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Transverse mechanical properties of collagen fibers from nanoindentation

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Abstract The mechanical properties of collagenous tissues, such as tendon and ligaments, are of particular interest as they are found extensively in the human body. In the present study the transverse mechanical properties of collagen fibers are reported for the first time. The elastic modulus was found to be 63 ± 4 MPa, while the viscosity was estimated to be $14 \text{ GPa} \leq \eta \leq 56 \text{ GPa s}$. Comparison with similar data in the literature, for bulk tendon and collagen fibrils, suggests that the apparent modulus of a network of interconnected building blocks is reduced as compared to the modulus of the individual building blocks; in particular $E_{\text{tendon}} < E_{\text{fiber}} < E_{\text{fibril}}$; this is due to the fact that as the scale of the microstructure increases (i) slippage and sliding between the respective building blocks (fibrils or fibers) increases, (ii) the volume fraction of the stiff collagen proteins decreases.

1 Introduction

Collagenous tissue (e.g. ligament, tendon, meniscus) is normally a multi-scaled, heterogeneous structure composed of collagen fibers and fascia that are interconnected in a "woven like" manner. The collagen fibers, which have physical dimensions on the micrometer-length scale, are composed of collagen fibrils. For increasing length-scale levels of collagenous tissue, the volume fraction of collagen decreases as the relative content of fascia increases. The constituent collagen fibers and fibrils are roughly aligned in a single direction (longitudinal direction), thus creating a tissue with transverse-isotropic symmetry on multiple length-scales. The elastic properties in the longitudinal direction usually receive the most focus in the scientific literature since it is the principle direction of loading in musculoskeletal motion. However, in many cases the elastic properties of collagenous tissue in the transverse direction can play an important role in the performance of the human musculoskeletal system, and, therefore, the biomedical community has recently began to focus on transverse properties as well.

The transverse elastic properties of various ligaments [1–4] and tendons [5, 6] have been determined through a range of mechanical test techniques. The results from the experimental characterization of tendons indicate that their response is essentially linear [6], which allows the elastic response to be accurately characterized simply with the elastic (Young's) modulus. While it may be straightforward to obtain the bulk transverse Young's modulus for relatively large specimen sizes, it becomes prohibitively difficult for sub-millimeter-sized specimens (and, thus, individual fibers or fibrils). To determine the transverse Young's modulus for such small specimen sizes, small-scale test methods must be used. Atomic force microscopy (AFM) has been employed to study the properties of fibrils in both the longitudinal direction (by fabricating a "composite" of fibrils and using beam theory [7]), and in the transverse direction through nanoindentation [8]. In [8] AFM nanoindentation measured the elastic modulus of a

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single collagen fibril (diameter between 20 and 100 nm) to be between 5 and 11.5 GPa in the transverse direction. This value is significantly higher than that of bulk tendon, 1–11 MPa [1–6]. However, the mechanical response of micrometer-scale fibers, whose dimensions lie between these two extremes of bulk-scale tendon and nano-scale fibrils, has not been determined. It is of interest, therefore, to measure the elastic modulus of collagen fibers in order to provide physical insight into the multi-scale response of collagenous tissue. In this connection it is noted that although the elastic modulus of tendon, fascicles, and fibers in the longitudinal direction depends significantly on the length and radius of collagen [9], such a size effect is not expected when studying the transverse direction as here.

One of the most convenient and accurate small-scale testing techniques for mechanical property characterization is nanoindentation. In the nanoindentation method, a nanometer-sized tip is indented into a material sample. By measuring the depth of the tip displacement into the sample and the corresponding applied load, the material stiffness and strength properties can be extracted through the well-documented Oliver–Pharr method [10]. Although nanoindentation was originally developed to test metallic and ceramic materials on sub-micron length-scales [10] it has been successfully extended to test softer materials such as polymers [11, 12] and biological tissues [13, 14]. In the present study, therefore, nanoindentation was employed to determine the transverse Young's modulus of collagen fibers of a tendon taken from the thigh of a calf. It should be noted that nanoindentation has also been used to measure the elastic modulus of pure collagen that was extracted from bovine tendon tissue [15], however, imaging was not performed and the loading direction (transverse or longitudinal) was not stated.

The structure of the paper is as follows: The sample preparation technique is described, followed by the elastic modulus, hardness and viscosity measurements through nanoindentation. The topology of the tendon is investigated with atomic force microscopy, and this is presented in the context of the nanoindentation results. The measured values of the aforementioned material parameters are compared to the values found in the literature using larger- and smaller-scale test techniques.

2 Materials and methods

The tendon sample was taken from the thigh muscle of an 18-month-old calf. Extraction of the tendon occurred 12 h prior to the experiment; storage took place in a refrigerator at 4°C. Once the bulk tendon sample was sectioned into a 1.5 cm × 1.5 cm × 4 mm piece in the transverse direction, using a blade, it was glued on a steel sample holder and placed under a CSM Instruments Nanoindenter. The specimen was kept moistened during the nanoindentation testing by depositing fluid droplets onto the surface. Once the nanoindentation measurements were completed the sample was placed under a Veeco Multimode AFM and images were acquired.

Nanoindentations were performed with a Berkovich diamond indenter tip, which had a tip radius of 150 nm. The tests were load controlled, in the trapezoidal loading sequence. The maximum applied load was 10 mN, and the loading and unloading rates were 10 mN/min; a holding time of 20 s was allowed once the peak load was reached. Ten indentations were performed, under these conditions, at a spacing of 0.05 mm, so as to ensure that each indentation was not influenced by that preceding it. The penetration depth, of about 10 μm, was well below 10% of the sample thickness and therefore the glue or steel holder did not affect the measurements.

In order to better interpret the nanoindentation results, the microstructure of the sample was imaged using AFM, immediately, after completing the indentations. AFM was performed using the Veeco Multimode, under tapping mode, with a SiC tip with a tip radius of 12 nm.

It should be noted that prior to testing the present sample, other similar tendon specimens were used to determine the appropriate testing conditions that would result in reliable load versus depth curves. Due to the roughness of the tendon surface, the inherent viscosity and "woven-like" structure of the fibers it was not possible to perform depth-controlled tests. The optimum load was 10 mN, resulting in an indentation depth of about 10 μm, indenting therefore several fibers. Only ten tests were obtained, since performing additional measurements would require the sample to be left for a sufficiently long time under the indenter, and that would result in aging and degradation of the mechanical properties; hence an inconsistency between measurements would be observed.

3 Results

Representative nanoindentation plots obtained for the sample at hand are shown in Figs. 1 and 2. In order to better interpret the nanoindentation results, the microstructure of the sample that was indented was imaged using AFM. From the AFM scans, the collagen fibers could be distinctly seen as illustrated in the 3D topography images of Figs. 3, 4 and 5. It is of interest to note the nanoindentation imprint in Fig. 5.

3.1 Elastic constant estimates

The CSM software automatically performed the Oliver–Pharr method and measured the elastic modulus of the

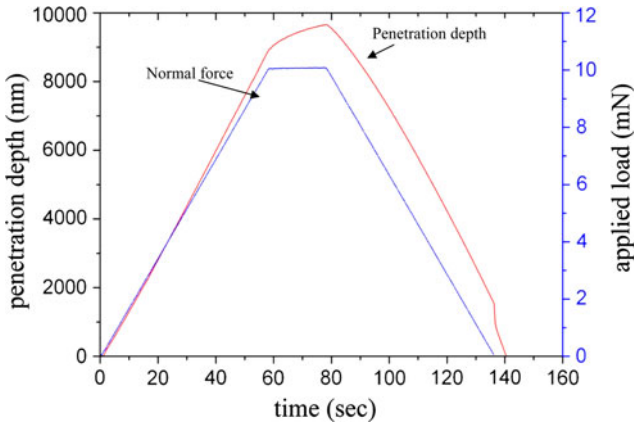

Fig. 1 Nanoindentation curves obtained for collagen fiber in the transverse direction. The *blue line* indicates the normal force exerted by the tip on the fiber as a function of time, whereas the *red line* indicates the penetration depth of the tip into the sample as a function of time (Color figure online)

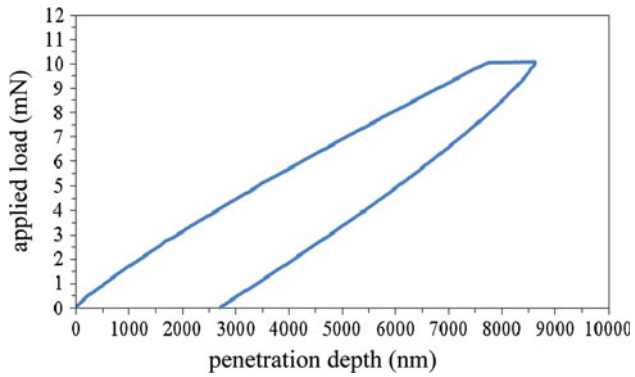

Fig. 2 Force versus penetration depth nanoindentation plot on collagen fibers in the transverse direction

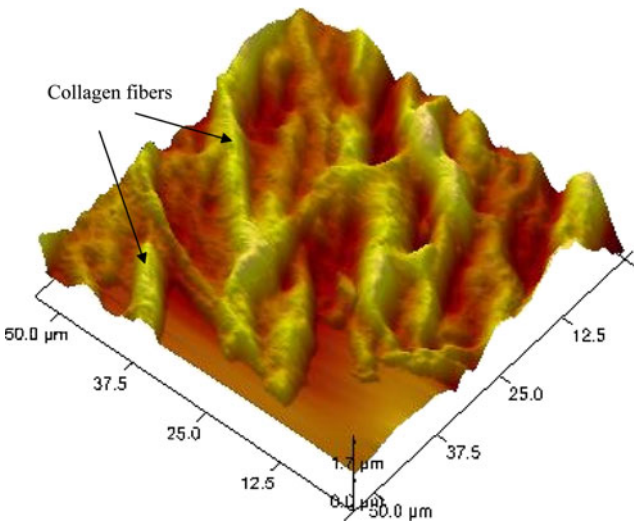

Fig. 3 3D Topography AFM image of tendon in the transverse direction, indicating the collagen fibers

indented area, which consisted of individual collagen fibers. Oliver and Pharr [10] expressed the reduced elastic modulus (E_r) of the tip-sample system as

$$E_r = \frac{\sqrt{\pi}S}{2\sqrt{A_c}}, \tag{1}$$

where A_c is the contact area, and S is the stiffness. The stiffness is obtained by differentiating the load-depth curve (Fig. 2) at the maximum depth, right before unloading takes place, and A_c is the contact area, which for a Berkovich tip is given by the pre-calibrated shape function

$$A_c = 24.56\, h_c^2, \tag{2}$$

where h_c is the contact depth given by [10]

$$h_c = h_{max} - \varepsilon\frac{P_{max}}{S}; \tag{3}$$

ε is a geometric constant (for a Berkovich tip $\varepsilon = 0.72$), while h_{max}, P_{max} are the maximum load and the corresponding maximum depth.

Once E_r is computed, the elastic modulus can be obtained from [10]

$$E = \frac{E_i E_r - E_i E_r v^2}{E_i - E_r + E_r v_i^2}, \tag{4}$$

where v is Poisson's ratio and the subscript i denotes the properties of the indenter tip. The Poisson ratio of the sample is taken as 0.4, while for the indenter tip, which is diamond: $E_i = 1000$ GPa and $v = 0.18$. Using the above-described Oliver–Pharr method the average value for the Young's modulus was determined to be 45.5 ± 1.3 MPa.

In Figs. 1 and 2, however, it was seen that during the hold time of 20 s at the maximum load of 10 mN, the indenter tip continued to be displaced into the sample, indicating that the sample underwent creep. Studies have shown that when materials exhibit creep, the Oliver–Pharr method does not estimate the modulus correctly and correction factors must be applied [16, 17]. According to [16, 17] the correct stiffness (S_c) that should be used in Eqs. 1–3 is given by

$$S_c = \frac{1}{S} - \frac{dh/dt}{dP/dt}, \tag{5}$$

where S is the apparent stiffness calculated during unloading as described previously, dP/dt is the unloading rate, which is 10 mN/min for the present experiments and dh/dt is the slope of the time versus depth plot at the maximum depth. A description on how to obtain dh/dt to the data of Fig. 1 is as follows: a fit (Fig. 6) is performed to the time versus depth data of Fig. 1 during the constant load holding time and then the resulting function ($h(t) = 8844.5 + 557.9\ (t - 55.3)^{0.39} - 13.43t$) is differentiated at the time when unloading began ($t = 75.3$ s).

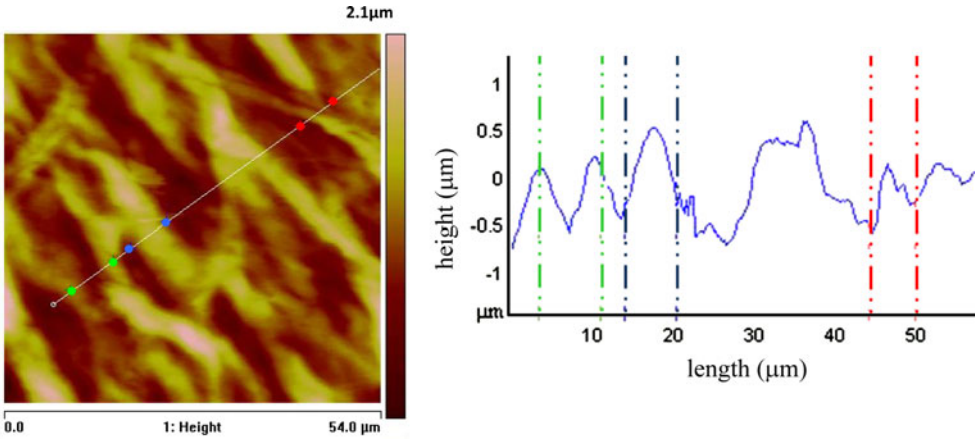

Fig. 4 (*left*) 2D topography image; (*right*) length and height profiles across the line of the left figure; it indicates the spacing between the collagen fibers, as well as their width and height. The *green*, *blue* and *red* markers on the left image, are indicated with *green*, *blue* and *red* vertical lines on the right image. And therefore the individual fiber topography is seen (Color figure online)

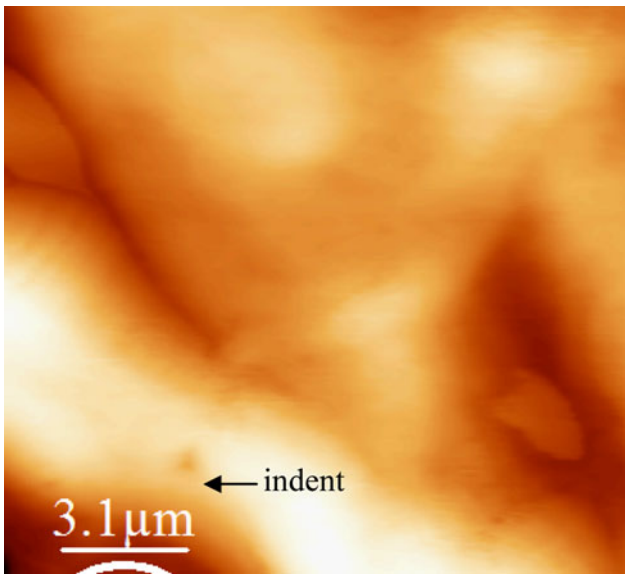

Fig. 5 Magnification of area depicted in Figs. 3, 4; the indent from which Figs. 1 and 2 were obtained is shown

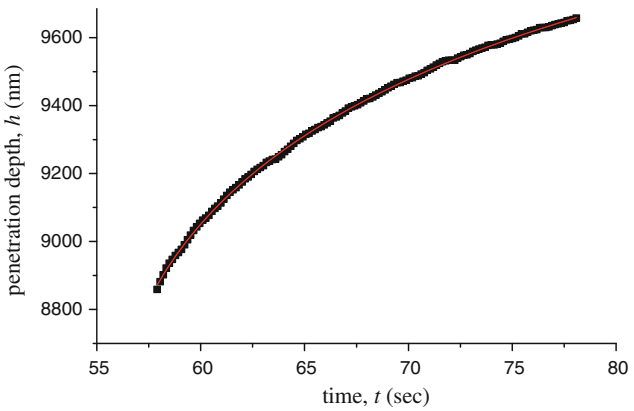

Fig. 6 Determination of *dh/dt* by performing a best fit to the experimental data of Fig. 1, during the constant load holding time. The fit of $(h(t) = 8844.5 + 557.9\ (t - 55.3)^{0.39} - 13.43t)$, lies on top of the data

The correct stiffness (Eq. 5) is therefore calculated and used in Eqs. 1–3 in order to obtain a correct elastic modulus (Eq. 4) that accounts for creep effects. Repeating this procedure for all indentation data gave an average Young's modulus of 63.1 ± 4 MPa.

In addition to the elastic modulus, the Vicker's hardness was estimated to be 7.4 ± 0.8 MPa by dividing the maximum applied load (10 mN) with the contact area that was obtained by inserting Eq. 5 in Eqs. 3 and 2. Then by using an approximate empirical conversion factor [18] this hardness value provided the ultimate tensile strength of the collagen fibers in the transverse direction as 25 ± 2.7 MPa.

3.2 Viscosity estimates

In addition to the elastic modulus and Vicker's hardness, which can be directly obtained from nanoindentation, it is possible to also obtain a value for the transverse viscosity of collagen fibers by further analyzing the data.

In [19] nanoindentation was used to examine viscous materials, and a theoretical model was developed in order to provide an estimate of their viscosity. The formulation of [19] is valid during infinitely-fast "step" loading, which is not the case in the present experiments. However, it is the only method that can provide a measure for the viscosity through nanoindentation with a Berkovich tip, and it will be employed in the sequel to estimate the viscosity of the collagen fibers, as no such values exist in the literature.

During creeping at a constant load, the penetration depth of the tip as a function of time is given by the expression [19]

$$h^2(t) = \left(\frac{\gamma_c^2(1-\nu)}{gk_c}\frac{P_0}{\eta}\right)t \Rightarrow h(t) = \sqrt{\left(\frac{\gamma_c^2(1-\nu)}{gk_c}\frac{P_0}{\eta}\right)t}, \tag{6}$$

where P_0 is the constant load at which creep took place, ν is the sample's Poisson's ratio and η is the viscosity of the

sample, while γ_c, g and k_c are geometric parameters for nanoindentation. For a Berkovich tip when the nanoindentation is elastic: $\gamma_c = \pi/2$, $g = 3\sqrt{3}\cot^2\beta$, $\beta = 24.7°$ and $k_c = \pi/(g\tan\beta)$ [19]; while when it is plastic $\gamma_c = \gamma_p = 1$ and $k_c = k_p = \tan\beta$. Letting $\nu = 0.4$ and $P_0 = 10$ mN (since that is the maximum load at which the holding time took place according to Fig. 1), the only unknown left in Eq. 6 is the viscosity.

The viscosity can, therefore, be obtained by fitting Eq. 6 to the nanoindentation data between $55.3 < t < 75.3$ s as follows. Eq. 6 has as its starting point the point at which creeping initiates, therefore the point (0, 0) of Eq. 6 corresponds to the point (h(55.3), 55.3) in Fig. 1. Hence, all the data points (h_i, t_i) between $55.3 < t < 75.3$ of Fig. 1 are normalized as (h_f, t_f) = ($h_i - 8844.5$, $t_i - 55.3$). Then a fit is performed to these data of the form

$$h(t) = K\sqrt{t}, \tag{7}$$

where all the constants of Eq. 6 are grouped in the constant K. Equation 7 fits very nicely to the nanoindentation data as illustrated in Fig. 7, where it can be seen that $K = 180$ nm/$\sqrt{\text{sec}}$, therefore, combination of Eqs. 6 and 7 gives

$$180 \times 10^{-9}\frac{\text{m}}{\sqrt{\text{sec}}} = \sqrt{\left(\frac{\gamma_c^2(1-0.4)10\times10^{-3}\text{m}}{gk_c \qquad \eta}\right)}. \tag{8}$$

Solving Eq. 8 for the viscosity, using the geometric parameter values for an indentation that is purely elastic (i.e. $\gamma_c = \pi/2$, $g = 3\sqrt{3}\cot^2\beta$, $\beta = 24.7°$ and $k_c = \pi/(g\tan\beta)$) provides a viscosity of $6.69 \times 10^{10}\frac{\text{N sec}}{\text{m}^2} = 6.69 \times 10^{11}$Poise, whereas for an indentation that is purely plastic ($\gamma_c = \gamma_p = 1$ and $k_c = k_p = \tan\beta$) the viscosity is 1.63×10^{11}Poise. The geometric parameters for a viscoelastic response are not known and therefore the two extremes (pure elasticity and pure plasticity) are considered here to illustrate that a significant difference in the viscosity estimation does not result. Repeating the process for measuring the viscosity for all indentations and averaging it is found that the viscosity of collagen fibers in the transverse direction is between 14 GPa s (elastic response) $\leq \eta \leq$ 56 GPa s (plastic response).

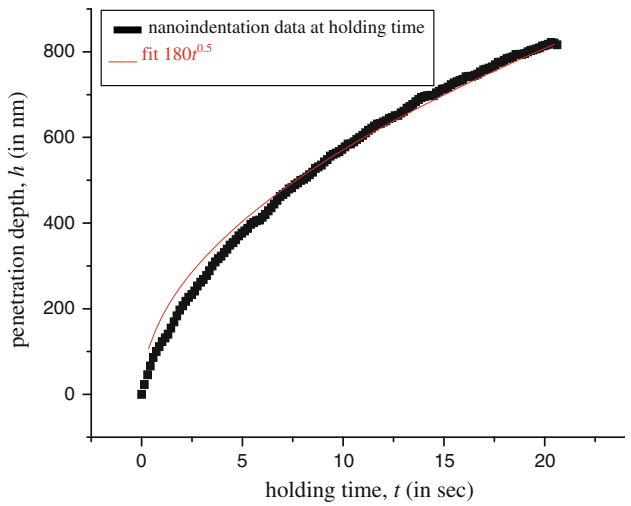

Fig. 7 Fit of Eq. 7 to normalized nanoindentation data of Fig. 1 during the holding time for determining the viscosity of collagen fibers

4 Discussion

Figure 3, which depicts the sample on which the nanoindentation was performed, clearly demonstrates that the collagen fibers were oriented perpendicular to the nanoindentation direction, indicating that the measured mechanical response was in the transverse direction. The line profile of the specimen surface is shown in Fig. 4. The profile indicates that the height of the fibers was between 0.5 and 1 μm, their spacing was about 15 μm, and their average diameter was about 7 μm. A magnification of the area under consideration is shown in Fig. 5, where the curvature of the collagen fibers is evident, and also the indentation imprint is visible. The imprint is very small compared to the contact area during indentation (for the indent in Fig. 2 it was estimated as 1200 μm^2) and hence the response of several fibers was accounted for. The small indent imprint is due to the significant elastic recovery that took place. Such a high elastic recovery was anticipated from the linear loading response observed in Fig. 2. Such a linear response has also been observed in nanoindentations of the soft polymer PDMS [20] which also has a "woven-like" structure.

The mechanical properties obtained from nanoindentation can be further analyzed in the context of the AFM images. As mentioned in the introduction, most previous studies have characterized the mechanical response of tendon using large-scale experimental techniques. These studies report that the values of the transverse Young's modulus of ligaments and tendons ranged between 1.1 and 11.0 MPa [1–6]. Small scale testing [8] conducted by AFM indentation estimated a Young's modulus between 5 and 11 GPa for a single collagen fibril (diameter between 20 and 150 nm). The present value of the transverse Young's modulus of 63 MPa reported herein lies between the values of bulk tendon and collagen fibrils; particularly the following trend is observed $E_{\text{tendon}} < E_{\text{fibers}} < E_{\text{fibril}}$. The reason for the discrepancies in the transverse modulus at different length scales is due to a scaling effect in tendon. There are two contributions to this scaling effect: (i) The fibrils comprise mostly of stiff collagen proteins, which leads to the relatively high elastic modulus. The fibers, in turn, comprise from an array of aligned fibrils along with

low-stiffness fascia. The volume fraction of collagen proteins in the fiber is, therefore, lower than that in the fibrils, leading to a reduced modulus. In forming tendon, the fibers are combined with additional fascia, and as a result the elastic modulus of tendon is further reduced. (ii) As the length scale of the microstructure increases and the collagen protein volume fraction decreases, the microstructure becomes more "woven-like" and, hence, the fibrils/fibers are given more "room" to slide and rotate with respect to each other, which, in effect provides for a decreased apparent material stiffness relative to the respective "building blocks". It should be noted that not accounting for creep effects, i.e. applying the Oliver–Pharr method [10] without the correction terms of [16, 17] gave a Young's modulus that was about 20 MPa lower.

Similarly to the elastic modulus, the ultimate transverse tensile strength of fibers was measured to be 25. ± 2.7 MPa, which is higher than that obtained for bulk ligaments and tendons, whose values range between 0.1 and 1.7 MPa [1–6]; corresponding values for fibrils could not be found. This increase in the ultimate tensile strength for microscale fibers as compared to bulk tendon is attributed to the above-described scaling effects for the elastic modulus.

The viscosity for tendon and collagen materials has not been studied much. The only other measurements to which the current viscosity of the collagen fibers could be compared to is to a viscous parameter that was computed for tendon and ligaments in the longitudinal direction [21]. This viscous parameter was $\sim$0.4 GPa s for tendon and $\sim$40 MPa s for ligaments. For the present fibers the viscosity estimate is 14 GPa s $\leq \eta \leq$ 56 GPa s; since the direction was transverse a higher value was expected than those reported in [21]. However, it should be noted that the viscosity measured presently, is not the same viscosity parameter as in [21] and also the method employed here provides a rough estimate as the loading conditions were not the same as in [19].

5 Conclusions

The present study employed nanoindentation to determine the transverse mechanical properties of a bovine tendon tissue, and AFM was used to image the indented area, which was on multiple collagen fibers ($\sim$3–7 μm in diameter). Previous studies in the literature have not reported the transverse properties of collagen fibers. Specifically, the measured elastic modulus was 63 MPa, which is between 5 and 500 times larger than that observed for bulk tendon, while the measured ultimate strength, which was 25.5 MPa is 3–70 times larger than that observed from larger-scale tests on tendon. This is attributed to the fact that large scale experiments capture the overall effect of multiple interconnected collagen fibers, and additional fascia, whereas nanoindentation captures the properties of a volume of fibers. In this connection it is noted that collagen fibers comprise of collagen fibrils, which are even smaller in diameter (20–150 nm) and contain a higher volume fraction of collagen protein, and in turn they have a higher elastic modulus than collagen fibers. Therefore, the elastic modulus of collagen fibers is found to be between that of bulk tendon and nano-fibrils, since as the scale of microstructure increases sliding and slippage among the collagen fibrils/fibers increases and the volume fraction of the collagen protein decreases giving $E_{\text{tendon}} < E_{\text{fibers}} < E_{\text{fibril}}$. Furthermore, the viscosity of collagen fibers, during nanoindentation in the transverse direction, was estimated to be approximately 14 GPa s $\leq \eta \leq$ 56 GPa s, which is the first viscosity estimate reported in the literature for the transverse direction.

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