Effects of Enzymatic Treatments on the Depth-Dependent Viscoelastic Shear Properties of Articular Cartilage

Darvin J. Griffin,[1] Josh Vicari,[1] Mark R. Buckley,[2,3] Jesse L. Silverberg,[3] Itai Cohen,[3] Lawrence J. Bonassar[1,4]

[1]Department of Biomedical Engineering, Cornell University, Ithaca, New York, [2]University of Rochester, Biomedical Engineering, Rochester, New York, [3]Department of Physics, Cornell University, Ithaca, New York, [4]Sibley School of Mechanical & Aerospace Engineering, Cornell University, Ithaca, New York

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ABSTRACT: Osteoarthritis (OA) is a disease that involves the erosion and structural weakening of articular cartilage. OA is characterized by the degradation of collagen and proteoglycans in the extracellular matrix (ECM), particularly at the articular surface by proteinases including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs).[1] Degradation of collagen and proteoglycans is known to alter shear mechanical properties of cartilage, but study of this phenomenon has been focused on bulk tissue properties. The purpose of this study was to assess microscale cartilage damage induced by trypsin or collagenase using a technique to measure the local shear viscoelastic properties. Safranin-O histology revealed a decrease in proteoglycans near the articular surface after collagenase and trypsin digestions, with proteoglycan depletion increasing in time. Similarly, confocal reflectance micrographs showed increasing collagen degradation in collagenase treated samples, although the collagen network remained intact after trypsin treatment. Both treatments induced changes in shear modulus that were confined to a narrow range (~400μm) near tissue surface. In addition, collagenase altered the total energy dissipation distribution by up to a factor of 100, with longer digestion times corresponding to higher energy dissipation. The ability to detect local mechanical signatures in tissue composition and mechanics is an important tool for understanding the spatially non-uniform changes that occur in articular cartilage diseases such as OA. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:1652–1657, 2014.

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Currently affecting more than 20 million Americans, osteoarthritis (OA) is the second leading cause of physical disability among adults in the United States.[1] OA involves the deterioration of cartilage in diarthroidal joints that can involve both enzymatic activity and mechanical factors. Diarthroidal joints are inevitably subjected to repeated stresses that leave disease cartilage susceptible to mechanical failure; thus, characterizing the changes in diseased cartilaginous material properties that occur in OA is integral to understanding the progression of this prominent disease. The compromised functionality of cartilaginous tissues can arise from very subtle changes in the organization of their structure. Furthermore, it has been reported that in OA structural damage to the collagen and proteoglycan network begins near the cartilage surface before progressing deeper into the tissue.[1,2] Degradation of the ECM by proteinases is known to alter the mechanical behavior of bulk cartilage samples,[2] but little is known about the specific changes in material properties near the tissue surface. The lack of knowledge of these changes is in part due to the fact that a reliable method for detecting microscale changes in early OA has not yet been established. As such, observing the effects of damage on the local mechanical properties of cartilage can provide information that may be obscured by measurements at the bulk level.

Previous studies have used degradative enzymes, such as matrix metalloproteinase-1 (MMP-1),[3,4,5] MMP-3[6,7] or inflammatory cytokines, including both α and β forms of interleukin-1 (IL-1)[5,6] and TNF-α[6,7] to selectively examine the effect of proteoglycan and collagen degradation on the mechanical properties of cartilage explants. While these studies demonstrated decreases in tensile,[8,9] compressive,[10,11] and shear stiffness,[12] the analyses were limited to bulk mechanical behavior. In contrast little is known about local mechanical changes (on the scale of $<100\,\mu m$) that occur in cartilage that has been degraded. Recently, our lab has developed a novel system for measuring the local dynamic shear properties of articular cartilage on the length scale of $20\,\mu m$.[13] This technique revealed that a narrow region of the tissue 100–$200\,\mu m$ below the articular surface has remarkably different properties than the rest of the tissue.[13,14] This surface region is also damaged or degraded early in the process of arthritis.[15] However the consequences of damage to this region on shear mechanical behavior are not well understood. The purpose of this study was to assess the effect of progressive proteoglycan and collagen degradation induced by trypsin and collagenase on the spatially localized shear properties of cartilage. Understanding the degradation of cartilage locally will provide a more comprehensive picture of the intricate relationship between tissue structure and properties as well as providing insight into the mechanical changes that occur very early upon surface damage.

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Correspondence to: Lawrence Bonassar (T: +607-255-9381; F: +607-255-7330; E-mail: lb244@cornell.edu)

MATERIALS AND METHODS

Tissue Sample Preparation

Fifty-six full thickness, 6 mm diameter explants were harvested sterilely from the patellofemoral groove of seven 1–3 day old calves (Gold Medal Packing, Oriskany, NY) with samples from each animal randomly assigned between control and experimental groups (Fig. 1A). The harvesting procedure produces cylinders with an undamaged articular surface.[16] After dissection, 56 samples were soaked in phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin for 30 min. Each cylinder was then cut along its long axis into two hemi-cylinders yielding a total of 112 hemi-cylinders (Fig. 1B). A small section (1–3 mm) of the deep region of each hemi-cylinder was removed with a razor blade to flatten the facet opposing the articular surface.

Enzymatic Digestion and Confocal Reflectance (CR) Microscopy

Cartilage samples designated for enzymatic digestion and CR imaging of collagen were coated with epoxy resin (Devcon, Danvers, MA) to ensure that enzyme exposure occurred only at the articular surface (Fig. 1C). CR microscopy was used to obtain real-time images of cartilage samples without the need for histologic stains,[17] where the fluorescence signal intensity is proportional to collagen density. A total of eight samples were imaged on a confocal reflectance microscope (Zeiss 710 Confocal, Germany) at 25× magnification in real-time at a frame rate of four frames/min. Samples were digested for 15, 30, and 90 min using two different enzymes, 2 mg/ml of bacterial collagenase to remove both collagen and proteoglycans (Worthington, type II collagenase, Lakewood, NJ) or 50 μg/ml of trypsin (Cellgro, 0.25% trypsin EDTA, Manassas, VA), which selectively cleaves the core proteins in proteoglycans without affecting the collagen network.[4] During degradation samples were imaged serially at 0, 15, 30, and 90 min with four samples observed for collagenase or trypsin exposure. After degradation, solutions were removed by serial washing with protease inhibitors in PBS. Additionally, 40 samples were fixed, embedded, sectioned, and stained with Safranin-O to observe proteoglycan distribution after 0, 15, 30, and 90 min of exposure to collagenase ($n = 6$ at each time point) or trypsin ($n = 4$ at each time point). In parallel, 44 samples were used for confocal strain mapping studies, with $n = 7$ samples at each time point of collagenase treatment and $n = 6$ samples at each time point of trypsin treatment.

Confocal Strain Mapping

The local shear modulus of samples was measured using grid resolution automated tissue elastography (GRATE) as described previously.[16] Samples were placed in PBS with 200 μL/ml 5-dichlorotriazinyl aminofluorescein (5-DTAF) (Molecular Probes®, Grand Island, NY) for two hours (Fig. 2A).[18,19] 5-DTAF modifies amines in proteins and fully stains the extracellular matrix (ECM). The deep zone of the hemi-cylinders were glued to a tissue deformation imaging stage (TDIS) and compressed to 10% axial strain. The TDIS was mounted on an inverted Zeiss LSM 5 LIVE confocal microscope and imaged using a 488 nm laser (Fig. 2B). To directly image applied strain, a line perpendicular to the articular surface was photobleached with the confocal laser. Sinusoidal shear displacements were applied to the articular surface by the TDIS at a frequency of 0.1 Hz and amplitude of 16 μm, and the resultant forces were measured with a load cell (Fig. 2C).

Custom software written in Matlab (The Mathworks, Inc., Natick, MA) was used to track the motion of the photobleached line as a function of depth.[13] These local displacements were fit to a sinusoidal function and differentiated with respect to z to calculate the strain amplitude $\gamma_0(z)$ and phase angle relative to the resultant stress $\delta(z)$. The magnitude of the dynamic shear modulus $|G^*(z)|$ was calculated as the ratio of stress amplitude τ_0 to strain amplitude γ_0, where τ_0 is the total applied stress required to deform the tissue from zero strain to γ_0. The rate of energy dissipation

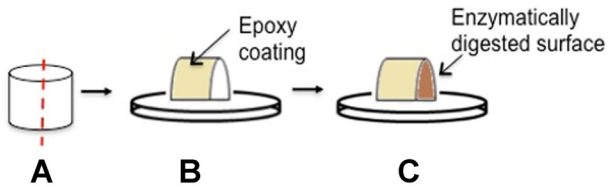

Figure 1. (A) 6 mm cylinders are bisected into hemi-cylinders (B) coated with epoxy resin (C) 50 μg/ml trypsin or 2 mg/ml collagenase are added to the tissue surface for 15, 30, and 90 min.

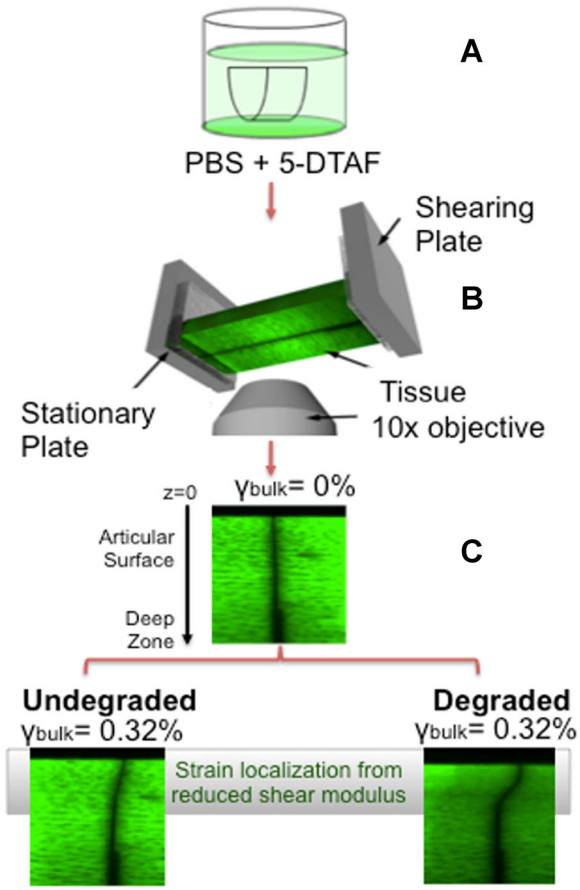

Figure 2. (A) Samples were placed in a 200 μL of 5-DTAF solution to fluorescently stain the ECM, (B) Schematic of the tissue deformation imaging stage; (C) A line was photobleached perpendicular to articular surface and subjected to 0.32 shear strain before and after digestion. The photobleached line for the degraded specimen exhibited a steeper slope from strain localization, implying inferior surface properties.

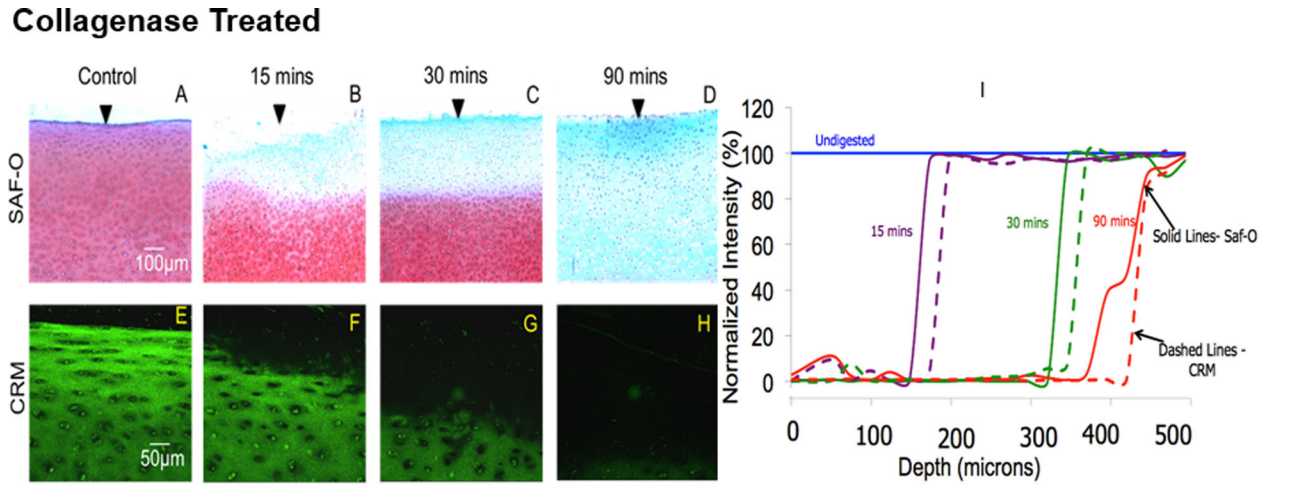

Figure 3. (A–D) Saf-O histology and (E–H) confocal reflectance micrographs of articular cartilage before digestion and after 15, 30, and 90 min of digestion with collagenase, (I) normalized intensity curves as a function of depth for SAF-O and CRM values ($n = 6$ at each time point).

per unit volume (Δv) at a depth z was calculated by the following equation:

$$\frac{\Delta E}{\Delta v} = \pi T_0^2 |G^*(z)|^{-1} \sin\delta$$

Statistical Analysis

Two-way ANOVA was used to evaluate the statistical significance of enzymatic treatments on the depth-dependent viscoelastic shear properties of articular cartilage.

RESULTS

Safranin-O staining revealed a progressive removal of proteoglycans near the articular surface after collagenase digestion with the depth of proteoglycan depletion increasing with time (Fig. 3A–D). Similarly confocal reflectance microscopy revealed degradation of the collagen network with a sharp border between degraded and undegraded regions (Fig. 3E–H). The depth of matrix removed increased from 150 μm at 15 min, 300 μm at 30 min, and 450 μm at 90 min. The depth of proteoglycan and collagen removal was similar at all times (Fig. 3I).

In contrast to collagenase, trypsin removed only proteoglycans (Fig. 4A–D) but not collagen (Fig. 4E–H). The depth of proteoglycan removal progressed from 100 μm at 15 min, 150 μm at 30 min, and 200 μm at 90 min (Fig. 4I). Confocal reflectance showed little to no change after trypsin treatment.

As previously reported shear modulus $|G^*|$ for healthy undigested samples exhibited a distinct spatial pattern[12,13,14,18,19] (Fig. 5A). The shear modulus was low in a narrow band (~400 μm) near the articular surface, with a well-defined minimum at ~100 μm. Beyond 500 μm in depth the shear modulus showed less variation. Collagenase treatment dramatically lowered the shear modulus at the surface. By 30 min $|G^*|$ at 50 μm was reduced by 20% relative to control, and by 90 min had decreased by more than 50-fold. The mechanical effect of degradation was confined to 350 μm at 15 min, 420 μm at 30 min, and 500 μm at 90 min. Notably the characteristic minimum $|G^*|$ was not present after 90 min of collagenase exposure (Fig. 5A). Collagenase treatment made the tissue more compliant and more viscous at the articular surface leading to an increase in both the local phase angle and local energy dissipation (Fig. 5B,C).

Similarly to collagenase, trypsin treatment samples displayed similar reduction in $|G^*|$ within the first 400 μm near the articular surface (Fig. 5D). The spatial pattern of change in $|G^*|$ was different between collagenase and trypsin. Trypsin-treated samples had the lowest $|G^*|$ at $z \approx 100$ μm from surface even after 90 min of exposure, similar to undegraded tissue (Fig. 5D). This is likely related to the fact that trypsin leaves the collagen network largely intact, as indicated by CRM data. The local phase angle after proteoglycan removal alone increased δ up to 2-fold locally in higher and longer exposure times. A similar mechanical trend was seen with ΔE, by 15 min displaying higher energy dissipation with longer trypsin exposure times.

DISCUSSION

This study evaluated the effect of progressive proteoglycan and collagen degradation on the depth-dependent viscoelastic shear properties of articular cartilage. In these experiments, enzymatic digestion by trypsin and collagenase at the surface resulted in local structural changes along with a significant reduction in the magnitude of the local shear modulus and a corresponding increase in the energy dissipation and localization. Safranin-O staining revealed a decrease in proteoglycan concentration near the articular surface after either trypsin or collagenase treatment, with proteoglycan depletion increasing with time. Enzymatic

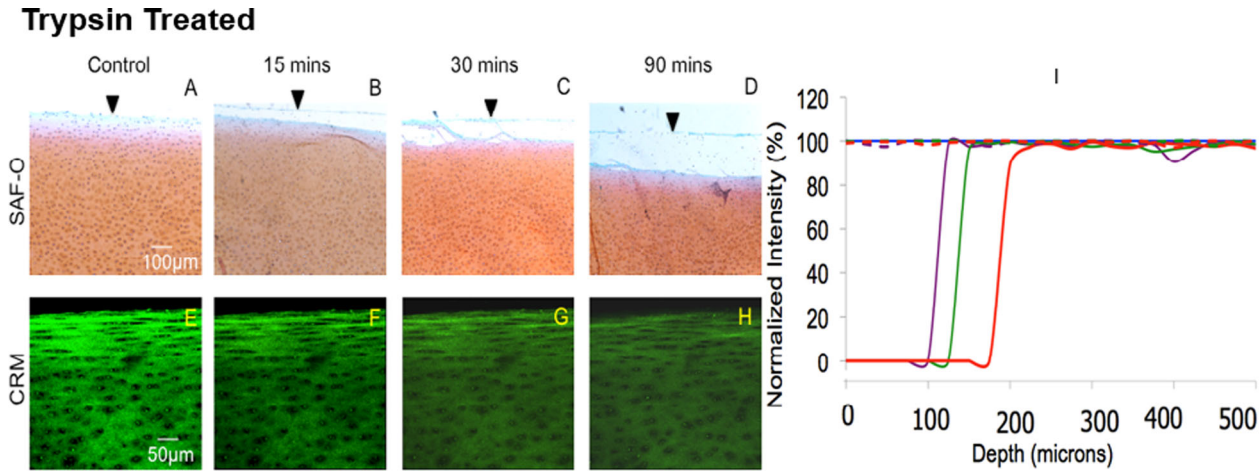

Figure 4. (A–D) Saf-O histology and (E–H) confocal reflectance micrographs of articular cartilage before digestion and after 15, 30, and 90 min of digestion with trypsin, (I) normalized intensity curves as a function of depth for SAF-O and CRM values ($n = 4$ at each time point).

treatments decreased the local shear modulus up to 50-fold, but increased tissue viscoelastic phase lag by up to 2-fold locally, and energy dissipation by up to 100-fold locally (Fig. 5). Because our measurement resolution was 20 μm, we were able to detect such mechanical changes in the tissue near the articular surface where enzymatic degradation occurred.

Histologic examination (Figs. 3 and 4) showed that the initial loss of proteoglycans and collagen were primarily near the articular surface and progressed deeper into the tissue with time. This progressive front of matrix removal by trypsin[20] and collagenase[21] has been previously documented. Confocal reflectance microscopy produced clear images of the collagen network near the articular surface and similarly, showed a progressive removal after collagenase exposure. In contrast, attenuation of confocal reflectance was associated with trypsin treatment; however, the collagen network appeared intact (Fig. 4E–H). As noted previously[22] collagen removal appeared to also cause proteoglycan removal, as evidence by the fact that collagenase treatment produced fronts that were co-located to within ≈25 μm.

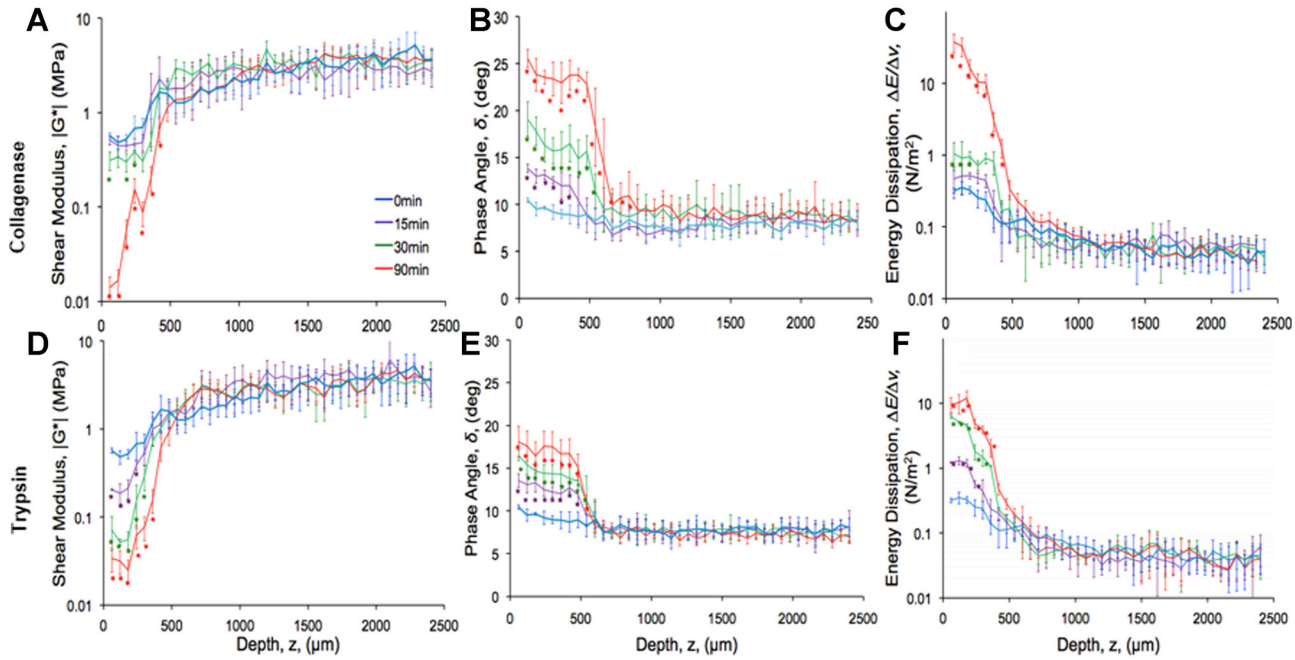

Figure 5. (A, B) Shear modulus G) vs. depth z from the surface (C, D) Phase angle (δ) vs. depth z (E, F) Energy dissipated (ΔE) vs. depth z (control $n = 5$, collagenase $n = 7$, and trypsin $n = 6$).

To assess the mechanical consequences of this degradation, we used confocal strain mapping to characterize the viscoelastic shear properties of the tissue. To extend our previous work,[13,14,18,19] this study investigated how removal of collagen and proteoglycans affects the mechanical properties in this surface region. Collagenase and trypsin treatments altered the shear modulus $|G^*|$ differently. Collagenase lowered $|G^*|$ dramatically at the surface (Fig. 5A). After 30 min of digestion, $|G^*|$ at 20 μm dropped by 30% at the surface, but by 90 min, $|G^*|$ surface had dropped so much that there was no visible minimum. In contrast, trypsin treatment lowered $|G^*|$ near surface, but the $|G^*|$ minimum was always at 100 μm, even after this region had been depleted fully of proteoglycans (Fig. 4D). These data suggest that both collagen and proteoglycans contribute to shear properties, but both play a distinct role on bearing shear loads at the articular surface. Particularly, proteoglycan removal lowers the overall magnitude of $|G^*|$ and increases the corresponding δ, but preserves the underlying microstructure that produces the characteristic minimum near the surface. Conversely, collagenase digestion, which is a harsher enzymatic treatment of the tissue, affects $|G^*|$ and δ the same way as trypsin but also destroys the microstructure that produces the characteristic minimum of $|G^*|$ near the surface.

We note this strongly non-linear drop-off in $|G^*|$ arising from collagenase digestion (Fig. 4A) is consistent with predictions of network-based models of cross-linked polymers.[24–27] In these models, a fibrous network's shear modulus is extremely sensitive to connectivity near the rigidity percolation phase transition. Above the percolation threshold, the cross-linked network is sufficiently connected that stresses can be transmitted through the system. Below the percolation threshold, the network is too fragmented, hence the system is unable to bears stresses and the shear modulus drops dramatically. The data can therefore be interpreted as an enzymatically driven transition that decreases connectivity in the collagen fiber network, and moves the system as a whole toward the percolation phase transition. The data also suggest the proteoglycan network effectively behaves as a simple viscoelastic medium that makes a contribution to $|G^*|$ in parallel to the collagen network. Indeed, the observations presented here are consistent with other data that has explored these models in greater detail.[28]

The local viscoelastic capacity of cartilage under shear has received little attention despite the fact that shear forces in articular cartilage correlate with tissue damage and disease.[23] In cartilage, the osmotic pressure exerted by the proteoglycans keeps the collagen network prestressed with tensile forces.[24] Furthermore, previous studies have shown that cartilage stiffens and becomes less viscous locally when deformed in shear.[18] Both of these studies therefore suggest that stressed or extended collagen networks are more elastic and less viscous, implying that removing such stress would make the network more compliant and more viscous. In the current study, specific removal of proteoglycans by trypsin treatment removed prestress chemically, making the tissue more compliant and more viscous at the articular surface leading to a very large local energy dissipation (Fig. 5F). Previous work reports that articular cartilage dissipates shear energy primarily near its surface.[13] The results of this study suggest that cartilage degeneration compromises this energy-absorbing surface region, therefore increasing tissue susceptibility to shear-induced damage into the remainder of the tissue.

Proteoglycan removal increased viscous behavior, which runs counter to the idea that proteoglycan–proteoglycan friction or collagen–proteoglycan friction is responsible for this viscous behavior. The combination of proteoglycan and collagen removal increased viscous behavior more than proteoglycan removal alone implying the removal of tension on the collagen network contributes to increased viscous behavior. Collagenase increased the rate of energy dissipation by cartilage up to 100-fold locally progressing higher at longer exposure times (Fig. 5C).

The mechanical effect of enzymatic digestion to the cartilage matrix was seen at depths beyond areas where histological staining was lost (~400 μm). Local proteoglycan and collagen removal by collagenase decreased $|G^*|$ up to ~500μm with increasing mechanical effects in δ and ΔE. Histology indicated initial loss of proteoglycans only up to ~200μm for trypsin-treated samples (Fig. 4D); however, mechanical reductions were seen up to ~500 μm in $|G^*|$, with increasing values in δ, and ΔE that extended beyond the boundary of degraded tissue. This suggest that local mechanical measurements are a sensitive measure of changes in cartilage shear properties because they can resolve changes in depth-wise variations as well as differences in the overall magnitude. This provides an attractive complement to histological imaging, which offers more explicit static structural information, and can be used in a synergistic fashion to develop a more robust understanding of healthy and disease tissue integrity.

Overall, our results provide novel information on cartilage mechanics and insights on the mechanisms for changes in matrix composition and functional properties associated with matrix degradation. As in early stages of OA, these treatments cause local superficial matrix damage, with concomitant reductions of surface mechanical properties on the microscale. Detection of such changes could reveal signatures of early onset OA that are not currently available with traditional imaging techniques, presenting new opportunities and new perspectives on cartilage therapies and repair.

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