51], [61], [98], [99], [119], [120]. Additionally, we have described the fabrication of porous and hollow porous PLA fibers with enhanced mass transport properties via incorporation of a water dispersible polymer into the primary PLA fiber matrix. Subsequent removal of the water dispersible component leaves behind a microporous network [51], [119].

The goal of this study was to fabricate and characterize both solid and hollow pure PLA, as well as porous and hollow porous PLA carded scaffolds as full thickness tissue engineering scaffolds validated using human adipose derived stem cells (hASC). Interest in hASC as a stem cell source has grown dramatically in the last decade due to their relative ease of harvest compared to other stem cell sources and their ability to differentiate into a variety of mesodermal lineages. We and others have reported on their use for a variety of tissue engineering applications [98], [100], [102–107], [122], [124], [135]. We hypothesized that solid, hollow, porous, and hollow porous PLA scaffolds could be created in a repeatable, scalable, high throughput manner via carding and that porous and hollow porous fibers would lead to enhanced hASC attachment, proliferation, differentiation, and spreading throughout carded scaffolds relative to pure PLA controls.
4.2. Materials and Methods

4.2.1. Fabrication of Carded Nonwovens

**Scaffold Fabrication**

PLA grade 6202D (NatureWorks LLC, Minnetonka, MN) was used as the primary fiber forming polymer for all nonwovens fabricated. AQ55S (Eastman Chemical Company, Kingsport, TN), a water dispersible sulphonated amorphous linear copolyester, was used as the sacrificial component. PLA/AQ55S pellets were fabricated by compounding PLA and AQ55S (blending in the melt state) at a ratio of 80:20 PLA:AQ55S. Pure polymers were mixed by hand and dried at 40 °C overnight to remove any adsorbed water prior to compounding. Continuous filaments were fabricated at the Nonwovens Institute Pilot facilities (NWI, Raleigh, NC) on a Hills multifilament research line. A 69 hole, 4 hollow slit (0.127 mm) spinnerette was used for hollow fiber production (**Figure 4.1a**). Hollow fibers were formed as molten polymer exited each orifice in a series of 4 rectangular openings arranged in a square pattern. The molten polymer streams fused just beyond the die exit, forming a hollow filament (**Figure 4.1a inset**). A 69 hole round orifice (0.35 mm) spinnerette was used for all round fibers, consisting of 69 circular openings to form each filament. Filaments were attached to a Lurgi gun (vacuum line) to form the spinline (bundle of 69 individual filaments) and wound around the collector godet roll (heated cylindrical variable speed roller). Melt temperature was fixed at 225 °C. Throughput was varied from 0.3–0.9 grams/hole/min (ghm). Maximum spinning speed was determined by ramping speed of the collector godet roll (**Figure 4.1a**) from 300 m/min until fiber breakage occurred and reducing the speed by 50 m/min below this value. Maximum spinning speed was then defined as stable spinning without fiber breakage for ten minutes. Ten pounds of fibers for
each condition (PLA solid, PLA/AQ55S solid, PLA hollow, and PLA/AQ55S hollow) were then collected at 700 m/min on bobbins (cylinders for winding filaments). Crimping (imparting a zag-zag pattern via heated rollers) was performed prior to the cutting process to aid in the subsequent carding step. Crimped filaments were then cut into 5 cm crimped staple fibers for subsequent carded fabric production (Figure 4.1b).

Carded fabrics were produced at NWI on a model carding unit to achieve a fabric basis weight of 200 g/m² (gsm) for each condition. Low Melting point Polyethylene terephthalate (PET) binder fibers were added at 20% by weight (w/w) to each fiber type to ensure mechanical stability during the washing procedure (Figure 4.1d). Fabrics were then bonded via needle punching on an Asselin A.50-RL 4 board needle loom (Andritz Asselin-Thibeau, Graz, Austria). The top and bottom fabric surfaces were each bonded at a needle punch density (npd) of 50 strokes/cm², for a total npd of 100 strokes/cm². The PET binder was then set in a through air Fleissner oven at 130 °C (Figure 4.1d) (Trützschler GmbH, Mönchengladbach, Germany).

**Removal of the Sacrificial Component**

The AQ55S sacrificial component was removed via washing scaffolds in deionized (DI) water at 85 °C for 10 minutes under gentle agitation. Fabrics were dried overnight in an oven at 40 °C prior to weighing to remove any absorbed water. Surfactant was added at a concentration of 250 µL per 1 L DI water in order to reduce surface tension and aid in AQ55S removal (referred to as “water washed”). Fabrics were subsequently washed in 6% sodium hydroxide (Thermo Scientific, Waltham, MA) for 60 seconds followed by rinsing in DI water for several minutes. Fabrics were then neutralized in a 1% by volume (v/v) acetic
acid (Thermo Scientific, Waltham, MA) bath for 15 minutes and subsequently rinsed for several minutes under DI water (referred to as caustic washed). Fabrics were allowed to dry in an oven overnight at 40 °C prior to weighing to determine weight loss (Figure 4.1e).

**Scaffold Characterization**

Fabric and fiber properties of carded nonwovens were evaluated prior to cell culture experiments. Basis weight was determined in accordance with Institute of Standards and Technology (IST) 130.2. Fabric and fiber surface and cross section images were captured on a Phenom G2 Pro Scanning Electron Microscope (SEM) (Eindhoven, Netherlands). Fiber diameter was determined using SEM surface images in ImageJ software (NIH, Bethesda, MD), using the average of at least 50 fibers for each calculation. A Hanatek FT3 Precision Thickness Gauge (East Sussex, UK) was used to determine fabric thickness (n=5), measured at random points across the fabric width and length. Pore size was determined using a Porous Materials INC advanced capillary flow porometer (Ithaca, NY) with GalWick as the wetting agent. Mechanical properties of fabrics were evaluated using a tensile strip test to failure in the machine direction on an Instron 4400R (Norwood, MA). The Brunauer-Emmett-Teller (BET) method was used to determine fiber surface area with a Quantachrome ASiQwin Automated Gas Sorption chamber (Quantachrome Instruments, Boynton Beach, FL) with Krypton as the analysis gas and 40 °C outgassing temperature.
4.2.2. Carded Nonwovens as Tissue Engineering Scaffolds

Scaffold Seeding

Scaffolds were seeded with an hASC superlot isolated from pre-menopausal donors containing five gender- and age-matched donor cell lines as previously described [107]. In brief, hASC were obtained from waste tissue from liposuction procedures performed at the University of North Carolina at Chapel Hill under an IRB exempt protocol (10-0201). 7/16 inch diameter circular scaffolds were sterilized for four hours in 70% ethanol in non-tissue culture treated polystyrene plates and rinsed in phosphate buffered saline (PBS). Sterilized scaffolds were cultured in complete growth medium (CGM: Eagle α-Minimum Essential Medium, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin) overnight. Second passage hASC were cultured to 75% confluency in 75 cm² tissue culture treated flasks and resuspended in CGM for scaffold seeding. Each scaffold was seeded with 30,000 cells/cm² at a concentration of 200,000 cells/mL and cells were allowed to attach for one hour in a humidified incubator at 37 °C and 5% CO₂. Scaffolds were subsequently flooded with CGM and cultured overnight prior to turning and seeding the remaining side with an additional 30,000 cells/cm². After twelve hours, scaffolds were carefully transferred to new tissue culture plates and the remaining cells were counted on a hemocytometer for seeding efficiency calculations. Seeded scaffolds were cultured in CGM for seven days with media changes every 3–4 days. After seven days, culture medium was changed to osteogenic differentiation medium (ODM; Eagle α-Minimum Essential Medium, 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 50 µM ascorbic acid, 0.1 µM dexamethasone, and 10 mM β-glycerophosphate), or adipogenic differentiation medium (ADM; Eagle α-Minimum
Essential Medium, 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 1 μM dexamethasone, 10 μg/mL human insulin, 100 μM indomethacin, and 500 μM isobutylmethylxanthine), or continued in CGM for an additional fourteen days.

**Analysis of Scaffold Performance**

Cell viability and proliferation was evaluated on days 1, 3, 5, 7, and 21 using a mammalian cell Live/Dead viability kit (Invitrogen, Molecular Probes, Eugene, OR) and the AlamarBlue™ (BioRad AbD Serotec, Oxford, UK) assay, respectively. Live/Dead scaffolds were stained with 4 μM ethidium homodimer-1 (dead cells stained red) and 3 μM calcein AM (live cells stained green) in PBS for 15 minutes and imaged on a Leica DM5500B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at 10x magnification. AlamarBlue™ in CGM (10% v/v) was added to scaffolds at each time point and incubated at 37 °C for three hours. 200 μL of media was then transferred to a 96 well plate and absorbance read on a GENios microplate reader (Tecan, Männedorf, Switzerland). Remaining AlamarBlue™ treated media was aspirated and scaffolds washed three times with PBS before being flooded with fresh media.

Adipogenic and osteogenic differentiation of hASC was evaluated on day 21 using Oil Red O and Alizarin Red S stains (Acros Organics, Geel, Belgium), respectively. Scaffolds were rinsed in PBS and fixed in 10% buffered formalin (Thermo Scientific, Waltham, MA) for 30 minutes. Scaffolds were stained in 40 mM Alizarin Red S or 0.4 mM Oil Red O for five minutes and excess stain was removed by washing in DI water several times. Stained scaffolds were imaged on a Leica DMDM5500B microscope under 10x magnification. Quantitative analysis of differentiation products was performed using a
colorimetric adipogenesis assay kit (BioVision, Milpitas, CA) for total triglycerides and a
calcium assay kit (StanBio Laboratory, Boerne, TX) according to the manufacturer’s
directions for total calcium. Triglycerides and calcium were normalized to total DNA
present by carefully cutting each scaffold in half with a razor. One half was analyzed for
triglycerides or calcium and the remaining half was analyzed for DNA content. DNA
scaffolds were digested in 1 mL of 2.5 units/mL papain (Sigma-Aldrich, St. Louis, MO) at 60
°C overnight. DNA was detected using 0.2 µg/mL Hoechst 33258 fluorescent dye (Fisher
Life Technologies, Waltham, MA) and read at 352/461 nm excitation/emission on a GENios
microplate reader.

SEM surface images were captured on day 7 in CGM and on day 21 across all media
types analyzed to evaluate hASC attachment and cell infiltration of scaffolds using a Phenom
G2 Pro desktop SEM. Acellular control scaffolds were dried overnight at 40 °C prior to
imaging. Cell seeded scaffolds were treated with a series of ethanol and
hexamethyldisilazane dilutions for 10 minutes each (Thermo Scientific, Waltham, MA) to
dehydrate scaffolds and prevent damage to hASC and ECM network (50, 70, 95, and 100%
ethanol followed by 1:3, 1:1, and 3:1 HMDS:ethanol followed by pure HMDS and allowed to
air dry in a chemical fume hood).

Statistical Analysis

An unpaired student’s t-test was used to analyze statistical difference for scaffold
characterization techniques. A one-way ANOVA was used for statistical analysis of
biological assays. Scaffold characterization, n=5 replicates and cellular quantitative assays,
n=3 biological replicates and n=3 technical replicates (n=9 per condition). Error bars
represent standard error of the mean. Statistical significance is indicated as p<0.05.
4.3. Results

4.3.1. Scaffold Characterization

Fabrication of Continuous Filaments

Optimal melt spinning temperature for the PLA/AQ55S blend was determined prior to determining maximum spinning speeds. Samples were collected at maximum throughput (0.9 ghm) using the hollow spinnerette (Figure 4.1a inset) at melt temperatures of 225, 235, and 245 °C. At 245 °C (optimal temperature observed for spinning pure PLA), the polymer melt resulted in sputtering at the spinnerette and could not be collected on bobbins. Fibers were collected at 235 °C with a reduction in sputtering; but with only a maximum of 300 m/min achieved. However, at 225 °C the sputtering effect was eliminated and filaments could be collected over a range of spinning speeds. The melt temperature was fixed at 225 °C for subsequent spinning speed trials for both pure PLA and PLA/AQ55S.

The maximum spinning speed attainable for samples tested is provided in Table 4.1. At the minimum throughput evaluated (0.3 ghm), filaments could not be collected at any spinning speed for both round and hollow spinnerettes and FreeFall fibers (fibers formed at the die and collected via gravity only, (not wound on collector godet roll (Figure 4.1a)) were collected directly from the spinnerette. A throughput of 0.6 ghm resulted in maximum spinning speeds of 700 and 1200 m/min for hollow and round filaments, respectively. There was no increase in maximum spinning speed achieved when throughput was increased to 0.9 ghm.
Table 4.1. Hollow and round multifilament maximum spinning speed. Maximum spinning is defined as stable spinning with no fiber breakage for ten minutes. Pure PLA controls were collected at the maximum spinning speed of PLA/AQ55S for direct comparison and do not reflect maximum spinning speed achievable for pure PLA. Melt temperature was fixed at 225 °C. FreeFall indicates fibers were not able to be collected at any speed.

<table>
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<tr>
<th>Cross Section</th>
<th>Polymer</th>
<th>Throughput [g/hm]</th>
<th>Max Speed [m/min]</th>
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<tr>
<td>Round</td>
<td>PLA/AQ55S</td>
<td>0.3</td>
<td>FreeFall</td>
</tr>
<tr>
<td>Round</td>
<td>PLA/AQ55S</td>
<td>0.6</td>
<td>1200</td>
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<tr>
<td>Round</td>
<td>PLA/AQ55S</td>
<td>0.9</td>
<td>1200</td>
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<tr>
<td>Round</td>
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<td>Round</td>
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<tr>
<td>Hollow</td>
<td>PLA/AQ55S</td>
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<tr>
<td>Hollow</td>
<td>PLA</td>
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*^g/hole/min

Fabrication of Carded Nonwovens

Ten pounds of each of the four fiber types (round PLA, round PLA/AQ55S, hollow PLA, and hollow PLA/AQ55S), hereinafter referred to as solid, solid porous, hollow, and hollow porous, respectively, were collected at 700 m/min. Stable spinning during the collection period was observed for all fiber types with no fiber breakage (Figure 4.1a). Filaments were unwound, crimped, and cut into 2 inch staple fibers for nonwoven production (Figure 4.1b). Carded nonwovens were produced from each fiber type on a model card at 200 gsm. Low melting point PET binder fibers were included for each fiber type at 20% by weight to improve mechanical properties, necessary during the washing procedure (Figure
Carded nonwovens were successfully fabricated from each fiber type, and subsequently bonded via needling on both sides at 50 strokes/cm$^2$. The PET binder was set in an oven at 130 °C (Figure 4.1d). Moderate shrinkage of pure PLA carded nonwovens was observed after setting the PET binder, however little to no shrinkage was observed for PLA/AQ55S scaffolds. Fiber and fabric properties of carded nonwovens are provided in Table 4.2.

Table 4.2. Fiber and fabric properties of carded nonwoven tissue engineering scaffolds.

<table>
<thead>
<tr>
<th></th>
<th>Solid</th>
<th>Solid Porous</th>
<th>Hollow</th>
<th>Hollow Porous</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET Binder [w/w]$^a$</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Needle Punch Density [st/cm$^2$]$^b$</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Basis Weight [gsm]$^c$</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Fiber Diameter [µm]</td>
<td>37.5 ± 1.5</td>
<td>34.2 ± 1.2</td>
<td>39.3 ± 0.9</td>
<td>38.0 ± 1.4</td>
</tr>
<tr>
<td>Thickness [µm]</td>
<td>2513 ± 45</td>
<td>2344 ± 31</td>
<td>3239 ± 55</td>
<td>2261 ± 27</td>
</tr>
<tr>
<td>Pore Size [µm]</td>
<td>71</td>
<td>91</td>
<td>76</td>
<td>79</td>
</tr>
<tr>
<td>Surface Area [m$^2$/g]</td>
<td>0.088</td>
<td>0.807</td>
<td>0.088</td>
<td>0.934</td>
</tr>
<tr>
<td>Peak Load [N]</td>
<td>48.5 ± 1.5</td>
<td>18.1 ± 2.7</td>
<td>41.9 ± 1.1</td>
<td>13.0 ± 0.9</td>
</tr>
<tr>
<td>Seeding Efficiency [%]</td>
<td>94.6 ± 2.6</td>
<td>86.7 ± 5.2</td>
<td>95.0 ± 2.2</td>
<td>89.2 ± 0.7</td>
</tr>
</tbody>
</table>

$^a$ weight/weight, $^b$ strokes/cm$^2$, $^c$ g/m$^2$

**Removal of the Sacrificial Component**

The washing efficiency of scaffolds was calculated to determine the percentage of AQ55S removed via the DI water wash. Washing efficiency of 100% would indicate
complete removal of the AQ55S component. Pure PLA solid and hollow control scaffolds were also subjected to the DI water wash to determine the extent of weight loss due to fiber loss and removal of spin finish applied during filament spinning. Spin finish is a lubricating liquid (of varied composition, depending on the type of polymer being spun) applied during spinning to prevent filaments from adhering to the collection rolls and facilitate fiber to fiber adhesion forming the spinline (Figure 4.1a). The DI water wash resulted in washing efficiencies of 1.0, 43.3, 0.8, and 36.8% for solid, solid porous, hollow, and hollow porous fiber scaffolds, respectively (Figure 4.2a). Scaffolds were then subjected to the caustic wash and the total weight loss from each washing procedure was calculated. The water wash procedure resulted in scaffold weight loss of 0.9, 5.5, 1.4 and 6.6%, while the caustic wash resulted in weight loss of 14.1, 35.6, 15.1, and 36.3% for solid, solid porous, hollow, and hollow porous scaffolds, respectively (Figure 4.2b).
Figure 4.2. (a) Washing efficiency of water wash only. A washing efficiency of 100% would indicate complete removal of the AQ55S component. Pure PLA solid and hollow control scaffolds were also subjected to the water wash to determine the extent of weight loss due to fiber loss and removal of spin finish. (b) Cumulative percent weight loss of water and caustic washes. It not possible to determine the extent of AQ55S removal alone due to the caustic wash because the caustic treatment removed both AQ55S and PLA. Different letters represent statistical significance (p<0.05). Error bars represent standard error of the mean.
SEM images of unwashed PLA scaffolds and water plus caustic washed PLA/AQ55S scaffold surfaces and fiber cross sections were captured (Figure 4.3). The smaller PET binder fibers were observed in surface images and were clearly distinguishable from experimental fibers (Figure 4.3, a₁–d₁). A hollow channel was successfully fabricated for both pure PLA and PLA/AQ55S fibers, with similar diameter of the hollow core (Figure 4.3, c₂ and d₂). The washing procedures resulted in a porous morphology for round and hollow PLA/AQ55S fibers with interconnected porous channels oriented in the fiber direction. The caustic wash resulted in removal of PLA from the surface of the fibers, exposing the internal pore structure and resulting in a rough, porous fiber surface.

Figure 4.3. Scanning electron microscopy images of scaffold surfaces (top row) at 500x magnification and fiber cross sections (bottom row) at 4000x magnification. Smaller diameter PET binder fibers were observed in surface images and were clearly distinguishable from experimental scaffold fibers. (a) Solid pure PLA, (b) solid porous PLA/AQ55S, (c) hollow pure PLA, and (d) hollow porous PLA/AQ55S. Water and caustic washes resulted in the formation of an interconnected porous morphology for PLA/AQ55S fibers (b₂ and d₂). Scale bars represent 100 µm (top row) and 10 µm (bottom row).
Scaffold Characterization

Prior to cell seeding, scaffold properties of acellular scaffolds were determined (Table 4.2). Thickness varied from 2513 ± 45, 2344 ± 31, 3239 ± 55, and 2261 ± 27 µm for solid, solid porous, hollow, and hollow porous scaffolds, respectively. The mean flow pore size of unwashed solid and hollow pure PLA control scaffolds and washed solid and hollow PLA/AQ55S experimental scaffolds was determined and resulted in average pore sizes of 71, 91, 76, and 79 µm for solid, solid porous, hollow, and hollow porous, respectively. Solid and hollow pure PLA scaffolds both resulted in specific surface areas of 0.088 m²/g as determined by the BET method [127]. Unwashed PLA/AQ55S round and hollow scaffolds exhibited surface areas of 0.11 and 0.08 m²/g. The washing procedure resulted in increased surface areas of 0.81 and 0.93 m²/g for solid porous and hollow porous scaffolds, respectively. BET surface area data is presented graphically in Figure 4.4. Mechanical properties of washed and unwashed experimental scaffolds and pure PLA control scaffolds were determined using a strip test in the machine direction. Representative engineering stress strain curves are presented in Figure 4.5a and resultant peak load for each scaffold type in Figure 4.5b. Round fiber scaffolds exhibited peak loads of 48.5, 26.3, and 18.1 N for solid PLA, unwashed PLA/AQ55S, and washed PLA/AQ55S, respectively. Hollow fiber scaffolds resulted in peak loads of 41.9, 18.8, and 13.0 N for hollow PLA, unwashed PLA/AQ55S, and washed PLA/AQ55S, respectively.
Figure 4.4. Surface area of acellular carded scaffolds measured via the Brunauer-Emmett-Teller method with Krypton as the analysis gas. Solid porous and hollow porous fibers exhibited over 600 and 1000% more surface area per square meter compared to their respective unwashed and pure PLA controls.
Figure 4.5. Mechanical properties of acellular scaffolds as measured via a strip test in the machine direction. (a) Representative engineering stress strain curves for each scaffold type. (b) Peak load for each scaffold type. As expected, PLA/AQ55S scaffolds exhibited reduced peak loads compared to pure PLA control scaffolds. The water and caustic treatments further decreased peak load for PLA/AQ55S washed scaffolds. Different letters represent statistical significance. Error bars represent standard error of the mean.
4.3.2. Analysis of Scaffold Performance

**Seeding Efficiency**

Human ASC attached to all scaffolds analyzed with seeding efficiencies of 94.6 ± 2.6, 86.7 ± 5.2, 95.0 ± 2.2, and 89.2 ± 0.7 % for solid, solid porous, hollow, and hollow porous scaffolds, respectively. Seeding efficiency does not account for any cell division during the seeding process. There were no significant differences observed in seeding efficiency among all scaffold types analyzed. Seeding efficiency data is presented graphically in Figure 4.6.

![Seeding Efficiency Graph](image)

**Figure 4.6.** Seeding efficiency of hASC on carded scaffolds. Seeding efficiency exceeded 85% for all scaffolds, attributed to the relatively thick (over 2 mm) nature of the scaffolds. Seeding efficiency does not account for any cellular division during the seeding process. There were no significant differences in seeding efficiency among all scaffolds analyzed. Error bars represent standard error of the mean.
**Proliferation of hASC**

Cellular proliferation on carded nonwoven scaffolds was quantified using the AlamarBlue™ assay on days 1, 3, 5, and 7 in CGM (**Figure 4.7a**) and on day 21 across all media evaluated (**Figure 4.7b**). Solid and hollow pure PLA scaffolds exhibited an increase in hASC proliferation over the first seven days of the experiment. However solid porous and hollow porous scaffolds exhibited relatively constant proliferation over the first five days, followed by a significant increase in proliferation on day seven (**Figure 4.7a**). At each time point, after day one, porous and solid porous scaffolds exhibited decreased proliferation compared to their respective pure PLA controls. At the end of the 21 day culture period, there were no significant differences observed among all scaffold types in CGM (**Figure 4.7b**). Although not significant, porous and hollow porous scaffolds exhibited a decrease in proliferation when treated with ODM compared to solid and hollow pure PLA controls. Similar trends were observed for all scaffolds cultured in ADM. All scaffolds exhibited a decrease in proliferation for ADM treated scaffolds compared to CGM controls and ODM treated scaffolds.
Figure 4.7. AlamarBlue™ cellular proliferation on carded scaffolds for day one to seven in complete growth medium (CGM) (a) and on day 21 across all media types evaluated (b). Human ASC proliferated over the first seven days on pure PLA solid and hollow fiber scaffolds. Proliferation on solid porous and hollow porous scaffolds remained relatively constant for the first five days, attributed to residual reactive species generated during the caustic treatment, followed by a significant increase on day seven (a). By day 21, there were no significant differences in proliferation between all scaffolds in CGM (b). Different letters represent statistical significance (p<0.05). Error bars represent standard error of the mean.
**Viability of hASC**

Live/Dead viability results indicated all scaffolds were capable of supporting viable hASC over the first seven days of the experiment (Figure 4.8). A relative increase in the number of live cells (stained green) was observed for solid and hollow pure PLA scaffolds (Figure 4.8, a_1–a_4 and c_1–c_4). Solid porous and hollow porous scaffolds exhibited a similar number of live cells over the first five days, with an increase observed on day 7 (Figure 4.8, b_1–b_4 and d_1–d_4). A greater number of dead cells (stained red) were observed on solid and hollow pure PLA scaffolds compared to solid porous and hollow porous scaffolds. At the end of the 21 day culture period, more viable cells were observed on all scaffold types across all media evaluated compared to earlier time points (Figure 4.9). CGM treated scaffolds showed a greater number of live cells compared to ODM and ADM treated scaffolds. Lastly, a greater number of dead cells were observed on solid and hollow pure PLA scaffolds compared to solid porous and hollow porous scaffolds across all media evaluated.
Figure 4.8. Live/Dead staining of hASC over the first seven days in complete growth medium at 10x magnification. All scaffolds supported viable cell growth (live cells green, dead cells red). Pure PLA solid and hollow scaffolds appeared to exhibit an increase in the number of viable cells for each time point (a\(_1\)–a\(_4\) and c\(_1\)–c\(_4\)), however, solid porous and hollow porous fiber scaffolds appeared to exhibit a relatively constant number of viable cells over the first five days (b\(_1\)–b\(_3\) and d\(_1\)–d\(_3\)), followed by an increase on day seven (b\(_4\) and d\(_4\)). Scale bars represent 100 µm.
Figure 4.9. Live/Dead staining of hASC at the end of the 21 day experiment across all media types evaluated at 10x magnification. All scaffolds supported viable cell growth (live cells green, dead cells red) with few differences observed in the number of viable cells among all scaffold types within each media type. Complete growth medium (CGM) treated scaffolds appeared to exhibit a greater number of viable cells compared to osteogenic and adipogenic differentiation medium (ODM and ADM) treated scaffolds. Scale bars represent 100 µm.
Differentiation of hASC

Adipogenic and osteogenic differentiation products (total triglycerides and cell mediated calcium accretion) were quantified on day 21 to determine the effects of fiber type on hASC differentiation capacity. Solid, solid porous, hollow, and hollow porous scaffolds resulted in total triglycerides normalized to total DNA of $13.4 \pm 2.5$, $12.9 \pm 2.1$, $14.2 \pm 2.3$, and $22.3 \pm 2.8$ nmol triglycerides/µg DNA, respectively (Figure 4.10a). There were no significant differences between solid, solid porous, and hollow scaffolds with respect to triglyceride levels in the ADM treated group, however a significantly increased level of triglycerides was observed for hollow porous scaffolds. As expected, CGM controls and ODM treated scaffolds exhibited significantly reduced triglycerides/DNA compared to ADM treated scaffolds. Adipogenesis results were confirmed with Oil Red O staining. All ADM treated scaffolds exhibited the presence of lipid vacuoles stained cherry red. Oil Red O results are provided in Figure 4.11. Similarly for osteogenesis, all ODM treated scaffolds exhibited significantly increased cell mediated calcium accretion compared to CGM and ADM treated scaffolds. Solid, solid porous, hollow, and hollow porous scaffolds resulted in total calcium normalized to total DNA levels of $0.345 \pm 0.014$, $0.904 \pm 0.054$, $0.456 \pm 0.066$, and $1.250 \pm 0.203$ µg calcium/µg DNA, respectively (Figure 4.10b). Amongst ODM treated scaffolds, there was no significant difference between solid and hollow scaffolds, or between solid porous and hollow porous scaffolds. However solid porous and hollow porous scaffolds resulted in significantly increased calcium/DNA compared to solid and hollow scaffolds. Osteogenesis results were confirmed with Alizarin Red S staining which revealed intense red calcium staining for all ODM treated scaffolds compared to CGM and ADM treated scaffolds (Figure 4.12).
Figure 4.10. Quantification of differentiation products normalized to total DNA content. (a) Total triglycerides and (b) cell mediated calcium accretion. All scaffolds treated with adipogenic differentiation medium (ADM) led to increased triglycerides compared to complete growth medium (CGM) and osteogenic differentiation medium (ODM) treated scaffolds. Hollow porous fiber scaffolds exhibited significantly increased triglycerides compared to solid porous scaffolds as well as solid and hollow pure PLA controls scaffolds (a). All scaffolds treated with ODM exhibited increased calcium levels compared to CGM and ADM treated scaffolds. Solid porous and hollow porous scaffolds led to significantly increased calcium compared to solid and hollow pure PLA controls (b). Different letters represent statistical significance (p<0.05). Error bars represent standard error of the mean.
Figure 4.11. Oil Red O lipid staining on carded scaffolds after 21 days in culture at 10x magnification. All scaffold types exhibited the presence of stained lipid vacuoles (cherry red spheres) when treated with adipogenic differentiation medium (ADM) (a₁–d₁). No lipid vacuoles were observed in complete growth medium (CGM) treated controls and osteogenic differentiation medium (ODM) treated scaffolds. Solid porous and hollow porous scaffolds absorbed the stain (b₁–b₄ and d₁–d₄), however lipid vacuoles are clearly distinguishable from stain absorbed into fibers (examples in each ADM pane indicated by arrows). Scale bars represent 100 µm.
Figure 4.12. Alizarin Red S staining on carded scaffolds after 21 days in culture at 10x magnification. All scaffold types exhibited the presence of intense red calcium staining when treated with osteogenic differentiation medium (ODM) (a\textsubscript{3}–d\textsubscript{3}). Low levels of calcium staining were observed in complete growth medium (CGM) treated controls (a\textsubscript{2}–d\textsubscript{2}) and moderate staining was observed for adipogenic differentiation medium (ADM) treated scaffolds (a\textsubscript{4}–d\textsubscript{4}). Solid porous and hollow porous scaffolds absorbed the stain as expected (b\textsubscript{1}–b\textsubscript{4} and d\textsubscript{1}–d\textsubscript{4}), however calcium staining is clearly distinguishable from stain absorbed into fibers (examples in each ODM pane indicated by arrows). Scale bars represent 100 µm.

**Infiltration of hASC**

SEM surface images were captured after one week in culture and at the end of the three week experiment in CGM, ODM, and ADM to assess the extent of cellular attachment and spreading on carded nonwoven scaffolds (Figure 4.13). After one week, very few cells were observed on the surface of all scaffold types (Figure 4.13, a\textsubscript{2}–d\textsubscript{2}). After three weeks,
cells were observed attached to all scaffold surfaces across all media types evaluated. Fine fibrillar cell generated ECM structures were observed spanning multiple scaffold fibers. Human ASC were observed attached to both PLA scaffolding fibers as well as small PET binder fibers. Lastly, small crystalline like structures were observed on ODM treated solid scaffolds (Figure 4.13, a4).

Figure 4.13. Scanning electron microscopy surface images at 2000x magnification of carded scaffolds after one week in complete growth medium (CGM) and at the end of the 21 day experiment in CGM, adipogenic and osteogenic differentiation medium (ODM and ADM). Few human adipose derived stem cells (hASC) were observed on scaffold surfaces after one week in CGM, however cells and cell generated extracellular matrix networks were observed for all scaffold types across all media types evaluated after three weeks in culture. Scale bars represent 10 µm.
4.4. Discussion

4.4.1. Fabrication of Hollow Porous Carded Nonwovens

Both hollow and round multifilaments were successfully fabricated using PLA and PLA/AQ55S polymers. Stable spinning of the PLA/AQ55S blend required reduced melt temperature compared to pure PLA (225 compared to 245 °C). This was expected as we have previously determined that the viscosity profile of the compounded blend is three times less than that of pure PLA (Figure 3.2). Spinning of pure PLA was carried out at 225 °C for direct comparison with PLA/AQ55S. Although not ideal, spinning of pure PLA at this temperature did not result in significant spinning defects such as fiber breakage and doglegging at the spinnerette (filament bending at the spinnerette/die exit). At the lowest throughput analyzed (0.3 ghm) round or hollow filaments could not be collected for PLA/AQ55S. Fiber breakage occurred at the spinnerette, not at the godet rolls or lurgi gun (Figure 4.1a), indicating that PLA/AQ55S lacked sufficient melt strength for filament spinning at this throughput. Increasing the throughput resulted in enhanced spinnability. Maximum spinning speeds of 700 m/min were achieved at throughputs of 0.6 and 0.9 ghm for hollow filaments, while round filaments could be collected up to 1200 m/min at these throughputs. Interestingly, increased throughput from 0.6 to 0.9 ghm did not result in faster maximum spinning speed for both cross sections. Fiber breakage occurred above the maximum spinning speed at the godet collection roll, and not the spinnerette.

Fabrication of carded nonwovens from multifilaments converted to staple fibers was successful for all fiber types. The inclusion of a PET binder fiber was required in order to provide sufficient mechanical integrity during the washing procedure, particularly the caustic wash (Figure 4.1c). Setting the binder fiber required heating the fabrics to 130 °C, resulting
in moderate shrinkage of the pure PLA fabrics, leading to an increase in average fiber size and scaffold thickness, as evidenced in Figure 4.3, a2 and c2. PLA/AQ55S fabrics did not shrink significantly during the setting process, as the AQ55S component dispersed in the PLA matrix prevented complete relaxation of the PLA phase. Future experiments to eliminate the requirement for a binder fiber, such as increased mechanical bonding via higher needle punch density or hydroentangling should be explored, both to reduce PLA shrinkage and remove the confounding effect of binder fibers on hASC behavior. However, the water and caustic washes developed led to the formation of an interconnected porous fiber morphology with small surface features conducive to cellular attachment, spreading, and differentiation for both solid and hollow fibers (Figure 4.3, b2 and d2). Surface areas of solid porous and hollow porous fibers were increased over 600 and 1000% compared to their respective pure PLA controls (Figure 4.4).

4.4.2. Hollow Porous Carded Nonwovens for Tissue Engineering

Human ASC were able to adhere to all scaffolds analyzed with no statistical differences observed in cell seeding efficiency. Very high seeding efficiencies exceeding 85% were observed for all scaffolds. This was attributed to the relative thick nature of the constructs, each exceeding 2 mm, which provided ample opportunity for cell-scaffold interactions as the hASC settled through the scaffolds and came into contact with scaffold fibers.

AlamarBlue™ cellular proliferation results indicated that solid porous and hollow porous scaffolds exhibited a decrease in hASC proliferation over the first seven days compared to solid and hollow pure PLA control scaffolds. Proliferation on solid porous and
hollow porous scaffolds remained relatively constant until day seven, when an increase in proliferation was observed (Figure 4.7a). These results were confirmed with Live/Dead staining. Although all scaffolds supported viable hASC, solid porous and hollow porous scaffolds exhibited a similar number of viable cells over the first five days, with an increase observed on day seven (Figure 4.8, b1–b4 and d1–d4) while pure PLA solid and hollow scaffolds increased in cell number at each time point (Figure 4.8, a1–a4 and c1–c4). We have previously shown that AQ55S does not significantly affect cellular viability and proliferation of hASC (Figure 3.11). The reduction in proliferation is likely due to the caustic wash treatment employed to expose the internal porous structure. Although scaffolds were neutralized in acetic acid after the caustic wash, residual reactive species may have been present that negatively affected early hASC proliferation. We speculate that these species are neutralized through reactions with hASC and media and this effect is eventually diminished. Future experiments to optimize the caustic wash procedure and characterize the resultant fiber surfaces should be conducted to minimize early negative interaction between hASC and fiber surfaces. Despite this effect, results indicated that after day five hASC proliferation increased on porous and hollow porous fiber scaffolds. At the end of the experiment, there were no significant differences in proliferation amongst all scaffolds treated with CGM (Figure 4.7b), and Live/Dead staining revealed similar numbers of hASC on each scaffold type in each media type evaluated (Figure 4.9). Further, SEM imaging revealed few hASC on the surface of all scaffold types analyzed after one week in CGM. However, at the end of the three week culture period hASC were observed throughout the scaffold surfaces for all fiber types. Human ASC attached to all fiber types, including PET binder fibers (Figure 4.13). These results suggest that despite negative interactions observed at early time points,
caustic treated scaffolds were capable of supporting viable proliferation of hASC over the 21 day culture period.

Quantification of differentiation revealed carded full thickness scaffolds were capable of supporting both adipogenic and osteogenic differentiation of hASC. Significantly increased levels of triglycerides and cell mediated calcium accretion were observed for all scaffold types when treated with ADM or ODM, respectively, indicative of the presence of adipocytes or osteocytes (Figure 4.10). Further, osteogenesis results indicated that solid porous and hollow porous scaffolds resulted in significantly higher levels of calcium accretion compared to pure PLA solid and hollow controls, indicating porous fiber led to increased levels of osteogenesis (Figure 4.10b). Increased levels of triglycerides were also observed for hollow porous fibers compared to pure PLA controls, although there was no difference between solid porous and pure PLA solid control fiber scaffolds (Figure 4.10a). These results suggest that surface morphology of scaffold fibers is an important factor capable of enhancing differentiation of hASC. Other groups have reported similar findings with regard to scaffold surface morphology effects on differentiation of a variety of stem cell sources [129–131], [136–140]. Trujillo and Popat have shown that surface morphology of polycaprolactone (PCL) scaffolds affects hASC differentiation. PCL scaffolds consisting of nanowires perpendicular to the substrate surface resulting in a porous nanotopography led to increased levels of hASC adipogenic differentiation and decreased levels of chondrogenic differentiation of hASC compared to PCL controls [137]. McMurray et al. have demonstrated that PCL scaffold nanotopography not only affects osteogenic differentiation of mesenchymal stem cells (MSC), but can also be tuned to promote proliferation of MSC while maintaining their stem cell phenotype. Our results of hASC cultured on porous and hollow
porous fiber carded scaffolds suggest that the porous surface morphology leads to enhanced adipogenic and osteogenic differentiation of hASC.

4.5. Conclusions

We have reported on the successful fabrication of solid and hollow pure PLA as well as porous and hollow porous PLA multifilament fibers and subsequent production of thick (2–3 mm), carded nonwoven tissue engineering scaffolds for full thickness defect repair. An interconnected microporous fiber wall network was realized via blending of PLA with AQ55S, a water dispersible copolyester. Removal of the AQ55S component in subsequent water and caustic washes led to the formation of porous fibers. The effects of fiber morphology on proliferation, viability, differentiation, and spreading of hASC were evaluated. After three weeks in culture, porous and hollow porous fibers led to similar levels of proliferation and viability compared to control solid fibers. All fiber types supported adipogenic and osteogenic differentiation of hASC. Hollow porous fibers resulted in a significant increase in adipogenesis compared to other fiber types, while both porous and hollow porous fibers exhibited significantly increased osteogenesis compared to control fibers, attributed to the rough surface texture and porous nature of the fiber surfaces. Lastly, hASC were able to spread and populate the surface of all scaffold types with cell generated ECM structures forming a three dimensional network. To the best of our knowledge, this is the first study to describe the production of porous and hollow porous fiber carded nonwovens at industrial scale speeds for tissue engineering applications. The rapid, repeatable, economical nature of the fabrication process makes carded scaffolds an attractive
candidate for clinical translation of full thickness tissue engineering scaffolds with enhanced mass transport properties.
CHAPTER 5 Novel High Surface Area Mushroom Gilled Fibers
Increase RUNX2 Expression of Human Adipose Derived Stem 
Cells Under Pulsatile Fluid Flow

This chapter has been submitted for publication to Biomaterials.

5.1. Introduction

The fabrication and characterization of novel high surface area hollow gilled fiber tissue engineering scaffolds via industrial relevant, scalable, repeatable, high speed, and economical nonwoven carding technology is described. Scaffolds were validated as tissue engineering scaffolds using human adipose derived stem cells (hASC) exposed to pulsatile fluid flow (PFF). The effects of fiber morphology on the proliferation and viability of hASC, as well as effects of varied magnitudes of shear stress applied via PFF on the expression of the early osteogenic gene marker runt related transcription factor 2 (RUNX2) were evaluated. Gilled fiber scaffolds led to a significant increase in proliferation of hASC after seven days in static culture, and exhibited fewer dead cells compared to pure PLA round fiber controls. Further, hASC-seeded scaffolds exposed to 3 and 6 dyne/cm² resulted in significantly increased mRNA expression of RUNX2 after one hour of PFF in the absence of soluble osteogenic induction factors. This is the first study to describe a method for the fabrication of high surface area gilled fibers and scaffolds. The scalable manufacturing process and potential fabrication across multiple nonwoven and woven platforms makes them promising candidates for a variety of applications that require high surface area fibrous materials.
Tissue engineering strategies for the creation of new functional bone tissue using a stem cell source require recapitulation of the chemical and mechanical environment of the native tissue being replaced. It is well known that chemical cues are capable of regulating differentiation of a variety of stem cell sources. However, in the past few decades, we and others have shown the critical role of the mechanical environment in controlling stem cell fate [122], [124], [141–156]. In the context of bone tissue engineering, compressive forces exerted on bones during normal day-to-day movement result in pressure driven flow of interstitial fluid through canaliculi, exposing mechanosensitive osteocytes to shear stress [141], [143], [144], [157], [158]. Compressive forces exerted on bones during normal day-to-day movement result in pressure driven flow of interstitial fluid through canaliculi, exposing osteocytes to shear stresses. Osteocytes have been shown to respond to these changes in shear stress and initiate an appropriate cellular response [141], [143], [157]. Bone tissue experiences shear stresses in the range of 8-30 dynes/cm² during normal physiological loading, with 30 dyne/cm² representing peak loading during extensive physical activity [159].

As a result, researchers have investigated the use of biomimetic magnitudes of shear stress for functional bone tissue engineering applications using stem cells. Multiple studies have evaluated the impact of fluid shear stress, both pulsatile and oscillatory, on osteocytes [143], [144], [157], [158], [160], [161] and bone marrow derived mesenchymal stem cells (MSC) [161]. They have shown that shear stresses consistent with shear stresses that occur in bone during normal physiological loading increase osteogenesis and new bone formation [143], [144], [157], [158], [160], [161]. Human adipose derived stem cells (hASC), a relatively more abundant and accessible stem cell source than MSC, have also been shown to
be mechanosensitive to shear stress [141], [142], [162]. Human ASC are an attractive candidate for a variety of tissue engineering strategies due to their relative ease of harvest compared to MSC and other stem cell sources. Human ASC can be readily obtained from routine liposuction and abdominoplasty procedures. We and others have shown that hASC are capable of multipotent differentiation, including adipogenesis, osteogenesis, and chondrogenesis [98], [100], [102], [104–107], [121], [122], [124], [135].

Shear stress has been shown to upregulate osteogenesis of ASC cultured on two dimensional substrates seeded with cells and exposed to pulsatile fluid flow (PFF) [163]. However, for the generation of three dimensional tissues, an initial biodegradable scaffolding structure is needed for early attachment of hASC, providing initial structural integrity, and capable of withstanding applied fluid flow. Our group and many others have reported on the use of poly(lactic acid) (PLA), an FDA-approved biodegradable polymer, for use in the creation of tissue engineering scaffolds [51], [61], [98], [99], [119], [120]. The degradation rate of PLA can be tuned by altering the polymer properties, such as molecular weight and crystallinity, to achieve the desired rate of degradation for a particular application [18].

While many studies have reported on the successful implementation and utilization of PLA and other polymers for tissue engineering scaffolds, critical challenges remain with large scale fabrication and scale up of biomimetic scaffolds. We hypothesize that nonwoven scaffolds composed of PLA are well suited to meet this need. In general, nonwovens are an arrangement of random or oriented fibers bonded together to create a web or fabric. The fibrillar structure of nonwovens mimics that of native extracellular matrix (ECM), and provides an ideal environment for cellular attachment and proliferation. In particular, we propose that, carded nonwoven fabrication techniques are capable of producing repeatable,
scalable, high speed, and economical scaffolds from a wide variety of fiber types, including PLA. In the carding process, staple fibers (short fibers of about 5 cm in length) are first separated into individual filaments and subsequently entangled together (Figure 5.1c). Fiber entanglement is achieved via a series of specialized combed rollers, and the resultant web can be layered many times to achieve a desired thickness (termed crosslapping). Finally, the structure is locked in place by bonding the fibers via a variety of methods, such as needle punching, hydroentangling, thermal bonding, or chemical adhesives [101], [132–134].

The goal of this study was to use carding technology to fabricate nonwoven scaffolds for bone tissue engineering applications and to test these scaffolds for their ability to support hASC viability, proliferation, and osteogenic differentiation while exposed to fluid shear stresses at magnitudes consistent with those that occur in vivo. Using this technology, we describe for the first time the fabrication of a novel fiber cross sectional morphology, which we have termed “gilled” fibers, for their resemblance to the underside of a mushroom cap. Gilled fibers consist of an outer solid PLA shell, with multiple finger like PLA projections extending toward an internal hollow channel. The high surface area and gilled structure makes these fibers an attractive candidate for nonwoven tissue engineering scaffolds seeded with stem cells and mechanically stimulated with PFF. We hypothesized that the gilled structure would lead to enhanced mass transport properties via capillary action, resulting in locally increased levels of shear stress magnitudes, and increased osteogenic differentiation of hASC seeded on the scaffolds.
Figure 5.1. Idealized schematic of gilled fiber multifilament spinning and carded scaffold fabrication. a) Bicomponent multifilament spinning: PLA and AQ55S polymer pellets are fed to separate extruders, melted, and forced through the bicomponent spinnerette/die. The spinnerette design directs the molten polymers through a series of specialized plates to form the winged cross section. Individual filaments are formed at each orifice and collected as a fiber bundle or spinline and wound around the collector godet roll via a vacuum gun (lurgi gun). Multifilaments are wound and collected on a bobbin or transferred via additional godet rolls for subsequent processing. b) Crimping and cutting: Fibers are crimped into a zig-zag pattern to facilitate transfer to carding rolls and cut into short (about 5 cm) individual staple fibers. c) Carding: Staple fibers are converted to an unbonded fiberweb via a series of specialized combed rollers. d) Bonding: Web structure is locked into place via needle punching. Barbed needles snare and entangle fibers through the scaffold thickness. e) The AQ55S component is removed via washing in hot deionized water. Finally, the scaffold is dried in an oven followed by winding on a collector roll. Fabrication is depicted as a continuous process; scaffolds fabricated in this study were processed in batch operations (bicomponent filament spinning, crimping and cutting, carding, bonding, and washing).
5.2. Materials and Methods

5.2.1. Fabrication of Gilled Fiber Scaffolds

Fabrication of Gilled Multifilaments

PLA grade 6100D (NatureWorks LLC, Minnetonka, MN) and AQ55S (Eastman Chemical Company, Kingsport, TN) were used for creation of all fibers. Prior to filament spinning, the rheological properties of pure PLA and AQ55S were determined on a Rosand RH7 capillary rheometer (Malvern Instruments, Malvern, UK). Polymers were dried overnight under vacuum at 85 and 40 °C for PLA and AQ55S, respectively, to remove any absorbed water. Approximately 35 g were loaded into the rheometer chamber fitted with a long die (16 mm length and 1 mm diameter) and subjected to a shear rate sweep from 20–10,000 1/s at constant temperature. PLA and AQ55S were each evaluated at 230 and 250 °C.

Gilled fiber multifilaments were fabricated using a winged fiber spinnerette (NatureWorks LLC, Minnetonka, MN) as previously described [164]. In brief, PLA was loaded as the core polymer with AQ55S forming the bicomponent sheath at a 50:50 ratio by weight (w/w). Multifilaments were extruded and collected on bobbins (cylinders on which filaments were wound). Round fiber cross section multifilaments composed of pure PLA were extruded and collected on bobbins under similar processing conditions for the fabrication of control fibers of the same fiber diameter as experimental gilled multifilaments (Figure 5.1a).

Fabrication of Carded Scaffolds

Gilled fiber and round PLA control multifilaments were unwound, crimped (heat set into a zig-zag pattern), and cut into 5 cm staple fibers for the fabrication of carded scaffolds.
(Figure 5.1b) at the Nonwovens Institute pilot facilities (NWI, North Carolina State University, Raleigh, NC). Staple fibers were carded on a model carding unit at NWI at a basis weight of 200 g/m$^2$ (gsm) (Figure 5.1c). Scaffold structure was locked into place via needle punching on an Asselin A.50-RL 4 board needle loom. Fabrics were bonded at a needle punch density (npd) of 50 strokes/cm$^2$ on the top and bottom surfaces for a total npd of 100 strokes/cm$^2$ (Figure 5.1d).

The gilled fiber cross section was exposed by washing the gilled fabrics in agitated deionized (DI) water at 85 °C for 15 minutes containing 250 µL surfactant per 1 L DI water to reduce surface tension and aid in removal of AQ55S (Figure 5.1e). Gilled scaffolds were dried in an oven at 40 °C overnight to remove any absorbed water prior to weighing before the washing procedure. Washed scaffolds were rinsed several times in fresh DI water and allowed to dry overnight at 40 °C prior to being weighed to determine weight loss.

**Scaffold Characterization**

PLA control, gilled unwashed, and gilled scaffold surfaces and fiber cross sections were imaged on a Phenom G2 Pro scanning electron microscope (SEM) (PhenomWorld, Eindhoven, Netherlands). Fiber cross sections were prepared by drying the scaffolds overnight at 40 °C followed by fracturing under liquid nitrogen using a razor. Scaffold SEM surface images were used to determine average fiber diameter using ImageJ software (NIH, Bethesda, MD) with an average of at least 25 fibers measured for each scaffold type. The fiber density of gilled fibers was determined empirically from direct observation of fiber cross sections using SEM imaging after washing. Representative fiber cross sections were converted to threshold images using ImageJ software. The total fiber cross sectional area as
well as the hollow internal area was then calculated using ImageJ to determine the percentage of hollow area in each fiber cross section. Fiber density was then calculated based on total area, less the hollow area, assuming a density of pure PLA (1.24 g/cm³).

Scaffold thickness was measured on a Hanatek FT3 precision thickness gauge (East Sussex, UK) (n=10 per scaffold) taken at random points along the scaffold width and length at least 5 cm away from the scaffold edge. Mean flow pore size of scaffolds was determined using a Porous Materials INC advanced capillary flow porometer (Ithaca, NY). GalWick was used as the wetting agent using the wet up/dry up no wait at dry method per manufacturer’s instructions. Peak load was determined for each scaffold type via a tensile strip test to failure on an Instron 4400R (Norwood, MA). Specific surface area of PLA control, gilled unwashed, and gilled scaffolds was determined using the Brunauer-Emmett-Teller (BET) method [127] on a Quantachrome ASiQwin automated gas sorption chamber (Quantachrome Instruments, Boynton Beach, FL). An outgassing temperature of 40 °C was employed to ensure temperature did not exceed the glass transition temperature of AQ55S (55 °C) with Krypton as the analysis gas.

5.2.2. Scaffold Cell Culture and Application of PFF

Scaffold seeding

Scaffolds were cut into 5/8 inch diameter circular scaffolds and were sterilized for four hours in 70% ethanol followed by rinsing five times in phosphate buffered saline (PBS) in 12 well non-tissue culture treated polystyrene plates. Scaffolds were then soaked in complete growth medium (CGM: Eagle α-Minimum Essential Medium, 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin) for 12
hours prior to cell seeding. Scaffolds were then seeded with a second passage human adipose derived stem cell pooled pre-menopausal, age and gender matched superlot isolated from five female donors that we have previously described [107]. Human ASC were obtained following an IRB exempt protocol (UNC 10-0201) from excess liposuction waste tissue from procedures performed at the University of North Carolina at Chapel Hill. Human ASC were cultured in 75 cm² tissue culture treated flasks to 75% confluency, trypsinized, and resuspended in CGM at 200,000 cells/mL. Scaffolds were seeded at 20,000 cells/cm² and hASC were allowed to attach for one hour in a humidified incubator at 37 °C and 5% CO₂. After the one hour attachment period, wells were flooded with CGM and cultured in the incubator overnight. CGM was then aspirated and scaffolds were carefully turned and seeded with an additional 20,000 cells/cm² and allowed to attach and incubated overnight. Scaffolds were then transferred to new tissue culture plates and allowed to proliferate in CGM for an additional seven days, with media changes every two days.

Cellular proliferation was tracked over the seven day expansion period on days 1, 3, 5, and 7 using the AlamarBlue™ assay (BioRad AbD Serotec, Oxford, UK). CGM was aspirated and scaffolds were carefully washed twice with phosphate buffered saline (PBS) and subsequently flooded with CGM containing AlamarBlue™ at a concentration of 10% by volume (v/v) and transferred to a humidified incubator at 37 °C and 5% CO₂ for three hours. 200 µL of AlamarBlue™ containing medium was the removed and transferred to a 96 well plate and absorbance read in triplicate on a GENios microplate reader (Tecan, Männedorf, Switzerland) at 570 and 600 nm. AlamarBlue™ containing medium was then aspirated, scaffolds were washed three times in PBS, and flooded with fresh CGM.
Cellular viability was analyzed on days 1, 3, 5, and 7 using a mammalian Live/Dead viability kit (Invitrogen, Molecular Probes, Eugene, OR). Live/Dead scaffolds were rinsed twice in PBS and incubated protected from light at 37 °C in PBS containing 3 µM calcein AM (live cells, stained green) and 4 µM ethidium homodimer-1 (dead cells, stained red) for 15 minutes. Scaffolds were then mounted on glass slides and imaged on a Leica DM5500B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at 10x magnification.

**Application of PFF**

The PFF shear reactor application system consisted of eight individual fluid shear chambers (Tissue Growth Technologies, Minnetonka, MN) connected to a fluid distribution manifold (Figure 5.2b). CGM was supplied from a reservoir (Figure 5.2a) through the manifold to the shear chambers (Figure 5.2d) via a MasterFlex L/S 7519-25 peristaltic pump (Cole-Parmer, Vernon Hills, IL) through gas permeable tubing (Figure 5.2c) (PharMed, Cole-Parmer) at a specified volumetric flow rate (Equations 5.1 and 5.2). All shear chamber and pump components were sterilized in an autoclave and assembled under aseptic conditions in a biological safety cabinet. After the seven day expansion period, hASC-seeded scaffolds were loaded into the shear chambers and connected to the bioreactor pump apparatus. The tubing lines were primed with CGM prior to being connected to the scaffold loaded shear chambers and the entire closed system was then transferred to a humidified incubator at 37 °C and 5% CO₂. Volumetric flow rates resulting in desired physiological shear stress magnitudes[159] of 3, 6, and 9 dyne/cm² were calculated according to Equations 5.1 and 5.2 and scaffolds were subjected to one hour of PFF at each shear stress.
Unstimulated/static controls were kept in the same system in the incubator but without application of PFF (referred to as 0 dyne/cm$^2$).

\[ v = \frac{\tau R}{2 \mu} \]  

\[ Q = A \nu \]  

**Equation 5.1.**  
**Equation 5.2.**

where

- $v$ = fluid velocity
- $\tau$ = shear stress
- $R$ = pore radius
- $\mu$ = dynamic viscosity
- $Q$ = volumetric flow rate
- $A$ = pore area
Figure 5.2. Pulsatile fluid flow (PFF) reactor setup. (a) Complete growth medium reservoir, (b) medium distribution manifold, (c) peristaltic pump, and (d) shear stress chambers containing scaffolds seeded with human adipose derived stem cells. No fluid leakage was observed during the experiment. Volumetric flow rates were validated for accuracy between chambers before application of PFF to confirm shear stress magnitudes of 0, 3, 6, and 9 dyne/cm².

Cellular viability of hASC on scaffolds subjected to PFF at each shear stress magnitude were analyzed immediately after application of one hour PFF in the same manner as Live/Dead scaffolds analyzed during the expansion period. Three scaffolds per scaffold
type and shear stress magnitude were collected in 1.5 mL RNase free tubes containing 350 µL lysis buffer RLT (Qiagen, Valencia, CA) and repeatedly ground with an RNase free pestle and frozen at -80 °C.

Human ASC seeded scaffolds exposed to PFF were thawed on ice and cell lysates were processed in QiaShredder homogenizers (Qiagen). Total RNA was isolated using an RNeasy mini kit according to the manufacturer’s instructions (Qiagen). Isolated RNA was quantified on a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Following RNA isolation, 48 ng of RNA for each sample was used for the generation of cDNA using an OriGene first strand cDNA synthesis kit according to the manufacturer’s instructions (OriGene, Rockville, MD) on an Eppendorf Mastercycler Nexus Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) and quantified using a NanoDrop 2000. Real time reverse transcriptase polymerase chain reaction (RT-qPCR) was performed using a TaqMan gene expression master mix (Applied Biosystems Fisher Life Technologies, Waltham, MA) with 2 µL of cDNA per sample (n=3) on an ABI Prism 7000 real time thermocycler (Applied Biosystems Fisher Life Technologies, Waltham, MA). Gene expression of runt related transcription factor 2 (RUNX2) was measured and normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT 1). Both primers were purchased from Applied Biosystems (Fisher Life Technologies, Waltham, MA). Fold change in expression was calculated using the comparative threshold (C_T) method as described by Schmittgen and Livak [165].
**Statistical Analysis**

An unpaired student’s t-test was used to analyze statistical difference for scaffold characterization and proliferation assays. A one-way ANOVA was used for statistical analysis of PCR data. Error bars represent standard error of the mean. Statistical significance is indicated as p<0.05.

5.3. Results

5.3.1. Fabrication of Gilled Fiber Scaffolds

*Fabrication of Gilled Multifilaments*

The rheological properties of PLA and AQ55S were determined prior to fabrication of multifilaments. Capillary rheometry results indicated that AQ55S exhibits a relative viscosity 2–6 times greater than that of pure PLA, depending on shear rate and temperature (Figure 5.3). As expected, viscosity decreased with increasing shear rate; with shear profiles exhibiting shear thinning behavior typical of polymer melts. Multifilaments were successfully fabricated using a winged fiber spinnerette with PLA loaded as the core polymer and AQ55S as the sheath polymer. Multifilaments were successfully fabricated and collected on bobbins for subsequent production of staple fibers and carded fabrics. Control pure PLA round cross section fibers were also collected under similar spinning conditions, resulting in PLA fibers of the same diameter compared to experimental gilled fibers (Figure 5.4).
Figure 5.3. Apparent shear viscosity (Pa s) versus shear rate (1/s) for pure PLA and AQ55S at 230 and 250 °C. AQ55S exhibited viscosities 2–6 times greater than pure PLA.

Fabrication of Carded Scaffolds

Multifilaments were processed into staple fibers for subsequent carded fabric production (Figure 5.1b). Filaments were unwound and crimped into 5 cm staple fibers. No fiber breakage during crimping and cutting was observed for pure PLA controls or for experimental gilled fibers. Staple fibers were successfully converted to carded fabrics on a small model carding unit (Figure 5.1c). A uniform basis weight of 200 gsm was achieved for both scaffold types. Scaffolds were bonded via needling on both sides at a total needle punch density of 100 strokes/cm² (Figure 5.1d).
Removal of the Sacrificial Component

Gilled scaffolds were washed in gently agitated DI water at 85 °C for 15 minutes. Mechanical bonding via needling was sufficient to withstand the washing procedure, with little fiber loss observed during the washing procedure. Washed scaffolds retained their nonwoven morphology with no significant changes in physical dimensions observed (Figure 5.4). Visual dissolution of the AQ55S component was observed within one minute of submersion in DI water as an opaque milky substance leaving the scaffold surface. The washing procedure resulted in fabric weight loss of 52.7 ± 0.4%, indicating an average washing efficiency of 107.61 ± 0.04%.
Figure 5.4. Scanning electron microscopy images of scaffold surfaces at 500x magnification (top row) and fiber cross sections at 2000x (middle row) and 5000x (bottom row) magnification. A winged fiber cross section was observed for unwashed gilled fibers (b₂ and c₂). Washing resulted in the formation of the gilled fiber morphology, consisting of a PLA sheath and finger like projections toward a hollow core (b₃ and c₃). Scale bars represent 100 µm (top row), 50 µm (middle row), and 10 µm (bottom row).

Scaffold surfaces and fiber cross section SEM images were captured to determine scaffold and fiber morphology (Figure 5.4). Fiber diameter of PLA control, gilled
unwashed, and gilled fibers was well conserved and resulted in fiber diameters of 23.8 ± 2.5, 24.4 ± 2.6, and 23.5 ± 1.2 µm, respectively. All fiber types resulted in a round outer diameter morphology (Figure 5.4, b₁–b₃ and c₁–c₃). Additionally, a portion of the gilled fibers exhibited cracks on the fiber surface, exposing the internal fiber structure (Figure 5.4, a₃–c₃). Unwashed gilled fiber cross sections revealed successful fabrication of a winged fiber cross section, with distinct polymer interfaces observed between PLA and AQ55S (Figure 5.4, b₂ and c₂). The washing procedure resulted in removal of the AQ55S component, leaving behind an inverted winged fiber cross section, consisting of an outer PLA skin and PLA finger like structures extending toward a hollow core (Figure 5.4, b₃ and c₃). The resultant structure resembled the gills on the underside of a mushroom cap; hence the name gilled fibers.

Gilled fiber density was calculated from representative SEM cross section images using ImageJ software (Figure 5.5). The cross sectional hollow area occupied a volume of 175 µm² with total area of the fiber occupying 446 µm². From these measurements, the fiber density of gilled fibers was calculated as 0.67 g/cm³, compared to a pure PLA fiber density of 1.24 g/cm³, representing a 46% decrease in fiber density. BET surface area measurements indicated PLA control, gilled unwashed, and gilled fiber surface areas of 0.066, 0.104, and 1.677 m²/g, respectively (Figure 5.6).
Figure 5.5. Empirical calculation of gilled fiber density. Representative SEM image of gilled fiber cross section (a) and (b). Cross sectional image was converted to a threshold image using ImageJ software (c) and converted to an area map to determine hollow area as a percentage of total fiber cross sectional area (d). Scale bars represent 10 µm.
Figure 5.6. Brunauer-Emmett-Teller surface area analysis of cell seeded scaffolds. The washing procedure and subsequent formation of gilled fiber scaffolds resulted in specific surface area of 1.677 m$^2$/g, compared to 0.104 m$^2$/g for unwashed scaffolds, representing an over 1500% increase.

Scaffold Characterization

Fiber and scaffold properties are provided in Table 5.1. Thickness of PLA control, gilled unwashed and gilled scaffolds agreed well and resulted in scaffold thicknesses of 2750 ± 72, 2894 ± 109, and 2600 ± 129 µm, respectively. Pore sizes of 73 and 84 µm were measured for PLA control and gilled unwashed scaffolds. The washing procedure resulted in slightly increased pore size of 92 µm for the gilled scaffolds. Mechanical properties of acellular scaffolds were analyzed via tensile testing to failure prior to cell seeding. Representative engineering stress strain curves are provided in Figure 5.7a. Tensile test data resulted in peak loads for PLA control, gilled unwashed, and gilled scaffolds of 31.7 ± 1.7, 27.3 ± 6.3, and 55.0 ± 10.1 N, respectively (Figure 5.7b).
Table 5.1. Fiber and scaffold properties of PLA control, gilled unwashed, and gilled scaffolds.

<table>
<thead>
<tr>
<th>Property</th>
<th>PLA Control</th>
<th>Gilled Unwashed</th>
<th>Gilled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber Diameter [µm]</td>
<td>23.8 ± 2.5</td>
<td>24.4 ± 2.6</td>
<td>23.5 ± 1.2</td>
</tr>
<tr>
<td>Needle Punch Density [st/cm²]</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Basis Weight [gsm]</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Thickness [µm]</td>
<td>2750 ± 72</td>
<td>2894 ± 109</td>
<td>2600 ± 129</td>
</tr>
<tr>
<td>Pore Size [µm]</td>
<td>73</td>
<td>84</td>
<td>92</td>
</tr>
<tr>
<td>Surface Area [m²/g]</td>
<td>0.066</td>
<td>0.104</td>
<td>1.677</td>
</tr>
<tr>
<td>Peak Load [N]</td>
<td>31.7 ± 1.7</td>
<td>27.3 ± 6.3</td>
<td>55.0 ± 10.1</td>
</tr>
</tbody>
</table>

a) weight/weight, b) strokes/cm², c) g/m²
Figure 5.7. Representative engineering stress strain curves for acellular scaffolds (a), and resultant peak load (b). Removal of the AQ55S component led to an increase in peak load for gilled fiber scaffolds compared to unwashed gilled scaffolds.
5.3.2. Human ASC-Seeded Gilled Scaffolds Subjected to PFF

*Proliferation and Viability of hASC*

Human ASC proliferated over the seven day expansion period on PLA control and gilled scaffolds (Figure 5.8). No significant differences were observed over the first five days; however on day seven gilled scaffolds exhibited a significant increase in proliferation compared to PLA control scaffolds. Little change was observed in the number of viable cells (stained green) for both scaffold types over the first five days (Figure 5.9a, a₁–a₃ and a₅–a₇); however an increase in the number of viable cells was observed on day seven (Figure 5.9a, a₄ and a₈). Further, a greater number of dead cells (stained red) were observed for all time points on PLA control scaffolds compared to gilled scaffolds.

**Figure 5.8.** AlamarBlue™ cellular proliferation on PLA control and gilled scaffolds. There were no significant differences between scaffold types observed over the first five days in culture, however a significant increase in cellular proliferation was observed on gilled scaffolds on day seven compared to PLA control scaffolds. Different letters represent statistical significance (p<0.05). Error bars represent standard error of the mean.
Figure 5.9. Live/Dead viability of human adipose derived stem cells (hASC). Live cells stained green, dead cells stained red. (a) Human ASC viability over the seven day expansion period. Little difference was observed in the number of viable cells over the first five days between scaffold types \((a_1-a_3 \text{ and } a_5-a_7)\), however gilled scaffolds appeared to exhibit a greater number of live cells on day seven \((a_4 \text{ versus } a_8)\). A greater number of dead cells were observed on PLA controls scaffolds compared to gilled scaffolds at each time point. (b) Human ASC viability immediately after application of one hour pulsatile fluid flow (PFF). A greater number of viable cells were observed on gilled scaffolds for all shear stress magnitudes compared to PLA control scaffolds. Further a greater number of dead cells were observed for all shear stress magnitudes on PLA control scaffolds compared to gilled scaffolds \((b_2-b_4 \text{ compared to } b_6-b_8)\).

**Application of PFF**

A representative image of the PFF apparatus is provided in Figure 5.2. Bioreactor assembly was carried out under aseptic conditions in a biological safety cabinet. Prior to
subjecting scaffolds to PFF, volumetric flow rate was validated with CGM. All chambers operated successfully and delivered equal volumes of CGM according to the specified flow rate (Equations 5.1 and 5.2). No leakage was observed during application of PFF. RT-qPCR was performed after the application of PFF to determine the effects on hASC mRNA expression of RUNX2. There were no significant differences between unstimulated scaffolds, however hASC seeded on gilled scaffolds exhibited significantly increased RUNX2 expression for shear stress magnitudes of 3 and 6 dyne/cm² (Figure 5.10). Both scaffold types led to a significant increase in RUNX2 expression for 9 dyne/cm² compared to unstimulated controls, however there was no statistical difference between the scaffold types at 9 dyne/cm².
Figure 5.10. Real time reverse transcriptase polymerase chain reaction (RT-qPCR) results. Fold change expression of runt related transcription factor 2 (RUNX2) normalized to hypoxanthine phosphoribosyltransferase 1 for each scaffold at each pulsatile fluid flow (PFF) magnitude. No statistically significant differences were observed between scaffold types on unstimulated controls (PFF = 0 dyne/cm$^2$); however hASC seeded on gilled scaffolds exhibited a significant increase in RUNX2 expression at shear stress magnitudes of 3 and 6 dyne/cm$^2$. There was no significant difference between scaffold types at 9 dyne/cm$^2$; however each scaffold type exhibited significantly increased RUNX2 mRNA expression compared to their respective unstimulated controls.

Live/Dead cellular viability images were captured immediately after application of PFF to determine the effects of applied shear stress on hASC viability (Figure 5.9b). Some reduction in the number of viable cells was observed on PLA control scaffolds for all shear stress magnitudes compared to unstimulated controls (Figure 5.9b, b$_2$–b$_4$). Some decrease in the number of viable cells also appeared to occur on gilled scaffolds, although to a lesser extent than PLA controls (Figure 5.9b, b$_6$–b$_8$). Little difference was observed between shear stress magnitudes applied with regard to the number of viable cells observed for gilled
scaffolds. A greater number of dead cells were observed on PLA control scaffolds compared to gilled scaffolds for all shear stress magnitudes.

5.4. Discussion

5.4.1. Fabrication of Gilled Fiber Scaffolds

We have described a method for successful bicomponent multifilament spinning of PLA and AQ55S using a winged fiber spinnerette, with PLA loaded as the core and AQ55S as the sheath. With this arrangement, an inverted winged fiber cross section (termed gilled fiber) was created, with the PLA component moving from the core and replacing AQ55S as the sheath component and vice versa. The gilled cross section was clearly defined and both polymers and the polymer interface could be observed under SEM imaging (Figure 5.4, b₂–b₃ and c₂–c₃). We believe that phase inversion of the polymer components was likely due to viscosity differences between PLA and AQ55S. AQ55S was measured to be 2–6 times more viscous than PLA. It has been demonstrated previously that in the case of two component polymer melt systems, phase inversion is likely to occur when there is a large difference in viscosity [87], [88], [166], [167]. Ratnagiri and Scott have demonstrated that phase inversion is favored in systems containing a low viscosity and low melting point minor component [166]. Li et al. have shown that the viscosity ratio, weight percentage, and interfacial tension between components as well as the processing conditions will affect the resultant blend morphology [88]. Blends composed of polymers with similar viscosities (viscosity ratio at or near) tend to form co-continuous structures for weight ratios between 30–70% [87]. In the present work, it is likely that the large difference in viscosity of the two components is responsible for phase inversion and formation of the gilled fiber cross section. Future work,
including loading AQ55S as the core component and PLA as the sheath during filament spinning, could shed further details on the observed phenomenon.

Gilled fiber multifilaments were successfully fabricated into staple fibers and subsequently carded to form nonwoven scaffolds. Staple fiber processing, carding, and bonding via needle punching did not affect the gilled fiber cross section. Needle punching provided sufficient mechanical stability to withstand the washing procedure. A dramatic increase of over 1500% in specific surface area was observed for gilled fibers compared to unwashed controls. Further, a washing efficiency greater than 100% was observed for gilled fiber scaffolds, indicating complete removal of the AQ55S component was likely achieved. The additional weight loss was likely due to individual fibers lost during the washing procedure, and removal of water-soluble spin finish applied during filament spinning. Spin finish is a lubricating liquid (of varied composition, depending on the type of polymer being spun) applied during spinning to prevent filaments from adhering to the collection rolls and to one another.

Although in this study we have focused on gilled scaffolds for tissue engineering applications, gilled fibers are attractive candidates for a variety of applications requiring high surface area, such as biomedical separation processes. In addition, while we have shown successful fabrication of carded scaffolds, our results suggest production of gilled fibers could be extended to other woven and nonwoven applications such as knits, woven fabrics, and direct fabrication of continuous filament nonwovens such as bicomponent spunbond fabrics, providing an array of fabrication platforms that could be tailored for specific applications.
5.4.2. Carded Gilled Fiber Tissue Engineering Scaffolds

The novel gilled fiber scaffolds described here exhibited promise as tissue engineering scaffolds using hASC. Cellular proliferation was significantly increased on gilled scaffolds compared to solid fiber controls after seven days in static culture (Figure 5.8). Further, fewer dead cells were observed on gilled scaffolds over the culture period (Figure 5.9a). SEM surface images of scaffolds exhibited the presence of linear cracks along the surface of a portion of fibers, originating between fingerlike PLA internal wing projections (Figure 5.4, a3). This effect was attributed to fracturing during the needle punching process. For the purposes of this study, these fracture lines may have served a beneficial purpose by exposing the internal hollow gilled channel, facilitating fluid flow. The hollow gilled channels may provide enhanced mass transport through capillary action, promoting better distribution of nutrients and oxygen throughout the thickness of the scaffolds, as well as providing a route for elimination of cellular waste products.

Human ASC seeded on gilled scaffolds exhibited an increase in RUNX2 mRNA expression, an early marker for osteogenesis [161], [168], for shear stress magnitudes of 3 and 6 dyne/cm² compared to PLA control scaffolds after one hour of PFF (Figure 5.10), even in the absence of chemical osteogenesis induction factors, such as β-glycerol phosphate, ascorbic acid, and dexamethasone. There was no statistical difference in RUNX2 expression between gilled and control scaffolds at 9 dyne/cm², although both scaffolds exhibited a significant increase compared to unstimulated controls at this shear stress magnitude. We speculate that the gilled scaffolds may have exposed hASC to local increases in shear stress magnitude compared to control scaffolds. Fluid flow through the gilled channels is expected to be increased compared to fluid flow through the macro pores in the scaffold, resulting in
increased shear at the fiber surfaces. This hypothesis could explain the increase in RUNX2 mRNA expression for low magnitudes of applied shear stress. At higher fluid velocities and shear stresses, hASC may be exposed to sufficient shear stress on both gilled and control scaffolds, resulting in similar levels of RUNX2 expression. Future empirical and computational analyses to more precisely quantify local shear stresses observed during PFF are needed to validate this hypothesis.

Finally, a greater number of viable cells were observed on gilled scaffolds at all shear stress magnitudes analyzed (Figure 5.9b), suggesting gilled scaffolds promote better adhesion of hASC compared to control scaffolds. Fracture lines observed on the fiber surfaces may have exposed the internal gill structures to attached hASC, providing an ideal microenvironment for cellular attachment.

This is the first study to describe the manufacture of novel gilled fibers and gilled fiber tissue engineering scaffolds. This study serves as a proof of concept experiment for the validation of hASC-seeded gilled fiber scaffolds as tissue engineering constructs using PFF as mechanical stimulation via shear stress. Future studies should be undertaken to further elucidate the effects of scaffold and fiber properties on the behavior of hASC, including longer PFF stimulation times over extended culture periods, other loading modalities, inclusion of chemical induction factors, analysis of early, transitional, and late osteogenic gene targets, analysis of end product expression such as cell mediated calcium accretion, and analysis of hASC spreading and infiltration throughout the scaffolds.
5.5. Summary

We report here for the first time the successful fabrication of novel high surface area gilled fibers. Gilled fiber carded scaffolds were successfully fabricated from multifilament fibers with sufficient strength to withstand washing procedures and application of PFF. A hollow internal cross section with finger like PLA projections resembling those of mushrooms was realized via bicomponent filament extrusion of PLA and AQ55S. The effects of fiber morphology on the proliferation and viability of hASC, as well as the effects of varied magnitudes of applied shear stress via pulsatile fluid flow for one hour were evaluated. Gilled fibers led to a significant increase in proliferation of hASC after one week in static culture, and a greater number of viable cells compared to round fiber PLA control scaffolds. Further, in the absence of any osteogenic induction factors in the culture medium, gilled fibers led to significantly increased mRNA expression of RUNX2, an early marker for osteogenesis, for applied shear stress magnitudes of 3 and 6 dyne/cm$^2$. This is the first study to describe gilled fiber fabrication and their potential for tissue engineering applications. The repeatable, industrial scalable, and versatile fabrication process makes them promising candidates for a variety of scaffold-based tissue engineering applications across a variety of platforms.
CHAPTER 6 Conclusions

6.1. Conclusions

In this dissertation we reviewed three of the most common nonwoven manufacturing methods used in the industry (spunbond, meltblowing, and carding). We have highlighted varying methodologies for the fabrication of fibers with enhanced mass transport properties, particularly, porous and hollow porous fibers. We have further reviewed relevant major studies implementing scaffolds composed of fibers with enhanced mass transport properties. We validated our hypothesis that scalable, industry standard nonwoven manufacturing techniques are suitable for the production of tissue engineering scaffolds. Further, we demonstrated a fabrication method for the production of Spunblown scaffolds composed of porous fibers. We extended this work to the fabrication of carded scaffolds composed of porous or hollow porous fibers. We have shown that porous and hollow porous fiber led to enhanced adipogenic and osteogenic differentiation of hASC, as well as enhanced cellular attachment throughout these scaffolds. Additionally, we have described the fabrication of novel gilled fiber scaffolds, which led to increased proliferation of hASC and increased expression of an early osteogenic gene marker in the absence of soluble osteogenic induction factors.

This body of works demonstrates that industry standard nonwoven manufacturing methods hold great promise as attractive candidates for commercial scale, repeatable, and economical fabrication of tissue engineering scaffolds with enhanced mass transport properties. Human ASC are capable of viable cellular proliferation, and adipogenic and osteogenic differentiation on high throughput scaffold described in this work.
6.2. Recommendations of Future Work

The scope of this dissertation was focused on the fabrication of tissue engineering scaffolds composed of fibers with enhanced mass transport. Using the polymer system and fabrication methods explored, porous and hollow fibers exhibited the presence of a PLA skin. Removal of this PLA skin via treatment in caustic resulted in early negative effects with respect to hASC proliferation. In future studies, optimization of the caustic washing procedure is recommended to eliminate or reduce this effect. Further, although caustic washing procedures are currently adopted in the industry for certain applications, it is desirable from an economical standpoint to eliminate this step all together. We have focused on AQ55S as the sacrificial component in a PLA backbone, however other polymer systems should be explored that result in porous and hollow porous morphologies without the need for caustic treatment. It is suggested that this may be achieved using a polymer blend in which the sacrificial component is less viscous than that of the primary fiber forming polymer under fabrication conditions. The ratio of sacrificial to primary polymer should also be explored further and is expected to affect the morphology of resultant fibers.

Further, we have focused on validation of enhanced mass transport nonwoven scaffolds using hASC. We believe the scaffolds developed in this work may be of interest for a wide range of stem cell sources for a variety of applications. Further studies to extend the scaffolding platform to other tissue engineering sectors should be explored. Further, with the exception of gilled fiber scaffolds exposed to pulsatile fluid flow, our validation approach of scaffolds developed was focused on static culture. It is know that the mechanical environment is capable of modulating stem cell response and is crucial for the generation of functional tissue. When considering strategies for scalable clinical translation of the
scaffolds developed in this work, application of appropriate mechanical cues should be considered.

While we believe these steps will increase the chances for success when implementing scaffold based tissue engineering strategies in clinical practice, the work described here demonstrates nonwoven manufacturing methods hold great promise as a platform for the generation of commercial scale scaffolds composed of a variety of fiber types with enhanced mass transport properties. This works represents the first study to fabricate porous PLA fibers using the Spunblown process. Additionally, we have for the first time described a method for the fabrication of high surface area gilled fibers. The findings in this work lead to a better understanding of the interplay between fiber and scaffold structure with human adipose derived stem cells.
REFERENCES


