NOVEL DEVICE TO QUANTIFY THE MECHANICAL PROPERTIES OF ELECTROSPUN NANOFIBERS

by

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A THESIS

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements of the degree of Master of Science in Biomedical Engineering

BIRMINGHAM, ALABAMA

2012
Electrospun biomaterials are gaining popularity as scaffolding for engineered tissues. These fibrous scaffolds of natural or synthetic polymers can mimic the nano-scale properties of the natural extra-cellular matrix. It is becoming clear that the mechanical deformation of any electrospun matrix plays an important role in cell signaling. However, electrospun biomaterials have inherently complex geometries due to the random deposition of fibers during the electrospinning process. This complex fiber geometry complicates any attempt at quantifying forces exerted on adherent cells during electrospun matrix deformation.

In order to quantify the mechanical properties of arrays of individual electrospun fibers in physiological conditions, a novel mechanical test platform has been designed and constructed. To facilitate wet testing, optical strain recording, and cellular substrate testing, the novel device is capable of testing in a cell culture environment and can keep the electrospun fibers within the focal plane of an inverted microscope. To limit the complications arising from the inherent random orientation of electrospun fibers, a method of manually depositing parallel electrospun poly(ε-caprolactone) (PCL) fibers was developed in this research. The designed micro-tensile testing platform was used to quantify the mechanical and viscoelastic properties of these parallel electrospun PCL fibers.
It has been shown that the novel device can perform direct observations of strain along an electrospun fiber using a non-contact optical strain recording method. The development of a device capable of recording true strain from arrays of individual electrospun fibers is significant in that an understanding of the materials used in designing tissue engineered implants can lead to improved engineered tissue substitutes.

Keywords: Electrospinning, Micro-Tensile Testing, Viscoelasticity, Biomechanics, Optical Strain Recording, Nanofibers.
DEDICATION

Soli Deo gloria
ACKNOWLEDGEMENTS

The author would like to thank:

The lab of Dr. Derrick Dean for assistance with electrospinning

Dr. Robin Foley for assistance with SEM imaging
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INTRODUCTION AND LITERATURE REVIEW

Tissue Engineering

One of the most exciting scientific advancements of the previous years is the progress in the field of tissue engineering. Tissue engineered substitutes for heart valves, skin, bone, cartilage, and blood vessels have been reported [1-5]. The primary goal of tissue engineering is to develop living engineered replacements for damaged and diseased tissues. The promise of tissue engineering is that it will return lost functionality without harmful drug side effects (as in traditional medicine), without fear of immune rejection (as in current organ transplantation), and without the prospect of a long-term foreign object in the body (as with the majority of medical implants). Tissue engineering seeks to achieve these long-term goals by formulating an engineered tissue that replaces damaged tissues with minimal drug intervention, developing biomimicking constructs that are produced from either the patient’s own cells or is acellular, and constructing a system from biodegradable materials that are naturally broken down as the body regenerates itself via the production of new tissue [6].

The main focus of tissue engineering over the years has been the development of a scaffold to fill the volume of the tissue and provide a framework for cells to infiltrate and be supported as they produce new tissue. The current generation of these tissue engineering scaffolds are made of biodegradable polymers and are frequently modified to promote specific cellular recruitment, adhesion, or function. Both natural and synthetic
polymers have been used to produce scaffolds; hybrids of natural and synthetic materials have been used as well [7]. While many of the goals of a perfect tissue engineering system remain to be fulfilled, researchers around the globe are taking incremental steps towards clinically acceptable tissue replacements. Despite intense research efforts, very few tissue engineered products are currently approved for clinical use.

Electrospinning and Nanotechnology

The production of a biomaterial similar to a specific naturally occurring extracellular matrix (ECM) is vital to the success of a tissue engineered construct. The natural ECM excreted by cells contains a highly organized multi-level structure. For example, a large organ, such as bone, is ordered on the macro-level by the organization of cancellous bone to support appropriate loads. On the micro-level, osteons are oriented with their long axis parallel to the primary loading direction of the bone. Finally, on the nano-level, it can be observed that individual osteons are composed of aligned collagen fibrils, and each collagen fibril is a collection of aligned collagen molecules with crystallized calcium phosphate and hydroxyapatite crystals. The example of bone ECM is one of a multitude of biochemical and structural arrangements of ECM that are highly specialized to the tissue function. Replicating the natural macro-to-nano multi-leveled structure of ECM in a tissue engineering scaffold could have profound implications on the success or failure of the implant.

Two basic approaches of producing nanostructured materials exist: top-down and bottom-up. The top-down approach takes a large-scale, or bulk, material and removes material until its dimensions reach the appropriate size scale. This process can be thought
of as similar to a sculptor taking a large block of stone and carefully chiseling it down to a much smaller statue. Bottom up production uses molecular-level building blocks for the construction of larger materials. The construction of nanofibers, nanofilms, and nanospheres has been reported using a bottom-up self-assembly technique [8]. Self-assembly is performed by taking a solution of molecular building blocks and changing the solution in some way (pH, ionic concentration, charge) to induce the molecules to form ordered structures. In addition to self-assembly, another bottom-up approach that is being extensively studied for tissue engineering scaffold applications is electrospinning.

Electrospinning was initially described in the early 1900s as a method of drawing fibers for the production of yarn [9]. However, the popularity of electrospinning increased dramatically after its re-discovery in the 1970s as a means of producing nanoscale fibers. The principle of electrospinning is simple: a polymer is dissolved in a volatile solvent which is extruded through a needle into a high-voltage static electric field. The combination of viscous and electrostatic forces causes the formation of a conical droplet at the end of the needle, known as a Taylor cone. From this Taylor cone, a thin stream of polymer solution is attracted towards a grounded collector some distance away. As the polymer stream moves to the collector, bending instability in the stream initiates a whipping motion of the polymer jet. Simultaneously, the volatile solvent evaporates forming solid polymeric fibers [10]. This combination of events leads to the formation of a randomly aligned non-woven mat of polymeric fibers on the collector (Figure 1).
The appeal of electrospinning as a means of producing nanofibers is due to the simplistic set-up of the electrospinning apparatus and the high degree of customizability [10]. The electrospinning set-up is comprised of only a syringe pump, a high-voltage power supply, and the appropriate polymers and solvents. Each of the parameters in the electrospinning process has been examined to understand how each factor influences the overall outcome. The parameters affecting the polymer solution include, polymer concentration, type of polymer, type of solvent, and viscosity of solution. Additional parameters used for customization include: polymer flow rate, diameter of needle, applied electric potential, shape of electric field, distance from needle to target, geometry of the target, and motion of the target [10, 11]. Additionally, electrospun fibers have been shown to have desirable mechanical properties, degradation properties, and have been functionalized to allow for binding of specific biomolecules [12, 13]. These properties are largely dependent upon the polymer selected for a specific tissue application.

Figure 1. Diagram of Electrospinning Apparatus. The polymer solution, syringe pump, high voltage power supply, and grounded collector are shown. Additionally, the diagram shows the polymer jet and the whip-like motion as it approaches the collector.
Traditionally, electrospinning is performed using a stationary conductive plate as the target. With this set-up, the electrospinning process deposits fibers in a non-aligned mat (Figure 2A). Altering the collector geometry, collector motion, and electric field has successfully been used to induce alignment in the resulting fiber mat [10, 14, 15]. However, there have been no reports of completely parallel and evenly spaced electrospun fibers. Producing even semi-aligned fibers is desirable for certain applications the engineering of artificial tendons and other anisotropic tissues (Figure 2B)[15, 16]. The ability to place cells in a mechanical environment that closely mimics physiological properties is a highly desired feature of any tissue engineered implant. Electrospun scaffolds have been shown to mirror both the fibrous and anisotropic nature of naturally occurring extracellular matrix [11, 16]. There is currently a significant amount of effort going into the mechanical characterization of electrospun materials.

Figure 2. SEM Image of Electrospun Nanofibers. (A) Randomly deposited electrospun nanofibers. (B) Aligned fibers collected on a rotating mandrel.
Mechanics

The mechanical properties of tissue engineering scaffolds are very important to adherent cell viability and function. Chemical manipulation alone is not enough to induce the desired function in cells growing on a tissue engineered scaffold. The mechanical properties of a material are typically quantified using the terms engineering stress and engineering strain. Engineering stress is defined as the applied force normalized by the initial cross-sectional area of the material. Engineering strain is defined as the change in length of the material normalized for its initial length. However, engineering strain is calculated based on the assumption that the overall dimensions of the sample does not significantly change. While this assumption is valid for most engineering materials (steel, concrete, etc.), materials that experience deformations greater than 1% no longer satisfy the assumptions of engineering strain. To account for reduction in volume that occurs over large deformations, the quantity “true strain” is used. True strain is defined as the sum of all instantaneous engineering strains. Therefore:

\[
d\varepsilon_{\text{eng}} = \frac{d\ell}{\ell}
\]

\[
\varepsilon_{\text{true}} = \int d\varepsilon = \int_{\ell_i}^{\ell_f} \frac{d\ell}{\ell} = \ln \frac{\ell_f}{\ell_i} = \ln(1 + \varepsilon_{\text{eng}})
\]

Materials that experience temporal dependent properties are known as “viscoelastic”. Most cells and ECM are viscoelastic. An example of viscoelasticity is when an object is suddenly hung from a material, the material quickly deforms to a certain length under the weight of the object, but as time passes, the material continues to slowly deform. This phenomenon is called “creep”. A creep test is performed by imparting a constant load to a sample and measuring the deformation over time. A stress-
relaxation test is conceptually similar to the creep test, however a sudden displacement is applied to the sample and the load is measured over time. Viscoelastic tests are illustrated in Figure 3.

Figure 3. An Illustration of Viscoelastic Mechanical Tests. The two different types of viscoelastic tests are shown. A stress relaxation test is shown on the top and a creep test is shown below.

Mechanical Properties of Electrospun Fibers

The efforts to characterize the mechanical properties of electrospun materials fall into two categories: testing the macroscopic properties of constructs on the millimeter scale and testing the microscopic properties of individual fibers on the micron scale.

Macro-Scale Testing

There are multiple reports of simple mechanical testing of various electrospun materials. Usually, the results are reported in terms of engineering stress and engineering
strain, despite the fact that electrospun materials typically experience large deformations [17, 18]. Some groups have performed more rigorous characterization of electrospun materials. Nerurkar et al. developed a constitutive model for aligned PCL scaffolds from uniaxial tests that describes the material as a composite of electrospun fibers and a fluid matrix [19]. Baker et al. developed a similar composite model for combinations of multiple fiber types in a fluid matrix from uniaxial testing data [7]. Others have performed biaxial testing of electrospun scaffolds and have developed elaborate macroscopic constitutive models that factor in the overall fiber alignment, direction of loading, density of fiber interactions, and fiber tortuosity [14, 20]. Duling et al. succeeded in characterizing the viscoelastic properties of macroscopic electrospun sheets using the quasi-linear viscoelasticity model developed by Fung [21, 22]. Barocas et al. developed a constitutive model for electrospun scaffolds by combining a representative volume element with the Cauchy stress tensor using uniaxial testing data [23]. The primary limitation of the constitutive models developed by these groups is the difficulty in linking the model to the deformations an adherent cell would experience on an electrospun substrate. While these models provide an excellent representation of the average properties of the material as a whole, the specific strains on a cell cannot be determined from the models alone due to the inherently complex scaffold geometry.

Testing of Individual Electrospun Fibers

Due to the multiple variables that influence the macroscopic mechanical properties of electrospun materials, testing of isolated fibers is the ideal method of determining the intrinsic mechanical properties of the material. Working with individual
micro and nanofibers presents a significant handling challenge. Consequently there are few examples of direct testing of individual electrospun fibers. Individual fiber testing was first reported by Tan et al. in 2004 using an AFM probe to apply micron level displacements [24]. Other methods of single fiber testing include: using AFM probe to bend fibers deposited over a microchannel, individual fibers in a tensile testing apparatus, and using microelectromechanical systems combined with AFM [25-29]. The majority of the reports of individual fiber testing characterize the fiber in terms of engineering stress and engineering strain [26, 28-30], however, Baker et al. has recently examined both the tensile and viscoelastic properties of dry electrospun fibrinogen fibers and recorded true stress and true strain [31]. The main limitation of single fiber mechanical tests is the inability to test fibers with adherent cells. Thus any cell-substrate interaction cannot be tested using this method.

Macroscopic testing of electrospun materials has the advantage of better matching the organ-level deformations that are seen in vivo. Nevertheless, microscopic testing seeks to understand the forces cells experience while seeded on the material. Understanding the cellular-level forces present in electrospun constructs is an important, and often overlooked, aspect of the biocompatibility of the material.

Theories of Mechanotransduction

The mechanical relationship between cells and substrate is emerging as a crucial indicator of the biocompatibility of a tissue engineered construct. This is because cells are sensitive to the mechanical forces they experience. The process by which cells receive stimuli from mechanical cues is known as mechanotransduction. However, the
mechanotransduction signaling pathway remains elusive. Currently, two main hypotheses exist to explain the transduction of mechanical signals into the cell. The first hypothesis is called “tensegrity”, a shortening of tensional integrity. Tensegrity holds that cells use their focal adhesions as anchors and microfilaments like ropes to support tensile forces and to control cell structure and cellular morphology. Tensegrity suggests that microtubules are used to support compressive loads (along their main axis) to aid in the overall cell structure[32]. This process of conducting forces along microfilaments and microtubules causes the entire cell to behave as a force transducer, that is, force on one side of the cell is transferred to the nucleus and every other part of the cell. The current alternative to the tensegrity hypothesis is the hypothesis of mechanosomes. The mechanosomes hypothesis says that only specialized regions (such as focal adhesions) transform the mechanical forces into a chemical signal [33]. It is clear that the nucleus is attached to some focal adhesions, but they claim that the mechanical signal must be transformed to a chemical one on the outside of the nucleus before alterations of gene expression occur.

Tensegrity

The term tensegrity was coined by the architect Buckminster Fuller (designer of geodesic structures including the Epcot center) in the early 1960’s, and is used to describe a structure composed of non-contacting compression elements supported by tension bearing elements [32]. It is of specific interest to note that a certain tensegrity structures form a sphere in their low energy state, but when bound to a flat surface, they produce a flattened morphology, not unlike that of a cell bound to a substrate. This
suggested that cell structure may be due to a tensegrity arrangement of the cytoskeleton [32]. A key point predicted by the theory is that perturbation of focal adhesions will cause a change in distant portions of the cell, specifically the nucleus. It has been confirmed that the nucleus is displaced by the motion of focal adhesions [32, 34]. The clearest example is an experiment where the distal end of a neuron was stretched away from the cell body; the nucleus was pulled out of the cell body and into the axon [35]. Other research has been done to suggest that the actin cytoskeletal filaments are used to support the tensile forces in the cell and the microtubules are used as the compressive elements. This was indicated by measuring the force a neural axon exerts on a force gauge. When the axon was exposed to microtubule dissolving drugs, the axon exerted a tension force; when microfilaments dissolving drugs were used, the tension force relaxed [35]. Despite the evidence for cytoskeletal elements directly connecting focal adhesions and the nucleus, there is not currently data to indicate whether the nucleus can decode the mechanical information transduced on these fibers.

**Mechanosomes**

An alternative hypothesis to tensegrity is the idea of mechanosomes. This hypothesis states that specific trans-membrane protein complexes detect mechanical signals and converts them to chemical second messengers within the cytosol; not unlike the principles behind many other signal transduction pathways [33]. One proposed mechanism for this type of communication begins with a mechanical signal being detected through integrin proteins, which then activates the cytoplasmic protein p130Cas. The activated p130Cas complexes with an adapter protein and induces the transportation
of Nuclear Matrix Protein 4 inside the nucleus where it is known to bind to gene promoter regions[33]. Though this pathway appears to be a prime candidate for causing nuclear response to mechanical signals, it remains unclear if it is the sole mechanotransduction pathway or if other mechanisms of mechanical signal transduction exist. One bit of evidence suggesting that other factors are involved is the fact that mechanical cues influence the cells morphology and differentiation, not just cell migration and adhesion. The Nuclear Matrix Protein 4 implicated in the above pathway is known only to bind with specific ECM associated proteins including various ECM degradation enzymes and collagen synthesis related proteins [33]. The control of these ECM associated proteins does not explain the full spectrum of effects that mechanical forces can have on cells.

Mechanical Sensitivity of Mesenchymal Stem Cells
While many types of cells exhibit mechanotransduction phenomena, Mesenchymal stem cells (MSCs) are of particular interest as they are frequently used in conjunction with a biomaterial to design an engineered tissue replacement. MSC’s are multi-potent adult stem cells with the capacity to differentiate into bone, fat, chondrocytes, and possibly myocytes [36]. It is known that these MSCs are sensitive to the mechanical properties of their substrate. Fu et al. showed that by altering only the substrate elasticity the differentiation of MSCs can be altered[37]. Cells also actively change their orientation in response to cyclic deformation and the surface characteristics of their substrate[38]. It has also been shown that MSCs are sensitive to the directionality of forces that are applied to them. Park et al. subjected MSCs to uniaxial tension and
observed a response different than MSCs subjected to equiaxial tension [39]. Additionally, it has been reported that the nucleus of MSCs undergoes shape changes when forces are applied to the aligned fibrous substrate [18, 40]. Mechanical deformations produce more than morphological changes in cells; several studies have indicated that gene expression and ECM production changes when cells are exposed to load [37-39].

Summary

The forces cells experience in electrospun tissue engineered scaffolds are not well understood. To quantify the properties of the electrospun fibers that many tissue engineered scaffolds are composed of as well as the forces cells experience on such scaffolds, a novel test platform capable of performing micro-tensile testing on arrays of individual electrospun nanofibers is needed. This platform would be the first of its kind that can: maintain a cell culture environment, record the dynamic mechanical properties of electrospun nanofibers, permit real-time optical strain recording, and impart highly controllable forces on cell substrates. This platform technology could be used in studies to help elucidate the mechanical influence of substrate deformation on cellular behavior in addition to many other experiments in mechanobiology.
MATERIALS AND METHODS

Electrospinning

Nano-scale fibers of poly(ε-caprolactone) (PCL) were produced via electrospinning. PCL was purchased from Durect Polymers (inherent viscosity =1.08 dL/g, Pelham, AL) and dissolved in a 50:50 mixture of Dichloromethane and Dimethylformamide (Fisher Scientific, Pittsburg, PA). After dissolving the polymer overnight on a magnetic stir plate, the solution was loaded into a syringe and extruded through a blunt tipped 25G stainless steel needle at a rate of 1.0 mL/hr. The needle was charged to +17kV relative to a grounded collector (rectangular aluminum plate) located 20 cm from the end of the needle.

The above description produces a non-woven mat of randomly oriented electrospun fibers (hereafter referred to as “Bulk PCL”). In order to produce highly aligned arrays of fibers, a forked metallic wand was rapidly waved just in front of the grounded target and individual fibers would collect between the parallel metal bars. From this non-aligned collection of individual fibers, all but one fiber was manually removed. This remaining fiber was manually deposited onto a separate set of parallel bars under a stereoscopic microscope in a controlled orientation. Using this procedure, arrays of individual electrospun PCL fibers were produced.
Device Design

Tensile testing of an array of individual electrospun fibers in cell culture conditions while in the focal plane of a microscope is impossible with nearly all of the existing commercial options for mechanical testing devices. Therefore, a novel micro-tensile testing platform was developed using the following design criteria: ability to measure low-forces, ability to produce micron-level displacements in the sample, ability to keep sample in the focal plane of an inverted fluorescent microscope, ability to perform testing at 37°C, and the ability to test wet samples in sterile tissue culture conditions.

The parts for the device were designed using the CAD software ProEngineer (Parametric Technology Corp., Needham, MA). This software allowed the iterative design process to be performed in a 3D virtual environment. The parts that would not come into contact with the cells or cell media were made from aluminum. This was due to its low cost and ease of machining. Parts that would come into contact with the cells or cell media were made from Delrin (DuPont, Wilmington, DE). This material can be machined, has good chemical resistance to ethanol, acetone, and heat; it is also light weight and has low elasticity. The UAB Undergraduate Machine Shop was contracted to manufacture the parts. Detailed engineering drawings of the parts and the design can be seen in Appendix A. A precision screw-type linear actuator was purchased from Newport (TRA12-CC. Irvine, CA) and a 0.25 LbsF (1.1N) load cell was purchased from Futek (LRF400, Irvine, CA) to complete the design. Both the actuator and the load cell interfaced with a custom LabView program (National Instruments, Austin, TX).
Mechanical Testing

Tensile testing was performed on the novel micro-tensile platform to characterize the mechanical properties of electrospun PCL. Because PCL fibers can extend well beyond 10% of their original length, the assumptions made for engineering strain are not valid. Therefore, the quantity “true strain” was used to describe the tensile behavior of the fibers. Due to the extremely small size of individual electrospun PCL fibers, the stress could not be accurately measured. As an alternative, the data was organized into load versus true strain measurements.

To quantify the viscoelastic properties of the electrospun samples, a stress relaxation test was performed. This test imparts a step strain to the sample and records how load changes over time. The stress relaxation response of the fibers was curve fitted using a least squares algorithm. The velocity of the actuator for the tensile tests was 0.1 mm/sec and the velocity for the stress relaxation test was 0.4 mm/sec.

Optical Tracking

To provide optical measures of the true strain present within electrospun fibers, an optical tracking system was implemented. Fluorescent micro-spheres (FluoroSphere Amine-modified 0.2 μm yellow-green, Invitrogen, Grand Island, NY) were sonicated for 30 minutes prior to use, then diluted in ultra-pure water in a 1:100 (v/v) ratio. A 4mL fine-mist spray bottle was purchased (PC 200 Professional Center Inc, Livingston, TX) and used to apply the fluorescent solution to the fibers. It was determined that a total volume of approximately 0.1 mL was dispersed with each pump, and 3 to 4 pumps were usually applied from a distance of about 15 cm. All applications of fluorescent spheres
were performed in a chemical fume hood to avoid any health concerns which could arise from the production of an aerosol.

The fluorescent spheres are composed of polystyrene, and it was assumed that the mechanism of adherence to electrospun PCL fibers was surface adsorption via hydrophobic interaction of the PCL and polystyrene.

Optical tracking of the fluorescent micro-spheres was done under an inverted fluorescent microscope connected to a dedicated computer. Video acquired during the test was processed using a custom MATLAB (Mathworks, Natick, MA) program which automatically tracked the motion of the fluorescent dots across the video frames. All custom MATLAB files are provided in Appendix B. The location of each fluorescent dot in time was used to determine the true strain along an individual electrospun PCL fiber.

Microscopy

*Scanning Electron Microscopy*

Scanning electron microscopy (SEM) was used to characterize the average fiber diameter as well to provide confirmation of the adsorption of the fluorescent microspheres. SEM imaging was performed on a Quanta scanning electron microscope (FEI, Hillsboro, OR) under high-vacuum conditions with an accelerating voltage between 5 and 20 keV. Prior to imaging, the samples were sputter coated with a thin (<10 nm) layer of gold. SEM images were saved as 8-bit tagged image format (tiff) files.
Optical Microscopy

Bright field and Fluorescent microscopy was performed on a Nikon TE-2000S inverted microscope (Nikon, Melville, NY). Fluorescent illumination was achieved using a C-LHGF I mercury lamp (Nikon) combined with the following filter sets: C-FL UV-2E/C DAPI, C-FL B-2E/C FITC, and C-FL G-2E/C TRITC. The microscope is equipped with four Nikon CFI Plan Fluor ELWD objective lenses: 4X, 10X, 20X, and 40X. This microscope has a dedicated computer equipped with Nikon NIS Advanced Research Software. All optical images were stored as 16-bit .tiff files or .nd2 files and then converted to .tiff using a MATLAB program developed by LOCI (Madison, WI)[41].

Polymer Characterization

To ensure consistency with literature reports and among different samples, several electrospun PCL samples were selected for characterization using differential scanning calorimetry (DSC). This testing was done on a TA Instruments Q series 100 DSC (New Castle, DE). DSC is one of the simplest ways to determine the crystallinity of a polymer. Crystallinity is calculated by subtracting the area of the crystallization peak (called the heat of crystallization) from the area under the melting peak (called the heat of fusion) and dividing by a reference value. The reference value used for this research was 135.44 J/g [42].
RESULTS AND DISCUSSION

Device Design and Construction

A novel micro-tensile testing apparatus has been developed as the first step toward performing mechanical testing on cell-seeded electrospun fibers. This device can detect low forces and produce micron-level displacements in fibrous samples. This novel micro-tensile testing apparatus is designed to facilitate mechanical testing of arrays of individual electrospun fibers using a real-time non-contact optical strain recording method in physiological conditions (wet samples at 37°C). Schematics of the design are given in Figure 4. An image of the completed device is shown in Figure 5. To the author's knowledge, this is the only device in existence which can: measure loads in the

Figure 4. Tensile Testing Device Schematics. A) Diagram showing the conceptual arrangement of the components of the novel device. B) Computer renderings of the design for the device.
milliNewton range, support the culturing of cells on the sample, test wet samples, perform testing in the focal plane of a microscope, and measure directly quantify strain using an optical method.

**Device Calibration**

To ensure the device produces accurate displacements in the samples, the accuracy of the actuator was quantified using optical microscopy. The actuator position was calibrated by imaging the position of the actuator tip as incremental changes in position were applied. The actuator was within 5 microns of its desired location after an applied displacement of 600 microns (Figure 6).
Figure 6. A chart showing the measured position of the actuator tip over the expected position of the actuator tip. The error is smaller than the chart data markers.

Production of Electrospun Fibers

Electrospun fibers of PCL were successfully produced and characterized using Scanning Electron Microscopy (Figure 7) and Differential Scanning Calorimetry (Figure 8). SEM analysis showed that the fiber diameter varied from 0.13 microns to 1.5 microns.

Figure 7. SEM Image of Collected Electrospun PCL Nanofibers.
Figure 8. Representative data from DSC of electrospun PCL. The melting point is 58.94°C and the enthalpy of melting is 73.22 J/g.

However, the manually aligned fibers ranged from 310 to 510 nm. DSC analysis indicated that the crystallinity of the PCL fibers ranged from 51% to 54%. This value is typical for PCL and similar to other reported values for the crystallinity of electrospun PCL and indicates that the fibers used in this study were not significantly different from those used in other studies [43].

Production of Aligned Arrays of Electrospun PCL Fibers

The current method of producing highly aligned electrospun fibers is by collecting them on a rotating mandrel spinning at high angular velocity. However, a highly-aligned array of individual fibers cannot be produced in this manner. Therefore, arrays of individual highly-aligned PCL fibers were fabricated manually (Figure 9). Image analysis revealed that manually aligned electrospun fibers can be deposited with a range of <2 degrees of angular variation and with a standard deviation of 0.75 degrees. The distance between fibers was measured to be 134 ± 15.6 microns (mean ± SD).
Electrostatic interactions between the residual charges remaining on the fibers inhibited deposition of aligned fibers at closer spacing. To the author’s knowledge, this is the first time that a highly-ordered array of individual electrospun fibers has been reported.

**Viscoelasticity Measurement**

The ability of the novel device to measure the viscoelastic properties of samples was tested by performing stress relaxation tests. Examples of the stress relaxation behavior of bulk PCL and individual PCL fibers are given in Figure 10. The viscoelastic behavior of the electrospun PCL is consistent with the expected viscoelastic behavior of electrospun synthetic polymers. The stress relaxation response was modeled using a double exponential equation (Figure 11) similar to work done by Baker et al [30, 31].
Figure 10. Viscoelastic Stress Relaxation Response. A) bulk PCL. B) individual PCL fibers (averaged response).

Figure 11. Model of the Viscoelastic Behavior of Individual PCL Fibers. The data was modeled using a double exponential curve.

\[ \text{Load} = a \cdot e^{\left(-\frac{\text{time}}{\tau_1}\right)} + b \cdot e^{\left(-\frac{\text{time}}{\tau_2}\right)} \]

\[ a = 0.003673 \quad b = 0.00657 \quad \tau_1 = 0.1386 \quad \tau_2 = 17.8253 \]

\[ R^2 = 0.9728 \]
Load vs. Strain Measurement

The dimensions of bulk samples of PCL were recorded and tested in the device to produce engineering stress vs. true strain data. This data was used to test the ability of the testing device to measure non-linear load-strain behavior (Figure 12).

![Tensile Properties of Bulk PCL](image)

Figure 12. Engineering Stress vs. Strain data for “bulk PCL” measured on the novel device.

Optical Strain Measurement

To quantify the strain a fiber is experiencing along its length, optical strain recording was done using a microscope to track the motion of fluorescent dots over time. The adherence of the fluorescent dots on the fibers was visualized using SEM (Figure 13A). The concentration of fluorescent dots was optimized to produce discreet strain markers visible along the length of the fiber (Figure 13B). Tensile tests were performed on arrays of individual electrospun PCL fibers and the load and applied strain was measured over time, along with the strain recorded optically. As seen in Figure 14, the macro-scale true strain (that is, the true strain calculated using grip to grip distance) is
Figure 13. Fluorescent Spheres Adhered to Electrospun PCL Fibers. A) SEM image showing fluorescent spheres adhered to the surface of two electrospun fibers. B) Fluorescent image of fluorescent spheres on two parallel electrospun PCL fibers.

Figure 14. Chart showing: optical strain recordings (red line), macro-scale strain recordings (blue line), and overall load (green line).
highly consistent with the strain recorded optically. This consistency is not surprising for PCL fibers due to the relative homogeneity of PCL. This data does illustrate that the optical strain recording method used in combination with the novel tensile testing device can accurately measure the strain present within a sample. The true utility of this optical strain recording technique is the ability to directly quantify the strain in samples that are not homogeneous along their length. For non-homogenous samples or samples with varying cross-sections the developed optical strain recording method is far superior to using the grip-to-grip strain.

In addition to calculating the true strain present in a single electrospun fiber, the developed system can also measure the force experienced by arrays of individual electrospun fibers. The combination of optical strain recording and overall load measurement permits the construction of load vs. strain curves to describe the mechanical properties of the sample. An example load vs. optically recorded true strain curve is provided in Figure 15.

![Load vs. Optical Strain Curve](image)

Figure 15. Load vs. Optically recorded true strain of electrospun PCL fibers produced using the novel tensile testing platform.
Single Fiber Properties

In order to determine the average properties of individual electrospun PCL fibers, arrays of fibers were subjected to tensile loading and the load vs. true strain of the sample was recorded. Then the load was normalized by the number of fibers present in the sample to reveal the average mechanical behavior of a single electrospun PCL fiber in tension. It was found that the average load per fiber at failure for electrospun PCL fibers was 5.96±2.09 μN/fiber. Representative data of the average mechanical properties of individual electrospun PCL fibers is shown in Figure 16.

![Average PCL Single Fiber Properties](image)

Figure 16. Load vs. true strain curve for an average single PCL fiber.

Significant Difference Observed in Maximum Extensibility of PCL and Reported Values

Individual fibers tested in tension using the novel micro-tensile platform showed an unexpected phenomenon: failure at relatively small strains. PCL is generally considered a hyperelastic material and has been reported in numerous sources to extend well beyond 100% strain. However, data collected during this study indicated that
individual electrospun PCL fibers fail prior to 25% strain. DSC data and SEM analysis both show no indications that the PCL fibers produced in this study were in any way different than the fibers electrospun by other groups. If we assume that the diameter of the fibers was 410nm (as determined from the median of the fibers imaged by SEM) then the engineering stress at failure can be calculated and compared with the values reported in the literature. The observed values for engineering stress at failure and the true strain at failure was compared to the corresponding values reported elsewhere and is summarized in Table 1. It can be seen that a statistically significant difference exists between the observed true strain at failure values and the values reported previously (p<0.0001). However, there was no statistical difference in the observed and previously reported engineering stress at failure values (p=0.0979).

Table 1. Comparison of Mechanical Properties of Individual PCL Fibers Observed and Previously Reported. The observed engineering stress was calculated by assuming that the fiber diameter was 410nm, which was consistent with the values observed in SEM images. P-value was calculated using student’s t-test.

<table>
<thead>
<tr>
<th></th>
<th>Eng. Stress at Failure (MPa)</th>
<th>True Strain at Failure (%)</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental Observations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>50.14</td>
<td>17.89</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>18.21</td>
<td>5.68</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td><strong>Reported values for single and highly aligned PCL fibers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>31.41</td>
<td>102.96</td>
<td>[26, 44, 45]</td>
</tr>
<tr>
<td>SD</td>
<td>21.2</td>
<td>36.24</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td>0.0979</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSION

A novel tensile testing device has been created which can perform direct observations of strain along an electrospun fiber and can be used for testing in cell culture conditions. To the author’s knowledge, this is the first time that direct measurement of strain in an electrospun fiber has been reported. The reported optical strain recording method is also customizable to measure the strain imparted to cells seeded on electrospun fibers. This method is robust in that it can be used for natural or synthetic electrospun fibers and can be used as a platform for the study of any cell type. Additionally, the information collected using this device can be used for the development of a constitutive model of the mechanics of electrospun fibers in various conditions, which can be used to computationally model the dynamic and complex geometries experienced by tissue engineered constructs in vivo. The development of a device capable of recording true strain from arrays of individual electrospun fibers is significant in that an understanding of the materials used in designing tissue engineered implants can lead to improved engineered tissue substitutes.
FUTURE DIRECTIONS

Through the above presented research and development of a novel micro-tensile testing platform, the foundation has been laid for future work in the area of mechanobiology. The platform was designed with the specific goal of being used to test the response of cells to the deformations of their substrate. Because this device can be extended to be used with any cell type or fiber type, the range of possibilities for this device is abundant. It is anticipated that this device will be used to determine the criteria needed for designing tissue engineering scaffolds with predictive cellular behavior.
REFERENCE MATERIAL


Virginia State University, Blacksburg, VA.


APPENDIX A

ENGINEERING DRAWINGS OF CUSTOM PARTS FOR NOVEL TENSILE TESTING DEVICE
Might want to make 2 holes and move them 2mm up.

Filename: ACTUATOR_MOUNT | Material: Aluminum | Version: 2 | Drawn By: Timothy Fee | Date: 5-31-11
Might want to make these holes slots
Filename: BATH_CONTAINER | Material: Delrin | Version: 3 | Drawn By: Timothy Fee | Date: 5/26/2011
NOTE: small details of this part have been changed, the overall shape is the same.
Don't counter-sink, just radius=2.2mm

QUANTITY: 2

no hole here

Filename: HOLDING_BARS | Material: Delrin | Version 3 | Drawn By: Timothy Fee | Date: 5-31-11
Filename: JIG_FOR_FORKS | Material: delrin | Version: 2 | Drawn By: Timothy Fee | Date: 5-31-11
SECTION A-A

Should reduce this dimension to 7mm

UNF #10-32 2-B

Filename: ADAPTER_LOADCELL_SETSCREW | Material: Aluminum | Version: 3 | Drawn By: Timothy Fee | Date: 5-31-11
Keyway depth = 1.1 mm

Stainless steel
- The fibers are suspended between the yellow bars and clamped between the yellow and red parts
- This assembly can be secured using a clip or a clamp
Final Assembly
APPENDIX B

CUSTOM MATLAB FUNCTIONS
OPENING_SCRIPT.M

%opening_script.m
%image extraction script
%this script calls the function "bfopen.m" (produced by LOCI, Madison, WI) and then extracts the images contained the a.nd2 file that is output by the microscope used for this research and writes each image to a tiff file.

%note: the function "bfopen.m" must be present in the current directory, as well as the file "loci_tools.jar". Both files are available from the LOCI website: http://loci.wisc.edu

%this function calls the custom matlab functions "timestampSort.m" and "jHashRead.m"

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NEGLIGENCE OR OTHERWISE) ARISING IN ANY WAY OUT OF THE USE OF THIS
SOFTWARE, EVEN IF ADVISED OF THE POSSIBILITY OF SUCH DAMAGE.

%% call bfopen.m

data=bfopen;

dataSetName=input('Please enter the name of the data set, the image
files will be of the form: name_seriesX_imageX.tif','s');

%% find number of series
numSeries=size(data,1);
fprintf('Found %d image series',numSeries)
numPlanes=zeros(1,numSeries);

for i=1:numSeries
    numPlanes(i)=size(data{i,1},1);
    fprintf('Found %d planes in Series %d',numPlanes(i),i)
end

%% write each of the images to a file (for each series)
% additionally, we need to extract the metadata

labels=cell(numSeries,[]);

for i=1:numSeries
    [m,n]=size(data{i,1}{1,1});
    imstack=zeros(m,n,numPlanes(i),'uint16');
    for j=1:numPlanes(i) %extract each image
        imstack(:,:,j)=data{i,1}{j,1};
        labels{i,j}=data{i,1}{j,2};%also get the image labels
    end
    for j=1:numPlanes(i) %write each image to file
        matFilename=sprintf('%s_series%d_image%05d.tif',dataSetName,i,j);
        imwrite(imstack(:,:,j),matFilename,'tif');
    end
end
if numSeries == 1
    timestamps=timestampSort(jHashRead(data{i,2})); %get and sort the
    %metadata
end
function out=jHashRead(in)
%out=jHashRead(in)
%this function reads the contents of a javascript hashtable,
%specifically, the one produced by "bfopen.m" it returns the two column
%matrix with the first being the field name and the second column
%being the value. This function calls the subfunction "hashDisplay"

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enum=in.keys();
i=1;
while enum.hasMoreElements
    key=enum.nextElement;
    value=hashDisplay(in.get(key));
    out(i,1)=key;
    out(i,2)=value;
    i=i+1;
end
end
hashDisplay

function blat=hashDisplay(entry)
[t,r]=strtok(entry,'^');
blat=[];
while ~isempty(t)
    blat=t;
    [t,r]=strtok(r,'^');
end
end

timестampsort.m

function out=timestampSort(in)
%out=timestampSort(in)
%this function takes in a 2 column cell array with field names in the
%first column and values in the second column. it searches the field
%names for the string "timestamp" and then sorts the corresponding
%times by the number at the end of the field name containing
"timestamp"

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l=length(in);
tfVect=zeros(1,l,'int8');
pattern='timestamp';

for i=1:l
    tfVect(i)=~isempty(strfind(in{i,1},pattern));
end
times=sum(tfVect); % total number of timestamps

stampdata=zeros(times,2); % preallocate the unsorted values

counter=1;
for i=1:l
    if tfVect(i)==1
        str=in{i,1};
        temp=textscan(str, '%*s %*s %*s %*s %d');
        temp=double(cell2mat(temp));
        stampdata(counter,1)=temp;
        stampdata(counter,2)=(in{i,2});
        counter=counter+1;
    end
end

% now sort the resulting data
out=zeros(times,2);
[holder1,holder2]=sort(stampdata(:,1));
out(:,1)=holder1;
ind=holder2;
for i=1:times
    out(i,2)=stampdata(ind(i),2);
end

POSITIONTRACKER.M

function out=positionTracker(imNames)
% out=positionTracker(imNames)
this function is used to read a series of tiff images one by one and allow the user to select points (bright on a dark background) to track over time. the returned variable "out" has the form: (N by P by 2) where N is the number of images, P is the number of points being tracked, the 1st layer is the x position of that point, and the 2nd layer is the y position of that point.

Note: the variable "test" contains the number of images that should be processed

the variable "box" contains the size of the bounding box that is used to track the point. therefore, each point being tracked must remain at least "box" pixels away from the edge of the image, any pixel being tracked should not move more than "box" pixels in any two sequential images, and no to points should be within "box" pixels of each other.

This function calls the subfunctions: "findPoints", "centroid", and "peak2".

the function "centroid" uses commands from the MATLAB image processing toolbox to find the intensity weighted centroid of the largest region of a thresholded region of interest. If your system does not have the image processing toolbox, you can replace each the centroid function with the peak2 function that tracks the maximum pixel value in a region of interest and uses the native MATLAB commands.

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%get a list of image files in the current directory
imNames=strcat('**',imNames,'*.tif');
files=dir(imNames);
numImages=length(files) %shows the number of images found.
pause(2)

test=100; %number of images to process, when out=numImages all the
files will be processed.
box=20; %number of pixels for the bounding box

%now call up the first image
I=imread(files(1).name);
set(gcf, 'OuterPosition', get(0, 'ScreenSize'));
imagesc(I);
title('image 1')
colormap(gray);

%prompt for a point
[X,Y,p]=impixel;
%call subfunction to locate each x and y dot
[X,Y]=findPoints(X,Y,I,box);

%show each point on the image
for z=1:length(X)
    line(X(z),Y(z),'Color','blue','Marker','o','LineStyle','none')
end
pause(0.5)
close
% pre allocate out
out=zeros(test,length(X),2);
%write the data
for i=1:length(X)
    out(1,i,1)=X(i);
    out(1,i,2)=Y(i);
end

% loop for the remaining images
if numImages==1
    error('only one image')
end

for i=2:test
    I=imread(files(i).name);
    set(gcf, 'OuterPosition', get(0, 'ScreenSize'));
    imagesc(I);
    goo=strcat('image number...',int2str(i));
    title(goo);
    colormap(gray);

    % call subfunction to locate each x and y dot
    [X,Y]=findPoints(X,Y,I,box);

    % show each point on the image
    for z=1:length(X)
        line(X(z),Y(z),'Color','blue','Marker','o','LineStyle','none')
    end
    pause(0.5)
    close
%write the data
for j=1:length(X)
    out(i,j,1)=X(j);
    out(i,j,2)=Y(j);
end
end
end

findPoints

function [xout,yout]=findPoints(X,Y,I,box)
%this function takes in an image I and two vectors of x and y
%positions. It finds the local maximum for each x-y pair and returns
%those points

l=length(X);
k=box;
xout=zeros(l,1);
yout=zeros(l,1);
for q=1:l
    ROI=I(Y(q)-k:Y(q)+k,X(q)-k:X(q)+k);
    [ix,iy]=centroid(ROI);
    xout(q)=X(q)-k+ix;
    yout(q)=Y(q)-k+iy;
end
end
peak2

function [x,y]=peak2(ROIin)
[rowstack, rowindex]=max(ROIin);
[~, col]=max(rowstack);
row=rowindex(col);
x=col;
y=row;
end

centroid

function [x,y]=centroid(ROIin)
filteredROI=medfilt2(ROIin,'symmetric');
level = 1.25*mean2(filteredROI);
[cols, rows]=size(filteredROI);
bw = zeros(cols,rows);
for col=1:cols
    for row=1:rows
        if filteredROI(col,row)>=level
            bw(col,row)=1;
        end
    end
end
stats=regionprops(bw,'Area','Centroid','PixelIdxList', 'PixelList');
biggest=0;
big=0;

for p=1:length(stats)
    if stats(p).Area>biggest
big=p;
end
end

if big==0
    [x,y]=peak2(R0Iin);
else

    %weighted centroid method
    k = big;
    idx = stats(k).PixelIdxList;
    pixel_values = double(filteredROI(idx));
    sum_pixel_values = sum(pixel_values);
    x_test = stats(k).PixelList(:, 1);
    y_test = stats(k).PixelList(:, 2);
    xbar = sum(x_test .* pixel_values) / sum_pixel_values;
    ybar = sum(y_test .* pixel_values) / sum_pixel_values;
    x=round(xbar);
    y=round(ybar);
end
end