Mechanical Properties and Cellular Response of Novel Electrospun Nanofibers for Ligament Tissue Engineering: Effects of Orientation and Geometry

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Abstract

Electrospun nanofibers are a promising material for ligamentous tissue engineering, however weak mechanical properties of fibers to date have limited their clinical usage. The goal of this work was to modify electrospun nanofibers to create a robust structure that mimics the complex hierarchy of native tendons and ligaments. The scaffolds that were fabricated in this study consisted of either random or aligned nanofibers in flat sheets or rolled nanofiber bundles that mimic the size scale of fascicle units in primary tensile bearing soft musculoskeletal tissues. Altering nanofiber orientation and geometry significantly affected mechanical properties; most notably aligned nanofiber sheets had the greatest modulus; 125% higher than that of random nanofiber sheets; and 45% higher than aligned nanofiber bundles. Modifying aligned nanofiber sheets to form aligned nanofiber bundles also resulted in approximately 107% higher yield stresses and 140% higher yield strains. The mechanical properties of aligned nanofiber bundles were in the range of the mechanical properties of the native ACL: modulus = 158 ± 32 MPa, yield stress = 57 ± 23 MPa and yield strain = 0.38 ± 0.08 . Adipose derived stem cells cultured on all surfaces remained viable and proliferated extensively over a 7 day culture period and cells elongated on nanofiber bundles. The results of the study suggest that aligned nanofiber bundles may be useful for ligament and tendon tissue engineering based on their mechanical properties and ability to support cell adhesion, proliferation, and elongation.

1. Introduction

The high incidence of tendon and ligament injuries necessitates the need for an efficient repair strategy. The anterior cruciate ligament (ACL) is frequently ruptured due to traumatic loading, and in the United States alone up to 200,000 ACL tears occur annually [1,2]. The most commonly injured tendons include the flexor/extensor tendons of the hand (incidence of 4.83 and 18/100,000 per year respectively) and the Achilles tendon (12/100,000) [3].

Following injury, tendons and ligaments have a low intrinsic healing capacity due to limited vascularization and thus surgery is required to repair or replace the injured tissue [4,5]. The most common surgical treatment options for tendon and ligament injuries are tissue replacement with allografts or autografts. Although autografts have been historically successful, the primary drawback associated with the technique is donor site morbidity that can result in pain, tendonitis, and weakness [6,7]. To avoid the complications associated with donor site morbidity, cadaver allografts have been used as a primary repair technique; however, limited tissue supply and a risk of disease transmission remain problematic [8]. Regardless of the type of surgical intervention, traumatic and degenerative tendon and ligament injuries still often lead to the development of osteoarthritis [9,10].

In order to improve surgical outcomes of tendon and ligament repairs, the field of tissue engineering has attempted to recapitulate the structure and function of the tendons and ligaments to create an artificial replacement tissue. However, there are currently no FDA approved tissue engineered tendon and ligament replacements [11]. To successfully replace native tendons and ligaments, biocompatibility and mechanical properties of the replacement should be considered [12]. Collagen scaffolds are advantageous because collagen is a major component of the native tendons and ligaments, however these scaffolds lack sufficient mechanical strength and may illicit an immune response [13,14]. Silk scaffolds have superior mechanical properties but suffer from limited cell attachment without extensive chemical modification [15,16]. Synthetic polymers are a popular option for scaffolds since many have already been FDA approved for other applications [11]. Biodegradable polymers can be utilized with a variety of fabrication techniques and the characteristics of the resulting scaffold, including degradation rate, cell compatibility, and mechanical properties, can be altered depending on the type of polymer used [12].

Tissue engineered replacements should take into account the unique structure and mechanical functions of tendons and ligaments which enables them to bear loads throughout complex joint loading regimes. The smallest structural unit of tendons and ligaments are aligned collagen fibrils, which range from 50-500 nm in diameter [4,17].

Collagen fibrils are organized into fibers approximately 10-50 μm in diameter. Groups of fibers are then organized into fascicles, approximately 50-500 μm in size, which are combined to compose the whole tendon or ligament. This hierarchical structure is likely important for the proper mechanical function of the tissue. It is generally thought that tissue engineering strategies employed to recreate tendons and ligaments must be able to accurately recapitulate complex the micro- and nano-scale structure in order to achieve proper function [18,19].

Electrospinning is a versatile tissue engineering technique that results in a non-woven mat of polymeric nanofibers [20,21]. One of the major benefits of electrospinning is the ability to produce nanofibers that may mimic the natural extracellular matrix of tissues, and thus can support cell adhesion, proliferation, and extracellular matrix production [22]. Furthermore, the structure of aligned electrospun nanofibers mimic the dimensionality of collagen fibrils that comprise native tendons and ligaments [23,24]. Electrospinning can be readily modified to achieve tunable characteristics such as fiber size, fiber alignment, and porosity [25–28]. Despite the promise of electrospun scaffolds in the field of tissue engineering there are a number of factors that have thus far limited their clinical applicability. Primarily, sheets of electrospun fibers are fragile and difficult to handle, making them difficult to use in a clinical setting despite architectural modifications in an attempt to improve handleability. Braided electrospun scaffolds seeded with human bone marrow derived stem cells have been shown to support cell adhesion and the upregulation of tenogenic markers [29,30], and stacked electrospun scaffolds with laser cut pores have successfully encouraged cell infiltration [31], but both demonstrated inferior mechanical strength compared to native ligaments.

Since previous attempts have been unable to achieve the cellular and mechanical behavior required for tendon and ligament replacement, it has become necessary to develop a more robust electrospun construct, while still maintaining the complex structure and the cellular compatibility. The hierarchical structure of tendons and ligaments has motivated the development of micro-scale bundles of nanofibers, which mimic the fascicles that compose native connective tissues. The objective of the following study was to assess the influence of nanofiber orientation on mechanical properties and cellular response of nanofiber bundles that have been designed to mimic the hierarchical organization of native tendons and ligaments. The overarching goal of this work is to create a more robust tissue engineered tendon and ligament replacement that closely replicates the native tissue structure.

2. Experimental Materials and Methods

2.1 Fabrication of nanofiber sheet and bundles

Poly(ϵ -caprolactone) (PCL) nanofiber sheets were fabricated using an electrospinning technique. The solution for electrospinning was prepared by dissolving PCL pellets ($M_w = 80,000$, Sigma 440744, St. Louis, MO) in chloroform and mixing the polymer solution with oleic acid sodium salt (OLA, TCI America O0057, Portland, OR) dissolved in methanol to ensure uniform fiber diameter. The final 10% PCL electrospinning solution was a homogenous mixture with a 3:1 chloroform to methanol volume ratio and a 97:3 PCL to OLA ratio of the solid portion. For electrospinning, a glass syringe (Cadence Scientific Micro-Mate, Staunton, VA) outfitted with an 18 gauge luer-lock blunt tip syringe needle (Hamilton 90134, Reno, NV) was filled with polymer solution and situated within an adjustable flow syringe pump (Genie Touch, Kent Scientific, Torrington, CT). The positive lead of a high voltage power supply (Acopian P030HP2, Easton, PA) was connected to the needle tip with an alligator clip. The collector consisted of an aluminum drum (NaBond, Shenzhen, China) 75 mm in diameter positioned 10 cm from the tip of the syringe needle and covered in aluminum foil. To create a randomly oriented flat sheet of nanofibers (Figure 1a) the collector drum was rotated at a speed of 300 rpm (linear velocity of 0.785 m/s), and to create a longitudinally aligned flat sheet of nanofibers (Figure 1b) the drum was rotated at a speed of 3000 rpm (linear velocity of 23.6 m/s). The volumetric flow rate of the PCL solution from the needle was 2.5 mL/h and the applied voltage was 15 kV. After fabricating a flat nanofiber sheet, nanofiber bundles were created by excising a rectangular section (approximately 5 cm x 10 cm) of either the random or aligned nanofiber sheet and rolling it into a cylindrical bundle composed of nanofibers (Figure 1c). The fabrication of nanofiber bundles required no special tools and was analogous to tightly rolling up a sheet of paper into a tube. However because the nanofiber sheets were very thin the center of the nanofiber bundle was not hollow. This fabrication technique allowed for the fiber architecture and the pores between the fibers to remain intact. After fabrication, the nanofiber sheets and bundles were soaked in methanol for 24 h to remove any remaining OLA and stored in a desiccator until use.

2.2 Characterization of nanofiber sheets and bundles

Nanofiber sheets and bundles were mounted on stubs using carbon tape, sputter coated with a 10 nm thick gold coating, and imaged using a field emission scanning electron microscope (SEM, JEOL JSM-6500F, Peabody, MA) operating at 10 kV to examine nanofiber architecture. To quantify nanofiber diameter using SEM images, 5000x magnification images ($n=6$) were taken from different samples of random and aligned nanofibers. Using Image J

v1.48 (NIH, Bethesda, MD), the diameter of 20 nanofibers in each image was manually (LG) measured. Diameters of random and aligned nanofiber bundles (n=15) were quantified by imaging samples with a CCD camera (Flea3, Point Grey Research, Richmond, BC, Canada) and measured manually.

The same 20 nanofibers used for diameter measurement were assessed for angular orientation to quantify nanofiber alignment [32]. A reference line was drawn along the horizontal plane and the angle of each fiber was measured relative to the reference line. To account for initial rotation of aligned samples the angles were normalized to the primary direction of sample rotation. The analysis yielded a distribution of fiber angles ranging from -90° to 90° where 0° was defined as the horizontal. Histograms were created from all angle measurements for both random and aligned samples. Angular deviation was calculated using the MATLAB circular statistics toolbox [33].

Cross-sectional area of nanofiber sheets and bundles was determined two ways - via measurements of “as-spun” nanofiber sheets and bundles with pores spaces left intact (Figure 2a, b), and of “compressed” nanofiber sheets and bundles with pores spaces removed (Figure 2c, d). As-spun nanofiber sheets are highly porous and using sample width and thickness to calculate cross-sectional area over estimates the true amount of material present in the sample, since the pores contribute significantly to the sample thickness. For nanofiber bundles, using the outer diameter to calculate cross-sectional area does not take into account pore space between nanofibers and additionally does not take into account any gaps between the rolled “layers” of nanofiber sheets.

As-spun thickness of nanofiber sheets (n=5) was determined by measuring the thickness at 10 locations on each sample from transverse SEM images (Figure 2a). As-spun cross-sectional area of nanofiber bundles was determined by manually measuring the diameter of nanofiber bundle and assuming a cylindrical shape (Figure 2b). To determine the compressed cross-sectional area nanofiber sheets (n=5) and nanofiber bundles (n=20) were compressed under a 10 lb weight for 10 min in order to eliminate pore space between nanofibers. Transverse sections of compressed nanofiber sheets and bundles were imaged via SEM to visualize true cross-sectional area of each sample after pore space had been eliminated. Thickness of nanofiber sheets was determined by measuring the thickness of 10 locations of each sample (Figure 2c). For nanofiber bundles, the diameter of each nanofiber bundle was measured prior to compression and then cross-sectional area was determined by outlining the area of the transverse cross-section in Image J v1.48 (NIH, Bethesda, MD) (Figure 2d). This allowed for the determination of a relationship between measured nanofiber bundle diameter and true cross-sectional area. Compression of nanofiber sheets and bundles was only conducted to assess true material cross-sectional area for calculation of material

properties. All mechanical tests and cell culture experiments were conducted on as-spun samples with pore spaces left intact.

2.3 Mechanical Testing

Uniaxial tensile testing was performed on random and aligned nanofiber sheets and nanofiber bundles. Samples ($n=5$) for testing from each group were culled from different nanofiber sheets to account for any batch-to-batch variability. Each sample was preloaded to 0.1 N before being strained at a set strain rate until mid-substance failure. Three strain rates were examined: 1%/s, 5%/s, and 25%/s to simulate approximate normal walking speeds, normal running speeds, and traumatic loading speeds respectively [34–36]. Prior to mechanical testing nanofiber sheets were punched into dog-bone shapes (Figure 3a) with a central width of 3.17 mm (ASTM standard #D638-05, Qualitest USA, Plantation, FL). Thin film grips covered in 100 grit sandpaper were utilized to clamp each specimen with a gauge length of approximately 4 cm. A servo-hydraulic mechanical test system (Bionic Model 370.02 MTS Systems Corporation, Eden Prairie, MN) equipped with an 8.9 N load cell (Futek LSB200, Irvine, CA) was used to test nanofiber sheets. Samples were speckle coated with India ink and surface images were captured during testing with a CCD camera (Flea3, Point Grey Research, Richmond, BC, Canada) at a rate of 15 frames per second. To calculate strain, images were analyzed using a Matlab-based Digital Image Correlation code to track the displacement of the speckle pattern in the central region of each dog-bone shaped sample [37].

For tensile testing of the nanofiber bundles, a custom fabricated testing fixture was used to secure each sample without creating stress concentrations (Figure 3b). The servo-hydraulic mechanical test system was equipped with a 44.5 N load cell (Futek LSB303, Irvine, CA) and prior to testing the diameter of each nanofiber bundle was measured to allow for calculation of cross-sectional area (as described previously for as-spun and compressed cross-sectional areas). Strain values for nanofiber bundles were calculated by dividing the crosshead displacement by the original gauge length. For all samples, stress was calculated by dividing force values by the either the as-spun or compressed cross-sectional area of each sample. Tensile modulus was determined by measuring the slope of the linear region of each stress-strain curve, with excellent agreement to the data ($r^2 = 0.98 \pm 0.02$). Yield stress and strain values were calculated based on the intersection of a 0.2% offset line.

2.4 Cell Culture

Adult human adipose-derived stem cells (Zen-Bio ACS-F, Research Triangle Park, NC) at passage 2 were expanded using standard cell culture techniques. The cell growth media consisted of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin. Nanofiber sheets and bundles were sterilized by soaking in 70% ethanol for one hour followed by exposure to ultra-violet light for 30 min prior to cell culture. Following expansion, cells in passage 4 were seeded on nanofiber sheets and nanofiber bundles at a density of 10,000 cells/cm² and growth media was changed every 2-3 days. After 1 and 7 days of culture cell, cellular response to nanofiber sheets and bundles was assessed by examining cell viability, cell adhesion, and cell morphology.

Cell viability was determined by a CellTiter-Blue Cell Viability Assay (Promega G808A, Madison, WI), which was used according to the manufacturer's instructions. 20 μ L of CellTiter-Blue dye was added to scaffolds (n=6) for every 100 μ L of culture media and samples were incubated for 4 h at 37°C and 5% CO₂. Blank scaffolds were also included as controls. Sample fluorescence was read in a microplate reader (Molecular Devices SpectraMax M3, Sunnyvale, CA) at 560 nm excitation and 590 nm emission.

At each time point cell adhesion was assessed by staining the cells with 5-Chloromethylfluorescein Diacetate (CMFDA, Life Technologies C2925, Carlsbad, CA), rhodamine-phalloidin (Cytoskeleton, Inc, Denver, CO), and 4',6-diamidino-2-phenylindole (DAPI, Life Technologies D1306, Carlsbad, CA). Nanofiber sheets and bundles were incubated in PBS and CMFDA stain at a concentration of 10 μ M for 45 min in a 37°C and 5% CO₂ incubator. Nanofiber sheets and bundles were then incubated in warm culture media for 30 min and fixed in 3.7% formaldehyde for 50 min at ambient temperature. All samples were rinsed with PBS between subsequent steps and protected from light exposure. Nanofiber sheets and bundles were exposed to 1% Triton-X 100 for 3 min to permeabilize cells. Samples were incubated at room temperature in a 5 μ L/mL solution of rhodamine-phalloidin for 25 min and DAPI was added for the final 5 min of the incubation period at a concentration of 300 nM. Samples were imaged using a Zeiss Axioplan 2 fluorescence microscope. Cell count and cell shape factor for each aligned and random nanofiber sheets and bundles (n=15) was determined from five images at 10x magnification. For cell counts, DAPI stained cell nuclei were counted (Particle count tool, Image J v1.48 NIH, Bethesda, MD) and cell shape factor was quantified using a ratio of cell length to cell width based on rhodamine-phalloidin stained cells [38,39]. Cell length was defined as the diameter of the smallest circle that would fit around the outer edge of a cell and cell width was defined as the diameter of the largest circle that would fit entirely within the cell.

SEM was used to visualize cell morphology and cell-nanofiber interactions. Cells and nanofibers were fixed for imaging in a solution of 3% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1 M sucrose for 45 min. Scaffolds were then soaked for 10 min in a buffer solution of 0.1 M sodium cacodylate and 0.1 M sucrose. Nanofiber surface and cells were processed in serial ethanol dehydrations for 10 min each and dehydrated in hexamethyldisilazane before being stored in a desiccator until SEM imaging.

2.5 Statistics

Data is presented as mean $\pm$ standard error of the mean. Data analysis was performed using Minitab (Minitab, Inc., State College, PA). For all quantitative measures, comparisons were performed using a two-way analysis of variance (ANOVA) models with Tukey's post-hoc test. Differences corresponding to a p-value of less than 0.05 were considered to be statistically significant.

3. Results

3.1 Fabrication and characterization of nanofiber sheets and bundles

Using the electrospinning methods described above, a non-woven nanofiber sheet was successfully created in a repeatable manner. Based on SEM images, the diameter of random and aligned nanofibers was significantly different ($p=.002$, Figure 1d). The mean fiber diameter of random nanofiber sheets and bundles was $334 \text{ nm} \pm 20 \text{ nm}$ and the mean fiber diameter of the aligned samples was $289 \text{ nm} \pm 19 \text{ nm}$. This size scale mimics the size of collagen fibers found in native tendons and ligaments. There was no significant difference in the average diameter of as-spun nanofiber bundles with random and aligned nanofibers ($490 \pm 60 \text{ }\mu\text{m}$ and $474 \pm 57 \text{ }\mu\text{m}$ respectively, Figure 1e). This size scale mimics the size of fascicles that compose native tendons and ligaments. As seen in the histograms of nanofiber angle, a collector drum rotating at a linear velocity of 0.78 m/s resulted in a wider spread of fiber angles (Figure 1f), while a collector drum rotating at 23.6 m/s induced nanofiber alignment and decreased the spread of nanofiber angles (Figure 1g). The angular deviation of random nanofiber sheets and bundles was 40.33° and the angular deviation of aligned samples was 17.31° . The as-spun thickness of nanofiber sheets was found to be $20.4 \pm 1.13 \text{ }\mu\text{m}$ and the compressed thickness of these same nanofiber sheets was found to be $5.6 \pm 0.41 \text{ }\mu\text{m}$. The

cross-sectional area of compressed nanofiber bundles was found to be linearly related to the measured diameter ($r=0.95$) (Figure 2e).

3.2 Mechanical Testing

All samples of nanofiber sheets and nanofiber bundles exhibited a linear elastic region followed by a larger region of plastic deformation (Figure 3c). Nanofiber sheets and bundles composed of aligned nanofibers displayed a slight toe region prior to the linear elastic region. Results are presented as material properties calculated using both the as-spun and compressed cross-sectional area measurements (Figure 3). Elastic modulus, yield stress, and yield strain results presented no strain rate dependence, thus data from the tests at the three strain rates were combined for statistical analyses.

The orientation of nanofibers within the scaffolds had a significant effect on elastic modulus (Figure 3d, Figure 3g). Sheets composed of aligned nanofibers had an elastic modulus 125% greater than sheets composed of random nanofibers ($p<0.0005$), and aligned nanofiber bundles had a modulus 105% greater than random nanofiber bundles ($p<0.0005$). Geometry, whether the electrospinning was a sheet or a bundle, only had a significant effect on elastic modulus for nanofiber sheets and bundles that consisted of aligned nanofibers; aligned nanofiber sheets had a significantly higher elastic modulus than aligned nanofiber bundles ($p<0.0005$).

Yield stress of all scaffolds (Figure 3e, Figure 3h) was significantly different depending on the orientation of nanofibers composing the scaffolds. Yield stress of aligned nanofiber sheets was 130% greater than yield stress of random nanofiber sheets ($p=0.006$). Similarly, aligned nanofiber bundles yielded at stresses 130% higher than the yield stress of random nanofiber bundles ($p<0.0005$). Geometry only significantly affected yield stress for scaffolds with aligned fibers: the yield stress of aligned nanofiber bundles was 95% greater than that of nanofiber sheets ($p<0.001$). Both fiber orientation and scaffold geometry influenced scaffold yield strain (Figure 3f, Figure 3g). Aligned nanofiber bundles yielded at strains 30% higher than random nanofiber bundles ($p<0.0005$). Additionally nanofiber bundles had significantly higher yield strains than flat sheets of the same alignment (aligned: $p<0.0005$, random: $p<0.0005$).

3.3 Cell Culture

The CellTiter-Blue cell viability assay revealed that cells proliferated and remained viable on nanofiber sheets and bundles for up to 7 days of culture (Figure 4a). Initially, cells appeared to adhere more readily to nanofiber bundles, as demonstrated by higher levels of viability at day one on nanofiber bundles compared to nanofiber sheets (aligned: $p=0.02$, random: $p<0.0005$). However by day 7 only random nanofiber bundles showed greater levels of cell viability compared to random nanofiber sheets ($p=0.004$) and no other significant differences between scaffolds were observed.

Fluorescence microscopy images revealed that nanofiber sheets and bundles were all able to support cell adhesion and proliferation for up to 7 days of culture (Figure 5). Results of cell counts indicate that neither nanofiber orientation nor geometry had a significant effect on the number of cells present, however for all nanofiber sheets and bundles cell number increased (random nanofiber sheet: $p=0.005$, aligned nanofiber sheet: $p=0.008$, random nanofiber bundle: $p=0.04$, aligned nanofiber bundle: $p=0.04$) over the 7 day culture period (Figure 4b).

To determine the influence of nanofiber orientation and geometry on cell morphology, cell shape factor (the ratio of cell length to cell width) was quantified via 10x magnification rhodamine-phalloidin stained images (Figure 4c). A lower shape factor indicates that the cell is more spherical and a higher shape factor indicates that a cell is more elongated. After 1 day of cell culture, cells on random nanofiber bundles were significantly more elongated than cells on random nanofiber sheets ($p=0.04$). Over the course of the 7 day culture period cells on aligned nanofiber sheets and nanofiber bundles continued to elongate while cells on random nanofiber sheets remained more spherical. At Day 7, cells on scaffolds (both flat sheets and nanofiber bundles) with aligned nanofibers were significantly more elongated than cells on corresponding scaffolds with random nanofibers (aligned: $p<0.0005$ and random: $p=0.002$). Geometry of nanofiber sheets significantly affected cell shape factor only for random samples; random nanofiber bundles had more elongated cells compared to random nanofiber sheets ($p<0.005$).

The results of cell shape factor quantification were supported by SEM images, which were used to visualize cell morphology on nanofiber sheets and bundles (Figure 6). In addition to visualization of morphology, SEM shows the interaction of cell extensions with the nanofiber surfaces. SEM results demonstrated that cells adhered and proliferated on nanofiber sheets and bundles after 7 days in culture and cells were evenly distributed on the nanofiber surfaces. Cells were noticeably elongated on aligned nanofiber sheets and random and aligned nanofiber bundles, while on random nanofiber sheets cells maintained a more distributed morphology. Based on high

magnification SEM images, cells on nanofiber sheets and bundles appeared to be interacting with nanofibers and neighboring cells via cellular extensions even after only one day of culture. Additionally, after 7 days of culture, cellular extensions appeared to be longer and the number of cell-cell interactions had increased substantially.

4. Discussion

Electrospinning is a simple polymer fabrication technique that allows for the creation of porous nanofiber sheets with controllable nanofiber size and alignment. Although flat electrospun sheets are suitable for cell growth, inherent limitations impede their use in tendon and ligament regeneration. It is challenging to produce flat sheets of electrospinning that are more than 1mm thick due to electrostatic repulsion properties that prevent excessive fiber deposition [40]. Although some research groups have attempted to laminate flat sheets of electrospinning and incorporate hydrogels to create more robust scaffolds, the mechanical properties are still relatively low [31,41–44]. In order to make electrospun scaffolds more applicable for large-scale tissue engineering purposes it is necessary to modify the geometry of flat sheets to better mimic the structure and mechanical properties of target tissues, in this case tendons and ligaments, both load bearing orthopedic connective tissues.

In the present study, we have investigated how to vary the structure of nanofiber sheets by modifying both the geometry and nanofiber orientation in order to better replicate the form and function of tendons and ligaments and improve mechanical properties. We present a novel technique for improving the mechanical properties of nanofiber scaffolds while maintaining the complex underlying fiber architecture. The electrospinning process was conducted using a rotating mandrel, which allowed for modulation of the nanofiber orientation. The parallel alignment of PCL nanofibers in aligned nanofiber sheets closely mimicked the size scale and structure of collagen fibers within the ACL [45,46]. Although the aligned nanofibers mimic the orientation of collagen fibers in the native ACL, the pores between the nanofibers are much larger than the spaces between the densely packed ACL collagen fibers. These larger pores in the electrospun scaffold are necessary for encouraging cell infiltration and production of extra cellular matrix. In addition to modifying the orientation of electrospun nanofibers, this study reports a method for modifying a flat nanofiber sheets into a three-dimensional bundle of nanofibers. The size of the nanofiber bundles (400-500 μm) mimics the size of collagen fascicles (50-500 μm) found in load bearing orthopedic tissues [4,45]. Modifying the geometry of electrospun sheets to create nanofiber bundles results in a novel

cylindrical scaffold that is remarkably robust, suggesting that it may be more feasible for clinical use. By fabricating nanofiber bundles from random or aligned nanofiber sheets we were able to modulate the mechanical properties as well as the cellular response.

In order for a tissue engineered scaffold to provide a suitable replacement for native human tendons and ligaments it is desirable for the scaffold to possess mechanical properties that are similar to native ACL. An important consideration in the calculation of material properties is how sample cross-sectional area is determined. Since electrospun sheets are highly porous, simply utilizing the width and the “as-spun” thickness of the nanofiber sheet to calculate the cross-sectional area overestimates the amount of material that is actually present and consequently underestimates the mechanical properties. To overcome this problem, in addition to calculating the as-spun cross-sectional area of nanofiber sheets and bundles, we compressed nanofiber sheets and bundles to eliminate pore space, and used additional SEM images to determine true material area. In this way it is possible to determine both the apparent properties of the bulk scaffold, as well as the inherent properties of the electrospun material. A limitation to this method was the difficulty in verifying if all pore space had been completely eliminated using only visual inspection of SEM images; however this work is still the first to calculate the mechanical properties of electrospun scaffolds by taking into account the true material cross sectional area.

Firstly, altering nanofiber orientation so nanofiber sheets consisted of primarily aligned nanofibers increased the elastic modulus by ~125% and the yield stress by ~150%. These differences in mechanical properties are possibly due to more fibers being present in parallel over which to distribute the applied loads, thus requiring more force to cause elastic deformation. Previous studies of the mechanical behavior of aligned nanofiber sheets of electrospun PCL have reported the elastic modulus to range from 20-40 MPa and the yield stress to range from 2-4 MPa [23,24,47,48]. Despite some expected variation due to spinning technique and duration, these previously reported values are relatively similar to values calculated in this study utilizing the as-spun cross-sectional area, but are up to an order of magnitude lower than the values calculated utilizing the compressed cross-sectional area. Similar to our findings, previous studies assessing the moduli of nanofiber sheets have shown aligned nanofiber sheets to have moduli 75-140% higher than the random nanofiber sheets ([23,47,48]).

In addition to altering nanofiber orientation, altering the geometry of nanofiber sheets significantly affected the mechanical properties. When random nanofiber sheets were used to create randomly oriented nanofiber bundles there was no significant difference in elastic modulus, however the elastic modulus of aligned nanofiber bundles was

observed to be ~55% lower than the elastic modulus of aligned nanofiber sheets. The decrease in elastic modulus of aligned nanofiber bundles may be due to the extent of nanofibers that were engaged during the tensile test and bearing the applied load. However it is difficult to speculate exactly why scaffolds with similar nanofiber alignment but different geometries possess different material properties. We hypothesize that these differing material properties may be due to interactions in the nanofiber bundles between the fiber layers and varying recruitment of the nanofibers to bear the applied loads as compared to the single layered sheets. Based on the low standard errors of the measured material properties, it is likely that these differences are not due to sample preparation and testing techniques. Further experiments will be necessary to elucidate the exact reasons for the variation in material properties with altered scaffold geometry.

One limitation of the mechanical testing techniques used in this study, which could limit the interpretation of the results was the different techniques used to measure the strain of flat sheets and nanofiber bundles. Speckle tracking of India ink dots was used to measure strain in the central region of the dog-bone shaped flat sheets, however due to the small size of the nanofiber bundles (~0.5 mm) and limited resolution of the camera used to track the samples the speckle coating technique could not be used. Instead strain for nanofiber bundles was measured using grip-to-grip displacement from the servo-hydraulic mechanical test system.

Previous studies have assessed the effects of modifying the geometry of electrospun scaffolds. Computational work conducted by Nerurkar et al. found that any modulus reinforcement effects from stacking layers of electrospun fibers only occurred when layers were oriented in opposing directions and fibers were forced to undergo rotation under tensile loading to align along the axis of the load, generating additional shear forces [49]. Similarly, Fisher et al., reported that when assessing the mechanical properties of stacks of sheets of electrospinning, only layers with “non-orthogonal” orientations showed evidence for a mechanical reinforcement effect compared to individual sheets [41]. Previous groups investigating the effects of twisting or braiding electrospun nanofibers have observed trends of increasing elastic modulus with more complex geometry, however this could be attributed to variation in the calculation of scaffold cross-sectional area [29,30,50]. Nerurkar et al. showed that layers of electrospun fibers stiffened after extended cell culture, likely due to cell matrix deposition between layers, which suggests that aligned nanofiber bundles may develop a higher elastic modulus over time in culture [51].

Modifying geometry also resulted in significant differences in yield stress and strain of the resultant scaffolds, particularly when nanofibers were aligned. Aligned nanofiber bundles had an average yield stress 90%

higher than aligned nanofiber sheets and an average yield strain 130% higher than aligned nanofiber sheets. This is promising for the proposed ligament tissue engineering applications because although ligaments typically only undergo less than 10% strain during normal loading, it would be valuable for scaffolds to be able sustain higher stresses and strains prior to plastic deformation [36]. Overall, modifying both geometry and nanofiber orientation to create a nanofiber bundle composed of aligned nanofibers resulted in a scaffold with modulus and yield stress and strain values within the range of the native human ACL (Table 1). Previous research has also assessed the mechanical properties of the sub-structures of the native human ACL, including the anteromedial, anterolateral, and posterior bundles as well as individual fascicles [52,53]. The anteromedial and anterolateral bundle and individual fascicles have moduli that are higher than that of the entire ACL and higher than that of aligned nanofiber bundles. This presents a limitation of the described scaffold since it does not precisely match the material properties of the structures that compose the native human ACL. However the modulus of aligned nanofiber bundles does closely match the modulus of the posterolateral bundle. Additionally the yield stress and yield strain of aligned nanofiber bundles are within in the range of the tensile strength and yield strain of ACL bundles as well as ACL fascicles. In the context of ACL replacement scaffolds, yield stress and strain are comparable to tensile strength and failure strain since a scaffold that has plastically deformed has essentially “failed” as it is no longer able to properly fulfill the role of the ACL. Notably, the results of the present study suggest that aligned nanofiber bundles may be suitable for use as a tissue-engineered scaffold, since the modulus and the yield properties of the scaffold meet the mechanical demands of the native ACL, and potentially other tendons and ligaments as well. Additional modification, such as combining multiple nanofiber bundles into a single construct may further alter scaffold mechanical properties. Preliminary work has suggested that securing together the ends of ~100 nanofiber bundles using solvent cast solid PCL produces a structure with significantly higher stiffness and failure load.

All nanofiber sheets and bundles were able to support cell adhesion and proliferation, which was expected since electrospun PCL nanofibers have previously been shown to support a variety of cell types [27,54,55]. However, quantitative measures indicated that there were some differences in both cell viability and cell morphology between nanofiber sheets and bundles. Higher fluorescence from CellTiter-Blue viability assays on nanofiber bundles may be due to the larger surface area of nanofiber bundles compared to nanofiber sheets. The results of cell counts based on DAPI stained nuclei indicated no differences in cell number on any nanofiber sheets and bundles at the Day 1 or Day 7 time points. Any discrepancies between CellTiter-Blue viability assay results and

cell count results may be because cells proliferated around the curved edges of the nanofiber bundles or migrated into the center of the nanofibers where they could not be imaged. The quantification of cell shape showed interesting effects of both geometry and nanofiber orientation on cell morphology. Similar to reports from other groups, after 7 days in culture cells on aligned nanofiber sheets were significantly more elongated along the primary nanofiber direction compared to cells on random sheets [23,27,56]. However, on nanofiber bundles cells were more elongated compared to nanofiber sheets regardless of fiber alignment. It is hypothesized that the cells may have detected the convex curvature of the cylindrical nanofiber bundles, regardless of fiber alignment, and consequently aligned along the length of the nanofiber bundle. This novel finding suggests that the micro-scale geometry of nanofiber bundles has a separate and significant impact on cell morphology. It has also been previously shown that substrate stiffness has important effects on cell development and differentiation [57,58]. Specifically the modulus of the substrate influences cytoskeleton assembly and; on less stiff surfaces cells have reduced spreading and increased rates of motility [59]. Additionally mesenchymal stem cells differentiation response has been shown to be acutely sensitive to the elasticity of the underlying substrate [60]. Because the scaffolds in this study demonstrate significantly different levels of stiffness and this may play a role in altering cell morphology. The ability to modulate cell morphology is important for tissue engineering applications because cell shape affects numerous cell processes, including cell migration and communication as well as cellular differentiation [61]. A limitation of the current nanofiber sheets and bundles is the low porosity (pore sizes of approximately 5 μm), which could potentially limit cell infiltration into the interior of the scaffolds. Cell infiltration could be encouraged by modifying the electrospinning technique to include sacrificial fibers/porogens or increasing the nanofiber diameter in order to increase overall porosity [62–64].

5. Conclusions

This work presents a novel cylindrical electrospun scaffold with robust mechanical properties and a complex underlying nanofiber structure. Nanofiber orientation and geometry of electrospun PCL were modified to mimic the fascicles of native tendon and ligament. Aligned nanofiber bundles had the highest yield stresses and strains, while still maintaining a relatively high elastic modulus. Remarkably, these mechanical properties are within the range of the native human ACL. For the first time in this work the mechanical properties of electrospun scaffolds were

calculated using a true material cross-sectional area, with pore space between nanofibers eliminated to prevent an underestimation of properties. Additionally all nanofiber sheets and bundles were able to support cell adhesion and proliferation for up to 7 days in culture; however nanofiber bundles are preferable for encouraging the parallel elongation of adipose derived stem cells. The mechanical properties and cell response of aligned electrospun nanofiber bundles suggest they are a promising scaffold for orthopedic tissue engineering applications. Future studies will investigate the utilization of growth factors to promote cell differentiation into a ligamentous phenotype and the effect of combining multiple nanofiber bundles into a more complex scaffold to further alter mechanical properties.

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7. Figures Captions

Fig 1 Nanofiber sheets with random (a) and aligned (b) nanofibers were used to fabricate nanofiber bundles (c). The diameters of nanofibers which compose random and aligned nanofiber sheets are significantly different (d, * indicates $p < 0.05$) however the diameter of random and aligned nanofiber bundles (NFB) are not significantly different (e). Randomly oriented nanofiber sheets have a wider spread of nanofiber angles (f) compared to aligned nanofiber sheets (g).

Fig 2 Transverse section of as-spun nanofiber sheet (a) nanofiber bundle (b). Transverse section of compressed nanofiber sheet (c) and nanofiber bundle (d). Linear relationship between measured nanofiber bundle diameter and compressed cross-sectional area (e).

Fig 3 Nanofiber sheets (a) and nanofiber bundles (b) during mechanical testing. Representative load displacement curves (c). Results of mechanical testing: scaffold elastic modulus (d, g), yield stress (c, h), and yield strain (d, i) for random nanofiber sheets (rNFS), aligned nanofiber sheets (aNFS), random nanofiber bundles (rNFB) and aligned nanofiber bundles (aNFB). Results were calculated using either as-spun (d, e, f) or compressed cross-sectional area (g, h, i). Due to a lack of strain rate dependence data from the tests at the three strain rates were combined for statistical analyses. * Indicates significant difference between random and aligned scaffolds with same geometry ($p < 0.05$). # Indicates significant difference between nanofiber sheets and nanofiber bundles with same nanofiber orientation ($p < 0.05$).

Fig 4 Quantification of cell viability, proliferation and morphology on random nanofiber sheets (rNFS), aligned nanofiber sheets (aNFS), random nanofiber bundles (rNFB) and aligned nanofiber bundles (aNFB) after 1 and 7 days of culture. CellTiter-Blue fluorescence (a), cell counts (b), and cell shape factor (c). * Indicates significant difference between random and aligned scaffolds with same geometry ($p < 0.05$). # Indicates significant difference between nanofiber sheets and nanofiber bundles with same nanofiber orientation ($p < 0.05$). & Indicates significant difference between time points for specific geometry and nanofiber orientation.

Fig 5 Representative fluorescent images of cells random nanofiber sheets (rNFS), aligned nanofiber sheets (aNFS), random nanofiber bundles (rNFB) and aligned nanofiber bundles (aNFB) after 1 and 7 days of culture.

Fig 6 Representative SEM images of cells on random nanofiber sheets (rNFS), aligned nanofiber sheets (aNFS), random nanofiber bundles (rNFB) and aligned nanofiber bundles (aNFB) after 1 and 7 days of culture.

Table 1: Material properties of aligned nanofiber bundles compared to properties of human ACL from previous studies.

		Modulus (MPa)	Yield Stress (MPa)	Yield Strain
Aligned nanofiber bundle		154 ± 28	54 ± 12	0.38 ± 0.03
		Modulus (MPa)	Tensile Strength (MPa)	Failure Strain
Noyes & Grood [65]	ACL, Old	65 ± 24	13 ± 5	0.49 ± 0.12
	ACL, Young	111 ± 26	38 ± 9	0.60 ± 0.07
Chandrashekar [66]	ACL, Male	128 ± 35	26 ± 10	0.30 ± 0.06
	ACL, Female	99 ± 50	23 ± 9	0.27 ± 0.08
Butler [52]	ACL fascicle	345 ± 22	36 ± 3	0.15 ± 0.008
Butler [53]	ACL anteromedial bundle	283 ± 114	46 ± 20	0.19 ± 0.03
	ACL anterolateral bundle	285.9 ± 141	31 ± 11	0.16 ± 0.04
	ACL posterolateral bundle	154 ± 119.5	15 ± 10	0.15 ± 0.05

8. References

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Figure 1

Figure 2