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High-Bandwidth AFM-Based Rheology Reveals that Cartilage is Most Sensitive to High Loading Rates at Early Stages of Impairment

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ABSTRACT Utilizing a newly developed atomic-force-microscopy-based wide-frequency rheology system, we measured the dynamic nanomechanical behavior of normal and glycosaminoglycan (GAG)-depleted cartilage, the latter representing matrix degradation that occurs at the earliest stages of osteoarthritis. We observed unique variations in the frequency-dependent stiffness and hydraulic permeability of cartilage in the 1 Hz-to-10 kHz range, a frequency range that is relevant to joint motions from normal ambulation to high-frequency impact loading. Measurement in this frequency range is well beyond the capabilities of typical commercial atomic force microscopes. We showed that the dynamic modulus of cartilage undergoes a dramatic alteration after GAG loss, even with the collagen network still intact: whereas the magnitude of the dynamic modulus decreased two- to threefold at higher frequencies, the peak frequency of the phase angle of the modulus (representing fluid-solid frictional dissipation) increased 15-fold from 55 Hz in normal cartilage to 800 Hz after GAG depletion. These results, based on a fibril-reinforced poroelastic finite-element model, demonstrated that GAG loss caused a dramatic increase in cartilage hydraulic permeability (up to 25-fold), suggesting that early osteoarthritic cartilage is more vulnerable to higher loading rates than to the conventionally studied “loading magnitude”. Thus, over the wide frequency range of joint motion during daily activities, hydraulic permeability appears the most sensitive marker of early tissue degradation.

INTRODUCTION

Cartilage, like many soft connective tissues, functions mechanically across a wide spectrum of daily loading frequencies (timescales) (Fig. 1a), from <1 Hz in slow activities such as walking, to 1000 Hz for high-rate activities such as jumping and impact sports (1–6). Poroelasticity is known to be a major mechanism underlying the macroscale mechanical functions of cartilage, based on theoretical and experimental tests including transient stress relaxation and creep (7–10) and dynamic compression (11). Poroelasticity is manifested via fluid-solid frictional dissipation and intratissue fluid pressurization, which underlie important mechanical functions of cartilage, especially frequency-dependent self-stiffening, energy dissipation, and hydraulic permeation. Recently, nanoscale methodologies have been employed to study cartilage functions under quasi-static and low-frequency loadings (12–14). However, the full frequency spectrum of poroelastic behavior, which is critically important to the understanding of nanoscale dynamic loading, has not been well studied. How does the molecular structure of cartilage provide optimal tissue-level function over the wide spectrum of daily joint motions? And how does impact loading induce molecular-level degradation of the extracellular matrix (ECM), which occurs at the earliest stages of posttraumatic osteoarthritis (15)? It has been demonstrated previously at the macrotissue scale that cartilage proteoglycans constitute the main resistance to fluid flow under quasistatic and low-frequency loading conditions (12,16,17). Here, we hypothesize that the glycosaminoglycan (GAG) chains of the proteoglycan aggrecan play a dominant role in the high-frequency loading of cartilage as the primary resistance to fluid flow at the nanoscale. Since proteolytic degradation of GAG-containing aggrecan occurs at the earliest stage of osteoarthritis (OA), it could lead to a significant alteration in the ability of cartilage to resist impact compressive loads. The consequences of this GAG loss for the dynamic functioning of cartilage over a wide frequency range have not been illuminated by previous observations regarding quasistatic behavior of the tissue (12,14,18). We have therefore quantified the nanoscale hydraulic permeability of both normal and GAG-depleted cartilage using a novel wide-frequency-range (1 Hz to ~10 kHz) nanorheology system. We show that this nanoscale hydraulic permeability is a sensitive indicator of the loss of cartilage function under high-impact loading frequencies.

MATERIALS AND METHODS

High-frequency AFM-based rheology system

To measure the complex dynamic modulus of cartilage over a wide frequency range (1 Hz to 10 kHz), we developed a high-frequency rheology system coupled to a commercial atomic force microscope (AFM) (MFP-3D, Asylum Research, Santa Barbara, CA). To obtain a vertical displacement on the order of 10 μm in commercial AFMs, a large piezo (on the
order of centimeters) needs to be chosen. As a result, the resonance frequency/bandwidth of the piezo is low (19), resulting in an upper frequency limit of ~200 Hz (13) or as low as 10 Hz (18) in commercial AFMs (for examples, see Fig. S1 in the Supporting Material). Our approach is to couple a high-frequency actuating system to the commercial AFM. The main component of the system is a small piezo, here called the secondary piezo (Fig. 2a). Unlike the z-piezo of the commercial AFM (the primary piezo), the secondary piezo is chosen to be small (2 × 2 mm) (PL022, Physik Instrumente, Auburn, MA) to maximize the feasible frequency range by pushing the resonance frequency of the combined piezo system to high frequencies. We made the displacement of the secondary piezo load-independent by applying a permanent prestress to the piezo by clamping it between a plate and the substrate (Fig. 2a). The clamp system has its own mechanical resonances, which were diminished by optimizing the plate geometry and material. To have a low weight/stiffness ratio, we fashioned the plate from carbon fiber, with dimension of 0.5 × 5 × 30 mm. We used polystyrene colloidal probe tips with end radius R ≈ 12.5 μm (Polysciences, Warrington, PA) attached to tipless cantilevers with nominal spring constant k ≈ 30.0 N/m (Budget Sensors, Sofia, Bulgaria). The colloidal probes were attached to the cantilever by the lift-off process: a dot of glue (M-Bond 610, Structure Probes/SPI Supplies, West Chester, PA) was placed on a tipless cantilever by making quick contact between the cantilever and a thin layer of glue (1 μl) spread over a 10 × 10-mm mica surface. We then made immediate contact between the tip of the cantilever and a colloid probe resting on a glass slide and waited for 1 min with the cantilever pushing against the colloid. This process was followed by heat curing for 2 h at 150°C.

Loading profile

To fully represent the loading frequencies that human cartilage experiences (Fig. 1a), we chose the indentation loading profile shown in Fig. 2b, a ramp-and-hold preindentation of ~2 μm followed by an oscillatory displacement with a dynamic amplitude of 2 nm. Thus, the dynamic amplitude is less than the dimensions of a single aggrecan (20) or collagen molecule (21). The preindentation is applied using the primary z-piezo of the commercial AFM, and the secondary piezo is used to apply the low-amplitude oscillatory displacement over a wide frequency range that is well beyond the capabilities of commercial AFMs. The secondary piezo is activated by an oscillatory frequency sweep signal (DC voltage of 0 V and oscillatory voltage amplitude of 0.5 V), which is generated in LabView.
Data acquisition and analysis

The z-piezo voltage from the secondary piezo and the deflection voltage from the MFP-3D were measured by the analog-to-digital converter NI USB 6351 and LabView. After the z-piezo and deflection voltages were converted to force, $F_{\text{osc}}$ and displacement, $\delta$, in the time domain, the frequency domain magnitude and phase were calculated from the fast Fourier transform (FFT) of the $F_{\text{osc}}/\delta$ ratio using MATLAB function etfe. The result was smoothed by applying a Hamming window with a 100-sample size, in which each time series contained $\sim 2 \times 10^6$ samples. All data processing was performed using MATLAB (The MathWorks, Natick, MA). The magnitude of the dynamic complex indentation modulus at each frequency was then obtained as (24),

$$|E'(f)| = \frac{F_{\text{osc}}(f)}{\delta(f)} \frac{1}{2(R\delta_0)^{1/2}},$$

where $R$ is the probe radius. $F_{\text{osc}}/\delta$ was normalized as above, based on a Taylor series expansion of the Hertz model to account for the spherical probe-tip geometry (24). The phase angle, $\phi$, of the dynamic modulus represents the phase of the resulting force, $F_{\text{osc}}$, with respect to the applied displacement, $\delta$ (Fig. 2 c).

Sample preparation

Eight cartilage cylinders (3 mm in diameter) were harvested from the femoropatellar groove of each of four knee joints from 1- to 2-week-old bovine calves (i.e., four different animals). The cartilage cylinders were harvested within 6 h postmortem and maintained at physiological ionic strength in phosphate buffered saline (PBS) afterward. Two sequential 0.7-mm-thick disks were cut from each cylinder. The intact surface of the first disk included the superficial zone of the cartilage, whereas the second disk consisted of middle-zone cartilage (the superficial zone constitutes the first 100–200 $\mu$m in young bovine cartilage (25)). From each joint surface, one set of four superficial/middle-zone disks was kept in PBS with protease inhibitors as normal cartilage, and the mechanical tests were then performed within 6 h after harvesting the samples. The second set was enzymatically GAG-depleted by incubation of the disks for 48 h in PBS containing 0.1 U/ml chondroitinase ABC (26) (Seikagaku Kogyo, Tokyo, Japan). After GAG depletion by this method, the collagen network is still intact (27) (see Fig. S2 for histological images and biochemical measurements of normal and GAG-depleted samples).

Histology

Light microscopy of toluidine-blue-stained disks was used to visualize the spatial distribution of the sulfated GAGs (sGAG) remaining in the disks (28). Axial (vertical) cross sections of normal untreated middle-zone disks showed that sGAG content was spatially uniform throughout the section (Fig. S2 a). In normal superficial-zone disks, GAG content decreased gradually from the bottom surface toward the topmost surface, where it is known to have the lowest GAG content of full-thickness cartilage (29) (Fig. S2 c). In contrast, after the enzymatic depletion, a spatially uniform loss of GAG was observed throughout disks from both middle-zone and superficial-zone cartilage (Fig. S2, b and d). For this histological examination, disks were fixed in 10% buffered formalin (Sigma-Aldrich, St. Louis, MO) overnight at 4°C and then dehydrated via a series of graded alcohol incubations followed by xylene. Samples were paraffin-embedded, and 5 $\mu$m sections were rehydrated and stained with 0.1% Toluidine blue O (Sigma-Aldrich) in deionized water (pH 7.1).

Biochemical measurements

We used the dimethylmethylene blue assay (30) to obtain a quantitative measure of sGAG content of 0.7-mm-thick disks from the middle zone and the topmost 0.2 mm of the 0.7-mm disks from the superficial zone. Treatment with chondroitinase ABC reduced GAG content by a factor of 8 in the middle-zone disks and by a factor of 3 in the superficial-zone disks.
normality of the data. We used 

\( E \) increased to 

the modulus of the fibrillar network is represented by 

an asymptote was 

but with overall lower values of 

modulus for the fibers is set to zero (31).

Fig. 3, b the force with respect to the applied displacement (e.g., Fig. 3, j and d, for the corresponding storage and loss moduli, \( E' \) and \( E'' \), respectively). \( [E'^*] \) for normal cartilage decreased to an asymptotic value at low frequencies of 

\( E_{L} \sim 0.15 \) MPa (Fig. 3 a). At high frequencies, \( [E'^*] \) increased by a factor of 15 to \( E_{H} \sim 2.5 \) MPa at \( f = 1 \) kHz.

We observed a similar trend for the GAG-depleted disks, but with overall lower values of \( [E'^*] \); the low-frequency asymptote was \( E_{L} \sim 0.1 \) MPa, whereas at \( f = 1 \) kHz, \( [E'^*] \) increased to \( E_{H} \sim 0.8 \) MPa, a factor of ~8 higher than \( E_{L} \).

The phase angle, \( \phi \), measured for normal cartilage peaked at \( f_{\text{peak}} = 55 \) Hz and tended toward 0° at lower and higher frequencies. A similar overall trend was observed for the GAG-depleted cartilage, but the peak frequency shifted dramatically upward to \( f_{\text{peak}} = 800 \) Hz. The important features of the magnitude and phase angle of \( [E'^*] \) for GAG-depleted cartilage were found at high frequencies (\( f \gg 100 \) Hz) beyond the frequency range typically accessible using commercial AFMs. The extended frequency range provided by the newly developed system made the observation of \( f_{\text{peak}} \) for GAG-depleted cartilage possible. The peak frequency of the phase angle, together with frequency dependence of \( [E'^*] \), are essential for extracting values of the intrinsic mechanical properties of cartilage by nanoindentation (see below) and for studying the link between these nanoscale mechanical properties and the molecular structure and composition of the ECM.

The protection of chondrocytes from impact loading is associated with the matrix poroelastic mechanisms of self-stiffening and energy dissipation. Self-stiffening, defined here as the increase in dynamic modulus \( [E'^*] \) with increasing loading frequency (Fig. 3, a, Normal), protects the soft chondrocytes and their pericellular matrix via an increase in the stiffness of the interterritorial matrix (ECM) in response to dynamic loads. Energy dissipation, which is proportional to the tangent of the phase angle, \( \phi \) (Fig. 3, b), involves dispersal of potentially destructive impact energy by poroelastic viscous dissipation. Consistent with our previous study (13), normal cartilage exhibits self-stiffening at the nanoscale, exemplified here by the increase in dynamic modulus from \( \sim 0.1 \) MPa to \( 3 \) MPa (Fig. 3 a) over the frequency range 1–1000 Hz, which encompasses most activities of daily life (Fig. 1 a). At high loading rates, such as those of running and jumping, appropriate self-stiffening increases cartilage’s ability to bear impact loads. This self-stiffening pattern is accompanied by an optimum frequency for the peak of the phase angle \( \phi \) (~50 Hz for normal cartilage, as measured here). It is important to note that we found that the self-stiffening and dissipative patterns of GAG-depleted cartilage did not occur until much higher frequencies (Fig. 3 b), by more than a decade compared to normal cartilage. As a result, GAG-depleted cartilage suffers a significant loss in its dynamic modulus at high frequencies (up to \( \sim 1000 \) Hz), a finding that to our knowledge has not been reported previously. In addition, the peak in phase angle is shifted from ~50 Hz to ~1000 Hz. Taken together, these results show that early GAG loss from an otherwise normal cartilage matrix results in a tissue that is unable to withstand impact loading, putting the collagen network at risk, as we describe further below.

Hydraulic permeability is a sensitive indicator of GAG loss

By fitting the fibril-reinforced model to the data obtained from normal and GAG-depleted cartilage (Fig. 3, a and b),
the corresponding poroelastic material properties were estimated (see Fig. 4 for averages over all four animals and Fig. S4 for data from the individual animals). For the middle-zone cartilage, where GAG content is normally high and relatively uniform across its depth (Fig. 1b), the equilibrium modulus was $E_{L,\text{normal}} = 0.13 \pm 0.042 \text{ MPa}$, and it decreased by a factor of 1.5 to $E_{L,\text{GAG-depleted}} = 0.09 \pm 0.024 \text{ MPa}$ for GAG-depleted tissue (Fig. 4c). However, the hydraulic permeability of these same middle-zone specimens increased by a factor of ~25 from $k_{\text{normal}} = 5.4 \times 10^{-15} \pm 2.0 \times 10^{-15} \text{ m}^4/\text{N s}$ to $k_{\text{GAG-depleted}} = 1.3 \times 10^{-13} \pm 0.7 \times 10^{-13} \text{ m}^4/\text{N s}$ (Fig. 4d).

Previous reports using macroscale (33–35) and nanoscale tests (12) have shown that the contribution of aggregan to the overall equilibrium compressive stiffness of cartilage is comparable to or greater than the contribution of the collagen network. Therefore, GAG depletion typically decreases the equilibrium modulus by a factor of ~2 or less, which is consistent with the results obtained here. In contrast, the hydraulic permeability of normal cartilage is governed predominantly by GAG chains (36). This is because the equivalent pore size for fluid flow is determined predominantly by the spacing between neighboring GAG chains (~3 nm (20,37)), which is much smaller than the average spacing between collagen fibrils (~100 nm (37)). Thus, depletion of GAG chains can greatly increase the hydraulic permeability, an increase found here to be ~25-fold for middle-zone cartilage.

To further confirm the hypothesis that GAG chains play a major role in the nanoscale poroelastic properties of cartilage, we quantified the effect of GAG depletion on...
the tissue and the AFM probe tip (13). Consistent with a mean of the modulus of specimens from individual animals are shown in Fig. S4. Wilcoxon signed rank test. The hydraulic permeability and equilibrium data are presented as the mean ± SE for 4 animals, based on a mean of N = 4 plugs from each animal. *p < 0.05, using the nonparametric Wilcoxon signed rank test. The hydraulic permeability and equilibrium modulus of specimens from individual animals are shown in Fig. S4.

superficial-zone cartilage. In the superficial zone, the overall GAG content is less than in the middle zone (29) and the GAG content is lowest at the uppermost tissue surface (e.g., see histological images in Fig. S2 for normal and GAG-depleted conditions). We observed that after GAG depletion, the equilibrium modulus decreased from $E_{c_{\text{normal}}} = 0.06 \pm 0.02$ MPa to $E_{c_{\text{GAG-depleted}}} = 0.04 \pm 0.02$ MPa, and the hydraulic permeability increased from $k_{\text{normal}} = 1.4 \times 10^{-14} \pm 0.6 \times 10^{-14}$ m²/N·s to $k_{\text{GAG-depleted}} = 2.6 \times 10^{-14} \pm 0.7 \times 10^{-14}$ m²/N·s (Fig. 4, a and b). Thus, the hydraulic permeability of superficial-zone cartilage increased by a factor of ~2 after GAG depletion.

We previously showed experimentally and theoretically that the characteristic peak frequency of the phase angle, $f_{\text{peak}}$, is related to the intrinsic material properties of a cartilage sample by the equation

$$f_{\text{peak}} \propto \frac{E_{c} k}{d^{2}}, \quad (2)$$

where $E_{c}$ is the equilibrium modulus, $k$ the hydraulic permeability, and $d$ the characteristic contact distance between the tissue and the AFM probe tip (13). Consistent with Eq. 2, the alteration in hydraulic permeability and equilibrium modulus caused by GAG depletion (Fig. 4) leads to the observed ~15-fold shift in $f_{\text{peak}}$ (Fig. 3 b).

Length-scale dependence of the peak frequency is an independent measure of GAG depletion

Using a second, independent approach, we further confirmed the hypothesis that Eq. 2 can be used to directly infer the product of hydraulic permeability and equilibrium modulus ($E_{c} k$) of a cartilage specimen. Here, we measured the variation in $f_{\text{peak}}$ as a function of the characteristic contact distance, $d$, by changing the static offset indentation depth, $\delta_{0}$, of $f_{\text{peak}}$ and $1/d^{2}$ is linear (Fig. 5) for both normal and GAG-depleted cartilage, but with a much larger slope for the GAG-depleted specimens. The consistency of this linear relationship confirms, first, that the frequency-dependent nanomechanics of GAG-depleted cartilage is governed
by linear poroelasticity, as previously shown only for normal cartilage (13). Second, the slope of each line represents the \((E_t k)\) product at the precise location of the AFM probe tip during each test. By comparing the experimentally measured slopes for typical normal and GAG-depleted disks (Fig. 5), we observed that the average \((E_t k)\) product for GAG-depleted cartilage was ~23 times greater than the \((E_t k)\) product for normal cartilage, on the same order as the ~15-fold increase obtained from the theoretically predicted values of \((E_t k)\) from our finite-element model (FEM), as well as the shift in \(f_{\text{peak}}\) observed in Fig. 3b. This measurement was repeated for most of the disks from other joints, and similar trends were observed.

Hence, the influence of aggrecan-GAG degradation on the nanoscale material properties of cartilage was estimated using Eq. 2 by two independent but self-consistent approaches: (1), the contact distance, \(d\), was held constant for both normal and GAG-depleted cartilage, and the shift in \(f_{\text{peak}}\) caused by GAG depletion was measured (corresponding to Fig. 3); and 2), the contact distance was experimentally varied for both normal and GAG-depleted cartilage, and the variation of \(f_{\text{peak}}\) with 1/\(d^2\) was measured, revealing alterations in the \((E_t k)\) product upon GAG depletion (Fig. 5). Interestingly, Eq. 2 has also been utilized to map the self-stiffening and energy-dissipation properties of cartilage at macroscopic length scales (~1 mm) (38,39). In previous macroscopic unconfined dynamic compression tests (38), the length scale chosen (~3 mm) was 200 times larger than the contact distance of the AFM tip used in this study, \(d \approx 14 \mu m\). As a result, the measured phase angle of macroscopic unconfined compression tests was shifted to lower frequencies by 5 decades. Equation 2 would therefore predict that \(f_{\text{peak}}\) would be shifted from ~50 Hz in this study down to ~0.5 mHz; however, this very low macroscopic peak frequency was not observable because of low-frequency measurement limitations (38) (see Fig. S5 for this comparison).

**Intratissue pressurization is reduced significantly by GAG depletion**

Using our fibril-reinforced poroelastic FEM (13), we studied the effects of GAG depletion on the nanoscale intratissue pressure distribution as a function of tissue depth and frequency. The values of \(E_t\) and \(k\) estimated for normal and GAG-depleted cartilage (Fig. 3 legend) were used in the model to predict the spatial and temporal variations in fluid pressure resulting from dynamic compressive displacement of the AFM probe tip (Fig. 2b). We found that the significant increase in hydraulic permeability, \(k\), after GAG depletion predicted a greatly reduced intratissue pressurization caused by nanoscale compression (Fig. 6). The fluid pore pressure in normal cartilage is higher by a factor of 5 than that of GAG-depleted cartilage simulated at the peak frequency of the normal cartilage, \(f_{\text{peak}}\approx 55\) Hz. In addition, in normal cartilage, the pressurization penetrates more deeply into the matrix and involves a larger volume of the matrix engaged in dissipation and self-stiffening at high loading frequencies. In contrast, in GAG-depleted cartilage, not only is the pore pressure reduced (Fig. 6), but because of the larger matrix pore size, the fluid velocity is higher at the location of loading (Figs. 1g and 6). GAG loss thereby reduces the self-stiffening and energy-dissipation properties of the ECM, and consequently, the chondrocytes may be more prone to damage caused by impact loads during the earliest stages of OA. In addition to these dissipative features, fluid pressure and flow velocity are also known to play significant roles in modulation of the cell-matrix biosynthesis (40). Thus, the ability of chondrocytes to respond to the proanabolic signals associated with moderate, low-frequency dynamic compression (41) could be significantly compromised by the consequences of high-frequency impact loading.

We believe that the information obtained from this study can aid in the fundamental understanding of the mechanisms involved in pathological processes and tissue regeneration of biological tissues at the molecular (42,43), cellular (44,45), and ECM levels (14,46–48). For example, in the ECM of tumors, the interstitial fluid pressurization compresses the blood vessels, which results in reduction of blood flow (46). Reduction of blood flow, accompanied by hypoxia, in turn promotes tumor progression. This study
thus may provide direct molecular-level insight into similar applications in the mechanopathology of tumors, where the fluid-solid interaction plays a critical role. Our wide-frequency approach in determining the nanoscale hydraulic permeability may also provide insight into the transport problems in hydrogels (49) and soft hydrated tissues with a wide range of poroelastic material properties.

CONCLUSION
In this study, we measured the high-frequency dynamic nanomechanics of normal and GAG-depleted cartilage, the latter representing a critical aspect of matrix degradation in the earliest stages of OA. The high-frequency measurements, which simulate loading rates during activities such as running and jumping, were made possible by developing a high-frequency nanorheology system coupled to commercial AFMs. Equipped with this system, we were able to investigate molecular-level fluid-solid interactions which manifest predominantly at higher frequencies. We quantified alterations in nanoscale poroelastic properties of cartilage caused by GAG depletion using a FEM-based fibril-reinforced poroelastic model. First, we showed that the nanoscale hydraulic permeability of cartilage is a more sensitive differentiator of early-OA-like matrix degradation compared to the nanoscale equilibrium modulus: GAG depletion increased the nanoscale hydraulic permeability of middle-zone cartilage by a factor of ~25, whereas the equilibrium modulus decreased by just 40%. Second, we demonstrated that GAG chains play a dominant role in the nanoscale resistance of cartilage matrix to fluid flow and intratissue pressurization, which, at the macroscale (50), is known to protect the tissue from high-loading-rate activities. At early stages of OA, during which GAG chains are being depleted but the collagen network may still be completely intact, the collagen network becomes more prone to damage caused by high-rate impact loading. Thus, we hypothesize that early-stage GAG-depleted cartilage is more susceptible to loading rate than the more conventionally studied load magnitude.

SUPPORTING MATERIAL
Five figures, methods, and references (51-54) are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(13)00277-4.

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