INTRODUCTION: The advent of new time-dependent nanomechanical methods has recently enabled the quantification of cartilage tissue poroelasticity and hydraulic permeability, \( k \), at the nanoscale [1-3] and holds great potential for early detection of pathological changes and diagnosis of osteoarthritis (OA). It is known that at the macroscale, tissue hydraulic permeability can undergo several order-of-magnitude changes due to OA [4] while the equilibrium stiffness may vary by only a factor of 2 [5]. This is because GAG chains are the main determinant of the pore size (consequently, hydraulic permeability) of cartilage while they contribute only partially to the compression stiffness of the tissue. Here, we extend the technique of atomic force microscopy-based dynamic oscillatory nanoindentation to a larger frequency range (1-10,000 Hz) and compare these data to finite element analysis simulations to study the effect of GAG content, relevant to early stage OA.

METHODS: Cartilage Sample Preparation: Middle zone cartilage disks (3 mm diameter \( \times \) 0.7 mm thick) were harvested from the medial femoropatellar grooves of 1-2-week-old bovine calves and maintained in 0.154 M sterile phosphate buffered saline (PBS) with protease inhibitors for less than 24 hours before testing. GAG-depleted cartilage disks were incubated in 0.1 U/ml chondroitinase ABC solution (Seikagaku, Japan) for 24 hours (5% CO\(_2\) incubator at 37°C), and then washed several times with PBS and kept in PBS solution with protease inhibitors prior to nanomechanical tests. Previous studies showed that such treatment caused \( \sim 71\% \) GAG loss, starting at the tissue surfaces [6].

AFM-Based dynamic oscillatory loading: Nano-indentation was performed using the MFP3D AFM (Asylum Research, Santa Barbara, CA) in conjunction with a newly developed activation device (Fig. 1a). Gold-coated polystyrene colloidal cantilever probe tips (end radius, \( R \sim 12.5 \mu m \), nominal spring constant \( k \sim 8.6 \text{ N/m} \), Novascan Technologies, Ames, IA) were employed. The cantilever deflection sensitivity \( \text{(nm/V)} \) was calibrated on a hard mica surface. The deflection profile was composed of pre-indentation and force relaxation, followed by sinusoidal displacements over a frequency range \( f = 1-5000 \text{ Hz} \) (Fig. 1b). The pre-indentation was applied by the MFP3D and the oscillatory displacement by the piezo of a newly developed device (Fig. 1a) which extended the high frequency cut-off from \( \sim 100 \text{ Hz} \) (typical of commercial AFMs [2]) to \( \sim 5000 \text{ Hz} \). This new device was comprised of a piezo with static pre-stress to enable load-independent displacements over frequency range of 4 decades (1 Hz - 10 kHz). The amplitude of the sinusoidal displacement, \( \delta = 15 \text{ nm} \), was chosen to be much less than the initial indentation \( \delta_0 \). Due to the broad frequency range, the load was applied at three different frequency intervals of 1-10 Hz, 10-810 Hz, and 810-5000 Hz with data sampling rates of 3 kHz, 300 kHz and 1 MHz, respectively. Data Analysis: A discrete Fourier transform (DFT) was used to obtain the fundamental frequency components of the z-piezo and deflection signals, from which the amplitude of the oscillatory force of the probe, \( F_{\text{osc}} \), and the oscillatory displacement of the probe, \( \delta \) (Fig. 1b) were calculated at each frequency, \( f \).

RESULTS: Fig. 2 shows averaged frequency-dependent dynamic mechanical properties for one joint cartilage with key features as follows: the low \( (E_L) \) and high frequency \( (E_H) \) limits of the dynamic modulus magnitude, and the characteristic peak frequency \( f_{\text{peak}} \) of the dynamic phase angle, were clearly observed for both normal and GAG-depleted cartilage (Fig. 2a,b). No significant difference in \( E_L \) or \( E_H \) between normal and GAG-depleted cartilage was observed (Fig. 3a,b). However, both the magnitude \( |E'| \) and phase \( \phi \) of the dynamic modulus were significantly shifted toward higher frequencies: \( f_{\text{peak}} \), increased from 27.2 \( \pm \) 13.2 Hz (intact) to 546 \( \pm \) 221 Hz (GAG-depleted tissue), a significant 20-fold increase in \( f_{\text{peak}} \) (t-test, using indentations at 10 different locations for each of two disks (Fig. 3c).

DISCUSSION: Previously, based on the length scale dependence of the measured peak frequency of the dynamic modulus phase \( f_{\text{peak}} \) in Fig. 2b) and finite element simulations, we showed that the observed frequency dependent dynamic behavior (Fig. 2a,b) is governed by linear poroelasticity [2]. Linear poroelasticity theory predicts the following length scale dependence between the peak frequency, \( f_{\text{peak}} \), contact distance, \( d \), between the probe tip and cartilage sample, hydraulic permeability \( k \), and the equilibrium modulus \( H \): 

\[
E_{\text{osc}} = \gamma (kfHd^2) \text{.}
\]

The constant \( \gamma \) (accounting for probe tip radius) and the contact distance \( d \) are the same for both intact and GAG-depleted samples. The values of \( E_L \) (accounted to \( H \)) and \( E_H \), were the same for both samples (Fig. 3a-b). Therefore, the \( \sim 20 \text{ fold increase in } f_{\text{peak}} \text{ is due to a } \sim 20 \text{ fold increase in } k \).

We conclude that changes in the hydraulic permeability dominate changes in the poroelastic properties of cartilage at the nanoscale, and these changes are excellent indicators of GAG depletion, which was selectively performed, here, using chondroitinase ABC.

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