Research Paper

Characterization via atomic force microscopy of discrete plasticity in collagen fibrils from mechanically overloaded tendons: Nano-scale structural changes mimic rope failure

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Abstract

Tendons exposed to tensile overload show a structural alteration at the fibril scale termed discrete plasticity. Serial kinks appear along individual collagen fibrils that are susceptible to enzymatic digestion and are thermally unstable. Using atomic force microscopy we mapped the topography and mechanical properties in dehydrated and hydrated states of 25 control fibrils and 25 fibrils displaying periodic kinks, extracted from overloaded bovine tail tendons. Using the measured modulus of the hydrated fibrils as a probe of molecular density, we observed a non-linear negative correlation between molecular density and kink density of individual fibrils. This is accompanied by an increase in water uptake with kink density and a doubling of the coefficient of variation of the modulus between kinked, and control fibrils. The mechanical property maps of kinked collagen fibrils show radial heterogeneity that can be modeled as a high-density core surrounded by a low-density shell. The core of the fibril contains the kink structures characteristic of discrete plasticity; separated by inter-kink regions, which often retain the D-banding structure. We propose that the shell and kink structures mimic characteristic damage motifs observed in laid rope strands.

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1. Introduction

Tendons are tension-bearing tissues that convert muscle contraction into skeletal movement used for every day locomotion. The functional attributes of tendon are the result of a highly organized hierarchical structure of extracellular matrix proteins. The most mechanically structural element is collagen, its most fundamental structural unit being the nano-scaled fibril (Benjamin et al., 2008; Kannus, 2001). Injury due to mechanical overload of a tendon results in the disruption of its ordered hierarchical structure, long-term alteration in its mechanical properties, and loss of function (Sharma and Maffulli, 2005; Spiesz et al., 2015). A structural motif for collagen damage due to tendon overload has recently been identified. It is characterized by a serial disruption (kinking) of collagen fibril structure along their length (Veres and Lee, 2012). Termed as discrete plasticity, such damage is associated with increased enzyme susceptibility, decreased thermal stability and denaturation of collagen, and cellular recognition of locally kinked fibrils (Veres and Lee, 2012; Veres et al., 2013, 2014, 2015).

The native structure of a collagen fibril (in bovine tendon) consists of tropocollagen molecules, self-assembled into five molecule cross-section microfibrils approximately 4 nm in diameter, these then assembled into sub-fibrils approximately 10–40 nm in diameter (Wess, 2005). It has been demonstrated that sub-fibrils and micro-fibrils are wound about the axis of the fibril in a manner similar to that in a laid rope strand (Bozec et al., 2007; Holmes et al., 1995; McKenna et al., 2004; Ottani et al., 2002; Silver et al., 1992; Zhao et al., 2011). Characteristic, periodic D-banding is observed along the length of native fibrils. This appearance is a result of the non-integer staggering of tropocollagen molecules within microfibrils, resulting in molecular density fluctuations along the fibril’s length. A single period of the D-band is ~67 nm in tendon fibrils and consists of a gap and overlap component where the gap has 4/5’s of the molecular density of the overlap region (Hodge and Schmitt, 1966; Orgel et al., 2001; Wess et al., 2005).

It is now clear that overload damage in a tendon involves alteration to molecular conformation or packing order at the nano-scale within individual collagen fibrils (Veres and Lee, 2012; Veres et al., 2013, 2014, 2015). In particular, two significant structural alterations to fibril morphology have been reported in association with discrete plasticity under scanning electron microscopy (SEM): (i) the appearance of a shell layer which obscures or replaces D-banding, and (ii) serial kink sites along the fibril’s length. These changes appear to relate to radial and longitudinal alterations respectively within the collagen fibril (Veres and Lee, 2012). To this point in time, discrete plasticity has been observed only under SEM, and that after dehydration and sputter coating to achieve nanometer resolution. In the present study, we have sought to circumvent these alterations to collagen fibrils before observation by application of atomic force microscopy (AFM).

We have previously demonstrated acquisition of high-resolution mechanical maps from hydrated collagen fibrils via AFM, using the peak force, quantitative nanomechanical mapping (PF-QNM) mode (Baldwin et al., 2014). From the acquired data we are able to calculate the maximum penetration depth of the tip into the collagen fibril, and the fibril’s modulus, all with a spatial resolution of around 10 nm. These mechanical properties were shown to be a measure of the local molecular density of the fibril. By this means, the penetration depth and modulus images visualize the interior of the fibril at distinct depths. In the present study, we have used this technique to analyze 25 control and 25 overload-kinked collagen fibrils, seeking to further our understanding of the molecular alterations in collagen fibrils associated with discrete plasticity damage. Based on the resulting data, and making use of previous description of the collagen fibril as a nanoscale rope and/or as a tube-like structure, we suggest a new model of kink formation which resembles damage motifs found in laid rope strands (Bozec et al., 2007; Gutsmann et al., 2003; McKenna et al., 2004).

2. Methods

2.1. Sample acquisition

Tissue harvesting and handling were approved by the Health Sciences Research Ethics Board of Dalhousie University. Bovine tails were acquired from 18 to 24 month old steers immediately after slaughter for meat at a local abattoir. The tails were stored for no more than 2 h at 4°C, after which the tendons were dissected from the dorsal region of each. In this study tail tendons from 5 animals were used, with a single tendon sourced from each tail.

2.2. Tendon preparation

A segment 1 cm in length was cut from each tendon to serve as a control and stored in phosphate-buffered saline solution (PBS) with a pH of 7.4 at 4°C. The remainder of the tendon was then clamped into an MTS Series 458 servo-hydraulic testing machine for mechanical loading. Each tendon underwent a pre-loading cycle at 1% strain per second to a maximum strain of 10%. This was followed by 5 overload cycles into the plastic region of the load-deformation curve under computer control as previously described (Veres et al., 2013). This repeated overload regime has been demonstrated to produce the most collagen fibrils displaying discrete plasticity in the tendon collagen while retaining a wide range of kink density among the individual fibrils. During loading, the tendon samples were kept hydrated by continuous application of PBS with a pipette. At completion of the loading procedure, the overloaded tendon was removed from the apparatus, and the clamped ends were cut away. Both the control tendon sample and the trimmed overload samples were then subjected to a decellularization process. (See Fig. 1.)

2.3. Decellularization

Decellularization was undertaken to allow clear visualization of individual collagen fibrils and has previously been demonstrated to preserve discrete plasticity damage (Veres et al., 2015). The process has been fully described previously (Ariganello et al.,...
data analysis

All data analysis was performed using SPIP 6.3.3 software (Image Metrology). From the acquired data, 6 properties of the collagen fibrils were of interest: the height and deformation of the fibrils in both hydrated and dehydrated states, the modulus of fibrils in the hydrated state, and the average serial kink density (kinks/μm) along each fibril over a 10 μm length. The deformation of a fibril at any point was defined from the force/distance data as the distance between the extension curve and maximum indentation at 15% of the peak force (max force). (Fig. 2). All height and deformation data were extracted from a 20 nm wide strip along the apex of the collagen fibril. The deformations in the hydrated and dehydrated states were summed with the corresponding height measure to provide a value termed the zero-force height. This measure represented the corrected height of the fibril in the absence of applied force due to the AFM tip (Baldwin et al., 2014).

Using the averages of the hydrated and dehydrated zero-force heights, a swelling ratio was calculated for each fibril as shown in Eq.(1):

\[ \text{Swelling Ratio} = \frac{H_{\text{Hydrated}}}{H_{\text{Dehydrated}}} \]

2.6. AFM data acquisition

In this study, ten fibrils from each of the 5 tendons were imaged along 10 μm of their length. We selected five fibrils from each control sample and five fibrils from each loaded sample which displayed sites of discrete plasticity that we will subsequently refer to as kinks. In total, we imaged 50 fibrils, 25 control and 25 kinked for a total axial length of 500 μm. Three essential steps were followed in this order for all data acquisition: (i) optical targeting of dehydrated collagen fibrils, (ii) AFM imaging of each dehydrated fibril using PF-QNM with a pixel size of 20 nm, and (iii) AFM imaging of rehydrated fibrils using PF-QNM imaging with a pixel size of 8 nm. Imaging of hydrated collagen fibrils was carried out in PBS with pH 7.4 and at room temperature. All collagen fibrils were exposed to a single dehyrdated step during sample preparation and dehyrdated imaging, followed by a single hydration step prior to hydrated imaging. Samples wereallowed 30 min to rehydrate prior to hydrated imaging.
Swelling Ratio = \frac{\text{Hydrated Zero Force Height}}{\text{Dehydrated Zero Force Height}} \quad (1)

This ratio provided insight into the fibril’s interaction with the surrounding PBS medium. The modulus along the radial direction of the hydrated collagen fibrils was calculated by fitting the Sneddon model as described in Eq.(2):

\[ F = \frac{2E}{\pi(1-\nu^2)} (\tan \delta^2) \quad (2) \]

where \( F \) is the applied force, \( \nu \) is the Poisson ratio, \( \alpha \) is the half angle of the cone, \( \delta \) is the depth of indentation into the sample and \( E \) is the elastic modulus of the sample (Maugis and Barquins, 1978; Sneddon, 1965; Stolz et al., 2004). The Poisson ratio of the fibril was taken to be 0.5, characteristic of incompressibility (Baldwin et al., 2014; Grant et al., 2009). The modulus obtained by fitting Eq. 2 serves as a probe for the molecular density of the collagen fibril (Baldwin et al., 2014). The Sneddon model was selected for modulus analysis due to the AFM tip geometry and a minimum indentation depth of \(~30\, \text{nm}\) upon the apex of the control collagen fibrils. The fit range of each force curve was determined using Bueckles’ rule, corresponding to a maximum fit range equivalent to 10% of the fibril’s zero-force height (Bueckle, 1973; Persch et al., 1994). This process produced maps of the radial modulus of the collagen fibril from which values were extracted along a \(20\, \text{nm}\) wide strip along the apex of the fibril. Outliers in the modulus of a single fibril were determined using JMP software (version 11.0.0, SAS Institute Inc.) and removed from the data set. The average modulus and the coefficient of variation of the modulus were calculated for each fibril. The average modulus was used to quantify the molecular density of the fibrils as justified in Baldwin et al., 2014, while the coefficient of variation of the modulus provided a measure of the heterogeneity of the molecular density for the collagen fibril.
The average, serial kink density along each fibril was determined by counting the number of kinks along a 10 μm length. This was performed on 10 μm x 10 μm dehydrated height images of kinked fibrils where a kink was classified as a transversal fault line on the fibril (Fig. 3). Kinked fibrils were split into two separate groups: kinked (average kink density less than 1.5 kinks/μm), and very kinked (average kink density greater than 1.5 kinks/μm).

2.8. Image analysis

To visualize the data, maps of height, deformation, and modulus were extracted using SPIP 6.3.3 and stitched together in Adobe Photoshop. All cross-sectional data presented were extracted along a 20 nm-wide strip along the apex of the corresponding collagen fibril.

2.9. Statistical analysis

Statistical analysis was performed using JMP software (version 11.0.0, SAS Institute Inc.). A 2-way ANOVA was performed for swelling ratio, modulus, and coefficient of variance data with variables of five animal tails and three kink density groups (control, kinked, very kinked). Where appropriate after the 2-way ANOVA, Bonferroni-corrected post-hoc testing was carried out using Fisher’s Least Significant Difference (LSD) test. All parametric data are presented as the mean ± the standard deviation.

3. Results

Topographic mapping of dehydrated collagen fibrils extracted from control tendon samples was free of curvature and showed the periodic D-banding pattern indicative of proper, native molecular packing within the fibrils (Fig. 3A). By contrast, fibrils extracted from overloaded tendon display discrete plasticity damage, following a twisted path with the D-band interrupted at sites of sudden directional changes in the fibril axis (Fig. 3B). These sites were consistent with the kink structures previously observed under SEM (Veres and Lee, 2012; Veres et al., 2013, 2014, 2015). The persistence of the D-banding between kinks suggests retention of ordered molecular packing in these regions (Fig. 3B, arrows).

Upon hydration in PBS, both control and damaged fibrils swelled significantly (Fig. 3). In the hydrated state, D-banding was barely observable for the control fibrils (5–10 nm fluctuation) and not observable for the kinked collagen fibrils (Fig. 3C and D). This observation suggests that the gap and overlap regions of the D-band have distinct swelling ratios in PBS, resulting in a nearly constant cross-sectional area along the length of control fibrils. The heterogeneity in swelling does,
however, permit observation of D-banding in the modulus maps of hydrated collagen fibrils (Baldwin et al., 2014; Spitzner et al. 2015). Indeed, in the present study, D-banding of control fibrils was observed in both deformation and modulus images (Fig. 4A and C). Kinked fibrils, by contrast, often showed no D-banding in either deformation or modulus images (Fig. 4B and D). Instead, we observed a highly penetrable shell layer enveloping the collagen fibril (Fig. 4B, arrow) and a new periodic modulus fluctuation occurring at the kink sites along the collagen fibril (Fig. 4D). An inverse relationship is expected between the deformation and modulus values of a collagen fibril. A highly deformed region should have a lower modulus and vice versa. This relationship broke down in the kinked regions of damaged collagen fibrils (Fig. 4B and D, rectangles) where a decrease in modulus was observed in the kinked region—without an increase in deformation. This observation is an artifact, due to the method in which the deformation is measured on force curves taken from an area where high penetration occurs.

Comparison of modulus images from control, kinked, and very kinked collagen fibrils demonstrated the progression of damage as serial kink density increased along collagen fibrils (Fig. 5). No correlation between tendon source and a region of the kink density range was observed, demonstrating no dependence on kink density and the macroscale loading of the tendons. Again, not all kinked collagen fibrils displayed a complete lack of D-banding when visualized via modulus (Fig. 5B and E). Plotting the modulus, coefficient of variation of the modulus, and the swelling ratio of the collagen fibrils against serial kink density revealed an increase in swelling ratio and decrease in modulus with increasing kink density (Fig. 6A, C and I). This is consistent with the notion that a decrease in modulus is due to a decrease in molecular density. Comparison of the mean properties from the three fibril groups showed significant, systematic differences between the control, kinked, and very kinked collagen fibrils (Fig. 6B, D and F; Table 1). The modulus was distinct between each of the three groups, while the swelling ratio increased significantly between the control and very kinked fibrils (Fig. 6B and F). It was striking that the heterogeneity in modulus changed so greatly with damage: the coefficient of variation for modulus in control fibrils was more than doubled in both kinked and very kinked groups, largely independent of kink density (Fig. 6C; Table 1).

4. Discussion

Our previous work has addressed two basic questions. First, what does it mean for collagen to be mechanically damaged in an overload event? Second are there identifiable structural motifs, at some characteristic scale, which are associated with damage? These are questions which are important for our understanding of evolutionary “design” in collagen and for biomedical identification and treatment of soft tissue injuries. Examining overload-damaged bovine tail tendons with SEM, we have identified a characteristic, nano-scaled motif for damage in the fundamental collagen fibril: local kinks which increased in serial density under increasing...
cycles of plastic overloading (Veres and Lee, 2012; Veres et al., 2013). As well, treatment with the acetyl trypsin, a serine protease, demonstrated that molecular collagen was denatured during this process, gradually obscuring D-banding in SEM images and occurring most intensely at the kink sites (Veres and Lee, 2012; Veres et al., 2013). Mechanical evidence suggests that these kinks may be part of a structural mechanism which absorbs overload energy via sacrificial destruction of certain subfibrils, toughening collagen at a very basic level. Due to the very local nature of the kink formation, we have termed this damage mechanism “discrete plasticity”. Culture of decellularized native and damaged tendons with immortal macrophage-like U937 cells has given the first evidence that inflammatory cells may be able to recognize and respond to discrete plasticity damage (Veres et al., 2015).

We recognized that the kink morphology observed by SEM only provides surface characterization of the collagen fibrils following significant alteration due to dehydration and metal sputtering. In the present study, we have sought to remove this barrier and to look at native and damaged fibrils in an unfixed, hydrated state. By comparing features in dehydrated and hydrated specimens, we directly examine the importance of water solvation in our imaging. Further, by carrying out nano-scaled mechanical examination of fibrils, we are able to probe the interior of damaged fibrils. In short, AFM offered the opportunity to determine whether the kink morphology of discrete plasticity damage was artifactual to any significant degree, and to greatly expand our structural understanding of accumulating mechanical damage at the fibril level in collagen.

### Table 1 – Fibril properties. Mean Sneddon modulus, coefficient of variation of the modulus, and the swelling ratio for the control, kinked, and very kinked collagen fibril groups. Mean ± standard deviation.

<table>
<thead>
<tr>
<th>Kink density</th>
<th>Sneddon modulus (MPa)</th>
<th>Coefficient of variation for modulus</th>
<th>Swelling ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.3 ± 3.9</td>
<td>0.20 ± 0.02</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Kinked</td>
<td>6.6 ± 2.1</td>
<td>0.43 ± 0.04</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Very kinked</td>
<td>2.1 ± 1.2</td>
<td>0.46 ± 0.03</td>
<td>2.4 ± 0.4</td>
</tr>
</tbody>
</table>

† Difference with value for control fibrils is significant with p<0.05, Bonferroni-corrected.

‡ Difference with value for kinked group is significant with p<0.05, Bonferroni-corrected.

4.1. **Hydration reveals structural features associated with discrete plasticity**

Comparing the dehydrated height, hydrated height, modulus, and deformation of kinked and control collagen fibrils revealed alteration of molecular packing due to tendon overload. Newly-validated structures include a low-density shell which encompassed a higher-density core in the damaged collagen fibril. SEM images under acetyl trypsin digestion suggest that this layer is composed of denatured collagen (Veres et al., 2013, 2014). Along the core of the fibril, the periodic kink structures of discrete plasticity have been confirmed, as have the relatively undamaged inter-kink regions spanning consecutive kinks (Fig. 4). The kink structures are mechanically characterized by a sharp decrease in modulus compared to the inter-kink regions and an increase in deformation by the tip (Fig. 4). The alteration in mechanical response of the shell and kink sites is most likely due to uptake of water associated with the loss of native molecular packing and local denaturation of triple-helical tropocollagen. This is supported by the preferential enzymatic digestion of material from the shell and kink structures by trypsin, as well as the decrease in thermal stability of the overloaded tendon (Veres and Lee, 2012; Veres et al., 2014).

The dramatic increase in the coefficient of variation of the modulus between the control fibrils and discrete plasticity-kinked fibrils (Fig. 6D) suggests that the kink/inter-kink modulus difference is conserved, independent of kink density—that is, a kink is a local structure which has limited influence on the mechanical behavior of neighboring regions in the fibril. This finding contrasts with the decrease in the mean modulus of damaged fibrils as kink density increases (Fig. 6A). Fitting the mean modulus versus kink density data with a linear ($r^2=0.042$) and an exponential fit ($r^2=0.62$) demonstrates the mean modulus is not solely due to an accumulation of discrete kink structures. It follows that, while the kink/inter-kink heterogeneity was captured by the coefficient of variation, the fall in mean modulus with increasing kink density is associated with both the formation of the shell structure and accumulation of kink sites (Fig. 7).

The D-banding seen in the modulus maps of hydrated control bovine tail collagen fibrils is similar to that seen in rat tail collagen fibrils (Baldwin et al., 2014). Increasing levels of...
damage in various tendon overload fibrils were associated with an increase in kink density, decreasing mean modulus, and disappearance of the D-banding in modulus images (Fig. 8). The loss of D-banding is tied to increasing thickness of the fibril shell which parallels increasing serial kink density. The swelling ratio in our experiments also increased with greater kink density (Fig. 6E). When the sample was dehydrated, this shell layer collapsed and the D-band could be seen in the topography of the inter-kinked regions of the kinked collagen fibrils (Fig. 3B and D). This suggests that the shell structure consists of a gel-like layer of denatured collagen molecules, highly solvated in water. This denatured collagen is likely not solubilized due to the presence of crosslinking between neighboring alpha chains (Eyre and Wu, 2005; Hormann and Schlebusch, 1971). The similarity in the mean dehydrated heights of control and kink collagen fibrils suggests that no significant molecular content is lost during kink formation, suggesting retention of the entire shell layer (Fig. S1).

The higher-density core inside discrete plasticity-damaged fibrils was primarily observed in the deformation maps rather than in the modulus maps. This is due to the difference in depth sensitivity of the two measurements. Modulus maps were calculated by fitting the force curves to a maximum depth of 10% of the fibrils zero force height (~40 nm indentation), while the deformation maps are calculated from the maximum indentation depth of the AFM tip into the collagen fibril (~100 nm). Therefore, the deformation maps have increased sensitivity to features buried deep within the core of the fibrils: e.g. the appearance of D-banding in some inter-kink regions of very kinked collagen fibrils (Fig. 9). The inconsistency of D-band observation in the inter-kink regions may be due to regional variation in the fibril shell thickness.

4.2. Laid rope damage motif model of collagen fibrils

It is clear that discrete plasticity damage results from tendon overload, and that the intensity of the associated structural changes increases with repeated loading into the plastic region of the tendon stress-strain curve. Nonetheless, the kinks which we have now observed in dehydrated and hydrated specimens, under both SEM and AFM, are rebound structures which result after unloading of damaged fibrils.

We know little about the mechanism via which the local damage occurs and how the kinks form. A collagen fibril consists of helically wound sub-fibrils and micro-fibrils composed primarily of crosslinked, triple-helical molecules (Hulmes et al., 1995; Ottani et al., 2002; Silver et al., 1992; Wess, 2005). The crosslinking of the tropocollagen molecules and the twisted nature of the collagen fibrils sub-components suggest that an apt macroscopic, structural analog of the collagen fibril might be that of a polymerized laid rope strand (McKenna et al., 2004). While not in the context of understanding damage, models of this sort have been previously proposed. Some investigators have suggested that collagen fibrils are nanoscale ropes, while others have suggested a tube-like organization or a liquid crystalline model for the fibrils structure (Bozec et al., 2007; Brown et al., 2014; Gutsmann et al. 2003). If we consider the collagen fibril as a laid rope strand within a larger rope, it is intriguing to consider whether the shell and kink structures observed in discrete plasticity samples might resemble damage motifs.
found in laid ropes, associated with internal abrasion and axial compression fatigue (McKenna et al., 2004).

The radial variation of the shell–core structures of kinked, damaged fibrils, coupled with the increases in both swelling coefficient and shell thickness with kink density, offers the possibility that the shell layer may be the result of forces acting directly on the surface of the collagen fibril. There is support in the literature to suggest that shear stress plays a strong role in the behavior of tendons under tension (Bruehlmann et al., 2004; Screen et al., 2004; Szczesny and Elliott, 2014). Indeed, interfibrillar stress has recently been calculated as being 32 kPa (Szczesny et al., 2015). In overload, if shear stress were applied to a particular collagen fibril, we would expect that any shear-induced damage might propagate inwards from the affected surface. Molecular alteration due to interfibrillar shear, would then explain the formation of the denatured shell layer of the damaged collagen fibril. One possible mechanism for such denaturation in the shell might be inter-strand delamination; a phenomenon wherein α-chains participating in a crosslink are pulled out of their respective tropocollagen molecules, thereby leading to a permanent, stable, denaturation (Marino, 2015; Svensson et al., 2013; Uzel and Beuhler, 2011; Veres et al., 2014). In shear loading of the fibril, such delamination would propagate radially inwards from the surface toward the core of the fibril. This mechanism is similar to internal abrasion within a rope strand, characterized by a low density shell and loss of substructure at the ropes surface, features seen in the shell of discrete plasticity damaged collagen fibrils (McKenna et al., 2004). In considering such a possibility, we must also consider an alternative hypothesis: that there is a pre-existing radial heterogeneity in the fibril structure which preferentially pre-disposes the outermost molecular layers of the fibril to damage: perhaps sacrificial damage which absorbs energy prior to fibril fracture, toughening the fibril. Such a heterogeneity has been proposed in the tube-like model of a collagen fibril (Gutsmann et al., 2003). It is not possible at present to differentiate which of these mechanisms may be at play, if not both.

The kink structures observed in association with discrete plasticity are thought to be caused by structural damage which occurred during the overloading of the tendon, but actually formed upon removal of load. Observation of the kink structures, now with both AFM and SEM, confirms their similarity to kink structures seen in laid ropes when their lower hierarchical components experience compression (McKenna et al., 2004; Veres and Lee, 2012; Veres et al., 2013, 2014, 2015). One experimental difficulty which emerges when intact tendons are mechanically mounted and overloaded is that the resulting damage is heterogeneous: that is, only sub-populations of the collagen fibrils within the tendon display discrete plasticity damage. Some regions appear to have been spared overload and remain entirely intact (Veres and Lee, 2012). Such heterogeneous damage could lead to regions which experience some degree of compression upon removal of external load, perhaps resulting in kink formation. Should this be the case, and morphological similarity between engineered rope and collagen fibril structure certainly does not confirm this mechanism, we might ask whether compressive damage would occur during physiological overloading of a muscle–tendon–bone complex, or whether it is peculiar to a testing machine sample mounted in grips.

One damage motif found in laid ropes, termed axial compression fatigue, is characterized by periodic Z-shaped kink structures along the longitudinal axis of lower hierarchical structures. These structures in some ways resemble the kink structures observed in discrete plasticity. Formation of rope kinks in axial compression fatigue has been shown to be the result of twisting of the hierarchical structures under tension, and a mismatch in component lengths and mechanical properties. Their locations are thought to be determined by regions of low torsional resistance or the non-uniform loading of rope components (McKenna et al., 2004). We have previously shown micron-scale variation in a collagen fibrils density along its length. This observation suggests weak points may exist along the fibril (Baldwin et al., 2014). Furthermore, there is heterogeneity in fibril diameter in tendon, which may imply accompanying heterogeneity in fibril mechanical properties correlated with diameter (Moeller et al., 1995). It may be the case that some fibrils or fibril regions are particularly susceptible to the effects of local stress concentration, whether via torsion or other modes. Whether the morphological similarities between laid rope failure motifs and discrete plasticity in tendon collagen fibrils are more than intriguing coincidences remains the subject for further study.

5. Conclusion

In this work, we have used AFM in a hydrated environment to quantify the effects of tendon overload on the structure of individual collagen fibrils. We have confirmed the morphology observed under SEM, but have now demonstrated the dependence of water uptake and mean fibril modulus on serial kink density. Further, we have shown that kink and inter-kink regions maintain a constant modulus ratio, independent of kink density, suggesting a truly local mechanism for kink formation. Our data supports the conclusion that the shell and kink structures are associated with molecular denaturation. We have also described a set of interesting resemblances between failures in engineered laid ropes and discrete plasticity kinking seen in tendon collagen. It may follow that discrete plasticity kink formation is an effect of fibril sub-structure and longitudinal, structural heterogeneities which play out under the sort of uneven loading which occurs during in vitro testing of tendon samples. It remains to be demonstrated if this is also the case during physiological overloads in high-load bearing tendons as in, for instance, strain injuries. Discrete plasticity in tendon collagen is an important and complex phenomenon, and atomic force microscopy is a particularly valuable tool for its examination.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jmbbm.2016.02.004.

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