Investigation into the Mechano-Chemistry of \textit{De Novo} Collagen Assembly

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I will never forget the day that Nector and I took a six foot stainless steel rod to an empty field and had a javelin throwing contest. Walking together to retrieve a throw, he stepped on a randomly placed, full Capri Sun juice pouch that had a straw in it, pointing right at me. He stopped to figure out what was under his foot, and I stopped to figure out what was hitting my leg. It took about two seconds of me getting drenched before either of us realized that we should move. I have found that taking the time to partake in the lightest moments of life is the only way to bear the heaviest.

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ABSTRACT

Collagen is the dominant structural protein in vertebrates, responsible for sustaining and transferring tremendous forces during joint activation and stabilization of the human musculoskeletal system. After decades of investigation, the embryonic developmental techniques employed by fibroblastic cells to control the monomer-level organization of collagen continue to remain a mystery. The proposed research in this dissertation is motivated by basic science to determine the essentiality of the cells in creating tissue in vitro. The results of this investigation strengthen the concept that upon completion of the initial anlage, cells are potentially relegated to mechanical stimulators / molecular production houses, and the mechanosensitive, physiochemical properties of the molecules drive tissue growth and maintenance. Successful demonstration will provide a significant opportunity for a generation of new engineering methods capable of acellularly recapitulating the development of load-bearing tissue.

This bottom-up approach to tissue engineering strongly emphasizes the necessity of maintaining monomeric control prior to, during, and subsequent to fibrillogenesis, in order to attain continuous, highly-aligned bundles of fibrils. The specific objective is to demonstrate the pronounced effect that mechanical guidance cues, in conjunction with critical-concentration molecular crowding, have on the kinetics, organization, and continuity of type I collagen structures. The over-arching hypothesis is that prior to polymerization, cells utilize the innate liquid crystalline behavior of collagen monomers to initially attain local organization, and this weakly stable property is globally and iteratively reinforced in the direction of applied mechanical stimulation.
CHAPTER 1: Introduction

In the field of tissue engineering, the terms mechanosensitive and mechanotransduction traditionally refer to the cell, whether it is cell-matrix interactions or cell-cell adhesions. The idea that the cell is a highly evolved living organism that can use external guidance cues to regulate its internal architecture and protein synthesis is readily accepted, and there are numerous investigations to support this. For review, see [1]. In our lab, we extend this concept by asking the question, “What if the extracellular matrix molecules are also mechanosensitive, in the complete absence of the cells. The proteins have had a nearly equivalent amount of time to evolve, and any built-in mechanisms to reduce the energy required to maintain the matrix would increase survivability in adverse conditions. Unfortunately, this line of thinking is commonly overlooked because equivalent structural systems on the macro-scale typically do not operate this way. There are no sets of conditions under which you can place a pile of wood and have it autonomously assemble into a house. However, we have long known that there are sets of conditions under which collagen monomers can spontaneously assemble into natively banded collagen fibrils [2].
Chapter 1.1: The Evolution of Collagen

The first recognition of the signature motif of the collagen molecule, a repetitive Gly-Xaa-Yaa tripeptide sequence, extends back approximately 800 – 1,200 million years to the unicellular choanoflagellate [3]. A choanoflagellate is a phenomenally evolved protist, recently recognized for genetic domains which were thought to be restricted to metazoans [3, 4]. In addition to C-terminus and triple helical collagen domains, choanoflagellates have the genetic information to encode cell adhesion and signaling molecules such as cadherin, C-type lectin, integrin-α, immunoglobulin, and tyrosine kinase as well as other extracellular proteins such as fibronectin and laminin [3, 4]. I have chosen to begin my dissertation with a description of choanoflagellates because an understanding of these protozoa offers a potential gateway to an understanding of how modern-day, multicellular life-forms began.

![Figure 1: The Choanoflagellate Structure. Image b shows the apical flagellum (arrow) and cell body. Image c shows the apical collar of actin-filled microvilli. Image d shows a composite image with the β-tubulin in green, the polymerized actin in red, and the DNA localization in blue. Scale bar = 2 µm. Image reproduced with permission from [3].](image)

The functional morphology of the choanoflagellates, described in exquisite detail by Leadbeater et al., comprises a slightly ovoid cell body 2-5 µm in diameter, a collar of up to 50 actin microvilli used for trapping food particles, and a microtubule flagellum for propelling the cell body [5-8], all shown in Figure 1. The choanoflagellate’s cell body maintains an organic coating of adhesive microfibrils (glycocalyx) so it can secure itself to a substratum, once in a nutrient rich region [5, 9]. By anchoring to restrict cell locomotion, the flagellum increases local water flow.
over the body by 10-20 times, which provides for an enhanced renewal of bacteria to be ingested [10, 11]. The attachment point is extended to a stalk-like structure (peduncle) to elevate the cell above the floating particle or seafloor to eliminate boundary layer effects [7, 11]. The choanoflagellate continues its extracellular matrix production with a single layer cup or triple layer flask-shaped, fibrillar structure (theca) that helps direct the fluid flow over the microvilli for feeding [5, 9]. The choanoflagellate then mechanically reinforces the entire system by secreting siliceous, rib-like structures (costae) [5, 6, 9, 12]. In the recent work of Gong et al., we learn that the siliceous shell is guided by an organic, proteinaceous core [13]. In addition to templating the biosilification process, the organic filaments serve as reinforcement to the links of the costal strips [13]. Each costal strip attaches at one end to the base of the peduncle, and at the other end to the tip of a microvillus (tentacle) [6, 12]. The actin-based tentacles project rigidly outward such that the costal strips are in a conical shape. The flagellum is then used to rotate the cell body, resulting in the helical twisting of the costal strips into an ornate structure akin to a ~100 µm long cornucopia (lorica) [6, 9, 12]. This process is displayed in Figure 2 for clarity. In other cases, the lorica is woven into more complex basket-like structures whose assembly process is still in question [5, 12]. The mineralization of fibrous proteins in the lorica of a billion year old species parallels the mammalian skeleton surprisingly well.
Figure 2: The Choanoflagellate Production of the Lorica. Images 1-3 and 4-6 display the production of the peduncle, the theca, and the early stage lorica for two different cells. Images 7-11 display the process of assembling the costal strips into the lorica. The cell utilizes a left-hand rotation to achieve the helical structure. Scale bars = 2 µm (1), 0.5 µm (7-11). Image reproduced with permission from [6].

While the primary focus of this document is collagen mechanochemistry, it is valuable to recognize the origin of the collagen molecule. This billion year old unicellular organism is the first known species with genes coding for the collagen motif that generates an organized extracellular matrix, which is then used to initiate colonization through cell division [14]. Within the confines of the lorica, the choanoflagellate produces excess costal strips just prior to cell division for the juvenile cell [5, 9]. The daughter cell stays if the nutrient supply is sufficient, and a colony forms through continual division [15]. Choanoflagellates require a dedicated use of their microtubule organizing center for either flagella-mediated locomotion or mitosis [15, 16], thus existing in a colony provides a symbiotic relationship in which the cells can alternate between flagellum-mediated nutrient turnover and cell division [15, 16].
This behavior is indicative of the evolutionary pathway of the sponge, the sister taxa of the choanoflagellate, a species with well-defined collagen applications [17, 18] and perhaps the first multicellular organism [3, 9, 16, 17]. The similarity between cells of a sponge, choanocytes, and choanoflagellates was recognized as early as 1866 [19]. Thus, the choanoflagellate offers an insight as to how the unicellular progenitor evolved into a multicellular diploblast. It is plausible that collagen contributed to the cell adhesion/signaling in the colony and the core proteins of the costal strips in the lorica, based on the genetic domains of the choanoflagellate and the sponge’s application of collagen in this precise manner [3, 4, 17, 18]. Since the Cambrian Explosion 530-570 million years ago, collagen has functioned as a load bearing material in sponges by providing mechanical anchorage to internal cells and a separating membrane from external cells, such that the diploblastic organism could evolve by tethering sponges down to the sea floor, and by providing a skeletal support in the form of short-chained collagen (spong) [20-24].

It may appear counterintuitive that it took 500 million years to transition from a choanoflagellate to a sponge, while in the same amount of time there was transition from a sponge to a human, but there are two primary reasons for this. First, the collagen molecule had to evolve to a length where it could functionally serve as more than just an adhesive. This process is the result of retained useful mutations, domain shuffling, and gene duplications [3, 16]. On average, 1% of genes duplicate per 1 million years, and the vast majority of changes are not retained in the species because they are not evolutionarily advantageous [25]. The genetic code for a collagen alpha chain began in the simplest form of 9 base pairs that coded for a single Gly-Pro-Pro sequence. Upon the initiation of gene sequencing in the 1970s, Yamada et al. demonstrated that this primary motif underwent gene duplication multiple times, until the exon length reached a preserved size of 54 nucleotides (6 Gly-Pro-Pro repeats) [26]. The molecule may have most efficiently served as a linker or stabilizing molecule between other proteins at this length [26].
1985 Runnegar extended this concept, benefiting from the advancements made in gene sequencing, with the realization that there are exons with a larger repeating structure of 702 nucleotides (13 sets of 54) [27]. Evidence suggests that during gene duplication of the 54 base-pair sequence, amino acid mutations occurred to replace certain proline residues with charged and hydrophobic amino acids [27]. This would aid in the stability of the molecule and perhaps also serve as a functional requirement for the splicing of the exons. In modern day vertebrates, collagen assembles in a staggered pattern with a D-periodic spacing of 67 nm (equal to 234 amino acids or 702 nucleotides) [27]. When working with collagen, it is useful to remember that the alpha chain likely had much less amino acid variation and may have even begun as glycine-proline-proline repeats [28], so every amino acid deviation from this could be an adaptation that was retained due to some evolutionary advantage. This must be appreciated when attempting to replicate the full function of collagen with a synthetic biomaterial.

The second reason for the limited role of collagen, as compared to its ubiquitous role today, is due to the concurrent evolution of the Earth’s atmosphere [29, 30]. Up until approximately 600 million years ago, the Earth was at a deficit for free molecular oxygen [31], as seen in Figure 3. This is essential because the enzymes which hydroxylate proline and lysine, prolyl hydroxylase and lysyl hydroxylase respectively, have an absolute requirement for molecular oxygen [32, 33]. Hydroxyproline leads to intramolecular stabilization and hydroxylysine leads to intermolecular stabilization, via lysyl oxidase (another oxygen-dependent enzyme) [34, 35]. Without these additional bonds to confer strength to the ECM, a load-bearing 3D matrix cannot be formed.
Figure 3: The Evolution of O$_2$. The top image depicts the level of atmospheric oxygen, and the lower two images depict the concentration of oxygen in the oceans. X-axis = billions of years ago. Image reproduced with permission from [31].

Putatively, there was a 500 million year gap between single celled organisms and multicellular organisms of substantial size because collagen was not yet capable of providing a large scale 3-D structural role. Using the 20 amino acids in the genetic code, there appears to be no other combination found which can replace the role of collagen. Collagen uses lysine, an essential amino acid, which heterotrophs must take in via dietary consumption, because they cannot synthesize it. Prolyl hydroxylase, lysyl hydroxylase, and lysl oxidase require molecular oxygen, which was not sufficiently present until nearly 600 million years ago [31], and furthermore this pathway is unfavorable because it is generally regarded as inefficient and competes with conventional respiratory pathways [36, 37]. Additionally, prolyl hydroxylase and lysyl
hydroxylase require ferrous iron, ascorbic acid, and \( \alpha \)-ketoglutarate [32, 34, 35]. The fact that collagen was still able to evolutionarily outcompete every other tested molecular arrangement is astounding. As stated by Robert Garrone, “From sponges to humans, collagen is always present, in all organisms, without exception,” [22, 38].

**Chapter 1.2: The Collagen Monomer and Fibril Structure**

A single collagen molecule is called a monomer, which centrally comprises three left-handed helical alpha chains [39], which intertwine to form a right-handed, 300 nm long and 1.5 nm diameter [40] coiled supramolecular assembly. Each alpha chain has a repeating sequence of Gly-X-Y [41-43], where X and Y are frequently proline (28%) and hydroxyproline (38%), respectively [44]. The triple-helical region persists for 1014 residues and is capped by an amino (16 \( \alpha_1 \), 9 \( \alpha_2 \) residues) and carboxyl (26 \( \alpha_1 \), 25 \( \alpha_2 \) residues) telopeptide extension [45]. There is a propeptide domain on either end of the molecule in addition to the telopeptide extensions. The propeptides serve as globular steric hindrance to prevent monomeric assembly into a fibril while the molecule is still intracellular. A diagram of the biosynthesis of a collagen monomer is depicted in Figure 4 [46].
Figure 4: Collagen Monomer Synthesis. Steps 1-6 occur intracellularly. The monomer retains its propeptides inside the cell to prevent premature fibrillogenesis. Once the monomer is transported outside the cell, the propeptidases remove the propeptides and the molecule becomes active. Image reproduced with permission from [46].
While more than 28 distinct collagen gene sequences for vertebrates have been identified [47, 48], the majority of attention has been placed on the fibril forming types (I, II, III, V, and XI) [49]. The fibrillar structure of collagen was first imaged in 1942 by Richard Bear, using low-angle x-ray diffraction, and Hall et al., using electron microscopy [50, 51]. Their investigation revealed that type I collagen fibrils, extracted from multiple species, all share a common D-periodic banding pattern of 64 to 67 nm. This pattern generates a regularly repeated gap that preferentially fills with stain during electron microscopy preparation and gives the collagen fibril its characteristic D-period, as shown in Figure 5. The gap region accounts for 0.54D and the overlap accounts for 0.46D [52].

![Collagen Fibril Periodicity](image)

*Figure 5: Collagen Fibril Periodicity. The fibril banding pattern is visualized through heavy metal staining during TEM preparation. The pattern is the result of a quarter-staggered arrangement of the molecules in the fibril. Image reproduced with permission from [53].*

The fibril was the smallest known subunit of collagen until 1954, when Gross et al. imaged single collagen monomers [39]. It was unknown how monomers organize within the fibrillar structure, until Hodge and Petruska put forth a five monomer intermediate structure, termed the microfibril [54]. Their 2D model utilized five monomers, progressively offset from each other by 67 nm, to account for the gap/overlap regions recognized within the banding pattern. In 1979 Hulmes et al. correctly posited that the triple helices are arranged quasi-hexagonally with respect to each other.
within the fibril [55]. More recently, Orgel et al. has added the first electron-density map, which confirmed that five neighboring monomers form their own right-handed microfibril [56]. This higher-ordered supramolecular structure is helical in nature and interdigitates with neighboring microfibrils to form the basis of the fibril [57]. Orgel and coworkers determined the axial location of the telopeptides in the microfibrils and found that neighboring telopeptides are covalently cross-linked, via lysyl oxidase, within and between microfibrils [58].

Chapter 1.3: Collagen in Tissues

Collagen is the predominant molecule in tissues under tension, compression, torsion, and hydrostatic pressure. The organization of the collagen is very tissue specific, with the morphology closely relating to the mechanical environment. Fibrillar type I collagen is either arranged into fiber bundles or into sheets with alternating direction, termed lamellae. The three categories of collagenous tissue are lamellae with a repeated angular offset from a reference line, uniaxial fiber bundles, and transversely isotropic fibrils.

Collagen is aligned in lamellae with alternating direction in blood vessel, bone, and intervertebral disc, represented by Figure 6. The fibrils within each lamella typically follow three primary directions: uniaxial, circumferential, and pitched at an angle to the long axis. The cornea shares a common structure, having orthogonal lamellae which are surrounded on the periphery by circumferential fibrils [59-63]. These tissues require such complex geometries to resist their complex loading profiles: tensile force, internal pressurization, and torsion. Since collagen is a rope-like molecule, the addition of hydroxyapatite is required in the bone to crystallize and resist the compressive forces. Figure 6 shows an osteon in cortical bone, where the haversian canals comprise helically pitched lamellae.
Collagen has a much simpler, yet no less hierarchical, conformation in tendon and ligament, as seen in Figure 7. Fibril forming collagens comprise 75 – 85% of their dry weight [64-66], and those fibrils are packed together into fibers, fiber bundles, and fascicles [67]. The collagen is densely packed in a predominantly uniaxial direction because these tissues are principally intended to resist and transmit uniaxial forces. Perhaps the most significant difference between tendon and ligament composition is the amount of elastin. Elastin comprises ~2% of the dry weight in human tendon, whereas ligaments can range from 5 – 47% of the dry weight, depending on the tissue [68-70].
Figure 7: Tendon Hierarchical Structure. Starting with the primary fiber bundle and increasing in size, each hierarchical level is surrounded by a sheath. The epitenon and endotenons have a high concentration of proteoglycans to facilitate lubricated sliding of individual subunits and to help bind the collagen fibers within the sheath. The sheath shares a common collagen structure to blood vessel, having fibrils oriented longitudinally, obliquely and traversly. Image reproduced with permission from [67].

The sclera and dermis are two tissues which exhibit predominantly transversely isotropic fibrils, although some semblance to laminar structure can be found in the sclera. Collagen constitutes 50 – 75% of the sclera [71, 72] and 80-90% of the dermis [73]. Skin and sclera are under substantially lower stresses than tissues such as bone and tendon, and as a result the structure is also under much less strict control. Fibrils are locally aligned but rapidly change direction with no evident global template, as seen in Figure 8. Interestingly, a very similar pattern emerges when 10 mg/ml collagen is extruded onto glass and allowed to dry down [74]. This may indicate a potential biological pathway, where the cells secrete collagen in abundance prior to cleaving of the propeptides.
Figure 8: Scleral Collagen, Dermal Collagen, and Extruded Collagen. Image A is of human scleral collagen, taken using SEM. Image B is of mouse dermis, using second harmonic generation. Image C is of 10 mg/ml collagen solution, extruded onto a glass coverslip, and allowed to dry down. In all images, the frequently changing direction is indicative of the lack of a prevailing guiding force, as seen in the tendon. Image A is reproduced with permission from [75]. Image B is reproduced with permission from [73]. Image C is reproduced with permission from [74].

Chapter 1.4: Tissue Engineering with Collagen

The type I collagen-based tissues in the human body are vastly different in their geometry and proteome (proteoglycans, glycosaminoglycans, and supporting collagen types), but can be grouped into three primary categories: lamellar, uniaxial, and transversely isotropic. Thus, the challenges associated with reconstructing a myriad of tissues, disregarding innervation and vascularization, reduce to controlling fibril diameter, fibril spacing, and fibril orientation. Adjustments to fibril diameter and spacing are energetically minimal tasks from the perspective that they involve the relocation of individual collagen monomers or water molecules. Collagen monomers sit on the precipice of stability/instability [76] and are highly mutable prior to the maturation of crosslinks [77, 78]. Lumican knockout mice can fully regain control over diameter and spacing with the addition of proteoglycans, even after tissue production [79, 80]. The crux of
tissue engineering is exerting strict control over the orientation of the collagen fibrils. The following subsections will review the methods implemented to generate organized collagen tissue, as well as the advantages and shortcomings.

**Chapter 1.4.1: Contact Guidance**

Contact guidance technique #1 uses dip-pen nanolithography. In 2001, Wilson *et al.* used an AFM in tapping mode to deposit a 40 mg/ml solution of collagen onto a gold-coated mica wafer [81]. Patterned lines were between 30 nm – 100 µm in length, 250 – 800 nm in width, and 50 – 200 nm in height. The authors found a banded periodicity in their lines when working in their upper range, but did not observe the same organization near the lower end of their range. Additionally, they had to thiolate their collagen in order for it to adhere to the gold surface, which resulted in the bulk solution no longer forming banding fibrils spontaneously.

Contact guidance technique #2 uses nanopatterning. In 2005, Denis *et al.* used a silicon wafer with tracks of monolayer methyl-terminated alkylsilane (CH$_3$) in between regions of monolayer oligo-(ethylene glycol)-terminate alkysilane (PEG) [82]. The CH$_3$ tracks were meant to attract collagen adsorption while the PEG was meant to prevent it. The CH$_3$ tracks were 30 – 90 nm wide and 30 µm long. Collagen solution was added to the wafer at 30 µg/ml and polymerized at 37°C. The resulting film was 5-9 nm high and 60 – 90 nm wide. There was no attempt to quantify fibrillar alignment except to say the track width was narrower than a monomer’s length and the edges of the films were fairly steep, so this indicates strong alignment. There was no comment on banding, and the author states a reduction in edge steepness in the wider CH$_3$ tracks.

Contact guidance technique #3 uses a microfluidic device. In 2006, Lee *et al.* used a silicon wafer to imprint tracks 1 cm long with widths ranging from 10 – 400 µm wide onto PDMS, which were then bonded to a glass coverslip [83]. Samples were filled with collagen at a concentration of 1.5
mg/ml, by injecting the solution at 5 – 10 mm/sec (flow condition) or by pipetting directly on to the PDMS and then sealing with the glass coverslip (no-flow condition). The 10 µm wide channels with the flow condition yielded the closest average alignment to the long axis of the channel, with over 40% of the fibers within five degrees of the axis. There was no variation in alignment along the width, despite the expected parabolic flow profile, and the thickness of the fibers was 0.3 – 0.5 µm. For channels with 200 µm widths or greater, there was no significant difference from collagen gelled on glass. The fiber lengths rarely exceeded 10 µm, thus the larger channel widths imposed a very weak boundary effect on such short fibers.

Contact guidance technique #4 uses a direct manipulation of the fibrils. In 2007, Cisneros et al. used an AFM tip to directly move fibrils, formed as a 3 nm thick gel on mica, into the desired orientation [84]. The collagen concentrations used throughout the experiments was unclear but one experiment used 12 µg/ml. The buffering solution was determined to have a substantial impact on fibril banding and initial alignment. The authors were unclear as to the optimal buffer, and instead use a variety of buffers across the experiments such as a buffer which mimics intracellular fluid, one that mimics extracellular fluid, and a 200 mM KCl – 50 mM Tris-HCl buffer. In order to manipulate the matrix, the gel was scanned in contact mode with forces set to 300 – 500 pN. The rate of manipulation and the maximum area manipulated was unclear, but the authors did state that there was only a 5 hour window of time after initial polymerization before the procedure became destructive.

Contact guidance technique #5 uses nothing more than the crystalline structure of mica. In 2007, Sun et al. showed that the crystalline structure and highly charged surface of mica strongly influences the orientation of polymerizing collagen fibrils [85]. In the absence of any external stimulation, 0.3 – 3.0 µg/ml collagen solution polymerizes directionally over a 90 x 90 µm² area.
Apparently, the mica lattice structure offers a preferential director to surface polymerization in a direction 60 degrees offset from the simulated diffraction pattern. The authors note that a sodium buffer was optimal for alignment and longitudinal aggregation, followed by an overnight incubation in a potassium buffer for lateral growth of the fibrils.

**Chapter 1.4.2: Electrochemically Induced Alignment**

Electrochemical alignment of collagen is unique method being performed principally by Dr. Akkus at Purdue University [86-88]. This method places two parallel wire electrodes, 0.254 mm diameter x 25 mm length, 1 mm apart in a solution of 6 mg/ml collagen suspended in deionized water. The electrodes are connected to a DC voltage source set to 6 V, with a 1 MΩ resistor set in series. The voltage measured between the electrodes was 2.5 V, which exceeds the 1.23 V electrolysis threshold for water. This electrolysis generated a pH gradient between the electrodes, causing an acidic pH at the anode and basic pH near the cathode. After 1 hour under these conditions, collagen had polymerized in the region that was ~ pH 7, with strong directional preference parallel to the electrodes. The authors claim a dehydrated collagen concentration of 1030 mg/ml [86]. The collagen fibrils lack D-banding at this time point and are incubated in PBS solution to trigger molecular shifting into periodicity. The concentrations tested are .5, 1, 5, and 10x PBS for times of 3, 12, or 96 hours in an incubator. For all time points, periodicity was only confirmed, via small angle x-ray scattering, in 1x PBS. The time study revealed little banding at 3 hours, fully recognizable banding after 12 hours, and no banding at 96 hours. The theory for the loss of banding at 96 hours is that the dibasic phosphate molecules bind to the positively charged amino acids, form salt bridges, and interrupt collagen molecule interactions [88].

**Chapter 1.4.3: Electrospinning**

There are many research groups involved in electrospinning collagen, but the process is largely unchanged. Typically, an electric potential is generated between the needle of syringe and a
rotating metal mandrel. Often times the spinning mandrel has round coverslips attached to the diameter to collect the fibrils for future cell testing. The syringe is filled with collagen monomers dissolved in 1,1,1,3,3,3 hexafluoro-2-propanol (HFP). The needle is kept a fixed distance from the mandrel, 4 – 6 inches, and a voltage of 15 -30 kV, in conjunction with a syringe pump set to 0 – 25 ml/hr, drives a jet stream of collagen through the air to the rotating mandrel. The HFP solvent evaporates in the air and a solid fiber is collected on the mandrel. A substantial advantage of electrospinning over other approaches is the ability to continuously produce fibers for hours at a time [89]. Collagen types I, II, and III have been successfully electrospun [89-92], but it remains very difficult to achieve strong fibrillar alignment, diameter control, or natively banded fibrils. Perhaps the most significantly changed parameter over the years is the collagen concentration. In the work of Matthews et al., the collagen concentration used was 0.083 g/ml [90], whereas Zhong et al. used 80 mg/ml [92]. This may be why Zhong et al. were able to reduce the angular distribution to ~ ±25°, but variable fibril diameter and frequent bifurcations were still an issue. Despite the benefits of rapid fiber production through electrospinning, Zeugolis et al. have put forth a decisive document on the matter, “Electro-spinning of pure collagen nano-fibres – Just an expensive way to make gelatin,” [93]. They demonstrate that HFP denatures collagen, destroys alpha chains, disrupts the triple helix, reduces the thermal denaturation temperature, and produces fibers void of internal structure [93].

Chapter 1.4.4: Magnetic Alignment
Magnetic alignment is a lightly explored field for collagen alignment, perhaps due its distance from physiological processes. In 1978 Worcester explained that aromatic residues are typically responsible for diamagnetic anisotropy [94]. However, only 1.6% of the total peptides are aromatic in collagen, and thus the diamagnetic anisotropy is a function of the peptide backbone. According to Torbet and Ronziere, the upper limit of the diamagnetic anisotropy of a single
monomer is $1 \times 10^{-25} J \cdot T^{-2}$ [95]. The aligning energy must exceed the randomizing thermal energy by a factor of 6, and yet the ratio for a single monomer is only $5 \times 10^{-3}$ for a 20 T magnetic field. Thus, the technique of magnetic alignment is restricted to applications aimed at the fibrillar level. The magnetic field is capable of inducing alignment once the fibrils exceed ~1000 molecules, but only until the steric hindrance of the network of fibrils begins to dominate. Additionally it should be noted that the magnetic force alone would only induce the fibrils to lie in a single plane perpendicular to the field, but would achieve no preferred directionality within that plane. This has led to the addition of confinement and contact guidance within that plane [96]. They physical boundaries as said to be on the order a few millimeters, but contact guidance investigations typically are on the order of tens of microns.

Chapter 1.4.5: Shear Flow
A shear flow microfluidic chamber is a common approach used to align collagen fibrils [97, 98]. Typically a neutralized collagen solution will be flown between parallel plates with a spacing of a 50 - 750 µm. Thermal control, initial concentrations, plate spacing, surface coatings, and flow rates are varied to optimize the deposition and growth of collagen fibrils on the substrates. Lanfer et al. have optimized their system by using a shear rate of ~200 s$^{-1}$ with collagen concentration of 0.8 mg/ml [97]. The amount of alignment was found to increase with shear rate, however higher shear rates yielded lower overall deposition. After an hour of continuous flow, 68% of the fibrils were within 5 degrees of the channel axis and the average fibril had a ratio of 40 times the length to diameter. While this study made no reference to banding, Saeidi et al. used TEM to investigate the structure and found a lack of native D-banding [98]. Their setup operated with 3 mg/ml collagen and tested shear rates up to 500 s$^{-1}$. Similarly, they noted an increase in fibril length / deposition with decreasing shear rates. As their fibrils grew in excess of tens of microns, the authors note a unique phenomenon where the fibril begins to hook, sometimes making a complete
180° turn. The misdirection of the fibril may be caused by a surrounding monomer network that initially coated the substrate, combined with the recoil of a tethered fibril if it fails to make attachment to the substrate upon elongation. Additionally, fibrils were often seen bifurcating and merging as growth progressed.

Shear flow has also been implemented to already polymerized collagen gels. In the work of Kureshi et al., a gel of 2 mg/ml collagen is held vertically, with the lower end making contact with filter paper [99]. As the fluid drains down the gel, the fibrils experience an orienting shear. The authors measure the fibril alignment using elastic scatter spectroscopy and conclude that the gels transitions from an anisotropy factor of 1 (isotropic) to 2, after one hour in this configuration. Local relaxation yields a factor of 1.55, which suggests the shear flows are enhancing the anisotropy. The author comments, “This is an indirect, slow and poorly controlled technique but alternatives using high-intensity magnetic fields or flow are equally difficult to apply,” [99]

Chapter 1.4.6: Other Methods
Two additional methods for orienting collagen are molecular crowding and fiber extrusion. These two methods more closely tie into the research of the following chapter, and thus will be discussed in the Introduction of Chapter 2.

Chapter 1.5: Limitations of Current Methodology
The most direct evidence that each method has significant shortcomings is in the fact that you cannot currently receive an engineered, collagen-based tissue for a surgical procedure. Perhaps the biggest drawback to contact guidance is the poor scalability. Most contact guidance techniques only produce a sample large enough for cell testing as a result. Using an AFM to deposit collagen greatly restricts the size of the tissue and is time consuming. Polymerizing collagen on tracks or mica surfaces imparts contact guidance, but the propagation of this
alignment is quickly lost. This limits the process to less than a 500 nm to 1 µm thick sheet/strip. Lastly, using an AFM to directly manipulate the fibrils is also a time-limiting process, which additionally is challenged by the small window of time before the gel sets and the method becomes destructive.

Electrochemical assembly is appealing because the SAXS images have scattering which is indicative of fibril banding after 12 hours of incubation. However, the few images shown of the actual fibrils reveal that the fibrils are fairly discontinuous, bifurcating, and quite often globular. The authors claim a dehydrated concentration of 1030 mg/ml, but there is no validation as to how this number was generated. The scalability of the process appears sufficient, in that fibers may be able to be produced at great lengths if the electrodes were made to great lengths. Based on the images of the fiber as a whole, the substantial changes in diameter make it questionable as to whether or not the fiber would stay together as one continuous unit.

The most obvious shortcoming of electrospinning is that the solvent used, HFP, appears to denature a substantial portion of the collagen monomers. However, more benign solvents have recently been used which are shown to retain the triple helical structure [100]. The diameter of the fibrils is held to a narrow distribution but the fibrils often have a spread in the alignment. Since there is very little literature pertaining to using benign solvents, this technique remains a pathway with future potential for tissue engineering.

Magnetic alignment of collagen has the advantage that it is a non-invasive technique. No harsh chemicals or synthetic material come into contact with the solution; however, immensely powerful magnetic devices are often required. The induced alignment is typically limited to a small window of time, once the fibrils comprise enough monomers to respond to the field but before too many fibrils are present and interfibrillar bonds / steric hindrances arise. Another
limitation of the system is the requirement of contact guidance to infer directional orientation in the plane. The use of contact guidance requires that structures are built at micron size scales and further assembly using a layer-by-layer technique is often necessary.

The final method discussed in this chapter, shear flow, does not exert sufficient control over the fibril formation process. Attention has been called to the fact that these fibrils hook / turn as they grow in length. The fibrils also lacked the native banding, which may be recoverable through proper post processing, but this has not yet been demonstrated. Even if periodicity is demonstrated, this technique may continue to be challenged by the 2D nature of the sheets of collagen that are produced. Once a monolayer of fibrils is formed, the all the surface sites of the treated substrate are now covered and no clear path to transition to a 3D structure has been put forward.

**Chapter 1.6: Conclusions**

Chapter 1 is designed to elucidate an appreciation for the complexity of a biological molecule, the collagen monomer. The triple helical structure has grown and evolved for approximately a billion years, which promotes it above any ordinary or synthetic polymer that we may apply to tissue engineering. Its first application in prokaryotes was likely the adhesive that held the lorica of the choanoflagellate together as well as the core fibers within the siliceous costal strips. Its first application in multicellular organisms was mechanical anchorage of the sponge to the seafloor as well as the structural molecule that allowed the sponge to sustain 3D geometries. Collagen has always provided a load-bearing function and no other biological molecules have out-competed it. Collagen has been evolutionarily tuned for one billion years to best serve as a structure molecule.

Chapter 1 does not delve into the mechanical properties of each tissue because the emphasis is placed on the packing, alignment, and hierarchy found in collagenous structures. The same
molecule, collagen type I, is predominant in all of the tissues discussed; thus, any interest is the mechanical properties should be reserved as a metric to infer the packing and alignment. It is well known that collagen mechanical properties are highly sensitive to the degree of crosslinking and can be grossly skewed upward despite a lack of recapitulating the fundamental structure. When tissue engineering, the goal must be to reproduce the fibrillar landscape of native tissue. There are three predominant structures which comprise type I, collagen-based tissues: lamellar, uniaxial, transversely isotropic. If one of these templates can be engineered, then the translation to making each tissue within that category might be as simple as incorporating the proper proteoglycans, glycosaminoglycans, supporting collagen types, and supporting molecules (hydroxyapatite).

The final portion of Chapter 1 was intended to reveal the challenges associated with assembling tissues and scaffolds using collagen, as well as the diverse ways that have been attempted. Mimicking native tissue requires control over fibril diameter, length, and orientation. The collagen fibrils require the D-periodic banding to exemplify proper monomeric position. The fibrils should not have bifurcations or spontaneously merge. The implemented process needs to be scalable such that full tissues can be generated and rapid enough to support commercialized production. The path to successful tissue engineering will be incredibly difficult, which is why over eighty years of investigation have already taken place [101]. I am of the opinion that if we try to engineer collagen as though it is any other building block, we will spend another eighty years. However, if we can discover the evolutionary advantages contained within the collagen monomer, fibril, and macrostructure, then we will achieve much more in much less time. Perhaps the two most important mechanisms are sensitivity to strain and critical concentration.
CHAPTER 2: Utility of an optically-based, micromechanical system for printing collagen fibers

Chapter 2.1: Introduction

This chapter begins by exploring two potentially biomimetic methods for organizing collagen: liquid crystalline assembly and fiber extrusion. Since both methods have their merits, I attempt to marry the two approaches by printing an acidic collagen solution out of a 100 µm ID needle into neutralized polyethylene glycol. The high-gauge needle offers a fiber-like geometry, and the concentration of PEG is designed to induce liquid crystalline alignment of the monomers. A recognized difficulty of liquid crystalline assembly is that the macroscale template often follows multiple directors [102]. Directors of liquid crystalline alignment take numerous forms, such as an impurity in the solution, a chemical gradient, a strain gradient, a concentration gradient, or confinement in a high aspect ratio space. The aim of this work was to reduce the overall geometry of the system down to a single fiber, such that directors would guide the system in one direction and uniaxial alignment could be achieved. This method would offer a rapid, continuous production system that could be easily scaled by adding a fiber collection stage.

Chapter 2.1.1: Vivo Collagen Organization Theory

In developing new techniques for assembling collagen, efforts have been made to understand how cells might secrete an organized collagen matrix in vivo. One leading theory suggests that cells are directly responsible for the placement of each collagen fibril. According to Canty et al., collagen propeptides are internally cleaved and trafficked to a plasma membrane protrusion, termed a fibripositor [103, 104]. It is here that the collagen is arranged into fibrils to be deposited in the extracellular matrix, aligned longitudinally along the tendon axis. However, Canty et al. state that this fibripositor structure only exists during an early period of embryonic development, requiring for at least one more method to explain continual growth and development [103].
second theory postulates that cells utilize the liquid crystalline behavior of high aspect ratio molecules, such as collagen, to produce self-aligned fibrils. Hukins et al. first recognized the smectic A and smectic C behavior of native tissue and theorized that this property could provide insight into tissue formation [105, 106]. Different laboratories have attempted to organize collagen in a manner mimetic of these two postulates.

Chapter 2.1.2: Liquid Crystal Based Approach
Giraud-Guille et al. demonstrated that monomeric solutions of collagen held at an acidic pH would display cholesteric, liquid crystal behavior once the concentration reached approximately 100 mg/ml [107, 108]. Following this work, Martin et al. asked the question whether this behavior had the potential to occur under native conditions [109]. Indeed, nematic, pre-cholesteric, and cholesteric characteristics were observed as the concentrations increased from 5 to 30 mg/ml, when using procollagen at physiological pH and ionic strength [109].

Chapter 2.1.3: Fibripositor Based Approach
Twenty years ago, the work performed by Kato et al. introduced a model design for extruding collagen into a fibril forming buffer (FFB) [110, 111]. Collagen was injected through tubing into an aqueous bath, which was held at physiological temperature, pH, and ionic strength. The fiber was transported via a conveyor belt to an isopropyl dehydration stage, next to a distilled water wash station, then allowed to air dry, and finally collected on a spool. Following this model, Kato et al. and Dunn et al. characterized the extruded collagen with mechanical testing of fibers produced from different size tubing as well as those exposed to various cross-linking techniques [111, 112]. In 1993, Cavallaro et al. made a significant modification by using polyethylene glycol (PEG) as the FFB. This hypertonic environment causes molecular crowding of the monomers, generating a denser collagen fiber which may be continuously extruded without fractures [113].
Since this work, numerous investigators have continued using variations of the model design in combination with the PEG FFB [114-121].

Chapter 2.1.4: Limitations to FFB Fiber Printing
While mechanical properties, biocompatibility, and production rates have improved greatly over the past two decades, experiments have still failed to attain complete control over fibril organization. In 2009, Caves et al. were able to print at 60 m/hr, however they state that their collagen alignment was not uniform [115] and their fibers were not comparable in strength to native tendon. In spite of significant gains in throughput and mass production and moderate gains in mechanical properties, achieving native collagen fiber strength remains a significant challenge [122]. Although the fiber modulus can be improved via numerous available cross-linking techniques, they are typically associated with cytotoxicity, integration, and degradative issues [123-125]. Furthermore, cross-linking does not address what we believe is the fundamental problem underlying the inadequate mechanical performance of FFB derived fibers: poor molecular/fibrillar alignment within the fibers. Thus, there remains a need to develop methods which exert control over the nanoarchitecture of the fibers, preferably at the time of drawing, when the collagen molecular kinetics may be more readily controlled. Failing to address this issue and simply using disorganized scaffolds may improperly template the cellular patterning via contact guidance [126]. As stated in 2011 by Caliari et al., “…a range of studies have suggested that successful regeneration templates for natively aligned tissues such as peripheral nerves, the myocardium, and tendon must provide tissue specific aligned contact guidance cues that recapitulate aspects of the tissue anisotropy [127].”

Chapter 2.1.5: Addressing the Limitations
To increase collagen alignment in printed fibers, we consider how this might be achieved in vivo. The coordination required for cells to communicate and orchestrate the handling and placement
of every collagen monomer and fibril appears excessively complex. We have proposed a simpler hypothesis, where in place of direct cellular control there exist guiding cues which make it energetically favorable for the collagen molecules and microfibrils to shift into alignment. We believe that this signal is potentially tensile strain and that it may be coupled with molecular crowding to produce organization. However, no system currently exists in which this hypothesis may be effectively tested. We have therefore designed an optically-based micromechanical testing and fiber printing device. While previous methods perform the mechanical testing after significant post-processing (fiber relocation through the surface tension of fluids, dehydrating, rehydrating, and pre-straining [110-121]), our device is located on an optical microscope for magnified, real-time imaging and for in situ mechanical testing. One additional benefit is that it scales down the use of consumables and size of the system (previously a fifteen foot long FFB setup [115], now a one inch long chamber). With this configuration, we can perform parameter manipulations and mechanical testing to infer how the fibril structure was affected by parameter manipulation, coupled with qualitative and quantitative information gathered through light microscopy.

Chapter 2.2: Materials and Methods

Chapter 2.2.1: Collagen Sources
The collagen printing and mechanical testing protocol was developed using bovine type I atelo-collagen in the form of monomeric solution (5005-B, Advanced Biomatrix, San Diego, CA) purchased at 3 mg/ml concentration in 0.01M HCL. Because this collagen source was pepsin extracted, the monomers lack intact native telopeptides [128]. For comparison, some experiments were performed using acetic-acid extracted, type I telo-collagen from 1 year old bovine sclera (Research 87, Boylston, MA). Acetic acid extraction of collagen retains the telopeptides which can influence the assembly kinetics and morphology of the assembled fibrils [129, 130].
Chapter 2.2.2: Isolation and Purification of Telo-collagen from Tissue

To isolate the bovine scleral collagen, the scleral bulbs were separated from the cornea, fat, muscle, optic nerve, and retina. The sclera was thoroughly washed with deionized water, diced, and placed in 0.4 M acetic acid for extraction at 4°C for 3 days. The solution was passed through a polystyrene 0.5 cm sieve and then through a 0.3 mm mesh to separate out the solid, cross-linked tissue. To further separate out the finer tissue material, the solution was centrifuged at 8,000 rpm at 6°C for 45 minutes and the supernatant was collected. Upon achieving a transparent solution, the acidic collagen solution was subjected to a sodium chloride precipitation at 3.5% wt./vol at 4°C for 12 hours. The precipitated collagen was then centrifuged at 8,000 rpm at 6°C, the supernatant was discarded, and the pellet was resuspended in 0.01 M HCl. This step was repeated to separate out the precipitated collagen that would not fully dissolve. The solution was concentrated through reverse-dialysis in 3,500 molecular weight cut-off tubing (133198, Spectrum Labs, Rancho Dominguez, CA) against 20% wt./sol. wt. PEG (Sigma Aldrich, St. Louis, MO), in 0.01 M HCl. The solution of collagen was then dialyzed in 50,000 molecular weight cut-off tubing (132129, Spectrum Labs, Rancho Dominguez, CA) against 0.01 M HCl to ensure that the solution was free of PEG and collagen fragments. Finally, the monomeric solution was passed through a 0.45 µm filter (09-719-007, Fisher Scientific, Waltham, MA). Solution purity was verified through an SDS PAGE (456-9036, Bio-rad, Hercules, CA), shown in Figure 9. Figure 9A displays the molecular weights found in commercially available PureCol collagen, while Figure 9B provides the molecular weight ladder associated with the gel. Figure 9C displays the extracted scleral collagen, demonstrating successful removal of impurities and partially digested protein. Both collagen sources were brought to a final concentration of 1.8 mg/ml in 0.01 M HCl for all testing, verified through a Sircol assay (S1005, Biocolor, United Kingdom).
Figure 9: SDS PAGE of collagen samples. A) Purchased atelo-collagen solution (PureCol). B) Molecular weight ladder. C) Acetic acid extracted bovine scleral telo-collagen

Chapter 2.2.3: Assembly Kinetics Assay
It is well documented that the intactness of the telopeptides has a significant impact on fibrillogenesis kinetics [129, 131, 132]. Thus, to investigate the success of the acetic acid extraction on preserving the telopeptides, a turbidity assay was performed using a Powerwave XS Spectrophotometer (BioTek, Winooski, VT). Performed at 37 °C, 200 μl of neutralized 0.5 mg/ml telo-collagen and atelo-collagen, n = 3 for each, was scanned for absorbance using a wavelength of 313 nm.

Chapter 2.2.4: Collagen Fiber Printing
Prior to printing collagen fibers, a 0.4 ml supply of collagen solution was seeded with 0.15 μl of 3 μm polystyrene bead suspension (09850, Polysciences, Warrington, PA), purchased at a concentration of 1.68 x 10⁹ particles/ml. These beads served as markers, embedded along the fiber length, to allow for the measurement of local strain. A custom printing apparatus, operated on a TE-2000E inverted microscope (Nikon, Melville, NY), facilitated the production of collagen fibers, as shown in Figure 10. The chamber was filled with 750 μl of 30 or 35% wt./sol. wt. PEG
in 1X PBS at a pH of 7.3. The PEG served as a molecular crowding agent to “force” molecular association of the collagen monomers. The PEG concentration was chosen based on the 30% PEG concentration used by Girard-Guille et al. to produce liquid crystalline collagen with high local alignment [102, 133]. Our intent was to extend the tested range to 35% and then to 40% (approaching the maximum solubility limit); however evaporation during time required to run an experiment caused the 40% PEG to precipitate PEG.

A calibrated glass micro-needle was lowered into the chamber to serve as an anchor point for the collagen printing. To deliver precise volumes and flow rates of the collagen solution, the printer assembly utilized a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA) with a stiff transmission line comprising 1) a glass syringe (81320, Hamilton Company, Reno, NV); 2) relatively inextensible polyether ether ketone (PEEK) tubing; 3) a steel extension tube (held in a micromanipulator) and 4) a 100 μm ID stainless steel needle (9990448, Integrated Dispensing Solutions, Agoura Hills, CA).
This printer assembly was primed with collagen solution and then lowered into the chamber, approximately 20 μm away from the calibrated micro-needle. Once in position, the flow was started and one to two seconds were allotted for the collagen solution to encompass the calibrated micro-needle. Next, the printer assembly began moving away at 1750 μm/sec for a distance of 12.5 mm, at which point the syringe pump was manually turned off. Seconds after completing the print, the collagen solidified within and around the printer needle, as well as around the calibrated micro-needle. The syringe pump was operated under one of three flow rates depending on the experiment. The flow rate was termed 100% when the average fluid velocity out of the printer needle was equal and opposite to the velocity of the printer assembly moving away from the calibrated micro-needle. The flow was termed 75% or 50% when the average fluid velocity was set to the respective percentage of the velocity of the printer assembly. These values would be analogous to draw ratios of 1:1, 1:0.75 and 1:0.50.

Chapter 2.2.5: Pipette Stiffness Calibration
A 1 mm diameter, borosilicate glass rod was pulled into a finely tipped micro-needle using a custom program on a P-97 Micropipette Puller (BR-100-10, Sutter Instruments, Novato, CA). Micro-needle stiffness was determined by following the protocol described by Flynn et al. [134]. Briefly, the profile of the micro-needle was imaged using a 20X objective on a TE-2000E inverted microscope. The moment equation for beam bending was numerically integrated over the length of the micro-needle, and a bending stiffness in terms of nN/μm of deflection was calculated for every x-location along the length. A finite element non-linear analysis (SolidWorks, Waltham, MA) was performed to determine at which magnitude of tip displacement the stiffness would shift by 5%. The micro-needle stiffness reduced to 95% original stiffness at approximately 450 μm tip displacement. Micro-needles were pulled with an appropriate stiffness to allow for a 10% collagen fiber strain, while having tip displacements less than 300 μm.
Chapter 2.2.6: Analysis of Cross-Sectional Area
The fiber printing process consistently yielded fibers of visually uniform diameter along the 12.5 mm length, with increased dimensions at either attachment point. Observed by z-scanning with differential interference contrast light microscopy, the cross-sectional geometry initially appeared circular, as expected due to the uniform hypertonic PEG environment acting on the printed column of collagen solution. However, shortly after printing, the structure had taken on folds, ridges, and crevices. To normalize for water content in the printed fiber and to facilitate comparison to well-packed tendons, the cross-sectional area was defined as the total cross-sectional area of the collagen molecules in the fiber cross-section, which is independent of the PEG concentration. The number of collagen molecules was calculated from the initial collagen concentration, flow rate, and printing velocity. Every 300 nm, approximately 6.74e6, 5.06e6, and 3.37e6 monomers were deposited into the fiber for 100%, 75%, and 50% flow rate, respectively. From this, the total collagen monomer cross-sectional area could be determined, using a monomer diameter of 1.5 nm. This approach resulted in an effective cross-sectional area of 11.92, 8.94, and 5.96 μm² for 100%, 75%, and 50% flow rate, respectively.

Chapter 2.2.7: Mechanical Testing

Chapter 2.2.7.1: Stepped, Static Mechanical Testing Protocol
Fibers printed into the PEG visually reached their final structural geometry within the first few minutes; however, each fiber was given 15 minutes to equilibrate with the PEG before mechanical testing. This was an attempt to permit any molecular shifting within the fibrils that could not be visually detected. The mechanical testing was a three step process, automated through custom code written in the microscope software, NIS Elements (Nikon, NY). First, a region of the fiber containing two marker beads separated by 150 – 350 μm was identified, imaged, and measured using edge detection algorithms. The algorithm required the recognized image of the bead to have a minimum diameter of 2.5 μm. This led to a maximum positional error
of 0.25 µm in locating the center of each bead, or equivalently, a total 0.50 µm error in estimating the length separating the two beads. Second, an image of the calibrated micro-needle was captured with respect to a constant position, which permitted tracking of the micro-needle displacement throughout testing. The point of applied load on the micro-needle was designated as where the long axis of the fiber intersected with the micro-needle. Third, the printer assembly generated strain by moving 100 µm away at a rate of 10 µm/sec. Once the 100 µm displacement was complete, the fiber was given 35 sec before the position of the calibrated micro-needle was captured and another 35 sec for the microscope stage to return to the region of fiber with the two marker beads. This process continued until the fiber had reached a 10% strain, as calculated by NIS Elements. Stress-strain testing was performed predominantly on the purchased atelo-collagen, PureCol, due to its high purity and commercial availability. A baseline stress-strain curve was produced using 30% PEG and 100% flow rate. Telo-collagen was printed under the same conditions to ensure no unexpected behavioral changes.

Chapter 2.2.7.2: Stress Relaxation Testing Protocol
Stress relaxation testing was implemented to determine whether the time invariant modulus or the viscoelastic behavior was primarily responsible for the change in stress-strain curves. The two conditions tested were those which yielded the shallowest and steepest stress-strain curve. Each fiber was printed under its respective conditions and given 15 minutes to polymerize. An initial positioning of two marker beads was taken prior to testing, such that the strain could be measured as described in the mechanical testing section. The printer needle was used to increase the length of the fiber by 250 µm, at a rate of 10 µm/sec, and the calibrated micro-needle was imaged every 5 sec for 300 sec. At 300 sec, the microscope stage shifted to the location of the marker beads and an image was taken to calculate strain. The process was repeated five times such that six stress relaxation curves were produced from each printed fiber.
The experimental data was fitted to a Generalized Maxwell-Weichert model, commonly used for modeling the viscoelastic behavior of collagen [135, 136]. The model serves as an adaptation of Fung’s quasi-linear viscoelastic model [137], which comprises a variable amount of Maxwell elements (a spring in series with a dashpot) in parallel with an additional spring. The multiple Maxwell-Weichert elements are used to account for different relaxation time scales resulting from different physical sources. The data was modeled using one through four elements and curve fit using a Levenberg-Marquardt approach. The model that produced the fewest coefficient sets with an r-squared above 0.90 was selected as the optimum fit.

Chapter 2.2.8: Scanning Electron Microscopy

Fibers produced for scanning electron microscopy (SEM) were printed, fixed, and dehydrated directly in the custom flow chamber at room temperature. Prior to using the flow chamber, we attempted to raise the fiber out of the PEG solution and place it into primary fixative. During extraction we noticed severe plastic elongation of the fiber as it exited the surface of the PEG. This technique was discontinued to prevent artificial, strain-induced alignment [138]. Instead, the fiber was fixed directly in the chamber via a series of fluid exchanges prior to extraction for SEM imaging. All fluid exchanges comprised a volume of 5 ml, transferred at 4 ml/hr. During exchanges the calibrated micro-needle was observed to ensure there was minimal loading produced by the flow. The tip displacement from the fluid flow was always less than 10 μm, resulting in insignificant strain for a 12.5 mm fiber.

The first fluid exchange was with the same concentration of PEG and the addition of 4% glutaraldehyde at 7.3 pH. The fixative was given forty five minutes to cross-link the collagen fiber, after the full 5 ml were flowed into the chamber. This was followed by a second fluid exchange to 35% ethanol, a third fluid exchange to 70% ethanol, and a fourth fluid exchange to 100% ethanol. Finally, the fiber was raised from the chamber at 10 μm/sec, placed on a glass
cover slip, sputter coated in gold-palladium, and captured for examination under the S-4800 scanning electron microscope (Hitachi, Japan).

Chapter 2.2.9: Fast Fourier Transform (FFT) Analysis
FFT is a powerful mathematical tool for image analysis. It converts an image from the spatial domain to the frequency domain to reveal edge alignment and repeating patterns. In our case, the FFT method was used to quantify the orientation of collagen fibrils from an SEM of a printed fiber. All SEM images were imported into NIH – ImageJ software for histogram stretching to enhance edge clarity. Custom coding was written and tested in MATLAB to perform FFT on our SEM images. A plot of the frequency domain was generated to qualitatively display the fibril alignment within the collagen fiber, as well as a polar plot of the angular distribution of fibrils for quantitative analysis. Histogram bin size was set to one degree, and angular orientations were considered between 0 to 180 degrees. The smallest range of orientations containing 70% of the fibril edges was reported, with a smaller range indicating a higher degree of organization.

Chapter 2.2.10: Statistical Analysis
Statistical analysis was performed in Excel using the Student’s T-Test to determine if two groups of data were significantly different. A two-tailed distribution and two-sample equal variance T-Test was selected. Data was considered statistically different for p-values below 0.05. With respect to the mechanical testing, statistical analysis was performed at each strain value when comparing two distinct collagen printing conditions and the highest p-value was reported.

Table 1: Results summary of the stepped, static mechanical testing

<table>
<thead>
<tr>
<th>Collagen Type</th>
<th>Starting Concentration (mg/ml)</th>
<th>Samples Tested</th>
<th>Flow Rate</th>
<th>Polyethylene Glycol Concentration (wt/wt%)</th>
<th>Average # of Monomers per 300 nm</th>
<th>Single Monomer Cross Sectional Area (μm²)</th>
<th>Total Collagen Cross Sectional Area (μm²)</th>
<th>Modulus at 2% Strain (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telo</td>
<td>1.8 pH 2.0</td>
<td>6</td>
<td>100%</td>
<td>30% pH 7.3</td>
<td>6.74E+06</td>
<td>1.77E-06</td>
<td>11.92</td>
<td>12.3 ± 1.13</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>100%</td>
<td>30%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>75%</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>50%</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atelo</td>
<td>1.8 pH 2.0</td>
<td>7</td>
<td>100%</td>
<td>35% pH 7.3</td>
<td>6.74E+06</td>
<td>1.77E-06</td>
<td>11.92</td>
<td>16.5 ± 2.12</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>100%</td>
<td>35%</td>
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<tr>
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</tbody>
</table>
Chapter 2.3: Results

The collagen printing setup, which was designed for mechanical testing and fluid exchange, is depicted in Figure 10. A strained fiber with marker beads and typical micro-needle displacements are shown in Figure 11 and Figure 12, respectively. All test conditions explored and physical/mechanical parameters found are presented in Table 1.

Figure 11: Typical collagen fiber with two marker beads used for strain measurement. The upper image is the fiber after printing. The middle image is the fiber at ~5% strain. The bottom image is the fiber at ~10% strain.
Figure 12: Calibrated micro-needle tip displacement during a stress-strain test. A) 0% strain. B) ~5% strain. C) ~10% strain.

Chapter 2.3.1: Assembly Kinetics

Shown in Figure 13, the telo-collagen reaches 50% of maximum absorbance approximately six times faster than the atelo-collagen. The parameters tested for significance were the time to reach 50% maximum absorbance, linear growth rate, and lag time. Linear growth rate was defined as the slope of the linear regression line during the growth portion of the curve, which was observed between the one-quarter and three-quarter times. Lag time was defined according to Silver et al., as the intersection of the linear growth phase regression line with the x-axis [139]. Using a two-sample equal variance t-test, 0.014 was the highest p-value calculated, indicating that there are substantially different assembly kinetics between the purchased atelo-collagen and the extracted telo-collagen, as expected.
Figure 13: Turbidity assay of collagen polymerization. Solutions made from refrigerated constituents were adjusted to pH 7.3 and scanned at 313 nm in a plate reader at 37 °C.

Chapter 2.3.2: Biomechanical Analysis

Chapter 2.3.2.1: Telo-collagen vs. Atelo-Collagen
While atelo-collagen is capable of forming normal cross-striated fibrils [140, 141], the telopeptides have additional benefits that may be essential for forming replacement tissues for biological applications. The telopeptides play a pivotal role in intermolecular covalent bonding [142, 143], as well as enzymatic resistance [144, 145]. However, this work focuses primarily on enhancing uniaxial alignment and molecular packing. Figure 14 compares the mechanical behavior of telo-collagen fibers with atelo-collagen fibers printed at the same conditions. The stepped, static mechanical behavior of printed atelo-collagen and telo-collagen indicates the telo-collagen produces a slightly stiffer fiber (for the condition tested). The two curves are statistically different but share the same curve shape. This is indicative that the impact of parameter changes on the commercially available atelo-collagen may be cautiously extended to the telo-collagen.
Figure 14: Stepped, static stress-strain curves for printed collagen fibers as a function of collagen type (atelo-collagen vs. telo-collagen). The telo-collagen appears to yield a stiffer fiber.

Chapter 2.3.2.2: Static, Stepped Mechanical Testing

Figure 15 shows the static, stepped stress strain curves as a function of varying draw ratio (flow rate). In Figure 15A, the condition of 30% PEG is shown while Figure 15B shows the results for fibers printed in 35% PEG. The trend clearly shows an increasing modulus as the draw ratio increases (flow rate decreases). In Figure 16 all of the stepped, static mechanical tests are plotted together. The trend of the curves reveals the influence of PEG concentration and draw ratio on the printed fiber mechanical properties. It can be readily observed that increased draw ratio and increased PEG concentration leads to stiffer fibers as expected. The strongest fiber was produced at 35% PEG and 50% flow rate (draw ratio of 2:1) while the weakest fiber was generated at 100% flow rate and 30% PEG (for atelo-collagen). Figure 17 compares the tensile modulus at 2% strain for the all of the conditions tested and Table I summarizes the results of Figure 17. Statistically significant differences in the moduli were reached between many of the conditions and are summarized in Table 2.
Figure 15: Stepped, static stress-strain curves for printed atelo-collagen fibers as a function of flow rate (draw ratio). A) Fibers printed in 30% PEG and B) Fibers printed in 35% PEG. The data suggest that increasing the draw ratio leads to stronger fibers.

Figure 16: Stepped, static stress-strain curves for printed collagen fibers at all conditions. Generally, increasing the PEG concentration and lowering the flow rate (increasing the draw ratio) led to stiffer fibers. The data presented is the average for each condition, but error bars were removed for clarity.
Figure 17: Comparison of the fiber modulus at 2% for the various conditions.

Table 2: Summary of the relevant statistically significant differences found between conditions.

<table>
<thead>
<tr>
<th></th>
<th>Telo 30% PEG 100% Flow</th>
<th>Atelo 30% PEG 100% Flow</th>
<th>Atelo 30% PEG 75% Flow</th>
<th>Atelo 30% PEG 50% Flow</th>
<th>Atelo 35% PEG 100% Flow</th>
<th>Atelo 35% PEG 75% Flow</th>
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<td>No</td>
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<tr>
<td>Atelo, 30% PEG, 50% Flow</td>
<td>---------------</td>
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<td>No</td>
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<tr>
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<td>---------------</td>
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<td>Yes</td>
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<tr>
<td>Atelo, 35% PEG, 75% Flow</td>
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<tr>
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<td>Yes</td>
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</table>

Chapter 2.3.2.3: Relaxation Testing

Mechanical relaxation testing was performed under conditions which generated the stiffest and the softest fibers (35% PEG/50% flow rate and 30% PEG/100% flow rate). Figure 18 shows a typical relaxation test performed under the conditions which produce the stiffest fibers. Data from the tests similar to that shown in Figure 18 were analyzed to extract the relaxation time constant, $\tau$, the equilibrium modulus, $E_0$ and the dynamic modulus, $E_1$. In Figure 19 we can see that the
extracted equilibrium modulus decays with increasing strain for both conditions. It should be noted that the equilibrium modulus found at 2% strain matches our static data very well. For the stiffer fiber we found an average equilibrium modulus of $22.45 \pm 3.60$ MPa, $\tau$ of $100.69 \pm 31.48$ sec and a dynamic modulus of $6.20 \pm 2.04$ MPa. While there was a significant difference in the aggregate $E_0$ and $E_1$ ($p << .01$), we found no difference between the fibers for the time constant ($p = 0.84$). Figure 20 shows the results of a typical Levenberg-Marquadt extraction analysis where the parameters which result in a high correlation coefficient ($>0.90$) are clustered for a single experiment.

Figure 18: A representative stress relaxation test performed on a fiber printed at 50% flow into 35% PEG. The fiber was pulled 250 µm at a rate of 10 µm/sec and given 300 seconds to relax, a total of 6 times.
Figure 19: Comparison of the equilibrium tensile modulus, $E_0$, at increasing strain for conditions which produced the stiffest and the softest fibers. The modulus found in the stepped, static mechanical testing has been inserted for comparison at 2% strain. Four fibers were examined at each condition.

Figure 20: A 3-D representation of the sets of $E_0$, $E_1$, and $\tau$'s in the equation $E = E_0 + E_1 e^{-\tau}$ that fit the data with an $R^2$ value greater than 0.90 (generated from the second relaxation curve of Figure 18). The blue volume comprises the sets of parameters which satisfy the correlation criteria, while the areas are projections onto their respective planes. The green and red areas clearly depict the narrow range of time invariant modulus values, $E_0$, which fit the model.
Chapter 2.3.3: Fiber Morphology

Many attempts have been made in the literature to resolve the surface fibrillar structure of extruded fibers. Unfortunately, most imaging attempts have shown a uniform, relatively featureless shell with macro-scale ridges and crevices [113, 114, 117-121, 146]. Figure 21 is a representative atelo-collagen fiber, printed into 30% PEG with 100% flow, which has been split and imaged by SEM. The outer surface structure was concealed by the external shell-like feature seen by others, but the fracture revealed the inner fiber structure to some degree. While the organization and orientation of the inner fibers were disrupted by the intentional fracturing of the sample, the relative uniformity in the diameter of the inner fibrils was apparent. In addition, there appears to be some degree of alignment of the collagen fibrils within the printed fiber.

![Figure 21: SEM of fractured fiber printed in 30% PEG at 100% flow. Note the external featureless “shell” and the internal fibrillar structure.](image)

Figure 22 shows another atelo-collagen fiber, printed into 30% PEG with 100% flow. In this case, the external shell was not evident and the structure of the smaller collagen fibrils packed into to the printed fiber is readily seen. The FFT algorithm was applied to the SEM images to describe
the anisotropy of the fibrils within the collagen fiber. Figure 22A shows the majority (greater than 70%) of macroscale features is aligned within ± 15° of the long axis of the fiber. Figure 22B shows the individual fibrils have a higher level of local disorder, with alignment to the long axis ranging ± 25°. The image suggests that fibril alignment is being positively influenced by the printing process, but there is opportunity for significant improvement.

Figure 22: Imaging and analysis of the orientation of the fibrils within the printed fiber at 30% PEG and 100% flow. In this case the external “shell” did not interfere with SEM imaging and the fibrillar structure within the fiber surface could be readily appreciated. A) Low magnification; B) Higher magnification. A1 and B1) SEMs of fiber; A2 and B2) FFT transform of the image; A3 and B3) Polar plot of the orientation of the structures within the image. Note that as magnification is increased, the orientation of the fibrils becomes more random. This type of alignment analysis could be used to tie fiber mechanics to the internal fibrillar structure.

Because it was difficult to optically measure the diameter of the fibers due to flattening or other shape changes in the cross-section, we only qualitatively report that increasing PEG concentration and decreasing flow rates visibly reduced the observable width of the final printed fiber. Figure 23 compares fibers for the conditions producing the stiffest and softest fibers, as seen via differential interference contrast light microscopy during live experiments.
Chapter 2.4: Discussion

The results demonstrate a new optically-based micromechanical measurement system which permits the in situ mechanical investigation of newly-assembled microfibers. The device allows both static and dynamic testing and utilizes calibrated glass micro-needles for optical force measurement in combination with optical strain measurements based on the location of embedded micro-beads. The device can reliably detect forces as low as 20 nN with local strain accuracy in the range of 99% for a fiber region with marker beads 200 µm apart. We have coupled the measurement chamber to a precision microfluidic delivery system which deposits collagen monomeric solutions within the chamber. The device was used to measure the micromechanical behavior of collagen fibers produced via molecular crowding in open solution with PEG. We used the system to examine the effect of atelo-collagen vs. telo-collagen, increasing draw ratio (decreasing flow rate) and increasing crowding force (increasing PEG concentration) on the resultant mechanics of printed collagen microfibers. The results indicate that stiffer, smaller fibers are produced as the draw ratio and the PEG concentration are increased. In addition the fibers did not show substantially different dynamic material properties indicating similar viscoelastic behavior. While the data were consistent with our expectations, there were other significant observations made during the process of fiber printing which deserve noting.
Chapter 2.4.1: Collagen Binding to Micro-needle
The binding of the collagen to the calibrated micro-needle is an interesting effect and quite fortuitous as an anchor point for the fibers. We noticed that the structure which formed between the collagen and the micro-needle could bear load nearly immediately (See Figure 12 for structure image). The structure is produced by first depositing a spherical bead of collagen around the micro-needle and then moving the collagen source away. While we are not sure how the structure comes about or why it forms so reliably, we believe it is due to a natural affinity of the collagen to glass and to the formation of a rapidly polymerized “shell” of collagen on the outside of the initial sphere. When the collagen depositor moves away, the polymerized collagen shell is pulled with it but catches on the micro-needle. The rapid polymerization of the monomer in the crowded solution then solidifies the micro-needle/collagen interface making it structurally robust.

Chapter 2.4.2: Kinetics of Polymerization of the Deposited Collagen Fiber
While it was not our main goal to examine the kinetics of polymerization of collagen in the crowding solution, we were somewhat surprised at the speed with which structure is formed. The collagen, once in the PEG, was capable of rapidly morphing into a load bearing solid structure, which began at the calibrated micro-needle and propagated through the PEG solution toward the moving printing needle. This could happen before the printing needle completed its deposition run if it was moved slowly enough. Once the polymerization front reached the printing needle, the new collagen being dispensed was no longer incorporated into the fiber and a large force was transferred to the micro-needle, displacing it off the computer screen and either breaking the collagen attachment or the fiber itself. Printing at a higher velocity and accordingly a higher flow rate was necessary to eliminate the problem. We chose, for this series of experiments to print at a high enough flow rate and at a high enough deposition needle speed to produce near-zero displacement of the anchoring glass micro-needle. Figure 24 demonstrates why this problem may arise. Even a small amount of PEG mixed with the collagen solution can reduce the
polymerization lag time to nearly zero. This reduction in lag time is most likely due to a combination of the molecular crowding and co-nonsolvency effects produced by the PEG.

![Graph](image.png)

**Figure 24:** A turbidity plot of collagen polymerization at room temperature when mixed with small percentages of PEG. As seen, at only a 2% concentration of PEG, the lag phase is near-completely eliminated.

**Chapter 2.4.3: Low pH Printing**

To isolate the effect of crowding from polymerization, we printed our collagen solution into PEG at a low pH (2.3). Our expectation was to generate a column of unpolymerized crowded collagen in the solution. The results (shown in Figure 25) were quite surprising. The column of collagen was deposited in a straight line, but then quickly buckled into a sinusoidal pattern. Measurements of the fibril length suggest that following buckling, the fiber was 1.4 times as long as a fiber printed into pH 7.3. The buckled fiber was weakly stable, being able to hold a small amount mechanical tensile force without appreciable straightening. The fiber, once straightened, was able to hold a substantial amount of load in the pH 2.3 environment. In Figure 26 the kinetics of the effect can be readily seen. The buckling wave proceeds from the anchor point towards the deposition needle at a fairly rapid pace. The kinetics suggests that charge shielding by excess protons produces a shift in organization of the collagen molecules. Thus, manipulation of pH to
isolate molecular crowding from polymerization was not a viable approach. Perhaps a better method is to print procollagen into the FFB at neutral pH and then trigger polymerization with propeptidases after the procollagen reaches organizational equilibrium. Because our micromechanical assay uses ~100 nl of collagen solution to form a 12.5 mm fiber, the usual difficulty associated with obtaining adequate amounts of procollagen and propeptidases can be greatly alleviated.

Figure 25: Buckling of printed collagen microfiber. (A) The collagen fiber, deposited into the molecular crowding solution (30% PEG) at low pH (pH 2.3) buckled into a fairly regular sinusoidal pattern. Surprisingly, the fiber was able to hold a substantial force (B) in spite of the buckling pattern and the low pH of the solution.

Figure 26: Kinetics of the buckling wave seen in collagen fiber printed at low pH. The series of images shows the progression of the buckling immediately after the printing needle passes the observation point at 7.79 seconds. The buckling wave appears within 0.38 seconds and is essentially stable within 1.5 seconds. The last frame shows the stable buckled collagen fiber (in focus). Because of the difficulty of capturing this effect, the first seven frames are slight out of focus which is why the fiber looks more diffuse.
Chapter 2.5: Conclusion

We designed, built, and demonstrated the capabilities of a custom, optically-based micromechanical assay and fiber printing system which captures both the mechanical properties of deposited fibers and the kinetics of their assembly. The system readily detects the effect of increasing draw ratio and changes in FFB/PEG concentration on the mechanics of the printed collagen fibers. Using this device, we tested the limits of the adjustable parameters used in the printing of collagen in FFBs (e.g. maximum PEG solubility and maximum draw rate without breaking the fiber). While we found modest improvements in mechanical properties, the fibers produced remained inferior to native tissue with respect to packing, alignment, and strength. Our findings suggest that simultaneous molecular crowding and polymerization may be a limited fiber forming procedure because fibrillogenesis is occurring while the collagen concentration is changing from dilute to highly-concentrated. The rapid polymerization potentially locks disorganization into the fiber because it occurs before the collagen can properly align and during dynamic changes in water, electrolyte, and proton concentration. In addition, there is the potential for PEG molecules to be incorporated into the collapsing fiber, further distorting the fiber structure. A better approach will likely arise if one can separate the crowding from the polymerization events, thereby permitting organization before polymerization locks in the structure. Regardless of the approach, the device we have demonstrated should permit detailed optical and in situ mechanical investigation of collagen microfibers which are directly printed into any fibril forming buffer.
CHAPTER 3: Collagen mechanosensitivity: applying elongational strain to trigger organized polymerization

Chapter 3.1: Introduction

Printing collagen solution into PEG in Chapter 2 was a bio-inspired approach to mimic fibripositor extrusion in the presence of a molecularly crowded environment. The aspect ratio of the long extruded cylinder was intended to set the director of alignment, through the innate liquid crystalline property of collagen. The initial experimental was designed to use acidic PEG to isolate the crowding step from the polymerization step. Holding everything in an acidic state would reproduce the effect of propeptides, since acquiring substantial amounts of procollagen is unfeasible. Unfortunately the acidic fiber still aggregated into a configuration that was able to withstand strain. The fibers rapidly buckled in a transverse manner, followed by a cross-sectional progression to more ribbon-like than circular.

In this chapter, I investigate if there are any evolutionarily built-in mechanisms that we can exploit to align fibrils within a fiber. Collagen had a billion years to optimize its function as a load-bearing molecule, which warrants research into whether any biological process has become automated in the absence of direct cell contact. The fact that collagen monomers readily self-assemble in a physiochemical environment motivates one to take a closer look. It must be considered that the collagen monomer has acquired mechanisms capable of autonomously assisting with assembly, growth, and turnover encoded into its molecular structure.

I extend this investigation to elastin and fibronectin under the pretense that there may be commonalities between extracellular proteins in general. The proteins may serve distinctly different purposes, but the cellular approach to manipulating the proteins may be quite similar.
Chapter 3.1.1: Assembly of Extracellular Molecules

Chapter 3.1.1.1: Collagen

The assembly of collagen monomers into natively-structured fibrils is long known to occur in the absence of cells [2]. However, fibril formation first requires the enzymatic removal of the C-propeptide, as this globular feature obstructs polymerization to prevent intracellular assembly. When the C-propeptide is present, the critical concentration for spontaneous assembly rises by a factor of 1000, as compared to collagen without propeptides [147]. The C-propeptide also serves to promote proper selectivity between two α1(I) chains and one α2(I) chain during trimerization [148-150]. Disulfide bonds join these subunits together, followed by a zipper like folding of the monomer from the C- to the N-terminus [148, 150].

A study by Gealy et al. labeled the two propeptides in the developing cornea and found that while the C-propeptide is only retained intracellularly, the N-propeptide is present both intracellularly and in the fibrils of the ECM during early development [151]. The N-propeptide can be observed even in the fibrillar collagen because it is a shorter, tighter sequence, due to the triple helical region in the middle of its domain [148]. The N-propeptide structure does impact the fibril diameter and assembly, but banded fibrils can still be produced [152]. As N-procollagen becomes an increasing percentage of the available collagen, the fibrils become more fluted and eventually form sheet-like structures [150, 152]. Hulmes et al. comments that the sheet thickness is incredibly uniform, which points at a precise limitation on growth [152]. Since the N-propeptides cannot fit in the core of the fibril, they are presented on the surface, which in high enough concentration blocks further growth by steric exclusion. The surface area to volume ratio of a sheet provides ample space for the N-propeptides, which may play a part in the initial formation of lamellar tissues such as intervertebral disc and cornea. Gealy et al. found the N-propeptide’s presence persisted until E14, at which point it was absent onward [151].
Having covered a few necessary details pertaining to the post-processing of the monomer prior to assembly, we can now look at what may be important for organized fibrillar assembly. This is not intended to prove that tissues are made a particular way, but rather to observe what properties are exuded that may be beneficial for a tissue engineer to recognize. The most significant piece of data in the literature, with respect to collagen assembly, is that the high aspect ratio of collagen makes it sensitive to liquid crystalline alignment [105, 153, 154]. Chiral nematic structures are formed when soluble collagen concentrations surpass 50 mg/ml, resulting in orthogonal lamella of alternating direction [155]. The geometry closely resembles the cornea, intervertebral disc, blood vessel, and bone [62, 156]. Smectic structures are formed at even higher concentrations, approximately 150 mg/ml, resulting in uniaxial alignment of the fibrils similar to the tendon or ligament structure [154, 157]. Liquid crystalline experiments with collagen are performed by keeping the collagen in the acidic state, but Martin et al. has demonstrated with droplets of procollagen that the same pronounced effect is observed at concentrations ranging from 5-30 mg/ml [109].

The liquid crystalline behavior of collagen is evident, but also weakly stable. The alignment tends to persist for short distance only, before following a new director [102]. The two pivotal relations to extract are: i) there are critical collagen concentration ranges necessary to recapitulate the supra-fibrillar architecture of tissues, and ii) absence of a global guiding cue results in failure to set a long range director for the liquid crystalline structures. Small fluid flows, thermal fluctuations, and impurities in the solution all re-template the liquid crystalline pattern, which suggests an external parameter must serve as a dominating director.

Chapter 3.1.1.2: Elastin

Mithieux et al. have provided a full review on elastin, from elastogenesis to applications in biomaterials [158]. This section will focus on the assembly of elastin, but some basic background
Elastin is a 60-70 kDa protein that is secreted as a globular molecule [158-160]. Each molecule is approximately 15 nm in diameter and comprises distinct alternating hydrophobic and cross-linking domains [161]. The molecules first aggregate and then undergo coacervation, a process similar to collagen monomer assembly. Coacervation is an entropically driven process where ordered water molecules surrounding the hydrophobic domains are displaced, which permits intermolecular hydrophobic segments to interact [158-160, 162, 163]. This process occurs in vitro as a result of an increase in temperature, but it is important to note that coacervated molecules still retain globular shapes. The lysl-oxidase mediated cross-linking can only occur after the molecules have properly shifted into alignment and formed hydrogen bonds. The formation of bifunctional, trifunctional, and tetrafunctional crosslinks is a strong indication that molecular aggregation and orientation has occurred equivalently to the physiological process [158]. Numerous groups have now achieved the recapitulation of coacervation and crosslinking in the absence of cells, noting a critical concentration of ~ 1-3 mg/ml [158, 162-164]. The process is temporarily reversible if the temperature is reduced soon after assembly, but progresses to a mature state where aggregates no longer separate upon cooling [160]. Unsurprisingly, this process occurs most efficiently at physiological pH, ionic strength, and temperature [160]. The inherent ability of tropoelastin to properly coacervate in an acellular environment indicates that the process is effectively driven by the structure of the molecule.
Figure 27: Stages of elastin assembly. Elastin monomers assemble into conglomerations through the displacement of ordered water molecules surrounding the hydrophobic sites. Once the molecules have aligned and formed bonds between their hydrophobic domains, lysine residues are oxidized to form allysine, which participates in bi-, tri-, and tetra-functional crosslinks. Image reproduced with permission from [160].

The final stage of elastin assembly is the transition from globular aggregates to fibers. It appears that microfibrillar proteins comprising fibrillin I, fibrillin II, microfibril-associated glycoprotein-1 (MAGP-1), fibulin, and proteoglycans may template the process [159, 160, 162, 163]. The binding of the elastin to these microfibrils has been shown to promote further coacervation and intramolecular alignment [165-167]. Cross-linking *in vivo* is believed to occur once the elastin globules have collected on the microfibrils, as fibulin-5 is thought to recruit lysyl oxidase [167, 168].
To understand how much of this process is cell driven and how much is molecularly automated, Kozel et al. have done carefully controlled experiments with live and killed cells [163]. The setup used bovine tropoelastin, rat cells that produced healthy tropoelastin fibers, and cells that only produced the microfibrils (no tropoelastin). When bovine tropoelastin was introduced into either setup, the bovine tropoelastin was successfully incorporated into the ECM, resulting in native fibers. When all cell lines were killed and then bovine tropoelastin was added, the bovine elastin fibers were visually different than previously. The structures formed were less filamentous overall and still retained more globules. Kozel et al. have extended this work with a dynamic investigation designed to reveal what the cells are physically doing, since the entire process up to the transition into a fiber can occur acellularly [169]. The authors claim that the cells are, “…likely responsible for the ultimate shaping of the fiber by way of mechanical manipulation of elastin at the cell surface.” It is here that I have to disagree with conclusions of the authors and instead infer that the cells are stretching the microfibrils, and this mechanical stimulation is leading to the proper transition from globules to coherent fibers, in the direction of the forces. Without the straining of the microfibrils, the transition from globular domains to elongated fibrils does not occur.

At this point that we have a sufficient body of information pertaining to elastin assembly:

i) At and above a critical concentration, initial aggregation and coacervation occurs automatically.

ii) Intermolecular alignment occurs automatically, as proven by the ability of lysyl oxidase to catalyze crosslinks.

iii) Microfibril contact guidance enhances alignment.

iv) Mechanical stimulation is essential to transition into well-oriented native fibers.
**Chapter 3.1.1.3: Fibronectin**

Fibronectin (FN) is a disulfide bonded dimer composed of two nearly identical 230 – 270 kDa subunits, which are in turn composed of three repeating modules [170, 171]. Fibronectin cannot spontaneously self-assemble, even at extremely high concentrations, because it is a compact molecule with cryptic binding sites in the third module [172]. The mechanisms behind FN conformational changes to initiate assembly are currently being explored. Fibronectin can be cell bound through its interaction with integrins, and the cytoplasmic domains of integrins become associated with the actin cytoskeletal network [173]. Contraction of the actin-myosin changes the conformational state of FN, reveals the cryptic binding sites, and enables subsequent polymerization [174]. However, Morla et al. demonstrate that incubation of soluble FN with the C-terminal two thirds of module III, called anastellin, induces polymerization even in the absence of cells [175, 176]. Thus, once FN fibrils are present, soluble FN molecules can spontaneously unfold and incorporate through homophilic interactions [177-179]. Fibrils that are created without any mechanical stimulation require stretching to transition from collapsed fibrils to those that have a native conformation [175, 176].

Ejim, Blunn, and Brown published a completely novel approach to fibronectin-based tissue engineering in the absence of cells [180]. They produced FN fibers and mats through the application of concentrating and applying a directional shear force. FN fibers were drawn from a 1 mg/ml neutralized solution of FN when a glass coverslip was dipped into the solution and manually pulled upwards. A single FN fiber would automatically attach to the edge of the coverslip and could be drawn for 5 mm from the fluid surface. The fibers ranged in diameter from 1 – 10 µm and showed a highly fibrillar composition in the region closest to the droplet, where the fibrils had yet to converge. There was no mention to a concentrating technique, which I can only infer was achieved passively through evaporation.
The FN mats, which are further detailed in [181], were made from 0.5 mg/ml neutralized FN solution, through the use of a pressure ultrafiltration cell. This device entailed a central shaft that rotated between 100 – 300 rev min\(^{-1}\) and an outer cylinder, lined with an ultrafiltration membrane (10,000 MWCO) to concentrate the solution at approximately 1 ml/min. The technique yielded a fibrillar mat that was visually aligned but had poor control over the fibrillar diameters (~10 to 200 \(\mu\)m), branching, and pore size. However, the FN mats were stable in media for at least two weeks, despite losing 50% protein content after 2 days of incubation. Heparin was added to the starting solution to enhance total fibrillar content to ~60%, but the resulting structure was less stable during incubation.

Brown continued this study by exploring cell interactions with the FN fibers [182-184] and most recently this has been explored by Vogel and coworkers [185, 186], but very little has changed with respect to fiber formation. The tool used to initiate the fiber pull has been a pipette tip, a micro-needle, a MEMs tip, and a coverslip edge. It appears nearly any fine point or edge is sufficient. In all of these studies, the common theme is critical FN concentration, achieved by pressurized filtration or evaporation, and mechanical stimulation, in the form of tension and strain.

**Chapter 3.1.2: Growth of Collagenous Tissues**

The earliest stages of *in vitro* fibril growth have been studied by Kadler *et al.* in 1990 [187]. Telo-collagen with cleaved propeptides was polymerized at 29°C, and the resulting fibrils had a distinct blunted end (C-terminal) and a pointed end (N-terminal). The fibrils rapidly reached a maximum diameter, and then grew exclusively from the pointed end without any change in diameter. Occasionally a “spear-like projection” would appear on the blunted end, and growth would progress from both ends of the fibril. Kadler concludes that there is a priority order for fibril growth: i) the highest affinity for binding new monomers is the pointed end; ii) the blunt
end has the second highest affinity, only accepting a subunit of monomers in the reverse orientation to form a new pointed end; iii) the lowest affinity for binding new monomers is the central region of the fibril, since no diameter increase is noted after initial fibril formation.

In 1995 Birk et al. provided a detailed analysis of fibril formation in embryonic chicken tendon [188]. E14 tendons had fibrils with an average length of ~30 µm, and 44 out of 49 of the fibrils were centrosymmetric, having two pointed ends. Each end of the fibril had the amino terminus pointed toward the end of the fibril and the carboxyl terminus pointed toward the center. Fibrils extracted after E16 were almost all broken at an end. Of the four intact fibrils collected at E17, the average length was 106 µm. This result, as well as extensive TEM imaging, indicated that after E16 growth was dominated by lateral fibril fusion involving extensive regions of the fibril segment length. Birk et al. correlated the fibril fusion with a drastic decrease in decorin content, where at E18 it was down to 1/3 the amount present at E12/14. Birk et al. concluded that an equivalent growth pattern has been seen in embryonic chick dermis and cornea, indicating a general mechanism in morphogenesis.

The information presented thus far on growth is insufficient to explain the mechanisms behind growth on the tissue-level. Tendon comprises continuous fibrils that connect bone to muscle, and it does not have free fibrillar ends for monomeric incorporation. In 2010 Holmes and Kadler crushed tendon in liquid nitrogen and demonstrated that the fibrils could then undergo tip growth when placed in monomeric solution [189]. This paper, directed toward tissue repair, does not comment on how this may pertain to tissue growth, but extrapolation is possible from the cited literature in this section. Once microtears occur from fatigue and overloading, the tendon may slightly lengthen but now have numerous broken fibrils as a result. These fibrils could possibly then grow until their ends were long enough to bridge the gap between each other, and
spontaneous lateral fusion could possibly occur to repair the microtear and grow the tendon. The loose ends may have the opportunity to meet through physiological motion, fluid flow, and smaller Brownian motions.

I am unaware of any widely accepted theory or even any proposed theory to explain the mechanisms behind tissue growth. However, I am inclined to believe that the growth mechanism is common in numerous collagenous tissues. The explanation of overloading, microtears, fibril extension, and fusion may be plausible for tendon, but this is an inappropriate model for tissues such as the cornea, sclera, blood vessel, and skin. Normal development and growth of collagen based tissues are critically dependent on the presence of appropriate biophysical cues [190-193]. The altered physical signals have an undeniable impact on the cell secretome, but the degree of stress and strain may also impact how the ECM responds irrespective of the changing secretome. This concept has yet to be well explored, but if strain can induce fibrillar assembly for fibronectin and elastin, then it may impact growth as well.

Chapter 3.1.3: Degradation of Collagenous Tissues
I have posited that physiological strains may directly affect the growth of collagenous tissues, but fortunately, the inherent mechanosensitivity of collagen pertaining to degradation is more thoroughly investigated. The two types of collagen degradation reviewed are thermal and enzymatic. Thermal degradation is the denaturation of the monomer into its 3 alpha chains and has been shown to occur in monomeric solution at temperatures slightly below body temperature [76]. In 1949 Lennox showed that the denaturation temperature of sheep skin and rat tail tendon increases when it is held in a loaded configuration [194]. In 1978 Snowden et al. compared the denaturation temperature of tendon sheath unloaded fibrils, unloaded tissue, and loaded tissue [195]. They found that the denaturation temperature is lowest in unloaded fibrils, intermediate in unloaded tissue, and highest in loaded tissue. This work has been now been confirmed multiple
times and on different tissues [196-201]. Miles et al. explain that collagen first unfolds at the thermally labile domain, a 65 residue long hydroxyproline-free sequence [202, 203]. Thus, there is a loss of configurational entropy when the molecule is placed under tension. Spatial confinement by the surrounding molecules, as well as a tightening down of the triple helix, reinforces the structure and enhances the resistance to thermal denaturation.

Strain protection against enzymatic degradation was first reported in 1977 by Huang and Yannas [204]. They showed that stretched collagen fibers were digested more slowly from bacterial collagenase than unloaded collagen fibers. This finding has been observed again in native tendon [205], corneal tissue [206], and collagen gels [207, 208]. In recent work, Ruberti and coworkers have shown that in acellular environment, strain acts as a director to preferentially, enzymatically degrade unloaded monomers [209], fibrils [134], and tissue [210], while greatly reducing the cutting rate on the loaded collagen. Markus Buehler and coworkers have modeled the enzymatic cutting event, which takes place within the thermally labile domain [211]. They concluded that mechanical strain protects against enzymatic cleavage via intramolecular stabilization of the triple helix.

**Chapter 3.1.4: Introduction Summary**

To quote from the work of Xavier Bichat, father of modern histology and descriptive anatomy, is perhaps the most appropriate way to summarize the Chapter 3 Introduction:

“Science requires […] considerations, which form the most beautiful part of the study of animal structure, which exhibit nature everywhere uniform in her operations, varying only in their results, sparing of the means she employs, prodigal only in the effects she obtains; modifying in a thousand ways a few general principles which, differently applied, preside over our economy, and constitute its numberless phenomena,” [212].
The extracellular matrix molecules form tissues, all throughout the body, with vastly different geometries, mechanical properties, and biological functions. However, there are only a few general principles which preside over ECM assembly, growth, and turnover. It is our hypothesis that the molecules of the ECM have been evolutionarily tuned to autonomously accomplish these functions, provided that the molecular concentration and application of strain are precisely regulated. This precise regulation is what the cells do during development; it is what they struggle to do during wound healing; and it is what tissue engineers are capable of doing.

The following sections of this chapter relate closely to the *in vitro* fibronectin assembly experiments. Collagen fibers are assembled from a concentrated droplet of monomeric solution through the application of elongational strain. The structure is investigated using differential interference contrast (DIC) microscopy, SEM, and TEM.

**Chapter 3.2: Materials and Methods**

**Chapter 3.2.1: Collagen Droplet Reagents**

Two bovine, dermal collagen sources were used in the creation of aligned collagen fibers. Pepsin extracted collagen (5010-D, Advanced Biomatrix) and acetic acid extracted collagen (5026-D, Advanced Biomatrix) were used with a starting concentration of 6 mg/ml. Each collagen solution was neutralized using an 8:1:1 ratio of collagen, 10X phosphate buffered saline (BP399-1, Fisher Scientific), and 0.1 M NaOH (12419-0010, Fisher Scientific) respectively. This resulted in a pH of 7.3 for both pepsin extracted collagen (atelo-collagen) and the acetic acid extracted collagen (telo-collagen). As shown in Figure 13, the telo-collagen fully polymerized in less than five minutes, even at substantially lower concentrations. The goal was to prevent any spontaneous polymerization during the experiment (~5 minutes), and so the amount of 0.1 M NaOH was increased until no fibrils were detected via DIC microscopy for 10 minutes. This resulted in a new neutralization recipe of 8:1:1.15, and consequently an increased pH of 7.7.
Chapter 3.2.2: Fiber Pulling Setup
The collagen fiber pulling setup, seen in Figure 28, was located on a Nikon TE-2000E microscope for live imaging and visual feedback. A custom nitrogen diffusing chamber was designed to provide humidity control and enhance the evaporation rate through convection. A nitrogen tank, regulated to 0.022 psi by a digital pressure controller (PCDAS-1PSIG-D / 5P, 5IN, Alicat Scientific), supplied the chamber’s one primary inlet which then branched off to six nozzles to provide a constant flow of dry nitrogen gas. A 125 µl droplet of neutralized collagen solution sat on top of an 8 mm coverslip, which rested on top of a 40 mm coverslip. The 8 mm coverslip was essential for pinning the boundary of the droplet, resulting in a repeatable initial geometry and evaporation profile. The 40 mm coverslip was used to provide the optical access to the inverted microscope. A glass micro-needle, held by a capillary holder and moved by an electronic micromanipulator (TransferMan NK 2, Eppendorf), was used as the probe to draw the collagen fiber. The chamber lid was mounted on the condenser lens of the microscope to minimize the effects of the local humidity in the room.
Chapter 3.2.3: Fiber Pulling Protocol

Prior to making the neutralized collagen solution, the 8 mm coverslip and glass micro-needle were properly aligned, the nitrogen gas was turned on to 0.022 psi, and the chamber lid was put in place. The system was then given a minimum of five minutes before an experiment was performed to equilibrate. The collagen solution was made by first combining the 0.1 M NaOH and 10x PBS components and then adding either 6 mg/ml atelo- or telo-collagen, suspended in 0.01 M HCL. This reduced the initial pH shock and prevented any instantaneous precipitation. The solution was then pipetted in and out (carefully avoiding introduction of air bubbles) 15 times to yield a homogeneous, neutralized 4.8 mg/ml solution. Next, 125 µl were pipetted onto
the 8 mm glass coverslip, again avoiding the addition of any air bubbles. The micro-needle was inserted 500 µm below the surface of the apex of the droplet, and then the system was untouched for 130 seconds. After the allotted time, a pre-written macro, in NIS Elements AR v4.13, raised the microneedle as follow:

<table>
<thead>
<tr>
<th>Absolute Position (µm)</th>
<th>Velocity (µm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,500</td>
<td>25</td>
</tr>
<tr>
<td>2,500</td>
<td>100</td>
</tr>
<tr>
<td>11,500</td>
<td>275</td>
</tr>
<tr>
<td>13,500</td>
<td>150</td>
</tr>
</tbody>
</table>

The initial wait step was required to pull a fiber of appreciable length (>10 mm), and the slower starting velocity was designed to initiate the formation of an attachment that could withstand the release of the meniscus. The 100 µm/sec velocity aided in smoothly transitioning to a 275 µm/sec velocity without breaking the fiber. The 275 um/sec velocity best paired with the speed of the surface enrichment, such that over the length of the fiber there was no visible thinning or thickening. The final step was included to ramp down the velocity as the source of enriched collagen was also equivalently depleted from the droplet.

Once the fiber pull had completed, the fiber was held in place, and the excess fluid was given ~1 minute to evaporate. The capillary rod, holding the glass micro-needle, was released from the micromanipulator and the fiber was plucked from the droplet. The fiber was then stored in a container, suspended in air and still attached to the glass needle.

**Chapter 3.2.4: Calculating the Surface Concentration**
The Péclet number was calculated to determine if the collagen was able to diffuse into the bulk during evaporation, or if the advection from the water molecules exiting the droplet led to surface enrichment. The Péclet number, a ratio of advective transport to diffusive transport, was
calculated as: \( P_e = \frac{L_c U}{D} \), where \( L_c \) is the characteristic length, \( U \) is the velocity, and \( D \) is the collagen diffusion coefficient. The characteristic length was calculated as: \( L_c = \sqrt{D t} \), where \( t \) is the time over which evaporation occurred. Fletcher measured the diffusion rate to be \( 7 \times 10^{-12} \text{ m}^2/\text{sec} \) for a 1 mg/ml solution [213]. This diffusion rate was steadily decreasing with increasing concentration, but no studies using higher concentrations were found [214, 215]. The velocity was calculated as: \( U = \frac{E_r}{A} \), where \( E_r \) is the evaporation rate (\( \mu \text{l/min} = \text{mm}^3/\text{min} \)) and \( A \) is the surface area of the droplet (\( \text{mm}^2 \)). The evaporation rate was measured by replicating the experiment on a scale instead of a microscope and found to be relatively constant. The surface area and change in height were calculated under the assumption that the droplet remained pinned to the 8 mm coverslip and maintained a spherical shape. The Péclet number was determined to be 2.15, using an evaporation time of 2.5 minutes. The calculation suggests that the collagen molecules are retained at the surface during evaporation without diffusion-driven escape into the bulk. If it is assumed that the evaporation occurred evenly across the surface of the droplet, then the calculated surface concentration, using the surface area at 2.5 minutes and the thickness of the layer as \( L_c \), is 15.5 mg/ml.

**Chapter 3.2.5: DIC Imaging**

The transitional region between the solid fiber and the liquid droplet has been termed the *necking region* for the purpose of discussion. The necking region was imaged using DIC microscopy at 200x magnification to investigate if any fibrillar structure could be detected. The necking region was imaged either by pulling the fiber horizontally from the top of the droplet or by pulling the fiber vertically and then laying it down on the glass coverslip. The fiber length was imaged by placing the fiber in a chamber with a glass bottom and adding 1x PBS to rehydrate. The fiber expanded and flattened down to the glass once the buffer was added, permitting clear visibility of the micro-structure.
**Chapter 3.2.6: Scanning Electron Microscopy**

Fibers produced for transmission electron microscopy (SEM) were glued at their two endpoints to glass micro-needles, attached to micromanipulators, and lowered into a 37°C chamber of 1x PBS. The fibers were incubated in a taut configuration for 4 hours, 48 hours, or 5 days. DI water was added every 15 minutes to replace evaporative loss for shorter experiments or through the use of a syringe pump for longer experiments. The fibers were then fixed with a recipe of 2.5% glutaraldehyde and 2.5% paraformaldehyde in a 0.1 M sodium cacodylate buffer for 1 hour at room temperature, followed by three fluid-exchanges with 0.1 M sodium cacodylate buffer every 15 minutes. The chamber fluid was then exposed to a graded series of ethanol exchanges: 35%, 70%, 90%, 100%, 100%, and 100% every 15 minutes. The fiber was raised out of the chamber and glued down to an 8 mm coverslip once the 100% ethanol evaporated off the fiber. Using a quick freeze deep etch device, the fibers were sputter coated with a carbon-platinum coating and taken to the SEM (S-4800, Hitachi, Japan) for examination.

**Chapter 3.2.7: Transmission Electron Microscopy**

Fibers produced for TEM were prepared the same as SEM. The fibers were incubated in a taut configuration for 0 - 48 hours. After fixation, the fibers were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour, followed by three fluid-exchanges with 0.1 M sodium cacodylate buffer every 15 minutes. The chamber fluid was then exposed to a graded series of ethanol exchanges: 35%, 70%, 90%, 100%, 100%, and 100% every 15 minutes. The fiber was raised out of the chamber and glued down to an 8 mm coverslip once the 100% ethanol evaporated off the fiber. The fibers were then placed in a small tin for resin embedding in Squetol:
<table>
<thead>
<tr>
<th>Squetol Reagents</th>
<th>Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quetol 651 (20440, Electron Microscopy Sciences)</td>
<td>1.40</td>
</tr>
<tr>
<td>ERL 4221 (15004, Electron Microscopy Sciences)</td>
<td>2.22</td>
</tr>
<tr>
<td>NSA (19050, Electron Microscopy Sciences)</td>
<td>6.38</td>
</tr>
<tr>
<td>DER 736 (13000, Electron Microscopy Sciences)</td>
<td>1.43</td>
</tr>
<tr>
<td>BDMA (11400, Electron Microscopy Sciences)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

The recipe was scaled up accordingly to yield the necessary volume. The fibers were exposed to a 6 hour 1:1 and 6 hour 2:1 ratio of Squetol to 100% ethanol, and finally to 100% Squetol. The 100% Squetol was exchanged after 12 hours and 24 hours. At the 24 hour mark, the tin was placed in the oven at 60°C for 24 hours.

After the fibers were successfully embedded, the blocks were trimmed and longitudinally thin sectioned on a microtome (Ultracut E, Reichert) using a diamond knife. The ~90 nm thin sections were plated on TEM grids and stained with 5% uranyl acetate and 5% Reynolds lead citrate. The sections were viewed with a JEOL JEM 1010 transmission electron microscope (JEOL, Japan) and images were digitally captured on a CCD camera system (AMT XR-41B, Advanced Microscopy Techniques Inc.).

**Chapter 3.3: Results**

**Chapter 3.3.1: Overview**

Fibers could not be pulled from the collagen solution until two to three minutes after placing the droplet of collagen into the setup shown in Figure 28. The evaporation was necessary to drive the surface concentration high enough that collagen monomers could continuously interact and one consistent fiber could be pulled from the droplet. A fiber could readily be pulled by piercing the droplet surface with a glass micro-needle and drawing away from the droplet once the evaporation time had passed. A collagen fiber being pulled horizontally is depicted in Figure 29.
Figure 29: The Initiation of a Collagen Fiber. A fiber is being drawn from a solution of 4.8 mg/ml starting concentration of monomers, after the solution had been kept in a dry nitrogen gas environment to enhance the surface concentration.

The fiber could be pulled to distances in excess of 10 mm on the microscope. The pull velocity was chosen to best maintain a constant droplet surface concentration such that the fiber was held to a tight diameter range. Figure 30 shows a full length collagen fiber. Fibers were also pulled to lengths in excess of 50 mm off of the microscope, through the use of a linear motor and a larger collagen source. The linear motor was needed to increase the available range beyond that of the micromanipulator, ~15 mm.

Figure 30: A Pulled Collagen Fiber. The fiber has been pulled to ~10 mm from a solution of 4.8 mg/ml collagen. The fiber maintains a fairly consistent diameter as a result of a pulling speed that yields a constant surface concentration during the drawing process. An attempt was made to balance the rate of depletion (drawing the fiber) with additional concentrating (surface diffusion and evaporation).
Chapter 3.3.2: DIC Structural Investigation
The substructure of the fibers was investigated using DIC microscopy by looking at both the necking region of the droplet and different regions along the length of the fiber. Fibers were pulled vertically from the apex of the droplet and then rotated around the droplet and laid down horizontally for imaging, as shown in Figure 31.

Chapter 3.3.1.1: The Necking Region
The necking region was imaged to provide an indication of what structure was being incorporated into the fiber length. Figure 32 shows three different fiber necking regions in different transitional states from vertical to horizontal. Figure 32A shows the necking region of a fiber that is still in the vertical configuration. Figure 32B shows the necking region of the fiber during the process of rotating it from vertical to horizontal. The radial orientation of fibrils along the surface of the
droplet clearly indicated that well organized fibrils are being incorporated into the fiber length. Figure 32C shows the necking region once the fiber was in the horizontal position and further drying had occurred. This image beautifully displays the exact location where fibrillogenesis begins. The fibrils propagate back into the droplet only a short distance, which strongly indicates that the mechanical stimulation is triggering the polymerization and as this force dissipates into the bulk of the droplet, it drops below a yet unknown threshold required to polymerize the monomeric solution. At the time of these images, no fibrillar structure could be recognized at any other location on the droplet surface or in the bulk.
Figure 32: The Collagen Fiber Necking Region. Image A shows the necking region of a fiber that is still nearly in the vertical configuration. Image B shows the necking region of a fiber as it is being rotated to the horizontal position. Image C shows the necking region of a fiber once it is in the horizontal position and further drying has occurred.
Chapter 3.3.1.2: The Fiber Length

Images of the length of the fiber further reinforced what had been observed in the necking region. The fiber was imaged after being rehydrated in 1x PBS at room temperature for a couple of minutes. The fiber expanded upon rehydration and also flattened as it spread across the glass cover slip. The substructure in the atelo-fibrils, seen in Figure 33, was very highly aligned with little to no visible structure in any direction other than the long axis. The alignment was persistent throughout the entire length of the fiber. Some areas appeared to have a greater thickness than others, which suggests a non-uniform assembly environment.

The substructure in the telo-fibers, seen in Figure 34, was slightly different. The very initial region of the fiber, shown in Figure 34A, was more disorganized than the rest of the fiber length. This image was taken immediately after PBS exposure, revealing the dissolution of crystalized salts on the fiber surface. The fibrils within the telo-collagen fiber were highly aligned, but some regions had a slight angular offset from the long axis of the fiber, as seen in Figure 34C. However, there was often little visual difference between a telo-fiber and an atelo-fiber.
Figure 33: Atelo-Fibers DIC Imaged Under 1x PBS solution. Fibers show distinct fibrillar structure, oriented in the direction of the fiber long axis.
Figure 34: Telo-Fibers DIC Imaged Under 1x PBS. The top image shows the very initial region of the fiber formed. The middle images show two regions along the mid-length of the fiber. The bottom images show two focal planes of the end of the fiber, cleanly cut by scissors from the droplet.
Chapter 3.3.3: Preliminary TEM Structural Investigation
The ultrastructure of an atelo-fiber was investigated without any incubation in PBS. The fiber was fixed, ethanol dehydrated, and embedded directly after being pulled from the droplet of monomer solution. The resulting structure observed in the TEM images, seen in Figure 35, lacked the native fibrillar look of any collagen tissue. This was counterintuitive based on the previous DIC imaging, where the only difference was the exposure to 1x PBS. Caves et al. found that their extruded collagen exhibited a much more fibrillar, banded state when incubated for 48 hours, and Cheng et al. found that their electrochemically produced fibers did the same after 12 hours of incubation [86, 115]. All future EM work would now include an incubation period prior to imaging.

Figure 35: TEM of an atelo-collagen fiber. The fiber was prepped for TEM without any incubation period. There is a sense of alignment from the top left corner to the bottom right in image A and B, but individual fibrillar structures are unrecognizable.

Chapter 3.3.4: SEM Structural Investigation

Chapter 3.3.4.1: Atelo-Fibers – PBS Incubation and Fixed
Scanning electron microscopy was implemented to observe the fibrillar substructure of the fibers. Atelo-fibers were given a 4 hour incubation period, fixed, and ethanol dehydrated before sputter coating for SEM imaging. Image A and B of Figure 36 showed little surface structure and
appeared to have a coating that masked the finer features. These images are representative of the thicker and thinner fibers generated. An atelo-fiber was fractured with a surgical scalpel to try and ascertain structural information underneath the surface coating. Figure 36C depicts the fibrillar orientation where the fiber was split longitudinally down the middle. In this figure, a fibril bundle is clearly depicted, confirming that the fiber does comprise oriented collagen fibrils.
Figure 36: SEM of atelo-collagen fibers that were incubated under a taut configuration for 4 hours, fixed, and dehydrated. The general fibril orientation can be detected in image A and image B, but clear fibril structures are difficult to recognize. Image C is of an area that was fractured by a scalpel prior to sputter coating. A fibril bundle runs in the direction of the long axis of the fiber. Deeper into the fiber the collagen appeared more loosely packed, which may be a result of the cutting process.

Chapter 3.3.4.2: Telo-Fibers – PBS Incubation and No Fixative
Telo-fibers were prepared with a 4 hour incubation, ethanol dehydration, but without exposure to fixative. This was an attempt at gaining more information about the structure of the collagen on the surface. As seen in Figure 37A and Figure 37B, the surface comprised highly aligned fibrils/micro-fibers, oriented in the direction of the fiber’s long axis. Figure 37C shows a higher magnification image of the surface where the fiber was fractured with a scalpel. The fibrils display a tight lateral packing but no banding was detected during SEM image. In many regions (not shown) the fiber had long cracks perpendicular to the fiber’s long axis, which was expected to be an effect of the dehydration without fixation.
Figure 37: SEM of telo-collagen fibers, incubated in a taut configuration for 4 hours, dehydrated, but not fixed. Images A and B show a fibrillar surface with uniform alignment in the direction of the long axis of the fiber. Image C shows a location in the surface that was fractured by a surgical scalpel.
Chapter 3.3.4.3: Telo-Fibers – PBS+PEG Incubation and No Fixative
A second set of telo-collagen fibers were imaged after 5 days of incubation in 2% polyethylene glycol in a buffer of 1x PBS. The concentration of PEG was chosen based on the turbidity kinetics shown in Figure 24. A concentration of 2% PEG was approximately the lowest amount required to virtually eliminate the lag time seen during collagen polymerization. Thus, we expected this hypertonicity to aid in holding the fibrils in close proximity to allow for the potential sharing of monomers to optimize the structure.

The fibers were prepared in parallel off of the microscope, and thus were in an unloaded configuration the entire time. The fibers were ethanol dehydrated, left unfixed, and sputter coated for imaging. Figure 38 shows representative images of the fibers, zooming in from image A to image C. Image A shows larger fibril bundles, some of which are discrete and others which have merged together. Image B demonstrates the kinks and waves of the more discrete fibril bundles, and the lower image shows the fibrils within each bundle. At the highest magnification, we see bifurcations and branching of fibrils in image C, but the general direction is still with the long axis of the fiber.
Figure 38: SEM of telo-collagen fibers, incubated for 5 days in 2% PEG, dehydrated, but not fixed. The images get progressively zoomed in from A to C, showing the hierarchical structures inherent in the fibers. The sharper kinks in the Image B are expected to be an effect of the dehydration.
Chapter 3.3.4.4: Telo-Fibers – PBS+PEG+Decorin Incubation and No Fixative
A third set of telo-fibers were imaged after 2 days of incubation in 1.63% PEG and 2% decorin in a buffer of 1x PBS. The 1.63% PEG was chosen as fine tuning from the previous experiment. After exploring the literature, it was determined that this pressure best matched the oncotic pressure of the interstitial fluid surrounding the tendon [216]. The value of 2% decorin was chosen based on physiological values. The tendon typically has an average of 1% (3 µg/mg) molar concentration by dry weight [217-219]. Twice the physiological value was chosen to increase ratio of the availability of decorin to the available binding sites and help drive the interaction to completion. The 2% decorin molar concentration, compared to the molar amount of collagen, was also included in the solution for pulling the fiber. This equated to 26 µg/ml in the solution, which was also the amount used for the incubating buffer.

Representative images of the resulting fiber structure are shown in Figure 39, with progressively increasing magnification. In image A, the fiber is truly more of a fascicle, composed of many fiber bundles. In image B, one fiber bundle is displayed, which itself is composed of many fibril bundles. In image C, one fibril bundle is shown and the individual fibrils can be identified. There is much less bifurcating of the fibrils and on all three hierarchical levels the structure is more clearly identified. Banding is not evident, but this may be an artifact of the sputter coating process.
Figure 39: SEM of telo-collagen fibers, incubated for 2 days in 1.63% PEG and 2% decorin, dehydrated, but not fixed. The images get progressively zoomed in from A to C, showing the hierarchical mimicry of the native tendon. The decorin appears to have strengthened each hierarchical level of structure, such that they merging and branching is minimized, and the fibrous structures persist uninterrupted.
Chapter 3.3.5: TEM Structural Investigation

Chapter 3.3.5.1: Atelo-fibers Incubated in a Taut Configuration
Transmission electron microscopy was implemented to gather information about the internal structure of the fiber. Atelo-collagen fibers were first incubated in 1x PBS for 24 hours in a taut configuration, before being processed for TEM as described in Chapter 3.2.7: Transmission Electron Microscopy. The regions with the highest degree of alignment and uniformity were always found on the outer shell of the fiber, as seen in the Figure 40A and Figure 40B. The fibrils were all oriented in the direction of the long axis of the fiber, with clearly defined spaces in between. Figure 40C and Figure 40D depict the center of the fiber. The center of the fiber typically consisted of this short, sparse fibril pattern or long wavy fibril pattern. Figure 40E and Figure 40F show a complete cross section of a fiber. The thickness of the outer, aligned shell varied from location to location, and sometimes the disorganized center of the fiber was quite small with respect to the thickness of the aligned outer region.
Figure 40: TEM of atelo-fibers after 24 hours of incubation in 1x PBS in a taut configuration. Images A and B show the outer shell of the fiber. Images C and D show the central core of the fiber. Images E and F show an entire cross section of the fiber.
Chapter 3.3.5.2: Telo-fibers Incubated in a Taut Configuration

Telo-collagen fibers were first incubated in 10 mM phosphate buffer containing 150 mM NaCl and 2% decorin for 48 hours in a taut configuration, before being processed for TEM as described in Chapter 3.2.7: Transmission Electron Microscopy. The change in buffer is only a very slight variation from 1x PBS, but Harris et al. show a very pronounced fibril banding pattern in this buffer [220]. Similar to the atelo-fibers, the regions with the highest degree of fibrillar alignment were found on the perimeter of the fiber, as seen in images A and B of Figure 41. The peripheral fibrils were all oriented in the direction of the long axis of the fiber, but their spacing was no longer well regulated. Figure 41C depicts a full cross sectional view of the fiber. After the shell of highly aligned fibrils, there was a region that typically took an ~ 45° angle to the axis of the fiber (best seen in Figure 41B). Figure 41D, E, and F depict all of the different patterns typically found in the core of the fiber. From left to right, these appear to be three predominant structures found at the core, depending on the concentration at that location, or perhaps on the flow profile.
Figure 41: TEM of telo-fibers after 48 hours of incubation in 10 mM phosphate buffer containing 150 mM NaCl and 2% decorin in a taut configuration. Images A and B represent the typical periphery of the fibers. Image C shows a composite of the entire cross section. Images D, E, and F show typical fibril patterns found in the core of the fiber.
Chapter 3.4: Discussion

The application of mechanical stimulation to trigger collagen polymerization was designed to test the concept that collagen has the same innate mechanosensitivity to strain as fibronectin and elastin. Evaporation was essential to reach a sufficient concentration to allow a fiber to continuously persist, which is why Harrington et al. were unable to generate structure only with equivalent mechanical stimulation [221]. The required concentration of 15.5 mg/ml is a value that will require much further investigation. Collagen has an overlap concentration at 2.7 mg/ml [215] and reaches liquid crystalline states at concentrations as early as 5 mg/ml [109]. Liquid crystalline collagen has also been observed at concentrations of 100 – 400 mg/ml [222]. The concentration of 15.5 mg/ml in the fiber drawing setup may be responsible for pre-organizing the collagen at the surface, or it may only be the concentration required for a continuous structure to form, break through surface tension, and mechanically sustain the drawing of the fiber. A rheological investigation will be required in the future to help clarify the role of the collagen concentration.

Strain and critical concentration appear to be a key way to control extracellular molecules, and this is further observed in macro-scale biological systems. The spider and silkworm both generate silk fibers through the simultaneous application of protein concentration and elongational strain, as the silk dope moves toward the spinneret. Scheibel and coworkers have provided a detailed schematic of a spider’s major ampullate [223], seen in Figure 42. The fibrin is first stored in the ampulla at concentrations up to 50% (w/v), where the dope possesses nematic liquid crystalline properties. The dope remains soluble at this concentration due in part to an elevated pH which is parallel to the function of collagen’s propeptides. The dope then experiences a change in ionic strength, pH, concentration, and extensional strain as it is exposed to an elongational flow. The precise control over these parameters, reinforced by an external application of strain, leads to a material with superior properties than can be achieved ex vivo by researchers [224-226].
Figure 42: The Process of Silk Secretion. The schematic shows the exchange of ions and water that takes place as the silk dope travels through the narrowing duct of the major ampullate. There is a final application of strain at the spinneret to enhance the structural properties. Image reproduced with permission from [223].

The flow that occurs inside the duct of the spider and silkworm has been modeled by Breslauer et al. to characterize the rotational and elongational components [227]. Both species possess a hyperbolic geometry which generates a pure central elongational flow such that the molecules can be automatically aligned. The silk dope experiences an extensional strain while in the liquid form and a mechanical strain as it exits the spinneret.

To compare the collagen fiber drawing technique to silk production, an image of the necking region was captured, seen in Figure 43A. The geometry of the necking region was plotted over a series of points, and the resulting curve was fit to the same two-stage exponential function used in [227]. The viscosity parameters were set to that of spider silk since collagen is similarly a non-Newtonian fluid, and the average outlet velocity was set to match the pull speed of the micro-needle. Figure 43B shows the flow profile, modeled in cylindrical coordinates, where a value of -1 equates to pure rotation, 0 equates to shear, and 1 equates to pure elongation. This preliminary model suggests the fiber drawing system is achieving pure extensional flow. This means that the molecules are experiences a strain along their axis and a rotational component only until they are aligned in the direction of the flow. The extensional flow is responsible for the regions of highly aligned collagen fibrils.
Figure 43: Modeling the Extensional Flow. The necking geometry was taken from image A and modeled according to the techniques used in [227]. -1 = pure rotation, 0 = shear, 1 = pure elongation.

The empirical results of the TEM images have shown that the entire flow is not under pure extensional flow, but instead there must be recirculating currents which are responsible for the patterns observed at the core of the fibers. The experiment is more complicated than the model because the model neglects the impact of phase shifting from a liquid to solid. If this transition happens from the outside radially inward, then the experiment transitions from a boundary-less pure elongational flow to a more complex flow. Further investigation into the physics will be required to achieve aligned fibrillar structures through the entire cross section of the fiber. Better understanding of the precise concentration and critical strain rate experienced at the highly aligned regions will undoubtedly lead to an embodiment that produces fibers with native morphology.
Chapter 3.5: Conclusions

Although the fibers currently being produced by the droplet-drawing method are inferior to what is required for tissue engineering applications, the fibrillar alignment on the periphery of the fibers is unparalleled by any other known technique. This study was designed to reveal that collagen is a mechanosensitive molecule, and that a parameter space exists where native morphology can be replicated. While it is unlikely that this approach is the physiological method for assembling tissue, it does take advantage of the two mechanisms which are likely involved: strain and concentration. It is known that strain can acellularly drive preferential collagen degradation, fibronectin assembly, and elastin assembly; it would be surprising if collagen assembly and growth were uniquely controlled.

Drawing collagen fibers from a droplet of solution has many associated challenges:

- The collagen supply is limited
- The surface concentration is increasing from evaporation
- The surface concentration is decreasing from fiber extraction
- The pH is changing
- The ionic strength is changing
- The entire source of collagen is neutralized, preventing long term production
- Gradients in concentration → gradients in viscosity → gradients in elongational strain

However, these challenges are largely due to working at a solution interface. The experimental results suggest that developing an extensional flow device to precisely concentrate, neutralize, and strain the collagen may solve many of the current limitations. It is interesting to note that the potential solution to this problem may be one that biology has already solved with the spider/silkworm ampullate and spinneret. This stock solution could eventually incorporate components of fibronectin, elastin, glycosaminoglycans, proteoglycans, and multiple collagen
types to yield more biologically relevant fibers. Control over collagen fibrillogenesis through the innate mechanisms already built-in, via evolution of the molecule, may be the most direct route to successful collagen-based tissue engineering.

CHAPTER 4: Fiber post-processing to achieve natively banded fibrils

Chapter 4.1: Introduction

D-periodic banding is often referred to as the characteristic fingerprint of fibrous collagen. It is common in type I fibrils in different tissues as well as different species [228, 229]. When trying to recapitulate the architecture of a collagen-based tissue, every hierarchical level must be replicated. The position of the monomers, packed into the fibril, is equally as important as the orientation of the fibrils that comprise the tissue. This chapter begins by acknowledging the importance of collagen banded and then covers the approach used to restore collagen banding to the droplet-drawn fibers.

Chapter 4.1.1: Bond Formation

The configuration of the molecules is essential for the formation of covalent crosslinks to occur between the lysines and hydroxylysines of the triple helix and telopeptides [230, 231], shown in Figure 44. It also places the 3-hydroxyproline sites into proper registration to enhance intermolecular stability through water-bridged hydrogen bonding or steroelectronic effects [232]. Interestingly the 3-hydroxyproline sites are also staggered D-periodically along the monomer [232].
Chapter 4.1.2: Gap Overlap Region – Energetic Characteristics

The biological importance of the gap-overlap region, which arises from periodicity, requires some background information. The gap-overlap region comprises 13 rigid and 12 flexible domains, depicted in Figure 45A as rods and springs respectively [233]. The flexible regions are identified with electron microscopy as dark vertical stripes across a fibril when the sample has been stained with heavy metals, shown in Figure 45B [234]. The flexibility plot of Figure 45A shows that the gap region has greater flexibility than the overlap region. This is further observed by the fact that the overlap region has approximately twice the elastic modulus as the gap region, despite only a 20% difference in fibril packing density [235]. The increased flexibility in the gap region is attributed to a randomly kinked network in the gap region, due to a sparsity of proline and hydroxyproline [57, 58]. This permits absorption of strain energy through molecular deformation and elastic unfolding of the kinks when the fibrils are placed under tension, which then converts back to kinetic energy upon elastic recoil [236, 237]. The amino acid spacing in the alpha chains increases ~1% for a 10% increase in tendon strain [238, 239]; thus, 90% of the energy is dissipated via molecular slippage, fibrillar slippage, water displacement, heat generation, and reversible bond ruptures [233, 240-242]. Energy storage through molecular stretching occurs up to ~2% strain in the alpha chain, which is ~20% tendon strain [236-238].
Figure 45: The Domains of Collagen Banding. Image A shows the multiple rigid (rods) and flexible (springs) domains within the gap-overlap region. Underneath is a plot of the flexibility at each domain location. Image B is a TEM of a fibril, which shows the collection of heavy metal staining at the flexible domains. Image A is reproduced with permission from [233]. Image B is reproduced with permission from [234].
Chapter 4.1.3: Gap Overlap Region – Mineralization

The efficiency of energy storage is further improved by an increasing degree of mineralization [233]. Tendons have a gradient of mineralization as they transition from the bone-tendon enthesis toward the muscle, which assists with uniform load distribution between neighboring collagen fibrils to prevent stress concentrations [243, 244]. This fact points toward another purpose of periodicity: mineralization nucleates in the gap regions [245-250]. This process is most succinctly explained in Landis et al.’s 2013 paper [251], which summarizes the finding of Silver and Landis in 2011 [252]. In the gap region, it is a common occurrence that all three alpha chains contain two or three adjacent amino acids. In addition there is a frequent pairing of negatively charged amino acids (glutamic acid and aspartic acid) in close proximity to positively charged amino acids (lysine and arginine), shown in Figure 46 [251]. The pattern of alternating charge-clustering leads to high charge densities at discrete locations and initiates the binding of calcium and phosphate ions. This process is believed to drive nucleation in the gap regions, followed by growth along the highly aligned collagen fibrils via contact guidance.

Figure 46: Charge Density Clusters in the e1 and e2 Domain of the Gap Region. D = aspartic acid (+), E = glutamic acid (+), K =lysine (+), R = arginine (+), and H = histodine (+). Landis notes that one positively charged residue may be replaced by hydroxylysine (+). Image reproduced with permission from [251].
The gap-overlap region also provides the binding sites for proteoglycan cores and GAGs such as chondroitin sulphate, dermatan sulphate, and keratin sulphate [253, 254]. Proteoglycans impact the hydration state, fibril shape, fibril spacing, and mechanical integrity of the tissue [255-259]. The proteoglycan decorin binds to the collagen in the d and e domains of the gap region, which is why little to no decorin is present in the bone [254, 260]. It has recently been recognized that decorin competes with the mineralization process [261, 262], but unfortunately there is not yet a supporting body of literature describing the fascinating dynamics that must occur at the bone-tendon enthesis, where a gradient of tendon mineralization occurs.

**Chapter 4.1.4: Periodicity and the Molecule Length**

Without knowing any of the previous reasons for the importance of the banded state, the importance could have been concluded from the simple fact banding is an evolved feature. Collagen, in its most primitive form, was nothing more than six repeats of G-P-P. Collagen progressively evolved to its current length and adapted the quarter stagger pattern to provide some advantage. If we consider the stagger between molecules to be something that was set early on through the pairing of complimentary amino acids, then the fact that the collagen molecule is 4.4 D-periods must also serve a purpose. Shown in Figure 47A, the length is not arbitrary but rather a consequence of functional design. At this length, the gap regions fall into perfect lateral registration. If the alpha chain was longer, as seen in Figure 47B, or shorter (not shown), then the resulting gap regions would fall out of lateral registration. This pattern may impact the mechanical properties, the hydroxyapatite crystal growth, the hydration spheres of the proteoglycans, and the GAG chain interactions.
Figure 47: Collagen Banding Pattern. The five colors used per ‘molecule’ are representative of the 5 exons that code for the monomer. Image A is a representation of the gap region using the actual monomer length, 4 complete exons (234x4 amino acids) and one exon truncated down to 0.4 the length of the others. Image B is a representation of the gap region if the molecule was 5 full exons (234x5 amino acids) long.

Chapter 4.1.5: Introduction Summary
If the goal is to produce functional replacement tissues, then the monomers have to be properly located in the fibrils, and the fibrils have to be properly oriented such that the native structure is replicated. Collagen is much more than a load bearing material; it has specific bond formation sites, protein binding sites, and cell binding sites. The fiber forming method presented in Chapter 3 has the potential to properly locate the fibrils within the fiber, but this came at a sacrifice of the monomeric positions. This method forms fibrils with largely increased kinetics when compared to collagen gelation, and then these newly formed fibrils are subsequently dehydrated. The remainder of Chapter 4 serves to develop a technique to recover collagen banding. It was mentioned earlier that other researchers have been able to recover fibril banding through incubation [86, 115], but the unique challenge here was recovering banding after complete dehydration. The mechanical properties of collagen are higher in the dehydrated state due to increased bond formation, and this may greatly inhibit the mutability of the matrix.
Chapter 4.1.6: Matrix Mutability
The underlying principle behind this chapter’s research is that surrounding environmental parameters can be varied to either stabilize or destabilize a monomer’s position within a fibril. There is a large multi-parametric space (conceptually depicted in Figure 48) that determines how strongly a monomer is locked into its current configuration. The environmental parameters are chemical (ionic strength, temperature, pH), molecular (PGs, GAGs, elastin, fibronectin, collagens), and physical (concentration, packing density, crosslinks, strain). There are ranges for a single parameter that stabilize as well as ranges that destabilize. There are multiple energy wells because there are numerous ways that stability can be inappropriately imposed, such as by adding fixative agents. The goal of this study was to navigate the landscape until all of the monomers had shifted into the proper energy well, such that the native configuration (fibril banding) was recovered. The dehydration step that occurred after drawing a fiber from a droplet of solution mispositioned the monomers, and a post-incubation step with carefully selected environmental parameters was applied to drive controlled matrix mutability.

Figure 48: An energy landscape representing the stability of a collagen monomer on a fibril, under the influence of multiple varying parameters.
The temperature was increased to 37°C to provide thermal energy and destabilize mispositioned monomers. The ionic strength and pH was brought to physiological values to weaken the electrostatic interaction between charged amino acids and destabilize the monomers. Decorin was added due to its periodic presence in the gap/overlap region and ability to compete for binding sites across multiple monomers. Decorin was expected to stabilize regions of the fibril that were properly configured, while destabilizing monomers that obstructed the native gap/overlap region. PEG was used with the intention to stabilize the entire system such that monomers would stay in close proximity to the main fiber and molecular shifts could occur without monomers completely dissociating.

**Chapter 4.2: Materials and Methods**

**Chapter 4.2.1: Collagen Fibers**
Collagen fibers were produced as described in Chapter 3.2.3: Fiber Pulling Protocol. The source material was acetic acid extracted, bovine dermal collagen, which preserved the telopeptides for biological relevance. Fibers were stored in a dry container, attached at one end to a glass micro-needle and suspended in the air. The collagen fibers were incubated, sometimes fixed, and tissue grinded for electron microscopy imaging. The tissue grinding was implemented to separate out single fibrils for clear analysis of fibril banding. The fibers that were fixed were exposed to a solution of 2.5% glutaraldehyde and 2.5% paraformaldehyde in a 0.1 M sodium cacodylate buffer for 1 hour prior to tissue grinding.

**Chapter 4.2.2: Fiber Incubation Protocol**
Each set of fibers was incubated for 48 hours in an unloaded configuration at 37°C. Four incubation conditions were explored to recover fibril banding:

1. 10 mM phosphate buffer with 150 mM NaCl
2. 10 mM phosphate buffer with 150 mM NaCl, 1.63% PEG
3. 10 mM phosphate buffer with 150 mM NaCl, 2% Decorin

4. 10 mM phosphate buffer with 150 mM NaCl, 1.63% PEG, 2% Decorin

The buffer was chosen based on the work of Harris et al. [220]. They investigated fibrillogenesis in varying buffers, ionic strengths, and pH conditions. This buffer provided the clearest visualization of banding on the TEM. The PEG condition was implemented to provide an environment that was more physiologically relevant. The work of Sverdlik et al. calculated the oncotic pressure of the interstitial fluid surrounding the tendon [216], which extrapolated to 1.63% at 37°C. The value of 2% decorin was chosen based on physiological values. The tendon typically has an average of 1% (3 µg/mg) molar concentration by dry weight [217-219]. Twice the physiological value was chosen to increase ratio of the availability of decorin to the available binding sites and help drive the interaction to completion. The 2% decorin molar concentration, compared to the molar amount of collagen, was also included in the solution for pulling the fiber. This equated to 26 µg/ml in the solution, which was also the amount used in the incubating buffer.

Chapter 4.2.3: Tissue Grinding Protocol

Each collagen fiber was placed in a glass tissue grinder (885470-0000, Kimble Chase), shown in Figure 49, with 100 µl of 10 mM phosphate solution with 150 mM NaCl. A helpful instruction for getting the fiber into the tube is to gently wrap the wet fiber around the very tip of the tissue grinding probe. Attempts to place the fiber in the tube with tweezers will only result in horrible devastation. The fiber was grinded until visible fibrous structure could not be recognized and the fluid had turned opaque.
Chapter 4.2.4: Sample Prep onto a TEM Grid

300 mesh Formvar coated TEM grids (01701-F, Tedpella) were used for imaging the tissue grinded collagen fibrils. The TEM grid underwent a series of droplet-treatments, depicted in Figure 50. From blue to yellow, the droplets rows are: 1% alcian blue, deionized water, 20 µl of grinded tissue solution, deionized water, and 1.5% uranyl acetate. The grid was sequentially placed atop each droplet (formvar side down) and then brought in contact with filter paper to wick off the excess fluid. The grid was first placed on the 1% alcian blue for 15 seconds to make the surface of the grid more hydrophilic. The grid was wicked dry with filter paper and moved between water droplets and filter paper to wick off the excess stain. The grid was next placed on the tissue grinded solution for 3 minutes to permit fibril adsorption. Then the three washes and filter paper drying steps were repeated to remove larger pieces of tissue and loosely bound material. Finally, the grid was continually held with tweezers and lowered onto the droplet of 1.5% uranyl acetate for a couple of seconds. The grid was wicked dry and brought back to the uranyl acetate for a total of 5 exposures. After a final drying with filter paper, the grid was stored for later imaging with a JEOL JEM 1010 transmission electron microscope. As a note, when the grid was brought into contact with the filter paper, a shallow angle was maintained such that only
the very edge of the grid was in direct contact with the filter paper. This was believed to aid in retaining the material on the grid.

Figure 50: Droplet Treatment for a Formvar-Coated TEM Grid. The blue droplet is 1.5% Alcian Blue. The aqua droplets are deionized water. The clear droplet is tissue grinded solution. The yellow droplet is 1.5% Uranyl Acetate.

Chapter 4.2.5: Measuring Banding Periodicity
Images of banded fibrils were rotated such that the lines separating the banding domains were oriented vertically. Next, the image was cropped down to the longest region of banding achievable and a Fast-Fourier Transformation was applied using MATLAB. The power spectral density was calculated for each row, averaged, and plotted. The resulting peak within the range of 50 – 70 nm was taken to be the banding. An example is shown in Figure 51.
Chapter 4.3: Results

Chapter 4.3.1: TEM of Fixed Collagen Fibrils
At the earlier stages of this research, fibers were incubated for 48 hours in 1x PBS, rather than 10 mM phosphate buffer with 150 mM NaCl. This was in accordance with Advanced Biomatrix’s protocol for neutralizing collagen solutions. Additionally, fibers were cross-linked with fixative in attempts of preventing any post-processing changes. The procedure resulted a complete lack of
any deposited fibrils on the TEM grid in ~98% of the hexagonal windows. Figure 52A shows a fibril that was formed in 1x PBS without any PEG or decorin and stained with uranyl acetate (UA). There is a hint of banding, but interestingly the banding pattern is at an offset angle, rather than perpendicular to the fibril length. Figure 52B shows a fibril that was formed in 1x PBS with 2% decorin and stained with UA. The fibril banding is clear enough to resolve some of the sub-bands, and the globular regions that are periodically attached to the fiber are suspected to be the decorin protein cores. Figure 52C shows a fibril that was formed in 1x PBS with 2% decorin and stained with 1.5% phosphotungstic acid (PTA). The different stain was used to as a test to determine if better resolution banding could be achieved. The banding was visible but not necessarily improved. Across the different samples imaged, the average periodicity measured 56.3 ± 3.4 nm. However, in all cases explored up until this point, 20 – 50% of the imaged fibrils looked like Figure 52D.1 and Figure 52D.2. In these images, the fibrils have been ruptured and damaged. The banding is typically weaker or completely undetectable.
Figure 52: Collagen Fibril Banding in 1x PBS after 48 Hours of Incubation. Image A is stained with 1.5% UA. Image B had the addition of 2% decorin and 1.5% UA. Image C had the addition of 2% decorin and 1.5% PTA. Image D.1 and D.2 represent 20-50% of all fibrils seen, although these exact fibrils were 0% decorin 1.5% UA.

Chapter 4.3.2: Control: A Dense Collagen Scaffold

Chapter 4.3.2.1: AFM of a Dense Collagen Scaffold
The broken, unbanded fibrils suggested there was an issue with the tissue grinding process, so a control sample was tested. The laboratory of Dr. Ruberti has extensive experience with dense
collagen scaffolds for cell testing experiments. These scaffolds are produced by neutralizing 3 mg/ml acetic acid extracted, bovine dermal collagen, injecting it into a dialysis cassette, incubating it for 12 hours for complete fibrillogenesis, and the dialyzing against 40% polyethylene glycol. These scaffolds have been characterized by AFM, as seen in Figure 53.

![AFM image of Dense Collagen Scaffold. The AFM imaging showed extensive collagen banding throughout the scaffold.](image)

**Figure 53: AFM image of Dense Collagen Scaffold.** The AFM imaging showed extensive collagen banding throughout the scaffold.

**Chapter 4.3.2.2: Tissue Grinded TEM of a Dense Collagen Scaffold with Fixation**

The fixation process, tissue grinding process, and TEM preparation was repeated for the dense collagen scaffold. Figure 54 demonstrates the same characteristic ruptures seen with the cross-linked tissue grinder fibers that were then UA stained. A lack of banding, globular regions, and frayed ends were equivalently prevalent. The average banding periodicity of the fibrils was 54.1 ± 3.6 nm. A second set of fibers that had been stored for a week in fixative was also processed (not shown) and nearly all of the fibers were ruptured and unbanded.
Figure 54: TEM images of Tissue Grinded, Fixed, Dense Collagen Scaffolds. Image A is a broken fibril that no longer demonstrates collagen banding. Image B has a fractured fibril end, but banding is retained.

**Chapter 4.3.2.3: Tissue Grinded TEM of a Dense Collagen Scaffold without Fixation**

As a final check, a dense collagen scaffold that was not cross-linked was imaged, shown in Figure 55. The vast majority of the UA stained fibrils appeared banded, undamaged, and without fraying fibril ends. The average banding periodicity was $57.7 \pm 2.9$ nm. The fixation process appears to have a slight impact on collagen periodicity, possibly constricting the entire fibril or the gap-overlap region.
Figure 55: TEM images of Tissue Grinded, Unfixed, Dense Collagen Scaffolds with UA stain. Image A reveals an interesting banding pattern that was uncharacteristic of the fibrils but noteworthy. Image B shows the typical fibril found when imaging this condition.

Chapter 4.3.2.4: Tissue Grinded TEM of a Dense Collagen Scaffold without Fixation with PTA Stain instead of UA Stain

The uncross-linked fibers from the dense collagen scaffold were also imaged after staining with PTA. The PTA had a destructive effect, causing fibril fraying throughout the entire length, shown in Figure 56. Some regions of the fibrils showed microfibrillar separation, but banding was still evident (Figure 56A). Other regions had completely disbanded and only subfibrillar structures remained, seen in Figure 56B.

Figure 56: TEM images of Tissue Grinded, Unfixed, Dense Collagen Scaffolds with PTA stain. Uncross-linked fibrils have begun to separate (image A), and in other cases have completely dissociated (image B).

For all future experiments, three major changes were imposed. First, the tissues were cross-linked. The added mechanical integrity of the cross-links was suspected to damage the fibrillar structure during tissue grinding and prevent clean separation into material thin enough to adhere to the grid and transmit through. Second, the UA stain was used for future experiments instead of the PTA stain. The fibrillar structure was highly susceptible to dissociation in the presence of PTA. Third, the incubating buffer was changed from 1x PBS to 10 mM phosphate buffer with 150 mM NaCl. The compelling images of Harris et al. visually suggested that this buffer was
superior to other buffers and salt solutions for yielding banded collagen fibrils [220, 263]. TEM sample dehydration and beam damage is known to distort the periodic spacing, but it was desirable to investigate if the different buffer could alleviate some of the discrepancy.

Chapter 4.3.3: TEM of Unfixed Collagen Fibrils

Chapter 4.3.3.1: Phosphate Buffer
Fibers were incubated for 48 hours in 10 mM phosphate with 150 mM NaCl, left uncross-linked, tissue grinded, and UA stained for TEM imaging. The resulting fibrils were nearly all banded, although the periodicity became very difficult to measure. The majority of the fibrils had banding patterns similar to those seen in Figure 57. The repeating subdomains could not be easily differentiated to define a banding periodicity. In addition, most fibrils appeared to lack a clear edge along the length of the fibril.
Chapter 4.3.3.2: Phosphate Buffer and Decorin

Fibers were incubated for 48 hours in 10 mM phosphate with 150 mM NaCl and 2% decorin, left uncross-linked, tissue grinded, and UA stained for TEM imaging. The resulting fibril banding spanned more of a range than the fibrils that were only incubated in phosphate buffer. Figure 58 shows fibrils that looked very representative to those that were incubated without decorin. However, Figure 59 shows fibrils with much more of a distinct fibril banding pattern. The periodicity was more easily recognized, and the banding could be measured. Figure 59E shows
two fibrils in very close proximity with greatly differing banding patterns. This eliminates stain exposure for causing such differences.

Figure 58: TEM images of tissue grinded, unfixed, fibers that were incubated in phosphate buffer + decorin and were stained with UA. The fibrils have banded domains but the periodicity is difficult to characterize.
Figure 59: TEM images of tissue grinded, unfixed, fibers that were incubated in phosphate buffer + decorin and were stained with UA. The fibril banding periodicity can be more feasibly measured.
The banding periodicity of the fibrils shown Figure 59 was measured according to Chapter 4.2.5: Measuring Banding Periodicity. Table 3 displays the results. The average fibril periodicity was 65.4 ± 2.2 nm. This value is in very close agreement with the periodicity of native fibrils. The small difference could be a consequence of the TEM process, particularly beam deformation.

<table>
<thead>
<tr>
<th>Image</th>
<th>Periodicity (nm)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>64.71</td>
</tr>
<tr>
<td>B</td>
<td>68.26</td>
</tr>
<tr>
<td>C</td>
<td>63.95</td>
</tr>
<tr>
<td>D</td>
<td>67.65</td>
</tr>
<tr>
<td>E</td>
<td>62.5</td>
</tr>
</tbody>
</table>

Chapter 4.3.3.3: Phosphate Buffer + PEG and Phosphate Buffer + PEG + Decorin
These two categories have been grouped together because the fibrils had a very similar appearance. Figure 60A and Figure 60C are images from phosphate buffer and PEG incubation. Figure 60B and Figure 60D are images from phosphate buffer and PEG and decorin incubation.

Figure 60A and Figure 60B are representative of almost all of the fibrils seen for these two samples. Fibril sizes were typically 30–40 nm, without any signs of banding. Figure 60C was the one single fibril imaged with any resemblance of fibril banding. It was rather faint without distinct periodicity, but the repeated pattern suggests that the monomers were very close to proper alignment. Figure 60D was also a unique picture, where the microfibrils could be seen within a larger fibril. This is reminiscent of the PTA deteriorated fibrils, however this fibril is likely progressing the other way. The PEG appears to retard the molecular shifting that leads to banding, so this fibril may have only progressed a small amount from the completely dehydrated stage.
Figure 60: TEM images of tissue grinded, unfixed, fibers that were incubated in phosphate buffer + PEG (A & C) and fibrils that were incubated in phosphate buffer + PEG + decorin (B & D) and were stained with UA. Image A and B represent the vast majority of the fibrils that were imaged. Image B is the one banded fibril that was found. Image D is an interesting larger fibril with recognizable microfibrils.

Chapter 4.3.4: TEM of Whole Fibers
The TEM work done in Chapter 3.3.5: TEM Structural Investigation was extended. The goal was to determine what the structure of the fiber looked like after an incubation period of only one hour. The one hour time frame was intended to give the fiber time to rehydrate without giving the extensive 48 hours to remodel.
Chapter 4.3.3.1: TEM of 1 Hour Incubated Fibers, Unloaded

A set of telo-fibers was prepared by incubating in 10 mM phosphate buffer containing 150 mM NaCl and 2% decorin for 1 hour in an unloaded configuration, before being processed for TEM as described in Chapter 3.2.7: Transmission Electron Microscopy. The objective was to determine if the 48 hours were necessary or the incubation lead to the same fibrillar structure almost immediately (1 hour). The samples were unloaded because they were processed in parallel in a six-well plate. This was not intended to have any impact on the process, but as seen in Figure 61, the unloaded configuration has quite an impact on the structure. The aligned peripheral fibrils were beginning to shed from the fiber, shown in the Figure 61A and Figure 61B. Figure 61C and Figure 61D show that the same core structures were present and unaffected by the change in loading state or the reduction to only one hour of incubation. Similarly, Figure 61E and Figure 61F show that central most structure was unaltered, but that fibrils had achieved a banded state in only one hour incubation. This is useful to know, since previous work has shown weak x-ray signal that suggests banding at 12 hours and TEM data only at 48 hours [86, 115].
Figure 61: TEM of telo-fibers after 1 hour of incubation in 10 mM phosphate buffer containing 150 mM NaCl and 2% decorin in an unloaded configuration. Images A and B depict external fibril shedding after only one hour in the buffer. Images C and D show an unaffected/unchanged structure from the 48 hour incubation in a taut configuration. Images E and F show that the fibrils were able to transition from unbanded to banded after only one hour of incubation.
Chapter 4.3.3.2: TEM of 48 Hour Incubated Fibers, Unloaded

A set of telo-fibers was prepared by incubating in 10 mM phosphate buffer containing 150 mM NaCl and 2% decorin for 48 hours in an unloaded configuration, before being processed for TEM as described in Chapter 3.2.7: Transmission Electron Microscopy. This was an investigation into what would happen to the structure, since only one hour caused a separation of the peripheral fibrils from the central fiber. Figure 62A shows that the peripheral fibrils had progressed in their separation from the fiber over the 48 hours. In many instances the aligned outer shell could no longer be recognized at all, shown in Figure 62B. Figure 62C shows that the central patterns were also affected after 48 hours. The packing of the fibrils was now much less dense and many fibrillar structures appeared to be located obliquely to the sectioning plane, shown in the higher magnification image of Figure 62D. This would imply that very free interfibrillar bonds persisted and a large portion of their length was able freely move/float. Figure 62E shows single banded fibrils in the core of the fiber, while Figure 62F shows banded fibrils on the periphery.
Figure 62: TEM of telo-fibers after 1 hour of incubation in 10 mM phosphate buffer containing 150 mM NaCl and 2% decorin in an unloaded configuration. Images A and B depict external fibril shedding after only one hour in the buffer. Images C and D show an unaffected and unchanged structure from the fibers incubated for 48 hours in a taut configuration. Images E and F show that the fibrils were able to transition from unbanded to banded after only one hour of incubation.
Chapter 4.4: Discussion

The fiber pulling technique discussed in Chapter 3 greatly accelerates polymerization and is followed by a non-physiological complete dehydration. The fibrils experience immense changes in ionic strength, pH, and bond availability from removal of water. Thus, the fibrillar alignment attained came at the expense of monomeric misalignment. This chapter served to examine the molecular mutability of the collagen monomer within a fibril, intended to restore native banding. Examination of the fibrillar structure after rehydration and incubation was performed with TEM using a tissue grinding technique and the conventional embedding / sectioning technique. Tissue that was known to display visually prevalent fibril banding was used to refine the tissue grinding protocol for the drawn collagen fibers. Once the approach was working satisfactorily, four buffers used during incubation were investigated for their success in yielding banded collagen fibrils. Conventional tissue preparation and imaging was later performed to investigate how quickly the 48 hour structure was achieved. A 1-hour incubation period was used to observe the changes from Figure 35 (TEM with 0-hour incubation) to Figure 41 (TEM with 48-hour incubation).

The tissue grinding experiments with dense scaffolds revealed two important factors. First, cross-linking of the tissue caused great difficulty in grinding the tissue down to single fibrils for imaging. Tissue grinded cross-linked fibers produced ~98% empty TEM grids. Additionally, the fibrils that did transfer to the grid were often ruptured or fractured with compromised banding. Once the cross-linking step was removed, the tissue grinded solution needed to be further diluted so the fibrils were not so densely packed on the grid. Numerous hexagonal windows were still empty, which implies the 1% alcian blue hydrophilic coating process can be additionally improved upon in the future. The second item learned was that PTA stain had a degradative effect on uncross-linked collagen fibrils. In the author’s opinion, it appeared as though the negative
staining of PTA was superior to UA, but UA was inert to the fibril structure and thus was the only viable choice.

The tissue grinding results were very conclusive. The fibrils that were incubated in only phosphate buffer were regularly found to be banded. The fibrils lacked clear physical boundaries, as well as clear periodicity despite the evident banding. The poorly defined edges of the fibrils suggest that the interaction with the negative stain may need to be improved.

The fibrils that were incubated in phosphate buffer with decorin had two distinct appearances. Some of the fibrils closely mirrored the previous set, while others had banding with well-defined banding periods. Figure 59E shows a fibril with each appearance side by side. This close proximity challenges the premise that the stain is leading to such stark differences. At this point it is unclear what the difference is between banded/not periodic and banded/periodic fibrils.

The phosphate-PEG group and the phosphate-PEG-decorin group yielded nearly identical results. The fibrils were almost all unbanded, but one important distinction is that the fibrils were all much smaller. This likely has to do with the molecular crowding of the PEG keeping the fibrils at a lower hydration state. Smaller diameter fibrils, such as those found in the cornea, often have undetectable bands. On the other hand, the oncotic pressure of PEG may have kept the monomers from being able to reversely bond from the fibril and then reposition appropriately. This cannot be determined conclusively without additional information such as x-ray scattering data.

The final two experimental sets may have produced the most intriguing results. It appears that in the absence of oncotic pressure, strain is a required parameter to sustain a densely packed fibrillar fiber. After only one hour in phosphate buffer and decorin, the fibrils began to separate from the fiber. This effect was even more pronounced after 48 hours, where even the core of the fiber had achieved a much lower density state. The reduction in interfibrillar interaction that decorin
produced likely encouraged this effect. The bonds holding the fibrils together may have a constant on/off rate. The fibrils on the perimeter have fewer total bonds due to their positions, and thus over time is may cause them to start to fray from the fiber. These results strongly suggest that strain imbues stability even after assembly, similar to that seen in a catch bond. Such stability was needed to hold a fiber together, without crosslinks or a surrounding hypertonic environment.

**Chapter 4.5: Conclusions**

It can be concluded that collagen monomers can recover from a state of dehydration and recover the native banding pattern. The average fibril periodicity for fibers incubated in phosphate buffer and decorin was 65.4 ± 2.2 nm. These experiments also showed that in order to achieve molecular mutability without sacrificing macroscale order, a taut fiber with low levels of strain was required. To extrapolate on this finding, it is likely that highly judicial amounts of strain or oncotic pressure may yield the same effect. While low degrees of cross-linking may serve the equivalent purpose, the cross-linked areas will suffer from the permanent positioning and possibly compromise the mechanical integrity.

Once the critical concentrations and elongational flow fields for pulling the fiber are properly defined, fibers can be made with highly aligned fibrillar cross sections and now can be post processed to achieve natively banded patterns. These fibers will immediately provide excellent scaffolds for live-cell, contact guidance, cell differentiation, and bone mineralization experiments. In the longer term, upscaling of the system should open the door to producing tissue replacements, especially those with minimal vascularization and innervation, such as the tendon and ligament. Stem cell based tissue engineering has an incredible amount of potential, but the appropriate scaffold may greatly accelerate the process. In addition, highly complex geometries,
such as those involved in craniofacial reconstruction may be best served by directly controlling the ECM.

CHAPTER 5: Dissertation Summary

“From sponges to humans, collagen is always present, in all organisms, without exception,” [22, 38].

“Science requires […] considerations, which form the most beautiful part of the study of animal structure, which exhibit nature everywhere uniform in her operations, varying only in their results, sparing of the means she employs, prodigal only in the effects she obtains; modifying in a thousand ways a few general principles which, differently applied, preside over our economy, and constitute its numberless phenomena,” [212].

These quotes are being revisited because they have provided the fundamental axioms upon which my Ph.D. has been built. When it comes to tissue engineering approaches, the three main thrusts are: synthetic materials, stem-cells, and collagen. The first quote serves to remind that collagen has been evolving and adapting for hundreds of millions of years. Designing a synthetic material to replace a collagen tissue is a short-term solution that assists with an immediate problem. Conditioning stem-cells to properly differentiate and replicate native tissue is an appropriate solution for the particular tissue of interest, but the solution is likely limited to that one tissue. Collagen is found in nearly a dozen different tissues and so this entire process may need to be replicated equally many times. This dissertation takes the approach of performing the basic science required to understand how to control the molecule directly, such that the technologies created can be translated to recapitulating any of the numerous collagen-based tissues. Each
approach has benefits and drawbacks, but there is a certain appeal to gaining a fundamental insight into a billion year old molecule that is found in every living organism.

The second quote is just over 200 years old now, but perhaps the concept has never been said more eloquently. If the goal is to learn how to assemble collagen tissues, then lessons can likely be learned from how these tissues are grown, how they are maintained and turned over, and how molecules of a similar class (extracellular proteins) do the same. Following the premise that in nature a few different principles are uniquely applied to constitute numberless phenomena is an ingenious way to look at the world. When you think in such a manner, you can find the similarity behind a growing tomato and an eye. If you remove the internal pressure, both fail to achieve their unaltered state because universally the pressure, i.e. the tissue strain, regulates the growth. Mechanosensitivity is also a universal principle, it is why trees that grow on hills still grow in direct opposition to gravity, and it is why small amounts of plaque built-up in arteries is so detrimental (the blood flow changes from laminar to turbulent and the shear stresses drastically change). Segueing back toward the work of this dissertation, the challenge is determining how much of the mechanosensitivity can be attributed to the cells and how much is actually attributed to changes in the proteins.

The first aim of this research, Chapter 2, focused on utilizing a confining geometry and the innate liquid crystalline quality that collagen possesses to determine if native fibers can be replicated. The question being pursued was: do cells make tissue by isolating small regions of space, secreting collagen monomers and water sequestering molecules (i.e. hyaluronic acid), and letting physics drive the reaction? Cells can leave the propeptides on the collagen molecules to inhibit polymerization until the concentrations are sufficiently high that a liquid crystalline state is achieved, but it is currently unfeasible to acquire quantities sufficient for these experiments. We
tried to mirror the effect by using an acidic environment, but this change had associated consequences. The monomers aggregated in a ribbon-like sinusoidal pattern that was not corrected upon neutralization. This setup was not able to properly test the hypothesis, and as an alternative the experiments proceeded with our hyaluronic acid substitute, PEG, being neutral pH rather than acidic. The results were modest, in that we achieved fibers with fibril angular distributions within ± 25° of the long axis of the fiber. However, we never truly tested the hypothesis because our working setup had fibrillogenesis occurring concurrently with changing pH, ionic strength, and collagen concentration. The use of a confining, high-aspect ratio geometry and liquid crystalline behavior has tremendous potential if premature assembly can be avoided without introducing undesirable side effects. Liquid crystalline materials can assemble uniaxially as well as form orthogonal lamella at certain concentrations; thus, the future applications of this technology could provide advances for numerous collagen-based tissues.

The second aim of this research, Chapter 3, sought to investigate the mechanosensitivity of the collagen molecule by using strain to trigger organized collagen assembly. Absence of tissue strain inhibits normal eye growth and increased strain is associated with the diseased state glaucoma, so potentially strain influences the ECM and not just cells. Chapter 3 discussed the role of strain in collagen degradation, elastin assembly, and fibronectin assembly (all in acellular environments). This background information led to the logical progression of testing collagen assembly in a likewise manner. The experiment took place at the interface of a droplet of collagen solution, where surface concentrations were increased prior to any stimulation. The mechanical strain was implemented in the form of elongational strain, and the post-process imaging (DIC, SEM, TEM) revealed that in certain regions this technique was able to demonstrate complete control over fibril alignment. The areas of high alignment, the periphery, persisted for the entire length of the fiber, which indicates a dominant assembly guiding cue. As with the eye, where there is only a
narrow window of appropriate strain for healthy tissue, the areas of disorganization represent where the concentration or strain rate wandered outside the desired range. This body of work requires a substantial amount of characterization and equation fitting to be optimized for tissue engineering applications, but there are no major foreseen obstacles. Other biological molecules such as decorin have already been included in the starting solution, which is another step toward making truly biological fibers. The next step will be to implement the mechanisms of this system in an embodiment that moves away from the complexities of a solution interface.

The third aim of this research, Chapter 4, was motivated by collagen’s inherent ability to form native D-periodic fibrils. The drawn fibers from Chapter 3 experience dehydration as they are raised out of the droplet, which consequentially mispositions the monomers with each fibril. The bonds that held the monomers together were non-covalent and so the mutability of collagen during an incubation period was explored. The hypothesis was that collagen monomers could settle into their lowest energy state if they were appropriately disturbed from their current state. Thermal energy was put into the system, and as the fibrils were rehydrated, water molecules had access to compete for hydrophobic binding sites. It was assumed that the lowest energy state was that of the banded fibril, which would explain why it could be spontaneously achieved. After 48 hours of incubation, fibrils that were incubated in phosphate buffer or phosphate buffer + decorin regained collagen banding. The decorin molecule is associated specifically with the gap region of D-periodic fibrils, so it was intended to destabilize and compete for the binding sites in this domain, aiding with mutability. The phosphate + decorin incubation had the greatest impact on restoring the periodicity, having an average D-banding of 65.4 ± 2.2 nm. Interestingly, fibers that were incubated in PEG failed to reliably demonstrate banding. The oncotic pressure of the PEG, although physiological, may have acted as a stabilizer to the current position of the monomers, and thus actually inhibited mutability.
An unexpected discovery during this experimentation was the fact that fibers incubated without a taut configuration began to lose their macroscale structure. The strain stabilized the interfibrillar bonds and allowed the fiber that was incubated for 48 hours to be structurally unaffected. The unloaded fibers had a visible dissociation of the peripheral fibrils after 1 hour and a complete dissociation of peripheral fibrils after 48 hours. The fibers also showed decreased packing density in the core of the fibers that were incubated for 48 hours.

Perhaps the overarching conclusion of this Ph.D. is there is a necessity to maintain judicial control over the strain field prior to, during, and subsequent to collagen fibrillogenesis. Other stabilizers exist which can be additionally utilized, such as proteoglycans (binding to multiple monomers), GAGs (contributing oncotic pressure), and cross-linking agents (adding covalent bonds); however many of these are not present in the earliest stages of tissue engineering, and thus strain is a valuable substitute. This author would predict that exertion of strict control over strain will eventually prove essential for replicating assembly, growth, and turnover.
REFERENCES


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