Raman Spectroscopy for Predicting Intervertebral Disc Composition and Functional Mechanical Properties

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INTRODUCTION: The intervertebral disc is responsible for maintaining and distributing large multiaxial spinal loads. The structure and composition of the healthy IVD are optimized for this mechanical performance. The IVD is comprised of a collagen (COL)-rich, fibrous outer annulus fibrosus (AF) ring that surrounds a glycosaminoglycan (GAG)-rich, gel-like nucleus pulposus (NP). The osmotic response to the anionic sulfated GAG of the NP, in conjunction with the restraining tensile properties of the AF, allows the multiaxial spinal loads to be distributed through pressurization of the interstitial fluid in the NP extracellular matrix (ECM). In response to aging and injury, the IVD undergoes detrimental changes to its composition and structure. Notably characterized by a significant loss of GAG and consequently pressurization of the NP, which leads to structural breakdown in the AF as the loading is altered from predominantly tension to compression within the AF. As there is a known association between disc degeneration and low back pain, the leading cause of years lived with disability in the US and around the world [1], there are emerging pharmacological and biomaterial therapies aimed at rescuing the composition and material properties of the IVD to restore its mechanical function. However, the ability to uniformly assess the efficacy of treatments that preserve and/or regenerate IVD is burdened by a lack of standardized biomarkers that can be applied across the spectrum of *in vitro*, *in vivo* and clinical testing platforms. Conventional biochemical and histological assays for composition are destructive, obviating repeated measures and clinical assessment. Raman spectroscopy is an inelastic light scattering technique that provides a non-destructive, quantitative, optical fingerprint of the molecular building blocks of key molecular constituents in the ECM of articular cartilage: GAG, COL, and H_2O. We recently demonstrated the capability of our novel Raman spectroscopic probe to perform high-precision assessments of the composition of articular cartilage both *in vitro* and *in vivo* with extremely high correlation to the articular cartilage mechanical properties [2,3]. In the current study, we examine the capability of our Raman spectroscopy to assess the composition and its correlation to mechanical properties for IVD specimens in health and disease. Assessments are performed using an *in vitro* model system of isolated bovine IVDs subjected to enzymatic degenerative treatment to model compositional changes manifest in IVD degeneration.

METHODS: Twenty-seven IVDs were isolated from six skeletally mature bovine oxtails and frozen until testing. The cartilage end plate was excised, exposing the NP and AF. For an initial batch of 15 disks, Raman measures were initially performed on the center of the NP and at three positions within the AF to differentiate between the spectral properties of these tissue regions. These IVDs were subsequently subjected to an enzymatic degeneration treatment, consisting of incubation in PBS supplemented with 2mg/mL trypsin at 21°C. To prevent IVD swelling during digestion treatment [4], IVDs were confined between two meshed acrylic plates. A polymer filter was inserted between the IVD and meshed plate to inhibit tissue herniation into mesh holes (Fig.1). IVDs were subjected to trypsin exposure for 0, 10, or 21 hours (n=5 IVDs per time point). *Ex vivo Raman Analysis* of NP for each disk is performed with a customized arthroscopic probe consisting of a NIR diode laser (ex=785nm, 500mW, B&W Tek), fiber-coupled spectrograph (EAGLE Raman-S, Ibsen Photonics), and needle probe ending with a 2mm sapphire ball lens [2]. Raman spectra were acquired with the probe lens in gentle contact with the disk surface over a 10s acquisition time (Fig.2A). The spectral fingerprint range (800-1800cm^{-1}) was preprocessed and subjected to a multivariate least-squares linear regression model to decompose the Raman spectra to calculate the relative contribution of the predominant IVD constituents (GAG, COL, and H_2O) to the composite IVD Raman spectra: $\mathbf{IVD}_{spectra} = GAG_{score}*(\mathbf{GAG_{REF}}) + COL_{score}*(\mathbf{COL_{REF}}) + H_2O_{score}*(\mathbf{H_2O_{REF}})$ where $\mathbf{GAG_{REF}}$, $\mathbf{COL_{REF}}$, and $\mathbf{H_2O_{REF}}$ are the component spectra of purified reference chemicals for each ECM constituent used to extract "scores" reflecting the contribution of each constituent. The NP is axially cored via a 7mm diameter biopsy punch and subjected to confined compression testing for the aggregate modulus. Mechanical testing consisted of three successive ramps of 5% applied strain (0.5µm/s), followed by 2-hour stress relaxation periods. An additional batch of twelve IVDs was subjected to trypsin exposure for 0 (n=8), 10 (n=2), or 21 (n=2) hours, after which the NP was cored and subjected to biochemical analysis for water content (gravimetrically) and GAG content (DMMB assay).

RESULTS: The cumulative spectral contribution of the extracellular matrix constituents by multivariate regression accounted for 94% of the variation in the IVD composite spectra (Fig.2B&C). Raman-derived biomarkers reflected the biochemical composition of the IVD, as marked by higher Raman GAG and H_2O scores in the NP relative to the AF (Fig.2D&E). In response to trypsin exposure, NP regions IVDs exhibited a trend of decreased Raman GAG score with digestion time (Fig.3A). For these specimens, Raman GAG score predicted 60% of the variation in aggregate modulus (Fig 3A, $p<0.001$). For the second experiment of digested IVDs, the NP further exhibited a trend of increased Raman H_2O score with digestion time (Fig.3B&C). For these specimens, the Raman GAG score predicted 80% of the variation in the assay-measured NP GAG content (Fig.3B) and 89% of the variation in the NP H_2O content (Fig.3C).

DISCUSSION: Results establish the capability of our Raman probe to achieve non-destructive measures of the composition and mechanical properties of IVD tissue specimens. Raman assessments are predicated on a spectral analysis that yields ECM-specific biomarker scores, which reflect the contribution of ECM constituents to underlying acquired Raman spectra. The Raman GAG score accounted for 80% of the variation in the assay-measured GAG content and 60% of the variation in the aggregate modulus of the NP. The Raman H_2O score accounted for 89% of the variation in the measured water content. The ability of Raman analysis to faithfully characterize IVD tissue composition was further supported by differential analysis of NP and AF tissue regions, whereby Raman biomarkers depict the GAG and water-rich composition of the NP in contrast to the collagen-rich makeup of the AF. These measures support using benchtop Raman probes as a novel research tool for rapid, non-destructive, repeated-measure assessments of IVD composition and material properties. Here, Raman can serve as a standardized objective measure of IVD degeneration and treatment response across a hierarchy of preclinical model systems, spanning from *in vitro* explant tissue studies to *in vivo* animal investigations, thus helping to guide new therapies into the clinic.

SIGNIFICANCE: This work supports Raman probe analysis as a standardized objective measure of IVD degeneration and treatment response across a hierarchy of preclinical model systems, spanning from *in vitro* explant studies to *in vivo* animal investigations, to guide new therapies into the clinic.

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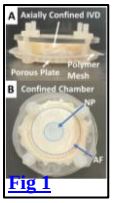

Fig 1

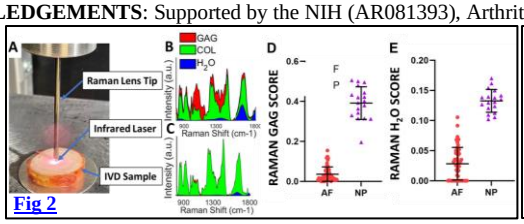

Fig 2

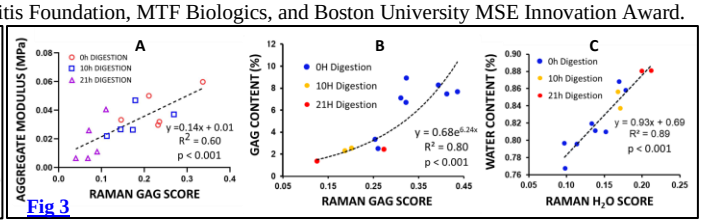

Fig 3

Fig 1: (A) Side view of IVD, which is confined between two acrylic plates to restrict swelling during enzyme treatment regimens. **(B)** Top view of IVD with a polymer filter between IVD and mesh plate to inhibit tissue herniation into mesh holes. **Fig 2: (A)** Raman probe in contact with IVD. Representative 2D stacked area plot of contributions of GAG, COL, and H_2O to IVD Raman spectra in **(B)** NP and **(C)** AF. **(D)** Raman GAG score and **(E)** H_2O score in NP and AF. **Fig 3: (A)** Correlation of Raman GAG score vs. aggregate modulus. Correlation of **(B)** Raman GAG score vs. GAG content, and **(C)** Raman H_2O score vs. water content.