Gel-spinning of Mimetic Collagen and Collagen/Nano-Carbon Fibers: Understanding Multi-scale Influences on Molecular Ordering and Fibril Alignment

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ABSTRACT

Synthetic gel-spun collagen and collagen/nano-carbon fibers were found to exhibit structural mimicry comparable to native tendons. X-ray scattering and microscopy analyses are used to characterize the molecular and fibrillar alignment in the synthetic fibers, where D-banding is observed throughout the spun fibers – consistent with native collagen. For the composite collagen/nano-carbon fibers, the morphology and dispersion quality of the nano-carbons within was found to play a significant role in influencing collagen molecular ordering and fibril alignment. Fibrillar and molecular alignment was also better preserved during elongation of the composites as compared to the control collagen fibers. These results show the structural

influence of a rigid inclusion on the collagen fibril structure. Both dry-and wet-state tensile testing were performed on the collagen fibers, and these results show behavior comparable to the native materials. Dry-state tests also reveal interfacial interaction between the nano-fillers and the collagen fibrils through theoretical analysis. Wet-state tensile testing indicates the *structure-property* behavior of the mimetic hierarchical structure within the synthetic fibers.

KEYWORDS

Collagen; fiber; self-assembly; mimetic; nanotechnology; gel-spinning.

1. INTRODUCTION

Currently there are several different types of collagens which fall into two subgroups (i) fibril-forming and (ii) non-fibril forming. Of the fibril-forming collagens, type I is the most predominant and is found in bone, tendon, ligament, skin, and connective tissue.[1, 2] Its abundance and relative ease of extraction has made it one of the most widely used collagens in research.[2] Studies using type I collagen include scaffold materials [3-5], wound healing[6, 7], cancer and cell behavior[8-11], and growing tissues[12, 13]. Type I collagen molecules may be extracted from several native sources including rat-tail tendon and bovine hide through solubilization and purification processes. The collagen fibril and fiber structure can be reconstituted/assembled from these source molecules using synthetic processing conditions that favor spontaneous assembly. Typical starting parameters for collagen assembly resemble the micro-environment of the human body in terms of temperature (warm (~37 °C)) and pH (neutral ~7).[1, 14] Although, it is well known that collagen fibrillogenesis (i.e., fibril formation) occurs via cues from nearby cells of the ECM, [15] the exact assembly pattern and growth mechanisms as well as the role of other collagen types and macromolecules present in this process are still not well grasped.[16-18] Understanding these mechanisms and factors will help in treating collagen related diseases, fabricating synthetic fibers closely mimicking native tissue (i.e., ECM), and developing materials for numerous tissue engineering applications.[19-22]

The major routes for producing collagen fibrils in vitro include electrospinning[4, 23, 24], thermoplastic spinning[25], and wet-spinning[26, 27]. The challenges associated with all methods for synthetically producing collagen fibers include, fiber discontinuity, misalignment, inability to achieve sufficient strength, failure to replicate the internal fibrillar structure consistent with native collagen, and reproducibility.[4, 23-27] Electrospun collagen scaffold

fibers are typically continuous and easily formed, but exhibit low strength (~2-12 MPa).[24, 28] These fibrous scaffolds also lack the characteristic D-banding associated with molecular packing due to the use of harsh spinning solvents.[29] In contrast, wet-spun fibers exhibit much higher mechanical properties (~55-225 MPa),[6, 18] and in some cases D-banding features are observed. However, the fibers from this processing route may not be continuous or reproducible with consistent quality.[26, 30] Depending on the final application of the collagen fibers, one processing route may be preferred over the other, since each spinning method shows promising aspects as well as limiting factors for forming fibers. In this work, a flow based gel-spinning approach is used to fabricate collagen fibers. The resultant fibers exhibit a multi-scale fibrillar structure (i.e., sub-micron) similar to native tendon.[31] This work aims to identify new processing conditions required to produce synthetic collagen-based materials with native structural mimicry.

In addition, nano-carbons are used to fabricate composite fibers in order to investigate how rigid nano-inclusions can influence and aid fibril alignment by acting as a template.[32-34] It is anticipated that due to these similar features as well as the inherent rigidity for the SWNT and CNC, these fillers may aid the self-assembly and in particular alignment of the collagen monomers and subsequent fibrils during fiber processing. SWNT are rod-like molecules which also exhibit liquid crystalline behavior and are also similar in size to the collagen monomer when dispersed as individual tubes. CNC are platelet-like materials with specific dimensions at the same order of magnitude to that of the collagen. The collagen monomer can be considered as a liquid crystalline biopolymer through characterization by polarized light microscopy and X-ray diffraction *in vivo* and *in vitro* [35]. Molecular solutions for this rod-like triple helix have exhibited behavior similar to lyotropic liquid crystals. Typically, aromatic polymers consist of

rigid and anisotropic molecular configurations that cause the formation of liquid crystalline regions. Polypeptide chain-based liquid crystalline polymers are extrinsic mesogens. The molecules of these extrinsic mesogens are conformationally flexible, and the formation of geometrically anisotropic mesogens is dependent on the appropriate micro-environment [36]. Considering the similarities of these materials components, it is expected that the inclusion of the rigid filler in composite fiber formation may lead to better fundamental understanding toward the ability to template ordering in biopolymers. This may provide some materials science insight into the formation of bone, which requires an interplay between collagen and a rigid inclusion for proper functionality.

2. MATERIALS AND METHODS

2.1. Materials

All reagents were used without further purification. PureCol[®] (Bovine collagen solution, type I, 3mg/ml) was purchased from Advanced BioMatrix. Phosphate buffered saline (PBS), 1× and 10× solutions (1.37M Sodium Chloride, 0.027M Potassium Chloride, and 0.119M Phosphate Buffer, pH 7.3-7.5), 0.5N Sodium Hydroxide Solution (NaOH), and OmniPur[®] polyethylene glycol (PEG) ($M_w \sim 7000-9000$) were purchased from Fisher Scientific. Super-purified SWNT (1.1 wt% Fe content, bulk density 0.14 g/cm³, lot #SP1001) were purchased from Continental Carbon Nanotechnologies, Inc. Carbon nanochips (CNC) (purity >99 wt% C; batch H700) were purchased from Catalytic Materials. All materials were used as received.

2.2. Sample Characterization

Scanning electron microscopy (SEM) images were taken on a Zeiss Supra 25 Field Scanning SEM (operation voltage 5 kV). Samples were sputter coated with chromium for imaging. Wide-Angle X-ray diffraction (WAXD) data was obtained using a Rigaku RAPID II curved detector system with a 3 kW sealed tube source (operating voltage 40 kV, current 30 mA, wavelength (λ) 0.1541 nm). Small-Angle X-ray scattering (SAXS) was performed on a Rigaku MicroMax-007HF system with CuK α radiation (operation voltage 40 kV and current 30 mA). Differential scanning calorimetry (DSC) was performed on a Q200 differential scanning calorimeter (manufactured by TA instruments). The heating rate was 10 °C/min from room temperature (25 °C) to 90 °C. DSC curves pertaining to the first heating cycle is provided for all collagen fibers. Mechanical testing of the dried fibers was performed on a RSA-G2 analyzer (TA Instruments). The gauge length and strain rate were 10 mm and 0.02 mm/s, respectively.

2.3. Fabrication of Collagen/Nano-carbon Fibers

SWNT and CNC were dispersed in either PBS, NaOH, or PureCol[®] using a Fisher Scientific SF30 bath sonicator (100 W, 42 Hz $\pm$ 6%) for 30 minutes to 24 hours at either room temperature (25 °C) or $\sim$ 0 °C. Succeeding dispersion, a collagen solution (pH $\sim$ 7) was created incrementally using eight parts collagen/nano-carbon (or solely collagen for the control fibers), to one part 2 M NaOH, to one part 10 $\times$ PBS (8:1:1). In order to prevent a pH shock to the collagen, first the parts consisting of NaOH and PBS were mixed followed by addition of the collagen dispersion. A vortex mixer was used to combine all parts. The collagen solution was left unperturbed for 15 minutes at room temperature (25 °C) for a gel solution to form. A glass syringe with a 22-gauge blunt tip needle was used to inject 1 mL of the collagen as well as collagen/nano-carbon solutions into the formation buffer (30 wt% PEG/1 $\times$ PBS bath at 37 °C) at

a rate of 1 mL/min. The syringe traveled at a rate of 0.1 m/s through the formation buffer during injection. The fibers formed immediately upon injection and were left in the formation buffer for 30 minutes. After formation, the fibers were transferred to a 1× PBS incubation bath for 2 hours followed by washing in a deionized water bath, both at room temperature (25 °C). The final fibers were air dried at room temperature for 24 to 48 hours before characterization.

Collagen/nano-carbon fibers were prepared at filler loadings of 0.5 and 5 wt%.

2.4. Fiber Elongation

Control, 0.5 wt% SWNT and CNC fibers were glued to cardboard tabs at each end using Duco[®] cement. After the cement hardened (~10 minutes), the fibers were individually mounted on the wet-tensile stage at initial gauge lengths ranging from 18 to 20 mm. Each fiber was rehydrated in 10× PBS for 10 minutes. Fibers were elongated to 0, 3, 6, 9, or 12% of their initial length at a strain rate of 0.02 mm/s. After elongation the PBS was immediately removed from the reservoir, the fibers were subsequently washed with deionized water, and dried under tension at room temperature (~25 °C) for 10 minutes. The fibers were removed from the tensile stage and allowed to dry for another 48 hours before further characterization.

2.5. Fiber Cross-linking

The control and composite fibers were cross-linked during the incubation step of the fiber spinning procedure. Gluteraldehyde (GA) solution was diluted using 1× PBS. Grade I, 25% in water/GA solution was purchased from Sigma Aldrich. After dilution the GA/1× PBS solution was stirred for 1 hour with a mechanical stirrer prior to being used. Fibers were transferred from the formation buffer after 30 minutes (i.e., PEG bath) to the GA/1× PBS solution for 2 hours before being washed and subsequently dried, both at room temperature (25 °C).

3. RESULTS AND DISCUSSION

The fabrication of collagen and collagen/nano-filler composite fibers is explored here in this work, considering the structural similarity (in size and geometry) between these component materials (Figure 1). Details associated with the processing approaches and characterization of collagen and collagen/nano-filler fibers are outlined the following sections.

Figure 1. Schematic representation showing the size and geometrical comparison between the collagen molecule and nano-carbon fillers used for fabrication of the composite fibers. The nano-carbon size is represented based on the size for the as-received individual SWNT and CNC platelet stack, as well as the actual dispersed SWNT bundle (determined experimentally).

3.1. Dispersion of the Nano-Carbons and the Resultant Fibers

In order to integrate the nano-carbons into the collagen fibers, SWNT and CNC were first dispersed by sonication in either PBS, NaOH, or the PureCol[®]. Each system was analyzed to

determine which solvent provided the best medium for dispersion stability and uniformity (in terms of particulate size). PBS and NaOH dispersions were sonicated for 24 hours at room temperature ($\sim 25^{\circ}\text{C}$), while PureCol[®] dispersions were sonicated for 30 minutes at $\sim 0^{\circ}\text{C}$. Limited sonication time was used for PureCol[®] dispersions to reduce potential molecular scission of the collagen monomer. Digital images of all dispersions were taken at 0 minutes, 5 minutes, and 24 hours, respectively after sonication (Figure 2). Immediately after sonication all dispersions appear to be fairly uniform (i.e., no obvious color gradients and no large agglomerations observed). However, after the dispersions were left unperturbed for 5 minutes then 24 hours both the PBS and NaOH suspensions show complete precipitation of the nano-carbons (Figure 2). Based on this dispersion study, the nano-carbons dispersed in collagen (PureCol[®]) remain much more stable with time (Figure 2) despite undergoing sonication for only 30 minutes. After standing for 24 hours these collagen dispersions only show minimal precipitation. For this reason, the collagen monomer solution is used as the dispersion medium for this work.

Figure 2. SWNT and CNC dispersed in PBS, NaOH, and PureCol®. Each dispersion is shown in the unperturbed state at 0 minutes, 5 minutes, and 24 hours after sonication. Immediately after sonication all dispersions show relatively good uniformity. After 5 minutes and 24 hours precipitation is observed.

Collagen dispersions are most stable.

Dispersion stability is important for processing composite fibers which exhibit uniform distributions of nano-carbons throughout. While each of the PBS and NaOH dispersions appear to be uniform immediately after sonication (Figure 2), quality after 24 hours of unperturbed time is a more important observation in terms of fiber spinning/processing times. During the spinning process used here, fibrillogenesis of the collagen is expected to occur continuously. An intact

collagen gel fiber takes ~30 minutes to form from the collagen solution used here as the spinning dope. During fiber gelation, dispersions that do not remain stable with time will lead to nano-carbon reaggregation throughout the as-spun fibers. Although not shown here, evidence of large agglomerations were found in fibers spun using unstable dispersions. Ultimately, these agglomerated particulates act as defects within the fabricated composite fibers. Defect formation due to poor dispersion of the fillers is expected to (1) reduce interfacial interaction between the collagen and nano-carbon, (2) inherently weaken the fibers by disrupting stress transfer between the matrix itself as well as with the fillers, (3) lead to development of voids, and (4) disrupt the collagen fiber assembly (fibrillogenesis) and growth processes during spinning. Therefore, agglomerations are not desired. In addition to using the collagen as a dispersion medium, to further mitigate this problem for the composite fibers, the nano-carbon loading/concentration was reduced to 0.5 wt%. At this loading a uniform dispersion of the nano-carbon in the PureCol[®] is achieved (Figure 2). Based on SAXS analysis of the 0.5 wt% collagen/nano-carbon dispersions the average SWNT bundle diameter, and CNC stack length ranged from 6 to 12 nm. Figure 1 shows that at this dispersion level the SWNT bundle has the most similar geometry to the collagen sub-fibril. Throughout this study it is shown that this geometrical similarity plays some role toward the development of the collagen microstructure (fibril formation and alignment) for the SWNT composites, and may also have implications regarding the level of interaction between the filler and biopolymer matrix.

3.1.1. Effects of Sonication on the Triple Helix

Since collagen is used as the dispersion medium, studies were performed to understand the effects of sonication on the molecules. X-ray analysis was used to evaluate the collagen molecular structure within spun fibers fabricated from as-received as well as sonicated

monomers (Figure 3). All samples showed the three primary wide-angle X-ray reflections expected for the collagen triple helix conformation. These peaks indicate the collagen molecular structures remain intact during the dispersion and spinning processes. The peak at $2\theta \sim 7.5^\circ$ indicates the lateral spacing between the molecules within the fiber (~ 1.1 nm) due to stacking of the triple helix.[37-39] A broad peak centered at 2θ of $\sim 20^\circ$ corresponds to the average distance between the three polypeptide chains.[37] The peak observed at 2θ of $\sim 31^\circ$ corresponds to the pitch of the collagen triple helix.[38] For the CNC fibers, a fairly sharp peak at 0.34 nm is also observed in the WAXD curves and is representative of the (002) graphitic inter-plane spacing. Based on this analysis, sonication for 30 minutes has a negligible effect on the fibril self-assembly process.

Figure 3. WAXD intensity curves for the modified control (non-sonicated, CONT0) and sonicated collagen (CONT) and 0.5 wt% nano-carbon composite fibers (0.5SWNT and 0.5CNC).

While the self-assembly process still occurs unaffected using sonicated monomer sources, in previous studies, there has been evidence that the collagen triple helix length can be broken during sonication.[40] The breakdown of the triple helix will have an effect on the stability (or melting temperature, T_m) of the sample. Therefore, along with WAXD analysis, DSC studies were also performed in order to investigate the effects that sonication has on the collagen molecular stability and fibril packing. Fibers fabricated using sonicated collagen monomer solutions were found to exhibit better fibril packing as compared to the fibers prepared from the non-sonicated samples. This is evidenced by an increase in enthalpy (ΔH) between the samples from 134.5 J/g to 247.1 J/g for non-sonicated and sonicated samples, respectively. DSC analysis also indicate that as the sonication time increases to 30 minutes, T_m decreases from 53.1 to 44.6 °C.[18] For this reason, while fibril packing was found to increase with sonication, the overall stability of the molecule does decrease. At 60 minutes of sonication time, T_m was found to decrease further even to 34.6 °C. Considering that sonication is a necessary step for the dispersion of the nano-carbons in the composites, balancing the effects of fibril formation/packing as well as molecular stability is needed. From the WAXD and DSC measurements, it was found that 30 minutes of sonication for the collagen solution and collagen/nano-carbon dispersions is considered most appropriate for the composite fiber preparation in this work.

3.2. As-spun Fiber Morphology

Upon spinning and washing the as-spun fibers, the morphology was examined for native mimicry. In order to investigate the fibril alignment in the as-spun fibers, a number of

characterization tools were used including electron (SEM) and optical microscopy measurements, as well as SAXS. SEM images (Figure 4) indicate that both control and composite fibers exhibit a predominant fibrillar structure inherent to some native collagen materials (e.g., rat-tail or bovine tendon).[31, 41] To quantify the average alignment of the fibrils in the collagen and collagen/nano-carbon fibers, the parallelism index (PI) was determined using intensity data obtained by polarized light optical microscopy (POM) (Figure 5). The PI for the collagen fibrils is a measure of the anisotropy or organization of the fibrillar network,[42] and can be calculated using Equation (1).

$$PI = \frac{I_{\max} - I_{\min}}{I_{\max} + I_{\min}} \quad (1)$$

$I_{\min}$ is the minimum polarized light intensity at 0° , while $I_{\max}$ is the maximum polarized light intensity occurring when the fiber axis is rotated at 45° with respect to the polarizer.[42] The PI values range between 0 and 1. A high PI value (~ 1) occurs when all the fibrils within a fiber run parallel to one another. A low PI (0) results when the fibrils have an unorganized or isotropic alignment with respect to one another. Coupling both SAXS analysis with PI results give information regarding the average fibrillar alignment for the spun collagen fibers.

Figure 4. SEM of (a) control (CONT), as well as composite (b) 0.5SWNT and (c) 0.5CNC as-spun fibers, showing the internal fibrillar structure. a_2 , b_2 , and c_2 are magnified images of the boxed regions in a_1 , b_1 , and c_1 , respectively, showing the fibril alignment.

Typically, polymeric fibers exhibit specific SAXS patterns consistent with a high or low alignment at the fibrillar level. Scattering along the equatorial direction is observed in the SAXS pattern when elongated objects are present with respect to the fiber axis (Figure 5).[43] These elongated objects include fibrils, elongated voids, as well as aligned nano-carbons. Based on the SEM analysis, a predominant fibrillar structure is observed in all fibers. In addition, the nano-carbon loading is very low – 0.5 wt%. For this reason, the equatorial and meridional scattering observed in SAXS is expected to be due to this fibrillar structure as opposed to voids or the

anisotropic nano-carbon filler presence. As fibril alignment increases, scattering in the equatorial direction becomes more pronounced and a streak develops. On the other hand, misalignment of fibrils results in a more diffused SAXS pattern with increasingly isotropic scattering in both meridional and equatorial directions as the degree of orientation decreases. Using both the PI and the SAXS intensity profiles (Figure 5) the fibrillar alignment was compared for all fibers processed in this work. In general, a fibril alignment is observed for all fibers. However, when comparing all fibers, the 0.5 wt% CNC fibers show the lowest degree of fibril alignment as well as an increase in void formation. As previously mentioned, the presence of void defects can affect the fibril alignment as well as the mechanical performance of the material. It should also be noted that the CNC filler geometry as compared to the SWNT exhibits a platelet structure (Figure 1), and this form may inhibit fibril alignment during spinning considering that fibrillogenesis and growth occur during such processing.

Figure 5. Bar chart and corresponding SAXS patterns for the as-spun fibers, comparing both the PI values fibril alignment. The equatorial (ED) and meridional (MD) directions are indicated on the SAXS patterns as well as the fiber axial direction for analysis.

Based on Figure 5, PI values are highest in the control fibers and the equatorial streak is also most pronounced. The PI values for 0.5SWNT and its corresponding SAXS intensity profile is comparable to control fibers suggesting good alignment and a high degree of anisotropy for the SWNT composite fibers. These strong similarities in alignment between control and 0.5SWNT fibers suggest that collagen fibril alignment is possible in the presence of the rigid fillers. The CNC do not have a similar effect, and this may be related to the structural similarities or lack thereof between the nano-material and the collagen molecules. This work shows that in addition to spinning control collagen fiber, incorporation of SWNT is also conducive to obtaining highly aligned collagen fibrils similar to native tendon, ligament, and bone. However, based on this analysis the nano-carbons are not necessary for fibrillar alignment during spinning, considering that control fibers show better overall alignment. The advantages for the incorporation of the SWNT in the collagen fibers is observed after further characterization where, (i) stabilization of fibrillar alignment during cross-linking processes, (ii) aiding of molecular assembly during elongation, and (iii) improvements in mechanical reinforcement to the collagen matrix as all observed. These results are discussed in the subsequent sections.

To further investigate the influence of the nano-fillers on collagen assembly, FTIR spectra were taken for control and composite fibers and compared to native tendon (Figure 6). The four spectra are almost identical with the exception of the additional peak at 1745 cm^{-1} and the more intense peaks at $\sim 2920\text{ cm}^{-1}$ and $\sim 2850\text{ cm}^{-1}$. These peaks can be attributed to lipids and complex carbohydrates present in mature tendon.[44, 45] There is also an additional small peak at $\sim 1160\text{ cm}^{-1}$ that can be attributed to lipids in the bovine tendon fibers. The synthetic fibers do not exhibit these peaks. This is related to the processing conditions for the synthetic fibers, which

lack additional fatty tissues and other molecules typically present in the body environment during collagen fiber formation. In all four spectra the signature peptide bond vibrations: amide A, amide B, amide I, amide 2, and amide 3 are present (Figure 6) indicating a similarity in chemical structure of the synthetic and native tendon materials. The similarity in the structures also suggests that the nano-carbons are not affecting the chemical or structural composition of the collagen during fiber formation, and that neither SWNT nor CNC are chemically reacting with the collagen molecules and/or affecting fibril formation. Additionally due to the minimal changes in bonding energies, it can be inferred that the individual collagen molecules are not wrapping the SWNT bundles. This is important considering that wrapping would lead to disruptions in lateral stacking of the collagen molecules to form the fibrils. This is expected considering that the size of the dispersed fillers is also on par with the sub-fibril diameter as opposed to the individual collagen triple helix molecule.

After analyzing the FTIR spectra further, some slight shifts in wavelength are observed between the synthetic and native tendon tissues. All three synthetic fibers experience a downward shift in the amide A ($\sim 3300\text{ cm}^{-1}$), amide I ($\sim 1630\text{ cm}^{-1}$), and amide II ($\sim 1540\text{ cm}^{-1}$) peaks (Figures 6a and 6b). These regions correspond to a NH stretch coupled with hydrogen bond[46], a C=O stretch[47], and a CN stretch/NH bend combination[48], respectively. These downward shifts signify an increase in the bond strength.[49] In the native tendon crosslinks are present throughout the fiber, which create conjugated double bonds next to C=O and CN bonds reducing their strength and causing the upward shift in the amine I and II peaks for the native tendon fibers. The decrease in the NH stretching and bending peaks signifies stronger hydrogen bonding in the synthetic fibers.[50] Similar to the C=O and CN peaks, covalent crosslinking in the native tendon causes a reduction in strength of the hydrogen bonding. Due to the lack of

crosslinks present in the as-spun synthetic tissue, the primary intermolecular interaction is ionic and hydrogen bonding. Amide B ($\sim 3075\text{ cm}^{-1}$), and amide III ($\sim 1200\text{ cm}^{-1}$) peaks do not show considerable shifts in wavelength (Figure 6). In addition, amide bands with lower frequencies signify less ordered proteins.[51] Beyond differences in crosslinking and hydrogen bonding strength, the native tendon tissue and the synthetic fibers have a very similar chemical makeup, and composite materials are unaffected by the nano-carbons.

Figure 6. Mid-infrared mean ATR-FTIR spectra of bovine tendon and composite fibers. a) 1000–4000 cm⁻¹, b) 2500–4000 cm⁻¹, and c) 1000 to 1700 cm⁻¹. Intense peaks at ~1750 cm⁻¹ (arrow), and ~2850 cm⁻¹ and ~2920 cm⁻¹ (dashed circle) signify complex carbohydrates and lipids found in the mature bovine tendon tissue due to its formation in a ‘bodily’ environment.

3.2.1. Observations of Mimetic Structure in the As-spun Synthetic Collagen Fibers

The hierarchical structure of tendon has been shown to consist of multi-scale ordering beginning from the collagen triple helix molecule to the varying level of fibril structure.[52, 53] Here, it has been demonstrated that synthetic collagen fibers spun from starting solutions of tropocollagen display several structural levels mimetic to native collagen.[52, 53] Based on the SEM analysis, the final fiber diameter is on the same scale as that of tendon fascicles (i.e., 50 to 300 μm) (Figure 7).[53] These fibers exhibit distinct fibrillar structure at two levels. Micro-scale fibrils are within the diameter range of 50 to 250 nm (Figure 7), while a sub-fibril structures between ~ 10 to 20 nm are also observed (Figure 7). The signature D-banding structure is also found within these synthetic fibers (Figures 7 and 8).

Figure 7. Fibril diameter ranges of native bovine tendon fibers and synthetic control (CONT) and composite (0.5SWNT and 0.5CNC) fibers as measured based on electron microscopy image analysis.

D-banding is inherent to native collagen fibrils and results from the periodic arrangement of the triple helix molecules during the fibril self-assembly process. SEM images of control and composite fibers (Figure 8) indeed show areas where D-banding is present. However, the banding structure is not present in all fibrils. This indicates that refinement of the fiber processing conditions is needed to improve the mimetic structure of the synthetic fibers toward approaching that of the native material. Comparing the relative amount of D-banding observed throughout the fibrils by SEM analysis, 0.5SWNT fibers show a large fraction of areas where banding is present (Figure 8 and Table 1). However, CONT and 0.5CNC fibers exhibit less D-banding as compared to the as-spun 0.5SWNT samples (Table 1). While not all fibrils in the synthetic fibers exhibit D-banding it is well-known that minor changes in pH and salt concentrations when forming the gel-solutions for spinning can help to promote the formation of the banding (lateral stacking) molecule structure for all fibrils.[54] Considering that all fibers discussed here were prepared from a spinning solution with similar pH and salt concentration, the banding structure present is relative between the samples. The presence of the SWNT is found to enhance the banding process during spinning based on the structural analysis performed.

Figure 8. SEM of fractured regions for the (a) CONT, (b) 0.5SWNT and (c) 0.5CNC fibers. a_2 , b_2 , and c_2 are magnified images of the boxed regions in a_1 , b_1 , and c_1 , respectively, showing the presence of D-banding at certain locations in the fibers. (a_3 , b_3 , and c_3) Show images of the internal fibrillar structure for CONT, 0.5SWNT, and 0.5CNC fibers show the presence of banded fibrils.

The comparative level of D-banding present in all three as-spun samples may indicate that the SWNT influences sub-fibril alignment, which in turn effects the lateral stacking of the collagen molecules. The presence of D-banding in the SWNT fibers also provides some evidence that the nano-filler geometry also plays a role in aiding mimicry for the bio-composite fibers. While both SWNT and CNC have been shown previously to act as rigid templates for polymer assembly, the SWNT material looks most similar to the collagen fibrillar structure. [34, 55] For this reason, tailoring the geometry of the nano-filler appears to be an important parameter for fabrication of mimetic bio-based composites. SAXS analysis proved a SWNT bundle size of ~6 to 12 nm. In addition, WAXD and FTIR characterization show evidence that the molecular structure of the collagen is unaltered with the presence of the fillers, and the molecules do not wrap around the SWNT. For this reason, the fillers are likely located between micro- or sub-fibrils within the collagen matrix. This level of interaction between the nano-filler and collagen has been shown to affect only the fibril alignment, and not the formation.

Table 1. Quantity of banded fibrils in non-crosslinked as-spun (0%) and 12% elongated control and composite fibers determined based on electron microscopy image analysis (>500 fibrils were counted per fiber sample).

Banded Fibrils (%)		
Fiber	0% Elongation	12% Elongation
Control	7 ± 6	6 ± 4
SWNT	16 ± 5	15 ± 7
CNC	6 ± 4	33 ± 15

Effects of Fiber Post-Processing Treatments

The as-spun synthetic fibers were subjected to two separate post-processing treatments, namely (1) hydrated/wet elongation and (2) GA crosslinking during spinning combined with hydrated elongation. Both treatments were examined for their influence on the final fiber morphology, properties, and provided insight regarding the spinning process as well as nano-carbon/collagen interactions. As discussed in the previous sections, the molecular structure and chemistry of the collagen is unchanged due to the addition of the nano-carbons. Therefore, fibril level interactions between the filler and collagen become more apparent with fibrillar alignment and/or developmental changes during elongation and crosslinking treatments.

3.3.1. Elongation in the Hydrated State

Rehydrated control and composite as-spun fibers were found to fracture at strains ranging from ~15 to 20% of their initial length. Therefore, to avoid fracture in the fibers a maximum elongation of 12% was chosen as the highest strain for these studies. After elongation, the fibers were dried and subsequently characterized in order to compare the fibrillar alignment and mechanical properties. WAXD analysis indicates that the intermolecular spacing of the collagen molecules decreases with elongation (Figure 9). This data indicates that the collagen molecules are forming closer-packed structures as the fiber is strained. In general, a fairly linear trend for the changes in intermolecular spacing as a function of elongation is observed (Figure 9), where the molecules are closest packed at 12% elongation (right before fracture was found to occur). In addition to the change in molecular spacing, the internal fibrillar structure of the collagen fibers was also examined as a function of elongation for the fibers. Based on SEM image analysis the relative quantity of banded fibrils was found to increase with elongation (Table 1). SEM allows for analysis of the fibers directly after spinning and hydrated elongation treatment

without the need for additional sample preparation techniques for imaging that may alter the as-fabricated fiber/fibril morphology. In addition, the area of analysis based on SEM allows for investigation over the macroscopic length of the fibers. After elongation the 0.5CNC fibers show the most pronounced increase for banded fibrils as compare to both 0.5SWNT and CONT samples. The CNC fillers possess a unique stack morphology allowing platelet sliding during drawing/elongation. It is believed that this process aids the fibril axial orientation and subsequently molecular lateral alignment toward banding.

Figure 9. Changes in collagen intermolecular spacing (at peak $\sim 2\theta$ of 7.5°) as a function of elongation obtained from WAXD analysis. As elongation increases, the molecules are found to pack closer together.

3.3.2. Combined Effects of Crosslinking Treatment and Elongation

Native collagen fibers exhibit chemical crosslinks at both the molecular and fibril levels. To understand the role of crosslinking in these spun fibers, crosslinking treatment studies were performed in conjunction with elongation post-processing. As shown in the previous section, just elongation of the spun fibers resulted in additional alignment of the collagen molecules (higher

degree of banding) and fibrils (increased PI values). For compatibility with the fabrication process used here, the crosslinking agent was introduced at a concentration of 1 vol% during the incubation stage of fiber spinning. These crosslinked fibers were subsequently elongated in the wet-state to their respective maximum strain, which was experimentally determined to be 8, 12, and 9% for the CONT, 0.5SWNT, and 0.5CNC fiber samples. The reduction in maximum strain for some samples was likely due to the introduction of the chemical crosslinking bonds between molecules and fibrils in the fibers. However, the 0.5SWNT fibers were still able to achieve high elongations both before and after crosslinking steps (12%). PI analysis was also performed on the non-crosslinked and crosslinked fibers with respect to elongation, and the values are shown in Figure 10. Based on these results, the crosslinked fibers do show a decrease in the overall local alignment of the fibrils. However, the composite fibers are able to maintain a higher level of fibril alignment as compared to the control fibers, and this may be related to the inclusion of the rigid nano-filler structures. Maintenance of fibril alignment is important for mimicry of the synthetic fibers as compared to native tendon.

Figure 10. PI values as a function of cross-linking and elongation (i.e., 0%, 6%, and maximum strain) in the wet-state.

Based on image analysis of the crosslinked fibers a large decrease in the fibril size distribution as compared to the non-crosslinked fibers is observed (Figures 7 and 11). The gels/dopes used in for all the spinning processes were found to exhibit fibrils with similar diameters to the range found for the crosslinked fibers (Figure 11). This finding indicates that during the spinning process used in this work, the fibrils continuously grow, and this occurs predominantly during the incubation stage. However, chemical crosslinking introduced at incubation arrests the fibril growth and development during this stage. In addition to the small fibril diameter in all treated fibers, no banding was also observed. In comparison to the untreated fibers, elongation appeared to have no further effect of the fibril or molecular rearrangement that could lead to fibril growth or banding. For these reasons, while incorporation of crosslinking steps is important it should be controlled to ensure fibril growth and development is also maintained during spinning process.

Figure 11. Fibril diameter ranges of as-spun and maximal elongated 1% GA cross-linked fibers. Averages values are shown with an asterisk. Elongation does not significantly alter the fibril diameter once the fibers are crosslinked.

3.4. Comparison of Tensile Properties for Fiber Samples

Mechanical testing was used to evaluate the modulus (E) and strength (σ) properties in both dry and hydrated states for the treated (crosslinked) and untreated fibers (see Table 2). In the dry-state, as-spun control samples (CONT) were found to have a maximum strength of ~500 MPa. This is comparable to the reported dry-state values for native collagen fibers[56-58] (Table 2). Previous reports regarding synthetic collagen fibers with no crosslinking also show strength values ranging from 10-225 MPa.[6, 59] It is anticipated that the mimetic fiber morphology in addition to the presence of rigid nano-carbons at this low concentration (0.5 wt%) contribute to the overall increase in strength for the fibers (Table 2). Both composite fibers with low loading (0.5 wt%) show an increase in strength properties as compared to the control fibers. However, at higher loading screen (5 wt%), poor nano-carbon dispersion lead to defects, and the strength values decrease accordingly as compared to the control fibers.

Table 2 also shows that wet-state mechanical properties for the fibers before and after treatment by crosslinking. This state of testing is more important for showing comparative mechanical behavior to that of a native tendon fiber. It is clear that the crosslinking step is very important toward improving performance of the spun fibers in the wet-state. It is anticipated that in the wet-state the fibers swell in diameter allowing the fibrils to move more freely past one another. If no intermolecular and intra-fibrillar crosslinking is present then the molecules and fibrils are easily pulled apart, resulting in much lower mechanical properties. The addition of crosslinking significantly improves the wet mechanical properties as compared to non-crosslinked fibers with Young's modulus and tensile strength values on the order of 200 to 300

fold higher. After crosslinking, deviations in mechanical behavior for each batch of control and composite fibers tested were found to be less pronounced as compared to what was observed for the non-crosslinked fibers (Table 2).

Table 2. Mechanical Properties and Rule of Mixtures Predictions of Control and Composite Fibers

Dry-State Testing					Wet-State Testing					
As-Spun Fiber			Rule-of-Mixtures Predictions		Elongated As-Spun Fibers (Non-Crosslinked)			Elongated As-Spun Fibers (1% GA Crosslinked Fibers)		
Fiber	E (GPa)	σ (MPa)	E (GPa)	σ (MPa)	%EL [†]	E (MPa)	σ (MPa)	%EL [†]	E (MPa)	σ (MPa)
CONT	7.5 – 11.3	300 – 600	–	–	12	33 ± 11	4 ± 1	8	810 ± 100	80 ± 9
5SWNT	7.2 – 10.3	200 – 300	7.7 – 46.8	500 – 2400		-	-	-	-	-
0.5SWNT	7.8 – 19.7	300 – 1200	7.6 – 10.7	400 – 600	12	92 ± 35	9 ± 1	12	840 ± 140	70 ± 8
5CNC	5.7 – 12.1	200 – 300	7.7 – 46.8	500 – 2400		-	-	-	-	-
0.5CNC	8.3 – 17.6	500 – 800	7.6 – 10.7	400 – 600	12	46 ± 4	5 ± 2	9	590 ± 50	75 ± 15
Native	2.2 ± 0.3	326 ± 21	–	–		1120 ± 230	126 ± 16	-	-	-

[†] Maximum elongation percentage used to stretch fibers before tensile measurements.

While the wet-state properties reveal the importance for the hierarchical structural and chemical mimicry between native and synthetic materials, dry-state properties provide some insight toward filler-matrix interactions. As shown, the mechanical properties of the 0.5SWNT and 0.5CNC fibers begin to surpass the mechanical properties of both native collagen as well as other synthetic collagen fibers measured in the dry-state (Table 2).[56, 59] A rule-of-mixture analysis was used to provide additional insight regarding the reinforcement and effective

contributions of the nano-carbon fillers in the composite based on the dry fiber properties.

Equations (2) to (4) were used to theoretically predict the composite fiber modulus (E_{Comp}) based on the moduli of the control fiber (E_M) (collagen matrix contribution) and nano-carbon (E_N) (measured and theoretically determined properties), as well as loading concentration of filler. The composite strength (σ_{Comp}) is also predicted based on the strengths of the matrix (σ_M) and filler (σ_N).[60]

$$E_{Comp} = fE_N + (1 - f)E_M \quad (2)$$

$$\sigma_{Comp} = f\sigma_N + (1 - f)\sigma_M \quad (3)$$

Where,

$$f = \frac{f_w}{f_w + (1 - f_w) \frac{\rho_N}{\rho_M}} \quad (4)$$

f is the volume fraction of the nano-carbons in the composite fibers and can be calculated from the weight fraction (f_w) of the nano-carbons in the composites using Equation (4). ρ_N and ρ_M are the densities of the nano-carbon and collagen matrix, respectively.

It is important to note the experimentally measured modulus value of CONT fibers was used as E_M . The use of control fiber properties helps to take into account the effect of spinning on the overall mechanical properties (e.g., presence of voids and collagen ordering) for the matrix portion. E_N values range from ~10 to 1000 GPa for both SWNT and CNC based on various measured and theoretical values.[61, 62] The tensile strength was estimated to range from ~1 to 50 GPa for both SWNT and CNC.[61, 63, 64] This large range is due to the

morphology differences as well as the exfoliation level (bundle size and platelet stack thickness) and orientation of the nano-carbons.

A modulus of 1 TPa corresponds to the axial properties for a fully oriented (in regards to the fiber axis) and exfoliated SWNT (i.e., individual tube)[61], while ~ 10 GPa corresponds to unoriented ropes (i.e., large bundles) of SWNT.[62] This bundle size as predicted from SAXS is around 6 to 12 nm. Similarly, the CNC also remain in stacks after dispersion (i.e., 25-30 chips per stack according to SAXS). Therefore the value of 1 TPa for the SWNT E_N value is not used in the analysis. The morphology of the CNC fillers is similar to layered graphite, where the modulus along the axial direction of a graphene sheet (i.e., basal plane) has been measured to be ~ 1 TPa.[65] Since the CNC in the composites remain as stacks, the inherent modulus value is estimated to be much lower. In order to calculate the theoretical mechanical properties, the density of the control fibers was also determined. For this calculation each fiber was weighed and the volume was determined from measuring the average diameter and length of each fiber. A circular cross-section was assumed in order to approximate volume of each fiber. The collagen density was estimated to be 1.3 ± 0.2 g/cc, which are comparable to native collagen.[66] The density of SWNT and CNC were taken to be ~ 2 g/cc as reported from the manufacturer.[67, 68]

Based on Equations (2) and (3) the modulus and tensile strength range for the 0.5 wt% composite fibers, modulus and tensile strength ranges were predicted to be 7.6 to 10.7 GPa and 0.4 to 0.6 GPa, respectively based on CNT fibers (i.e., E_M values). Comparing the theoretical and experimental values (Table 2), the maximum rule-of-mixture predictions for both the Young's modulus and strength are consistent for the 0.5 wt% composite fibers. This provides

evidence of interfacial interaction between the nano-carbons and collagen matrix. This collagen/nano-carbon interaction is only achieved at low loading (i.e., 0.5 wt%), and is anticipated to contribute to structural changes observed during fiber processing.

The spinning process outlined in this study is able to produce mimetic collagen and collagen/nano-carbon composite fibers with short processing times (less than four hours). Based on the hierarchical structure, alignment, and high mechanical properties for these synthetic fibers, native tendon is the most suitable tissue for comparison. The fibers spun in this work were compared to other wet-spun collagen fibers in the literature, and based on the mechanical properties and features reported, the synthetic fibers here are most comparable to tendon fascicles (Table 3).

Table 3. Comparison of the synthetic collagen fibers in this study and other wet-spun fibers from the literature. All mechanical properties listed are measured in the hydrated/wet-state.

Reference	Spinning method	Incubation	Process Time	Cross-linking Scheme	Diameter (μm)	Young's Modulus (MPa)	Tensile Strength (MPa)	Features
Caves et al.[18]	Wet-spinning, 5mg/mL RTT collagen, 10 wt% PEG buffer, strain	48 hours PBS	70+ h	GA vapor	53 ± 14 dry	775 ± 173	94 ± 19	~30 nm fibrils, banding, poor alignment
Wang et al.[69]	Wet-spinning, 0.33 wt% RTT collagen,	4 h isopropyl alcohol	5+ h	DHT	-	-	92 ± 31	No banding
Kato et al.[70]	Wet-spinning, 1 wt% corium collagen, no PEG	4 h isopropyl alcohol	5+ h	DHT GA vapor	-	170-200 384-503	50-66	No banding
Pins et al.[26]	1 wt% RTT collagen, strain	24 h PBS	49+ h	DHT	74 ± 11 dry	252 ± 72	38 ± 13	Poor alignment, some banding
Pins et al.[71]	1 wt%, 10 mg/mL RTT, PBS formation 24 h	24 h PBS	49+ h	None DHT	327 ± 34 94 ± 12 wet	1.8 ± 0.3 383 ± 112	0.9 ± 0.2 46.8 ± 17	Banding on outside, fibrils on outside align during stress
Cavallaro et al.[6]	5 mg/mL BAT, 20 wt% PEG 4 min, 3 baths, pump moving formation buffer	PBS 10 min, isopropyl unknown time	times vary	None, 2.5% GA/2% paraFA solution 10 s, EDC 8 h	25-75 range, 55 average	-	1.2 ± 0.2 28 ± 3 24 ± 3	Banding at surface only
Zeugolis et al.[72]	BAT, tubing into formation buffer, 20 wt% PEG static 10 min	10 min PBS, overnight: DI water, PBS, isopropanol or tris	~ 24 h	none	110-160 dry, 260-300 wet	3.8-16.5 wet 150-1250 dry (SEM)	3-3.9 wet 112-356 dry (SEM)	Some banding
Zeugolis et al.[14]	20 $\mu\text{g/mL}$ BAT, 20 wt% static PEG formation 10 min	10 min PBS, 10 min wash (3 baths)	30 min	none	123 ± 21 (dry) – acid treatment 52 ± 8 (dry) – pepsin treatment	1.5 ± 0.6 1.7 ± 0.6 @ 2% (dry)	168 ± 62 169 ± 53 (dry)	Poor alignment, some banding in larger fibrils, non-uniform diameter
Zeugolis et al.[59]	7 mg/mL BAT, 20 wt% PEG static	15 min PBS	45 min non-CL, ~24 h CL	None EDC 0.625% GA solution	171 ± 6 167 ± 11 260 ± 26 (dry)	3.8 ± 1.1 1.8 ± 0.3 7 ± 3 @ 2% strain wet	3.0 ± 0.9 3.2 ± 0.6 6 ± 3	Very non-uniform diameter, no banding in CL fibers
This work	3 mg/mL bovine hide	2 h PBS	225 min	GA solution	30-60	840 ± 140	70 ± 8	Banding in many fibrils, fibril size mimics native tendon

4. CONCLUSIONS

In summary this work shows the ability to form synthetic collagen fibers, which exhibit mimetic multi-scale structure similar to native collagen. This study also shows the ability to use

nano-materials effectively in synthetic collagen fiber processing, where these rigid fillers may be expanded to other components (i.e., non-carbonaceous) to increase functionality and compatibility for subsequent biological applications/processes. For example, bone tissue is a collagen-based native composite material. It provides insight into the ability of nano-carbons to sustain collagen molecular and fibril alignment. Dispersion quality of the nano-carbons plays a large role on the morphology and mechanical properties of the resultant fibers. Several factors including collagen solution preparation, spinning route, filler-addition, incubation processes, crosslinking, and elongation are also shown to influence the ability to replicate native collagen structures by synthetic processes. Crosslinking was shown to be necessary toward completing structural mimicry in collagen materials. However, crosslinking was also shown to prevent fibril growth in this spinning process. For this reason, the introduction of this processing stage is highly important. These materials may also contribute to understanding key fundamental mechanisms for collagen fibril assembly.

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