Mechanical characterization of collagen fibers and scaffolds for tissue engineering

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Abstract

Engineered tissues must utilize scaffolding biomaterials that support desired cellular functions and possess or can develop appropriate mechanical characteristics. This study assessed properties of collagen as a scaffolding biomaterial for ligament replacements. Mechanical properties of extruded bovine achilles tendon collagen fibers were significantly affected by fiber diameter, with smaller fibers displaying higher tangent moduli and peak stresses. Mechanical properties of 125 mm-diameter extruded fibers (tangent modulus of 359.67 28.4 MPa; peak stress of 36.07 5.4 MPa) were similar to properties reported for human ligaments. Scaffolds of extruded fibers did not exhibit viscoelastic creep properties similar to natural ligaments. Collagen fibers from rat tail tendon (a well-studied comparison material) displayed characteristic strain-softening behavior, and scaffolds of rat tail fibers demonstrated a non-intuitive relationship between tangent modulus and specimen length. Composite scaffolds (extruded collagen fibers cast within a gel of Type I rat tail tendon collagen) were maintained with and without fibroblasts under standard culture conditions for 25 days; cell-incorporated scaffolds displayed significantly higher tangent moduli and peak stresses than those without cells. Because tissue-engineered products must possess appropriate mechanical as well as biological/chemical properties, data from this study should help enable the development of improved tissue analogues.

Keywords: Collagen Science Ltd. All rights reserved.

1. Introduction

Many efforts to construct engineered tissue analogues in vitro have utilized systems of cells cultured on biomaterial scaffolds. Cell/biomaterial constructs which possess appropriate biological and mechanical function will be of great clinical use for tissue replacements. For example, it has been estimated that as many as 150,000 Americans suffer an injury to their anterior cruciate ligament (ACL) each year [1]. The ACL plays a critical role in knee stability and heals poorly, often necessitating surgical reconstruction to restore knee function [2]. The most commonly used surgical ACL reconstructions (autografts of patellar or hamstring tendons) yield good results in general but are still greatly limited by impaired knee function, morbidity at the donor site, secondary pain, and other complications from the autograft harvest [2].

Natural tendon and ligament tissue consists of a hierarchical structure of collagen fibrils and fibers [3], providing a specific microenvironment for incorporated cells and governing the mechanical properties of the tissue. Collagen—the most prevalent structural protein in the human body—is therefore a natural biomaterial to evaluate for ligament replacement, as well as other tissue engineering efforts [4–10]. Collagen gels have been evaluated for use in ligament tissue engineering, and showed promising biological results in that cells cultured on or within these gels produced extracellular matrix, and aligned longitudinally with the long axis of the tissue equivalent (mimicking cell alignment in ligaments in vivo) [11,12]. Unfortunately, collagen gels do not possess the mechanical strength that would be needed for a functional ligament replacement in vivo. Due to their potentially greater mechanical strength, collagen fibers and fiber scaffolds have been used as an

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alternative to gels. Fibroblastic cells have been shown to
attach to and function on collagen fibers in vitro [13–15]
and fibroblast-seeded collagen fiber scaffolds have been
evaluated in implantation studies [15–17], showing
promising biological results. Biomechanical analysis of
collagen fibers and scaffolds remains an area of interest.

The literature contains some mechanical characterizations
of collagen fibers [13,18–20] and scaffolds [16].
However, parameters such as ultimate force or breaking
load [13,16,20], a structural property that depends on the
scale of the specimen being tested, are often reported.
Assessing and reporting material properties (which
should not depend on specimen size) such as tangent
modulus [19,13,16,20] provides information valuable to
many applications of the material in question—espe-
cially critical in tissue engineering efforts, where ‘scaling
up’ from laboratory experiments to human tissue size
may be necessary in order to meet clinical needs, and
where scaffolds must be designed to meet or withstand
specific mechanical conditions in vivo or in vitro (e.g.
[21,22]). Furthermore, like mechanical properties, the
time-dependent or viscoelastic properties (as demon-
strated by creep and stress-relaxation tests) of ligaments
and many other soft tissues affect in vivo tissue function.
This study, therefore, assessed structural, material and
viscoelastic properties of single- and multi-fiber collagen
scaffolds, addressing issues of fiber diameter and source.
Understanding the fundamental mechanical properties
of the fibers and scaffolds allowed the development and
preliminary characterization of a collagen fiber-em-
bedded gel scaffold, with and without the incorporation
of living cells. The results provide motivation for
continued and thorough experimental characteriza-
tion—biomechanical as well as chemical/histological—
of collagen as a biomaterial for ligament and other
tissue engineering applications.

2. Materials and methods

2.1. Preparation of single collagen fibers

Extruded collagen fibers were formed according to
procedures adapted from published protocols
[23,19,13,16]. A 1% (w/v) solution of Type I collagen
from bovine achilles tendon (Sigma, St. Louis, MO) in
HCl (pH 2.0) was mixed with a blender for 4 min,
allowed to rest for 10 min, re-mixed for 4 min and
centrifuged (5 min, 5000 rpm/4000g). After centrifuga-
tion, the collagen dispersion was stored at 4 °C for up to
days. Collagen fibers were formed by extruding the
dispersion through microbore tubing (inner diameters of
0.051, 0.102, or 0.127 cm; Cole-Parmer, Vernon Hills,
IL), at a rate of 0.5 ml/min, into a 37 °C bath of fiber
formation buffer (composed of 2.75 g N-tris(hydroxy-
methyl)-methyl-2-aminoethane sulfonic acid, 3.16 g
NaCl, and 1.7 g Na2PO4 in 400ml distilled water; pH
adjusted to 7.5; all chemicals from Sigma) [23,19,13,16].
The fibers remained in the buffer for 45 min, after which
they were transferred to a room temperature bath of
95% ethanol and allowed to dehydrate for 4 h. Fibers
were then dipped in distilled water to rinse and hung to
air-dry overnight, thereby reducing their diameters to
approximately one-tenth of the original tubing diameter.
The resulting fibers were crosslinked by soaking in a 1%
(w/v) solution of 1-ethyl-3-(3-dimethylaminopropyl)-
carbodiimide (Sigma) in distilled water for 24 h at 4 °C,
and then rinsing in distilled water for an additional 24 h
at 4 °C. Crosslinked fibers were then air-dried and stored
at room temperature in an airtight container until use
for construction of scaffolds and/or mechanical testing.

Rat tail tendon collagen fibers were used as a well-
characterized [24–28,18] comparison material. Briefly,
tails of sacrificed Sprague–Dawley rats (44–48 days old)
were removed, skinned, and placed in phosphate-
buffered saline (PBS). A dissecting probe was used to
pull individual tendon fibers through the surrounding
cutis and out from the tail. Some fibers were cut away
from the tail, placed between layers of surgical gauze
soaked with PBS, and used immediately for assembly
into scaffolds and mechanical testing. Some fibers were
cut away from the tail, crosslinked in a 1% (w/v)
solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-
imide (Sigma) in distilled water for 24 h at 4 °C, rinsed,
air-dried and then used in construction of scaffolds and/
or mechanical testing.

2.2. Preparation of multi-fiber scaffolds

2.2.1. Fiber scaffolds

Each extruded collagen fiber scaffold was formed by
aligning 10 fibers (each 7.6 cm long) into a parallel array.
The ends of the fibers were secured with a rolling hitch
knot of 4–0 suture silk (Ethicon, Somerville, NJ).
Scaffolds of rat tail tendon fibers were formed similarly,
with either 10 crosslinked (7.6 cm-long) fibers or 14 non-
crosslinked fibers of equal lengths in each scaffold.

To confirm their viability as cell culture substrates,
some scaffolds of extruded collagen fibers were seeded
with fibroblasts and cultured for various lengths of time;
cells on the scaffolds were then visualized with the
commercially available “Live/Dead” fluorescent stain-
ing kit (Molecular Probes, Eugene, OR). Specifically,
scaffolds of extruded collagen fibers were tied onto
custom-built acrylic frames, placed in tissue culture
plasticware petri dishes (120 mm diameter, Fisher
Scientific, Pittsburgh, PA), and sterilized [23,16] by
soaking for 1 h in a dilute solution of the lactic acid-
based sterilant Exsor (Alcide Corporation, Redmond,
WA) (1:1:10:24, v/v/v/v, Exsor base: Exsor activator:
distilled water: phosphate buffered saline). The Exsor
solution was removed and scaffolds were then sub-
merged in cell culture media (Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 20 U/ml penicillin, 20 mg/ml streptomycin and 0.5 mg/ml fungizone; all chemicals from Invitrogen, Carlsbad, CA) for 24 h under standard culture conditions (i.e., humidified, 37 °C, 5% CO₂/95% air). After removing this media from the petri dishes, each scaffold was covered with 0.7 ml of a high-density (approximately 2 × 10⁶ cells/ml) suspension of rat skin fibroblasts (CRL-1213, American Type Culture Collection, Manassas, VA) in cell culture media, and placed under standard culture conditions for 20 min. An additional 8 ml of cell culture media was then added to each dish and the cell-seeded scaffolds were cultured for 1, 4, 8, or 16 days, after which the “Live/Dead” fluorescent staining kit was used according to the manufacturer’s instructions (Molecular Probes) to visualize cells on the scaffolds. The “live” indicator stain in this kit is Calcein AM, which readily passes through cell membranes where it is enzymatically converted into fluorescent (green) calcein. The “dead” indicator stain in this kit is Ethidium homodimer-1 (EthD-1). EthD-1 cannot pass through the intact membrane of live cells, but instead enters cells through damaged membranes and binds to nucleic acids, producing a red fluorescence.

Extruded collagen scaffolds used in creep testing experiments were sterilized for 18 h in a 70% ethanol solution with deionized water. After sterilization, the scaffolds were soaked in sterile deionized water for 10 min, and then vigorously rinsed in sterile deionized water prior to testing.

2.2.2. Fiber-embedded gel scaffolds

Extruded collagen fibers were combined with a collagen gel to make fiber-embedded gel scaffolds. Briefly, 50 extruded collagen fibers (each 2.5 cm long) were tied in a parallel array and secured at each end of the array with a rolling hitch knot of 4–0 suture (Ethicon). The scaffolds were sterilized by soaking for 1 h in a dilute solution of Exspor (1:10, v/v, activated Exspor; distilled water), rinsed in 3 consecutive 20-min baths of sterile PBS, and allowed to dry under sterile conditions. These scaffolds were placed into individual channels of a custom-built mold (mold dimensions 5.4 × 4.45 × 0.64 cm³, L × W × H; containing 8 channels of dimensions 3.18 × 0.318 × 0.318 cm³, L × W × H). Rat skin fibroblasts were then enzymatically lifted from flasks and suspended (5 × 10⁶ cells/ml) in a solution of Dulbecco’s Modified Eagle Medium (1X and 5X concentrations; Invitrogen), 10% fetal bovine serum (Invitrogen), 2.77 mg/ml acid-soluble Type I rat tail tendon collagen (Upstate Biotechnology, Lake Placid, NY), and 2 m NaOH (Sigma). After filling each channel of the mold with this cell/collagen suspension (over and around the fiber scaffold in each channel) the mold was incubated under standard culture conditions for 30 min to allow the cell/collagen suspension to gel. The mold was then placed in a 120 mm diameter plastic petri dish (Fisher Scientific), covered with cell culture media, and cultured under standard conditions. Fiber-embedded gel scaffolds were examined using the “Live/Dead” assay kit (Molecular Probes) and, after 25 days of culture, mechanically tested. To assess the effects of constituent cells on the mechanical properties of fiber-embedded gel scaffolds, a second set of scaffolds was fabricated similarly but without fibroblasts, maintained under identical conditions, and mechanically tested after 25 days.

2.3. Mechanical testing and data acquisition

2.3.1. Determination of fiber diameter

In order to calculate the stress developed in the collagen fibers during mechanical testing, it was necessary to determine the cross-sectional areas of the single fibers and the multi-fiber scaffolds. The wet diameters of 17 randomly selected single rat tail tendon collagen fibers were measured using a laser micrometer (Keyence, Woodcliff Lake, NJ). Each fiber was rotated to measure the diameter from three different angles; the average of these three measurements was considered the specimen diameter. Single extruded collagen fibers were too small to measure with the laser micrometer. However, the wet diameters of collagen fibers extruded from various tubing sizes had previously been measured and reported [20]; linear regression of the previously published data allowed extrapolation to predict the wet fiber diameters for the tubing sizes used in this study. The predicted fiber diameters were confirmed by manually measuring extruded fibers using a micrometer slide and a light microscope. All collagen fibers were assumed to be circular, allowing calculation of fiber cross-sectional areas from fiber diameters. The load-bearing diameter of all multi-fiber scaffolds was assumed to be the sum of the cross-sectional areas of the individual fibers.

2.3.2. Tensile testing

To facilitate gripping during testing, the ends of collagen fibers and fiber scaffolds (non-gel-embedded) were placed in cylindrical molds of a low-temperature, slow-curing cement (Bondo®; Atlanta, GA). Lengths of 4–0 suture silk (Ethicon) were tied to the ends of single-fiber specimens with a rolling hitch knot. The surgical silk on single fibers and on multi-fiber scaffolds was used to pull specimens carefully into embedding molds filled with freshly mixed Bondo® (80:1, v/v, base and activator; this mixture did not exceed 37 °C while hardening) such that the sutures and end of the fibers were firmly embedded up to the hitch knot, leaving fibers above the knot untouched. After the Bondo® had hardened, the embedded specimens were removed from
the molds and placed in PBS for 30 min to rehydrate. The embedded ends of specimens were fixed in custom-made clamps and loaded into a computer-controlled testing system (Model 1122, Instron, Canton, MA).

To maintain gel hydration, fiber-embedded gel scaffolds (both with and without cells) were not placed in slow-curing cement, but rather were fixed to plastic tabs with cyanoacrylate to facilitate gripping. The sutures and the ends of the gel scaffolds were coated with several drops of cyanoacrylate and pressed between a pair of Plexiglas tabs (2 × 3 cm², L × H, 0.318 cm thick) such that the ends of the scaffolds were affixed between the plastic tabs, but the region of the scaffolds between the suture knots remained free. The prepared tabs and ends of the fiber-embedded gel scaffolds were fixed in standard compression grips and mounted in the computer-controlled Instron 1122 system for testing.

All specimens were tensile-tested at a loading rate of 12.7 cm/min, with the exception of some scaffolds constructed from non-crosslinked rat tail tendon fibers, which were loaded at a rate of 2.54 cm/min. All specimens were kept hydrated with phosphate-buffered saline throughout testing. Force data were collected in TestWorks® (MTS, Eden Prairie, MN) software at a frequency of 50 Hz. Strains (change in length divided by initial length) were calculated using crosshead displacement; stresses were calculated by dividing force data by the cross-sectional area of the specimen (assumed to remain constant). Stress–strain curves for biologic soft tissues often display both linear and non-linear regions; the linear region is often considered indicative of the stiffness of the material, and thus the slope of a line tangent to this region is usually reported as the modulus. Because of the non-linear behavior of these tissues, it would be incorrect to call this modulus “Young’s modulus,” which is reported in traditional (linear elastic) materials testing.

2.3.3. Viscoelastic testing

Extruded collagen fiber scaffolds composed of 10 fibers each were loaded into a custom-designed tensile creep-testing device. The device utilized a two-pulley system to ensure that a uniaxial vertical load was applied to the scaffold without any horizontal or torsional forces. The length of the scaffold between the clamps was measured to the nearest 0.01 mm using digital calipers. Tensile creep testing was performed on each specimen under a load of approximately 2.5 MPa. During testing, scaffolds were hydrated with a misting spray of room temperature PBS applied every 60 s. Elongation was continuously measured using a linear variable differential transformer (LVDT) mounted at the top of the device on the linear motion slide. Analog output of the LVDT was recorded with an analog-to-digital board on a PC-compatible computer. Voltage measurements from the LVDT were correlated to the actual elongation distance by calibrating the device with steel blocks of known dimensions. Strain was calculated as the elongation normalized to the undeformed length of the scaffold. This undeformed length was the clamp-to-clamp length of the scaffold in the device as measured with digital calipers. The strain data were plotted as a function of time to produce creep curves.

In order to compare the data from the tests, two parameters of creep (equilibration time and equilibrium strain) were computed. The time needed for the collagen scaffold to reach equilibrium was determined by calculating the rolling standard deviation of the strain data over a period of 1 min. The time at which this standard deviation value fell below and remained below 0.0005 was deemed the equilibration time. The equilibrium strain was determined by averaging the strain values for a 3-min period after the equilibration time for each scaffold.

2.4. Statistical analysis

Means of tangent modulus and peak stress data were compared using two-tailed t tests, without the assumption of equal variances. To test for correlations between tangent modulus and initial length of scaffolds made from rat tail tendon fibers, simple regressions were performed using StatView software (SAS Institute, Cary, NC). Statistical significance was defined as p < 0.05:

3. Results

Single collagen fibers derived from rat tail tendon had diameters measured by the laser micrometer ranging from 0.2229 to 0.2887 mm. The average value of 271 mm was used for calculations in this study. Fitting a linear regression to previously published data on the wet diameter of extruded collagen fibers [20] yielded the equation:

Wet fiber diameter $d_{mm}$

$\frac{3}{4} \, f_{0.1298}$ Extrusion tube diameter $d_{mm}$ 6.79 mm

Using this equation it was predicted that, in this study, collagen fibers extruded through microbore tubes with inner diameters of 0.051, 0.102, and 0.127 cm would possess wet diameters of 59, 125, and 158 mm, respectively.

The tangent moduli and peak stresses of crosslinked, single extruded collagen fibers decreased with increasing fiber diameter (Table 1). The mean tangent modulus and peak stress of crosslinked, single rat tail tendon collagen fibers were significantly (p < 0.01) higher than the mean tangent moduli and peak stresses of all the crosslinked, extruded collagen fibers (Table 1). The stress–strain curves obtained from tensile testing of extruded collagen
Table 1
Mechanical properties of crosslinked, single collagen fibers

<table>
<thead>
<tr>
<th>Source</th>
<th>Diameter (nm)</th>
<th>n</th>
<th>Modulus (MPa)</th>
<th>Peak stress (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extruded</td>
<td>59</td>
<td>8</td>
<td>484.7 7.76.3</td>
<td>50.0 7.13.4</td>
</tr>
<tr>
<td>Extruded</td>
<td>125</td>
<td>11</td>
<td>359.6 7.28.4</td>
<td>36.0 7.5.4</td>
</tr>
<tr>
<td>Extruded</td>
<td>158</td>
<td>10</td>
<td>269.7 7.11.9</td>
<td>24.7 7.2.9</td>
</tr>
<tr>
<td>Rat tail tendon</td>
<td>271</td>
<td>12</td>
<td>1174.9 7.283.3</td>
<td>114.6 7.51.0</td>
</tr>
</tbody>
</table>

Tangent modulus and peak stress values are reported as means ± standard deviations. The peak stress values of the 125-nm diameter fibers were significantly (p<0.05) different from those of the 59-nm diameter fibers. All other possible comparisons of peak stress mean values, and all tangent modulus mean values, yielded significant differences at the p<0.01 level.

![Fig. 1](representative-stress-strain-plots-generated-from-tensile-testing-of-crosslinked-single-extruded-and-rat-tail-collagen-fibers-wet-diameter-of-the-extruded-collagen-fiber=59-mm-wet-diameter-of-the-rat-tail-tendon-collagen-fiber=271-mm.png)

Fig. 1. Representative stress–strain plots generated from tensile testing of crosslinked, single, extruded (A) and rat tail (B) collagen fibers. Wet diameter of the extruded collagen fiber=59 mm; wet diameter of the rat tail tendon collagen fiber=271 mm.

![Fig. 2](tangent-modulus-of-scaffolds-created-from-non-crosslinked-rat-tail-tendon-collagen-fibers-as-a-function-of-initial-scaffold-length-a-scaffolds-tested-at-an-extension-rate-of-2-54-cm-min-b-scaffolds-tested-at-an-extension-rate-of-12-7-cm-min-the-regression-shown-in-frame-b-indicated-that-tangent-modulus-was-significantly-p<0-01-correlated-with-initial-scaffold-length.png)

Fig. 2. Tangent modulus of scaffolds created from non-crosslinked rat tail tendon collagen fibers, as a function of initial scaffold length. (A) Scaffolds tested at an extension rate of 2.54 cm/min. (B) Scaffolds tested at an extension rate of 12.7 cm/min. The regression shown in frame B indicated that tangent modulus was significantly (p < 0.01) correlated with initial scaffold length.

fibers were shaped differently than the stress–strain curves obtained from similar testing of rat tail tendon fibers (Fig. 1).

The tangent moduli of scaffolds created from 14 fresh, non-crosslinked rat tail tendon fibers depended on the initial lengths of the scaffolds (Fig. 2); this dependence increased as the rate of load application increased from 2.54 to 12.7 cm/min and became statistically significant (p<0.01).

The tangent modulus and the peak stress of scaffolds constructed from 10 crosslinked, 125-mm diameter extruded collagen fibers were significantly (p<0.01) lower than the tangent modulus and peak stress of single, crosslinked, 125-mm diameter extruded collagen fibers (Table 2). In contrast, the properties of scaffolds constructed from 10 crosslinked rat tail tendon collagen fibers were not significantly different from those of single, crosslinked rat tail tendon collagen fibers (Table 2).

The mean (± standard deviation) equilibrium time for creep-tested 10-fiber extruded collagen scaffolds was 30.02 ± 1.33 s and the mean (± standard deviation) equilibrium strain was 0.095 ± 0.024.

Sample photomicrographs of rat skin fibroblasts adherent to and viable on extruded collagen fibers, as well as within fiber-embedded gels, are given in Fig. 3. After 25 days of culture, fiber-embedded gels containing cells exhibited significantly (p<0.01) higher tangent
Table 2
Mechanical properties of collagen scaffolds as a function of fiber number

<table>
<thead>
<tr>
<th>Source</th>
<th>Number of fibers</th>
<th>n</th>
<th>Modulus (MPa)</th>
<th>Peak stress (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extruded</td>
<td>1</td>
<td>11</td>
<td>359.67 28.4</td>
<td>36.07 5.4</td>
</tr>
<tr>
<td>Extruded</td>
<td>10</td>
<td>12</td>
<td>261.27 63.5</td>
<td>19.97 7.2</td>
</tr>
<tr>
<td>Rat tail tendon</td>
<td>1</td>
<td>12</td>
<td>1174.97 283.3</td>
<td>114.67 51.0</td>
</tr>
<tr>
<td>Rat tail tendon</td>
<td>10</td>
<td>13</td>
<td>995.17 144.0</td>
<td>106.17 13.9</td>
</tr>
</tbody>
</table>

The diameter of the extruded fibers was 125 nm and that of the rat tail tendon fibers was 271 nm; both types of fiber were crosslinked prior to assembly into scaffolds. Tangent modulus and peak stress values are reported as means ± standard deviations. All possible comparisons of either peak stress or tangent modulus mean values yielded significant (p < 0.01) differences except for the properties of 1-fiber and 10-fiber rat tail tendon scaffolds, which were not found to be significantly different from each other.

Fig. 3. Representative micrographs of cells on scaffolds of extruded collagen fibers (wt diameter = 125 nm). All cells stained positively with the “live” stain (1.2 nM ethidium homodimer-1) and not the “dead” stain (1.2 nM calcein AM) of a commercially available “Live–Dead kit” (Molecular Probes, Eugene, OR), indicating intact cell membranes. (A) Cells on collagen fibers, after 24 h of culture. Dotted lines at left delineate the contours of two fibers; other fibers are also visible to the side of and behind these two fibers. Original magnification 200 (scale bar = 100 nm). (B) Cells in collagen fiber-embedded gels, after 2 days of culture. Original magnification 100 (scale bar = 100 nm).

Table 3
Mechanical properties of collagen scaffolds cultured with and without cells

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Modulus (MPa)</th>
<th>Peak stress (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without cells</td>
<td>5</td>
<td>49.67 3.3</td>
<td>2.97 0.9</td>
</tr>
<tr>
<td>With cells</td>
<td>6</td>
<td>83.47 10.8*</td>
<td>5.47 0.4*</td>
</tr>
</tbody>
</table>

The diameter of the (extruded, crosslinked) fibers was 125 nm; scaffolds contained 50 fibers each. Tangent modulus and peak stress values are reported as means ± standard deviations. Mean tangent modulus and peak stress values of scaffolds cultured with cells were significantly (p < 0.01) different from those of specimens cultured without cells.

modulus and peak stress values than did fiber-embedded gels which did not contain cells (Table 3). Additionally, stress–strain curves obtained from tensile testing of fiber-embedded gels that incorporated cells were more uniform and displayed fewer incremental failures than curves obtained from fiber-embedded gels without cells (Fig. 4).

4. Discussion

Interest in tissue engineering for ligament replacement has been driven by concerns about autograft donor site morbidity and the potential for allograft disease transmission. Collagen is a good candidate for this application as it is a biodegradable, natural material which may undergo tissue-remodeling and ultimately be replaced with neo-collagenous tissue in vivo. While other ligament constituents contribute to the mechanical properties of the tissue (elastin, for example, plays a role in tensile resistance and elastic recoverability, although it comprises less than 5% of the dry weight of ligament tissue) the hierarchical structure of collagen comprises the majority of the dry weight of ligament tissue (>90%) and is primarily responsible for ligaments’ tensile strength. The current work therefore provides a mechanical characterization of collagen as a biomaterial for ligament and other tissue engineering applications, focusing on material and viscoelastic properties of single collagen fibers and multi-fiber scaffolds. Establishing baseline mechanical properties of the fibers and scaffolds subsequently allowed the development and preliminary characterization of a collagen fiber-embedded gel scaffold, with and without the incorporation of living cells.

Tensile testing of single, extruded collagen fibers produced a classic stress–strain response generally observed for soft biologic materials (e.g., ligaments): a non-linear, initial region (the “toe region” or a “J-shaped curve”) that changes to a linear region of greatly increased tangent modulus which persists until failure [29]. Single collagen fibers from rat tail tendon also
produced a characteristic tensile testing stress–strain curve in agreement with previous studies of this tissue [18], but in marked contrast to the response of extruded collagen fibers. Rat tail fibers displayed strain-softening, or decreasing tangent modulus with increasing tensile strain, especially after approximately 10% strain. While this agrees with previous work on rat tail tendon [18] and could be an inherent biological feature of these fibers, some of the observed reduction in tangent modulus may be due to the common practice of using engineering stress (i.e., stress determined using initial cross-sectional area of the specimen).

The tangent moduli and peak stresses of single, extruded collagen fibers found in this work are in general agreement with previous reports [19]. Fiber diameter had a significant effect on the mechanical properties of extruded collagen fibers, with smaller fibers displaying greater tangent moduli and peak stresses. This confirms previous work on peak stress [20], and there are two likely reasons for this relationship between fiber diameter and mechanical properties. First, the larger a fiber is, the more likely it is to contain defects. Second, larger fibers have a smaller surface to volume ratio, which results in a smaller percentage of fiber cross-section undergoing carbodiimide crosslinking (in addition to the natural crosslinks which already exist in the collagen) compared to a smaller fiber exposed to the same treatment [20]. If needed, stronger forms of crosslinking might be used to increase fiber moduli.

The tangent moduli presented here for crosslinked rat tail tendon fibers are comparable to those reported by Haut for non-crosslinked rat tail fibers [18], despite differences between this study and Haut’s, including the type of rat (i.e., Sprague-Dawley vs. Fischer), age of rat (48 days vs. 9 months), original fiber location within the tail, etc. Scaffolds of non-crosslinked rat tail fibers in the present study were found to have substantially lower tangent moduli than the crosslinked, single rat tail fibers. Interestingly, data obtained in this study from scaffolds of non-crosslinked rat tail fibers support the non-intuitive finding that the tangent moduli (a material property which should not depend on overall specimen size) of rat tail collagen fibers are dependent on specimen length [18]. One possible explanation for this behavior is the occurrence of varying regional strains within the fibers [30,18,31], as have been observed in tensile tests of bone–ligament–bone complexes [30]. In the present study, increases in specimen length were significantly correlated with increases in tangent modulus at a higher rate of load application, and the tangent modulus of the tissue increased slightly with loading rate, as has been noted for many viscoelastic soft tissues [32].

Despite the notable elastic properties of the collagen scaffolds, viscoelastic creep of 10-fiber collagen scaffolds occurred very rapidly in comparison to actual ligaments. Equilibration time was less than 35 s for all scaffolds tested; previous studies indicate that creep continues beyond 20 min in native ligaments [33]. The relative speed with which the fiber scaffolds reach equilibrium may provide insight on the causes of creep behavior in ligaments. It has been suggested that soft tissue viscoelastic behavior is the result of the interactions of collagen and extracellular matrix components. Other studies purport that creep properties of ligaments are, at least in part, the result of collagen fiber recruitment [34]. The present study provides evidence for an additional causal mechanism for viscoelastic creep behavior in ligaments beyond the presence of collagen fibers.

While rat tail tendon provides a biologically derived, well-studied collagen fiber for use as a control or reference biomaterial, the ultimate intent of engineered ligament analogues is to replace normal ligament tissue and to restore normal function. For this reason, the mechanical properties of single- and multiple-fiber collagen scaffolds should be considered relative to the mechanical properties of human knee ligaments. Despite intense research on knee ligament reconstruction over the last 25 years, there does not exist a substantial body of literature on the mechanical properties of knee...
ligaments, owing to the relative complexity of fiber alignment in these tissues and their relatively low aspect ratio (diameter/length). By dissecting the ligaments down to bone-fascicle-bone units (cross-sectional area between 1 and 2 mm²), Butler et al. [29] determined the mechanical properties of the anterior cruciate ligament, the posterior cruciate ligament, and the lateral collateral ligament from young donors. The modulus and peak stress of these ligaments averaged 345 and 36.4 MPa, respectively [29], and modulus and peak stress values reported for the human medial collateral ligament are in the same range [35]. The mechanical properties of all extruded, single collagen fibers in this study compare favorably to reported properties of knee ligaments [29]; the 125 μm-diameter fibers exhibited properties (tangent modulus of 359.6 ± 28.4 MPa; peak stress of 36.0 ± 5.4 MPa) similar to those reported for human knee ligaments [29]. However, the tangent moduli and peak stresses of multi-fiber scaffolds formed from 125 μm-diameter fibers were found to decrease as the number of fibers increased. This reduction is likely due to non-uniform distribution of tension between the various fibers composing the scaffold, allowing certain fibers to carry more load and fail sooner than others.

Fiber-embedded gel scaffolds (composed of 50 fibers) displayed an average tangent modulus of 83 MPa and peak stress of 5.4 MPa when cells were incorporated and the entire scaffold was maintained in static culture for 25 days. These values represent improvements of 68% and 86% in tangent modulus and peak stress, respectively, relative to fiber-embedded gel scaffolds without cells, and are on the same order of magnitude as (but lower than) properties of normal knee ligaments [29]. The application of in vitro cyclic mechanical stimulation (e.g., [21,22]) may provide a way to additionally strengthen these scaffolds. The reasons for the altered mechanical properties of the cell-seeded (as compared to the cell-free) scaffolds are unknown, but may be associated with cells functioning within the three-dimensional environment in such a way that, ultimately, external loads were applied more uniformly across fibers in the scaffold. This would produce the strong single peak observed at failure of the cell-seeded scaffolds (Fig. 4B), and the increased peak stress. In contrast, Fig. 4A shows multiple break points, indicating that failure of the cell-free scaffolds occurred in stages. Although elucidating the specific mechanisms by which cells affect load distribution within a scaffold is beyond the scope of this study, it seems reasonable that collagen-producing cells distributed throughout a gel between fibers might strengthen the gel between fibers, increasing the chances that adjacent fibers would act in concert. The cells did not preferentially populate the fibers within the fiber-embedded gel scaffolds (Fig. 3B). This is logical given the cell/collagen suspension method used to create the gel, and since both the fibers and the gel were made from Type I collagen. A non-uniform distribution of cells in the constructs might have indicated a biocompatibility problem. In natural ligament, fibroblasts lie in the space between collagen fibers where they remodel the collagenous tissue and produce extracellular matrix. Therefore, the architecture shown in Fig. 3B may be preferential in the development of ligament analogues.

While peak stresses are reported in this study for sake of comparison to previous data, peak stress is probably not the key mechanical property which will drive ligament analogue design efforts. The peak stress tolerated by an analogue must certainly be high enough (factor of safety) that the tissue is not forced to perform near its breaking point. However, matching the mechanical behaviors of natural and engineered tissues on the low end of the stress–strain curve may be equally important, since this is the region of normal, day-to-day ligament function. Obtaining an appropriate modulus and implementing a functional ‘slack zone’ (mimicking fiber recruitment and elongation in the toe region of the stress–strain curve) will probably be key aspects of designing clinically successful tissue engineered ligament replacements. Failure to include a suitable ‘slack zone’ during the implantation of a ligament replacement could lead to a prosthesis (with an acceptable modulus) which is either functionally too tight or too loose, and which therefore develops loads which are too high or too low, respectively.

5. Conclusions

A tissue-engineered product with excellent biological/chemical compatibility but which cannot withstand the mechanical loads incurred during typical conditions of use will not be clinically useful. The development of novel collagen gel/scaffold constructs requires a clear understanding of the mechanical properties of the constituent biomaterial, and data reported in this work should therefore enable the development of improved tissue analogues that meet specific mechanical demands. Even though collagen fibers are simple biomaterials, important structure/function relationships observed in this study still need to be developed and explained, including the effect of gauge length on apparent modulus, specific mechanical and biological contributions of included living cells, etc. Finally, the present work demonstrates that combining collagen fibers with collagen gels constitutes a straightforward approach to designing ligament analogues, maintaining the important flexibility in scaffold design offered by the gel (e.g., to embed cells during gel polymerization, entrap factors conducive to cell function, etc.) and improving the mechanical properties of the resulting construct.
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