Small-angle light scattering to detect strain-directed collagen degradation in native tissue

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It has been demonstrated that there is a mechanochemical relationship between collagen and collagenolytic enzymes such that increased tensile mechanical strain reduces the enzymatic cutting rate. This mechanochemical relationship has the potential to permit directed remodelling of tissue-engineered constructs in vitro and to shed light on the generation of load-adapted collagen-based connective tissue. In this investigation, we demonstrate that small-angle light scattering (SALS) has the sensitivity to dynamically detect the preferential enzymatic degradation of a subset of unloaded collagen fibrils within differentially loaded native tissue. Detection of the difference in the relative degradation rate of unloaded fibrils versus loaded fibrils was manifested through changes in the spatial distribution of the SALS signal. Specifically, we found a linear increase in the eccentricity of the SALS data that was consistent with preferential retention of the collagen fibrils aligned with the applied tensile strain. We conclude that SALS is simple, inexpensive and may provide a useful optical screening method permitting real-time monitoring of strain-controlled tissue and construct remodelling.

Keywords: collagen; strain-stabilization; light scattering

1. INTRODUCTION

In vertebrate animals, there are four classes of extracellular matrix (ECM) molecules whose primary functions are to resist mechanical force—they are: collagen, elastin, proteoglycans (PGs) and hydroxyapatite (HA). In general, collagen and elastin resist tensile force while PGs and HA resist compressive forces. These load-bearing molecules can be found distributed throughout the vertebrate musculoskeletal system ECM in varying concentrations (e.g. HA 65% dry weight of bone [1], PGs approx. 3% dry weight of femoral head cartilage [2], collagen 70–80% dry weight of tendon [3], elastin 7% dry weight of ligament [4]). Of the four classes of ECM structural molecules, collagen is the most abundant load-bearing material in vertebrates and contributes more than 25 per cent to the total protein mass [5]. In addition, collagen is considered to be the embryonic template molecule, arriving in the embryo during the early formation of load-bearing structures and providing a guiding rudiment (e.g. for mineralization of bone). Thus, understanding the behaviour of collagen as a load-responsive material is of paramount importance if we are to gain insight into the development, growth, maintenance and pathology of load-bearing ECM in vertebrate animals.

There is substantial evidence that collagenous tissue, fibrils and monomers gain resistance to some collagenolytic enzymes even when a small mechanical tension (<3 pN per monomer) is applied along their main axis [6–14]. The protective behaviour has been termed a ‘low-force switch’ by Camp et al. [7]. The existence of such a switch suggests that collagen would tend to accumulate and be retained in the path of mechanical force, with the collagen fibril long axis typically aligning with the principal direction of tensile stress (e.g. tendon [15]). Such a property would greatly simplify the initial construction and subsequent remodelling of load-bearing collagenous matrix (both in native tissue and in engineered constructs). However, it remains to be demonstrated that such a mechanism is at work in vivo or in native tissue in vitro using physiologically relevant matrix metalloproteinase (MMP) enzymes. Part of the difficulty resides in the fact that there are few methods capable of detecting preferential, strain-directed collagen remodelling in tissues.

Our laboratory has previously used the corneal stroma as a test matrix to probe the concept of strain protection of collagen against enzyme proteolysis by bacterial collagenase (BC) [11,13]. In our initial paper [11], we theorized that applied load or strain could
preserve a loaded subset of fibrils relative to less loaded set of fibrils in the presence of the collagenolytic enzyme from Clastriodium histolyticum. The cornea was chosen because it possesses a unique matrix which is well suited to test the strain-stabilization theory. This is true principally because the cornea comprises arrays of uniform diameter, aligned fibrils packed into lamellae which alternate in direction [16–19]. Further, the lamellae, which are mechanically uncoupled, can be preferentially strained in one direction, leaving a population of less loaded or unloaded ‘control’ lamellae. By the strain-stabilization theory, the fibrils under lower tensile load should be preferentially attacked by the collagenolytic enzyme. In a time-stop series of experiments, one may periodically (and destructively) examine the matrix morphology following the application of a judiciously chosen mechanical force (or strain) with simultaneous exposure to enzyme. In their paper, Ruberti & Hallab [11] performed such an investigation and found that application of mechanical force preferentially preserved fibrils in the direction of the applied load. However, the limitation imposed by morphological assessment at fixed time points (tissue processing for transmission electron microscopy (TEM) takes multiple days in practice) and effectively prevents real-time feedback during the remodelling process. Thus, the dynamics of the enzymatic ‘remodelling’ process were not captured. Quantifying strain-controlled enzyme/collagen degradation dynamics in bulk tissue can provide insight and direction both to tissue engineers seeking to produce organized constructs for load-bearing applications and to basic scientists seeking to understand the development, remodelling, homeostasis and pathology of connective tissue.

Small-angle light scattering (SALS) offers a relatively simple, inexpensive technique to provide semi-quantitative structural information about dense fibrous connective tissues. Other forms of microscopy such as scanning electron microscopy (SEM) and TEM provide highly detailed information over an extremely small sample area and require intensive sample preparation that inhibits dynamic imaging. Unlike SEM/TEM, SALS has the ability to give dynamic information on the macroscopic scale, as typically an incident laser beam samples an area of the order of hundreds of square micrometres. SALS is also simpler to use and less expensive to obtain than second harmonic generation imaging, which is a plausible alternative to SALS [20–22]. Owing to the inherent nature of light scattering, SALS is only capable of detecting structures that are of the order of the wavelength of the incident light. Studies on calfskin [23] and connective tissues [24] have shown that structural information obtained by SALS is in close agreement with small-angle X-ray scattering (SAXS), which is generally accepted as an accurate technique to map out collagen architecture [25,26]. This indicates that SALS is a quicker, simpler alternative to SAXS, but incurs the cost of reduced structural resolution. Nonetheless, because the operative structural element in native tissues often comprises bundles of fibrils acting in parallel (lamellae in cornea and fascicles in tendon), SALS information may accurately reflect the structural competency of the tissue. Previous studies using SALS on connective tissue [27,28] treat the collagenous fibre network as a two-dimensional assembly of single slits, where the effective slit width is an average of the fibre diameters and inter-fibre spacing. In this simplified model, light is scattered perpendicular to the fibre axis so that a fibre with an angular orientation of $\phi$ scatters light intensity at $\phi + \pi/2$, with each fibre population at an angular orientation of $\phi$ and $\phi + \pi/2$, each population contributes scattered light intensity at $\phi + \pi/2$ and $\phi$, respectively. Similarly, if there is a random or pseudo-random distribution of fibre populations, light is scattered pseudo-isotropically.

Figure 1. A diagram illustrating light scattering-off of collagen fibres. The parallel aligned fibres act as diffraction slits and scatter incident light perpendicular to the fibre axis. If one fibre population is at an angular orientation of $\phi$, that population contributes scattered light intensity to $\phi + \pi/2$. If this fibre population dominates the total fibre population (i.e. a majority of fibrils lie in one direction), the scattered light will take the shape of an ellipse with the major axis at an angular orientation of $\phi + \pi/2$. If two perpendicular populations exist at angular orientations of $\phi$ and $\phi + \pi/2$, each population contributes scattered light intensity at $\phi + \pi/2$ and $\phi$, respectively.

Collagen fibrils in the cornea are approximately 35 nm in diameter [18,29], far below the diffraction-limited resolution of SALS. However, the fibrils are bundled together to form fibrous structures of several micrometres wide called lamellae [16,30]. In each lamella, collagen fibrils are aligned parallel to each other with inter-fibril spacing much less than the wavelength of light, resulting in the scattered light being nearly in phase for adjacent fibrils. Researchers have used SALS to explore the corneal structure in the past. Most notably, McCally & Farrell [31] used SALS with crossed-polarizers on bovine cornea under uniform pressure. They deduced that the lamellae are not randomly oriented in the cornea, but rather that there exist two predominant fibre populations of unequal number that lie orthogonal to each other at roughly 45° and 135° with respect to the nasal-temporal direction. This agrees reasonably with more recent SAXS studies conducted on bovine cornea by Hayes et al. [32], which found an excess of collagen oriented at slightly more than 90° with respect to the nasal-temporal direction. Thus, our SALS system is well suited to test our strain-stabilization hypothesis in native tissue containing arrays of collagen fibrils.

2. EXPERIMENTAL SECTION

2.1. Specimen

In bovine corneas, monodisperse diameter type I collagen fibrils, packed into lamellae run parallel to each
other in one plane, and are approximately orthogonal to adjacent planes (although there is a substantial number of adjacent lamellae which are not strictly orthogonal [17]). The structure provides an opportunity to generate a strained fibril population (experimental) and an unstrained fibril population (control) in the same specimen. Corneas were obtained from cows of 20 to 45 kg provided by Research 87 Inc. (Boylston, MA, USA). Corneas were removed from the ocular globes and prepared for loading and exposure to enzyme as described in Zareian et al. [13]. Briefly, corneas were excised, debrided of epithelium and endothelium and stored at −80°C. Multiple freeze–thaw cycles (to −80°C) were used to devitalize the tissue for further control over the samples. A custom-made cutting die was used to generate repeatable vertically oriented corneal strips (superior–inferior direction) approximately 0.75 ± 0.1 mm thick × 17.5 ± 2.5 mm long × 6 mm wide. The samples were always kept moist with 37°C Dulbecco’s Modified Eagle medium (DMEM; Mediatech Inc.) during all excision and mounting into the test chamber.

2.2. Mechanical loading apparatus

An environmentally controlled uniaxial bioreactor [13] was used to explore the effect of mechanical strain on the preferential degradation of fibrillar collagen tissues via SALS. The device comprises a load cell (Honeywell Sensotec, model 31; maximum 2.26 kg; resolution 0.9 g), a uniaxial motor (Zaber Technologies T-LA60; resolution, 16 μm; maximum speed, 4 mm s⁻¹; speed resolution, 0.001 mm s⁻¹), and is controlled using a custom LabView program. The system has an accuracy of ±0.01 N, and adjacent specimen chamber walls comprise quartz glass to permit incident laser light to illuminate the sample, while an integrated proportional-integral-derivative controller–driven temperature control system maintains the chamber at 37 ± 1°C. Each corneal strip was positioned between two cam grips inside the chamber and immersed in DMEM. Cyanocrylate glue was applied to the sample grip interface and permitted to dry quickly to provide a firm hold of the cornea during testing. The strain, \( \varepsilon \), is calculated from the grip displacement \( l \), using the initial specimen gauge length, \( l_0 \), as the reference: \( \varepsilon = (l - l_0)/l_0 \).

2.3. Digestion protocol

Crude BC from \( C. histolyticum \) (Clostridioppeptidase A; Sigma–Aldrich, no. C0130) with a molecular weight range from 68 to 125 kDa was used. Each mole of collagenase requires 4 moles of calcium (Ca²⁺) in order to activate the solution. DMEM contains enough Ca²⁺ to support the BC at the concentrations used in our system. To initiate enzymatic cleavage in an experiment, the DMEM buffer solution was replaced with solution containing BC (DMEM and 0.05 mM BC) for the remainder of the experiment. All BC solutions were made previously and stored in separate containers at −80°C until use for each experiment. At the onset of each experiment, the BC was preheated in a water bath for 30 min until a temperature of 37°C was reached, at which point the BC was injected into the chamber. Collagenase activity can vary from batch to batch and can decline with time. However, this is not an issue because of the short duration of our experiments (approx. 1 h).

2.4. Loading protocol

All experiments were conducted in strain-control mode of the bioreactor. Cornea strips were first positioned in the bioreactor chamber with DMEM buffer and loaded until a reference load of 0.01 N was detected, which we used as our initial specimen gauge length \( l_0 \). To test our hypothesis that strain preferentially preserves loaded fibrils, three types of experiments were conducted:

- **Loaded, exposed to BC (n = 10).** The strain was held at a constant 6 per cent while the load and SALS intensity distributions were recorded. Loaded cornea samples were allowed 20 min for the stress to ‘relax’ owing to the viscoelastic nature of the corneal tissue. After 20 min elapsed, the loaded cornea samples were subjected to degradation by BC for 40 min to explore the effect of strain on enzymatic cleavage of collagen fibrils/fibres.

- **Unloaded, exposed to BC (n = 9).** The strain was held at a constant 0 per cent for the full 60 min while the SALS intensity distributions were recorded. After 20 min, the unloaded cornea samples were subjected to degradation by BC to explore the effect of enzymatic cleavage on the SALS intensity distribution.

- **Loaded, unexposed to BC (n = 5).** The strain was held at a constant 6 per cent for the full 60 min, while the load and the SALS intensity distribution were recorded. The loaded cornea samples were not subjected to degradation by BC to explore the effect of stress-relaxation on SALS intensity distributions.

Figure 2. Experimental set-up used to image bovine cornea dynamically under uniaxial load subject to enzymatic cleavage: A, Green HeNe laser linearly polarized to 135°, \( \lambda = 534.3 \) nm, \( d = 0.8 \) mm. B, Adjustable mirror mounts to scan through sample. C, Bovine cornea strip in bioreactor under uniaxial strain (6%). The inset shows both a cornea pre-degradation (smaller inset with C1) and post-degradation (C2). D, Biconvex lens, \( f = 30 \) cm, to redirect scattered light for imaging and analysis. E, Ground glass to display SALS patterns. F, Charge-coupled device to capture and analyse SALS data.
2.5. Small-angle light scattering dynamic imaging

An optical bread board was used to mount a linearly polarized green HeNe laser (Melles Griot; model 05-LGP-173; 0.3 mW) with a beam diameter \( d = 0.8 \) mm and \( \lambda = 534.3 \) nm, with its polarization axis at 135° with respect to the x-axis (figure 2). A linearly polarized laser was chosen over an unpolarized laser since in the latter, the polarization orientation randomly shifts owing to temperature drifts, and collagen is known to exhibit birefringence. Two mirrors on kinematic mounts were used to direct the beam to the sample such that the incident beam was normal to the tissue sample (angle of incidence = 0°). A biconvex lens (focal length, \( f = 0.3 \) m, Thorlabs) was positioned behind the sample to redirect scattered light from the sample onto a ground glass where it was captured by a long focal charge-coupled device (CCD) camera (Prosilica, model CV640; black and white; frame rate 120 fps; resolution 9.9 \( \times \) 9.9 \( \mu \)m per pixel). At the onset of each experiment, the incident beam was directed to the centre of the corneal strip, and scanned in the vertical direction until a nearly isotropic scattering location was found. Once the ideal location was identified, the sampling area remained constant throughout the duration of the experiment.

Previous methods of analysing SALS of collagen networks [27,28] involved collecting the scattered light intensity distribution along the azimuthal direction, \( I(\Phi) \) at a constant scattering angle \( \theta = \theta_0 \). The width of the \( L(\Phi, \theta = \theta_0) \) versus \( \Phi \) distribution is proportional to the degree of fibre orientation. Fibre architectures that have fibres loosely oriented in one direction will have a wider \( L(\Phi, \theta = \theta_0) \) versus \( \Phi \) peak than fibre architectures that have tightly oriented populations in one direction. However, this analysis is unsuitable for degradation experiments. As fibres are cleaved by BC and removed from the cornea, the scattering angle decreases as there are many fewer collagen fibres to scatter the incident light (see figure 5). Over long enough times (\( t \approx 75 \) min) in the unloaded experiments, the scattering angle reduces to nearly zero, leaving only the intensity distribution of the incident laser beam. The method of Sacks et al. [27,28] can give drastically different results depending on the scattering angle \( \theta_0 \) used to take the intensity distribution over, and hence is not ideal in degradation experiments in which the scattered light distribution is dynamic.

To analyse SALS intensity distributions in the ensemble of loading protocols used, we implement a simple image moment technique [33]. Image moments offer a robust way to quantitatively analyse scattered light distributions as it evolves over time; it requires no manual selection of a scattering angle \( \theta_0 \) over which to measure intensity distributions. The image moments are defined as

\[
\bar{y} = \frac{\sum_i I_i y_i}{\sum_i I_i}, \quad (2.1a)
\]

\[
\bar{x} = \frac{\sum_i I_i x_i}{\sum_i I_i}, \quad (2.1b)
\]

\[
\Sigma = \sum_i I_i y_i^2, \quad (2.1c)
\]

\[
\Sigma = \sum_i I_i x_i^2, \quad (2.1d)
\]

and

\[
\Sigma = \sum_i I_i x_i y_i. \quad (2.1e)
\]

The image moments can be used to calculate the covariance matrix of each SALS image:

\[
\text{cov}(I(x, y)) = \begin{bmatrix} \Sigma - \bar{x}^2 & \Sigma - \bar{x}\bar{y} \\ \Sigma - \bar{x}\bar{y} & \Sigma - \bar{y}^2 \end{bmatrix}. \quad (2.2)
\]

It is then straightforward to calculate the eigenvalues of the covariance matrix

\[
\lambda_i = \frac{\Sigma - \bar{x}^2 + \bar{y}^2 - \bar{y}^2}{2} + \sqrt{\frac{4(\Sigma - \bar{x}\bar{y})^2 + (\Sigma - \bar{x}^2 - \Sigma + \bar{y}^2)^2}{2}}, \quad (2.3)
\]

which represent the major and minor axis of the ellipsoidal SALS intensity distribution. From this we can find the eccentricity

\[
e = \sqrt{1 - \frac{\lambda_2}{\lambda_1}}. \quad (2.4)
\]

We can quantitatively analyse the collagen structure by treating the collagen fibre populations (lamellae) as a two-dimensional assembly of slits, contributing scattered light intensity perpendicular to the orientation of their fibre/fibril axis. The eccentricity then is a direct measurement of the degree of collagen fibre/fibril organization in one direction. Higher values of eccentricity correspond to a larger percentage of the sampled fibres/fibrils that are oriented perpendicular to the direction of the major axis of the ellipse. By using the eigenvalues of the covariance matrix to find the eccentricity of each SALS intensity distribution, the subsequent structural changes of collagen fibres/fibrils owing to loading and enzymatic cleavage can be analysed.

2.6. Transmission electron microscopy

TEM was implemented to provide an alternative method which could be used to verify the SALS results at the ultrastructural level. Specimens from two of the protocols (\( n = 2 \) from groups 1 and 2) were gently removed after the experiment and collected from the bioreactor chamber. Once safely removed from the BC, the samples were fixed in Karnovsky’s fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4) and processed for TEM as described in Guo et al. [34]. Briefly, all specimens were removed from Karnovsky’s solution and were exposed to 1 per cent osmium tetroxide and then dehydrated in graded ethanol. After the dehydration process, all
Figure 3. (a) Scattering geometry used to model collagen fibrils as infinite cylinders. (b) Predicted scattered irradiance-off of two cylinder populations, one that is parallel to the x-axis and another parallel to the y-axis. The value $a$ is defined as the ratio of the number of cylinders in the x-direction to the number of cylinders in the y-direction ($a = 1$ when the two populations are equal). The polar plot shows the irradiance for values of $a$, starting at $a = 1$ on the outside, and $a = 0.8, 0.6, 0.4, 0.2$ towards the inside.

specimens were embedded in Epon-Araldite and were sectioned perpendicular to the applied load in the tensile specimens. At the end, all sections from specimens were stained with uranyl acetate and lead citrate in methanol and mounted on JEOL 1010 electron microscope (JEOL, Tokyo, Japan).

3. RESULTS

A simple model was developed to capture the predicted SALS intensity distributions of collagen fibrils/fibres based on treating collagen fibres/fibrils as a system of two-dimensional diffraction slits. The collagen fibres were treated as uniform cylinders, with the scattering geometry shown in figure 3a. Light is incident along the z-axis on a cylinder much smaller than the wavelength and parallel to the x-axis. The field of scattered light at an angle $\theta$ from the z-axis in the y–z plane is independent of $\theta$ if the polarization is parallel to the x-axis and proportional to cosine of $\theta$ if the polarization is parallel to the y-axis. In either case, at a large distance $z_o$, the field is proportional to the cosine of the angle $\varphi$. For a collection of cylinders all oriented in the same direction along the x-axis, assuming incoherent addition resulting from random positioning of the cylinders, the irradiance will be proportional to the $\cos^2(\varphi)$. Likewise, for cylinders oriented along the y-axis, the irradiance will be proportional to $\sin^2(\varphi)$. If the density of cylinders in the x-direction decreases, being proportional to $a$, where $a = 1$ when the density of cylinders in the y-direction is equal to that in the x-direction, then the irradiance will be proportional to $a \cos^2(\varphi) + \sin^2(\varphi)$. The polar plots in figure 3b show this function for a range of values of $a$, in which $a$ decreases, the intensity distribution becomes more anisotropic with the longer axis parallel to the x-direction.

The typical mechanical response of a cornea under all three types of loading/enzyme experiments run in this study is shown in figure 4. In group 2, the unloaded samples exposed to BC are strained until a force of 0.01 N is reached, and kept constant at that strain (approx. 0%) for the entire experiment. In groups 1 and 3, the loaded samples exhibit significant stress-relaxation and reach equilibrium at approximately 20 min after initial loading. At $t = 20$ min, the BC is added to group 1 and there is a significant reduction in load-carrying capability in the cornea due to enzymatic cleavage. However, by the end of the experiment ($t = 60$ min), the strained corneas in group 1 still have an appreciable load (approx. 0.02–0.03 N), indicating that at least some fibrils/fibres still remain. A series of filtered, thresholded SALS intensity distributions of a loaded cornea exposed to BC are shown at specific time intervals in figure 4b–f. Altogether, figure 4 shows that a majority of the load-carrying capacity of the cornea, and most likely the cornea fibres/fibrils, are cleaved in the first 15 min after exposure to 0.05 mM concentration of BC. During this exposure to BC, the SALS intensity distribution becomes more and more anisotropic, taking the shape of an ellipse with the major axis perpendicular to the direction of strain.

Typical raw images of SALS intensity distributions of the unloaded, degraded cornea from group 2 are directly compared with the loaded degraded cornea from group 1 in figure 5. After 40 min of exposure to BC, both the loaded and unloaded cornea samples undergo a decrease in scattering angle. However, in the case of the unloaded cornea (figure 5a,b), the decrease in scattering angle is fairly uniform, indicating that fibres in all directions are degrading at roughly the same rate. For the loaded cornea (figure 5c,d), the decrease in scattering angle along the direction of strain is noticeably greater, indicating a preferential removal of fibres unaligned with the direction of strain.

It should be noted that the eccentricity is a direct measurement of the degree of fibre orientation, however, it is the change in eccentricity that allows us to use SALS to dynamically test our hypothesis that strain prevents collagen fibril/fibre degradation by BC. The lamellar architecture of the bovine cornea is not constant across the centre superior–inferior strip [32], and can possibly vary slightly from one cornea to the other. It is also likely that sample preparation and loading into the bioreactor slightly affected the lamellae architecture of the cornea. It is not uncommon then to get fairly distinguished eccentricity values from different corneas, even under the same loading/strain.
protocol and approximate sampling locations. We thus use statistical analysis to ensure that the change in SALS intensity distribution is significant by implementing a paired, two-tailed Student’s \( t \)-test to evaluate the relative change in eccentricity during the degradation of the corneal stromal tissue. After approximately 20 min, the sample is ‘relaxed’ and bacterial collagenase (BC) at a concentration of 0.05 mM is added, indicated by the dashed line. As the enzyme cleaves collagen fibres, the load decreases until it reaches a minimum (approx. 0.02 \( \pm \) 0.01N). (b) An individual SALS intensity distribution from a sample from group 1 at \( t = 20 \) min thresholded for clarity. (c) SALS intensity distribution at \( t = 30 \) min; (d) \( t = 40 \) min; (e) \( t = 50 \) min; (f) \( t = 60 \) min.

Figure 4. (a) Typical load versus time behaviour of corneal tissues in the ensemble of loading protocols used in this study. Squares represent group 1, the triangles represent group 2 and the circles represent group 3. In the loaded samples from groups 1 and 3, the cornea is quickly strained to 6% and the load is recorded. Owing to the viscoelastic nature of the cornea, stress-relaxation is observed. Though obvious, this shows no significant changes in SALS intensity distributions (\( p > 0.05 \) throughout the duration of the experiment). Though obvious, this shows

Figure 5. Typical SALS patterns from cornea strips subject to enzymatic cleavage from both unstrained control tests (group 2) and strained tests (group 1). Both cornea samples were allowed 20 min to relax before BC was added to initiate cleavage. (a) 0% strain and 0 min exposure to BC. (b) 0% strain and 40 min exposure to BC. (c) 6% strain and 0 min exposure to BC (strain direction indicated by white arrows). (d) 6% strain and 40 min exposure to BC.

The SALS distributions in the form of the relative change in eccentricity, \( \Delta e \), for the ensemble of loading configurations are shown in figure 6. \( \Delta e \) is equal to the difference in the initial value of \( e \) at \( t = 20 \) min (the time when BC is added) and the value of \( e \) at the specific time of the measurement. We can see that the eccentricity of the loaded samples unexposed to BC from group 3 show no significant changes in SALS intensity distributions (\( p > 0.05 \) throughout the duration of the experiment). Though obvious, this shows...
that the SALS intensity distribution is constant when: (i) enough time has elapsed and the cornea is sufficiently ‘relaxed’ and (ii) the collagen fibre/fibrils do not undergo any changes in the structural arrangement after approximate equilibrium is reached.

Similarly, we see that the SALS distribution from the unloaded samples exposed to BC from group 2 shows no significant changes over time \( (p > 0.25) \) throughout the duration of the experiment. It should be noted that once BC is added to the cornea, the scattering angle decreases \( (\text{figure 5a,b}) \) indicating fibre/fibril structural changes (enzymatic cleavage).

For the loaded samples exposed to BC in group 1, there is a significant change in the SALS eccentricity \( (p < 0.02) \) from 35 min onward \( (15 \text{ min after BC addition}) \). Over the course of 40 min of exposure to BC at a constant strain, the eccentricity increases linearly as the overall scattering angle decreases, although not uniformly \( (\text{figure 5c,d}). \)

Typical high-magnification TEM micrographs of corneal tissue cross sections (perpendicular to the loading direction) from both groups 1 and 2 are shown in figure 7. Figure 7a,b shows lower magnification micrographs of the loaded and unloaded samples following exposure to BC. The loaded sample exhibits a considerably altered architecture from that of a typical cornea, which comprises alternating arrays of aligned fibrils. Close examination of figure 7a shows that the fibril populations that survive the BC exposure were predominantly aligned with tensile load. Areas that appear to be remnants of fibril arrays and were transverse to the applied load (black arrows) can be seen in the figure. In figure 7b, there are significantly fewer fibrils and more open spaces than the loaded sample for the same time of exposure to the enzyme. Moreover, populations of fibrils that were both aligned with and transversely to the tensile load exhibit degradation (white arrows). Figure 7c,d show higher magnification micrographs of loaded and unloaded samples, respectively. In the loaded case, there are fairly distinct boundaries between subsets of fibrils aligned with and transversely to the load (black arrows), with the transverse (unloaded) fibrils preferentially degraded. In the unloaded case in figure 7d, there is general fibril degradation with no discernable orientation preference.

4. DISCUSSION

The uniform decrease in scattering angle exhibited by unloaded samples \( (\text{figure 5a,b}) \) indicates that the degradation rate \( (\text{loss of fibril scatterers}) \) is consistent across fibrils/fibres for all orientations in the absence of a directional load. The SALS data are supported by high-magnification TEM micrographs \( (\text{figure 7b,d}) \), in which fibril populations orthogonal to each other and under similar load \( (\text{approx. 0 N}) \) both undergo substantial degradation. Our null hypothesis specified that applying a unidirectional load to the tissue should not alter this uniformly decreasing pattern in the SALS signal, indicating no effect of load on the rate of degradation of the tissue. The first problem encountered in testing the hypothesis is the fact that mere application of the uniaxial load, in the absence of the enzyme, changed the SALS pattern to suggest a more vertical fibril alignment \( (\text{i.e. with the load; figure 5c}) \). Because of this unexpected complication \( [35,36] \), it was necessary to compare the eccentricity of the initial SALS pattern with all subsequent patterns at different time points within the same experiment. Figure 5d shows that following degradation under mechanical strain, there appears to be increasing preferential alignment emerging from the initial eccentric pattern in the same sample. Figure 6 confirms this observation statistically and also provides quantification of the rate of differential degradation of the two sets of fibrils \( (\text{loaded and unloaded controls}) \). The overall increase in eccentricity exhibited by experimental group 1 is approximately linear \( (r^2 = 0.951) \), with a slope of \( 8 \times 10^{-3} \text{ min}^{-1} \), which is directly related to the relative rate of preferential degradation of fibrils perpendicular/unaligned with the load. To characterize this, we assume that after 40 min of exposure to BC, the amount of fibrils perpendicular to the load has significantly decreased. This is partially justified by figure 5b, in which after approximately 40 min of exposure to BC, the scattering angle of the unloaded fibrils decreases almost to that of the incident laser beam, indicating that a very low number of fibrils is capable of scattering the beam.
fibrils is being disrupted at a faster rate than the loaded
set of fibrils within the same sample. The preferential
disruption supports the idea that strain can be used to
sculpt tissue in the presence of the highly active
enzyme, BC. The two other control groups (0% strain,
enzyme and 6% strain, no enzyme) do not produce a sig-
nificant difference in the eccentricity, further supporting
the conclusion.

High-magnification TEM micrographs generally
corroborate the SALS data and indicate that there is pre-
ferential retention of loaded fibrils. In figure 7, it is clear
that there are more fibrils, which are less degraded, remain-
ing in the loaded sample compared with the unloaded
sample. Furthermore, within the loaded sample, fibrils
aligned with the load appear to be retained preferentially
over those which were transverse to the load.

As outlined in detail in §1, there have been numerous
investigations which demonstrate that small applied
loads have a marked effect on the rate of enzymatic
degradation of collagen at the tissue, reconstituted net-
work and single molecule scales. However, the precise
mechanism behind the strain-dependent activity of
the enzyme on collagen has yet to be elucidated. In
whole tissues such as the cornea and tendon, it is pos-
sible that applied mechanical tension could exclude the
enzyme preferentially from loaded fibres or lamellae
through a pseudo-Poisson effect. This could lead to
what appears to be preferred cleavage of unloaded
tissue. However, volume exclusion of enzyme as the
mechanism of preferential degradation in loaded
cornea was discounted in the study of Zareian et al.
[13] through arguments based on porosity changes.
Nabeshima et al. [10], through a separate series of diffu-
sion experiments on loaded tendon, showed that
enzyme retention in the tissue was actually enhanced
(not reduced) by the application of mechanical strain.
At the fibril and molecular level, applied strain could
(i) expose or conceal enzyme-binding sites; (ii) shift
the α-chains to prevent enzymatic cleavage; or
(iii) enhance the stability of the helix itself (possibly
by bringing the alpha chains into closer proximity and
increasing the hydrogen bonding). Currently, the mech-
anism of strain-stabilization remains an open question.

In spite of the positive and statistically significant
results that were obtained, there are several limitations
of the approach used in this investigation. First,
the measurement method cannot actually detect collagen
fibrils in the cornea (approx. 35 nm in diameter), but
rather it is most likely detecting lamellae packed with
hundreds to thousands of fibrils in uniform alignment.
Therefore, the perceived loss of signal is possibly related
to the disruption of the lamellar structure in a

Figure 7. Low- and high-magnification TEM micrographs of cross sections from corneal samples exposed to BC. Each cross sec-
tion is perpendicular to the direction of the applied tensile load. (a) Low magnification taken from a sample subject to 6% tensile
strain and 40 min exposure to BC from group 1. Black arrows indicate areas where there appears to be remnants of fibrils trans-
verse to the direction of load. (b) Low magnification taken from sample subject to 40 min exposure to BC from group 2. White
arrows indicate remnants of fibrils both aligned and transverse to the direction of load. (c) High magnification of sample from
group 1. Black arrows indicate what appear to be boundaries between fibrils aligned to the direction of load and remnants of
fibrils transverse to the direction of load. (d) High magnification of sample from group 2. White arrows highlight areas of
fibril degradation both aligned and transverse to the direction of load. Scale bars (a,b), 2 μm and (c,d), 1 μm.

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5. CONCLUSION

The results strongly support previous work which suggests that mechanical strain stabilizes collagen against enzymatic attack by BC [6,7,11,13,38]. The detection of preferential enzymatic disruption of unloaded fibrillar/lamellar structures demonstrates that SALS may be a simple low-cost method capable of dynamically tracking and quantifying distinct enzyme-induced changes in tissue-engineered constructs and in native tissue, which has previously eluded researchers [13]. To produce load-bearing, tissue replacements which require arrays of collagen fibrils that are highly organized at the nanoscale, tissue engineers could potentially modulate strain and collagenase concentration in combination with SALS feedback to controllably sculpt collagen architecture. Such an advance could enable large-scale production of engineered tissue replacements with optimized collagen organization.

This project was partially funded by NIH grant NEI-ROI-EY015500. Part of this work by M. Robitaille and K. T. Wan is supported by the National Science Foundation under grant no. 0757140. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. The authors are grateful to William Fowle of Northeastern University for his expertise and help with the transmission electron microscopy.

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