Raman Spectra Predict the Composition, Structure, and Viscoelastic Properties of Articular Cartilage

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INTRODUCTION: Hyaline cartilage is a porous, viscoelastic, biphasic composite material comprised of an anisotropic type-II collagen (COL II) fibril network (5-20% wet weight) that affords structure and tensile strength, complemented by a negatively charged, sulfated glycosaminoglycan (GAG) matrix (5-15% wet weight) that retains interstitial water [1]. These components act synergistically, bestowing the rheological and tribological material properties essential to cartilage function. With joint loading, compression pressurizes interstitial fluid, which the COLII network resists, decreasing pore volumes while relatively increasing the local fixed negative charge density. This collective effect hinders interstitial fluid transport. Subject to this strain-dependent decrease in tissue permeability, the increased viscous drag of interstitial fluid flowing through the porous COL II network accounts for the tissue's non-linear, viscoelastic behavior [2]. >90% of the applied joint load is supported by pressurization of entrapped interstitial fluid, i.e. interstitial fluid load support (*IFLS*). Osteoarthritis (OA) is characterized by cartilage degeneration. GAG depletion reduces *IFLS*, transferring load to the COL II fibril network, which subsequently breaks down, culminating in increased hydraulic permeability, decreased cartilage stiffness and lubricity. A hierarchy of model systems is used to assess the efficacy of OA therapies: *in vitro* studies on explanted tissue, *in vivo* preclinical animal studies, and controlled clinical trials. However, the ability to uniformly assess the efficacy of OA treatments to preserve or regenerate the cartilage ECM is burdened by a lack of standardized biomarkers that reflect tissue function and can be applied across the spectrum of testing platforms. Raman spectroscopy is an inelastic optical light scattering technique that provides a quantitative, optical fingerprint of a tissue's molecular building blocks, allowing quantification of the predominant molecular constituents of cartilage: GAG, COL, and H_2O. We developed a novel Raman needle probe capable of performing both benchtop *ex vivo* and arthroscopic *in vivo* measurements of these ECM-specific compositional biomarkers for cartilage with a high degree of accuracy (R^2=0.8-0.94) that reflect the material properties intrinsic to its physiologic function [3]. Using an equine explant model, we demonstrate that Raman-probe-derived biomarkers can measure cartilage composition (GAG&COL content), anisotropy (collagen fiber parallelism index [PI]), that account for the functional viscoelastic material properties (equilibrium modulus [E_{eq}], dynamic modulus [G^*], phase shift angle [θ], and hydraulic permeability [k]).

METHODS: Osteochondral plugs (∅8.5×10 mm; n=30) were extracted from the femoral condyle (load-bearing) and trochlear notch (non-load-bearing) of 15 skeletally mature equine knees with no macroscopic cartilage degeneration. *Raman measures* were performed with a custom needle probe using a tip with a sapphire ball lens (∅2mm ball lens; ~270μm depth of penetration), a NIR diode laser (ex=785nm), and a fiber-coupled spectrograph (QEPro, Ocean Optics). The cartilage spectra (800-1800cm^{-1}) were fit to a multivariate linear regression model: $\mathbf{Cart_{Spectra}}=GAG_{Score}*(\mathbf{GAG_{REF}})+COL_{Score}*(\mathbf{COL_{REF}})+H_2O_{Score}*(\mathbf{H_2O_{REF}})$, where $\mathbf{GAG_{REF}}$, $\mathbf{COL_{REF}}$, and $\mathbf{H_2O_{REF}}$ are the reference spectra of purified chemicals for each ECM constituent, and the "scores" are the regression coefficients reflecting the relative contribution of each constituent (Fig.1A&C). The high-wavenumber range spectra (2700-3800cm^{-1}) were used to compute the area under the OH peak, reflecting tissue hydration (Fig.1B&D) [4]. *GAG content* was assessed via Digital Densitometry (DD) from Safranin O-stained sections using a CCD camera (SenSys, Photometrics Inc.). *Collagen content* was estimated from FTIR spectroscopy (Perkin Elmer Spotlight 300) via integrated amide I absorbance (1585–1720cm^{-1}). *PI* was determined via polarized light microscopy (Leitz Ortholux II POL). DD, FTIR, and PI profiles were averaged over the superficial zone 300μm chondral layer for consistency with the Raman interrogation region. E_{eq} was determined via the slope of the stress-strain indentation (d = 0.55 mm) response after 4 stress-relaxation steps (4% strain; 100%/sec ramp; 600-sec relaxation). G^* was determined from the ratio of stress to strain amplitudes during a sinusoidal 4% peak-to-peak strain amplitude @ 1Hz frequency. A Fourier transform was employed to analyze θ. k was determined analytically from a fibril-reinforced poroelastic finite element model fit to a cartilage stress relaxation test [5].

RESULTS: The multivariate spectral regression model accounted for 92±2% of the variation in the cartilage Raman spectra (Fig.1). Compositionally, Raman GAG_{score} accounted for 82% of the variation in densitometry-measured GAG content; the COL_{score} accounted for 55% of the variation in the FTIR-measured COL content (Fig.2&3). The GAG_{score} accounted for 76%, 38%, 45%, and 25% of the variation for viscoelastic properties E_{eq}, G^*, θ, k, respectively. Multivariate linear regression combining GAG_{score} and OH_{area}, accounted for 81% and 55% of the variation in E_{eq} and θ, respectively, while a multivariate linear regression combining COL_{score} and OH_{area} accounted for 62% of the variation in PI.

DISCUSSION: Compositional biomarker scores derived from multivariate spectral regression analysis of cartilage ECM Raman spectra measured non-destructively using a Raman needle probe accurately portrayed the relative contribution of GAG, COL, and H_2O to the biochemical composition and structure of hyaline cartilage: GAG [R^2=0.82], COL [R^2=0.55], anisotropy (PI) [R^2=0.62]. The influence of specific cartilage constituents on the tissue's viscoelastic properties was predicted by specific combinations of the biomarker scores: E_{eq} [R^2=0.81], G^* [R^2=0.38], θ [R^2=0.55], k [R^2=0.25]). Notably, the combined GAG_{score} and hydration-associated OH_{area} predicted E_{eq} and θ, representing the elastic and proportion of elastic to viscous behavior. Thus, the GAG_{score} and OH_{area} portray that the loss of anionic GAGs from the ECM is associated with a reduction in equilibrium and dynamic moduli due to reduced Donnan osmotic swelling pressure. Additionally, this decrease is correlated with an increase in hydraulic permeability resulting from expanded interstitial pore space. The correlation between Raman COL_{score} and PI was unanticipated given the lack of polarized spectral measures for this version of the Raman probe [6]. A potential explanation is that COL fiber orientation varies with cartilage zonal tissue composition, the superficial zone forms 10-20% of cartilage volume; water content is highest (~80%) and anisotropic collagen (type II and IX) fibers arranged parallel to the articular surface likely enhance the correlation with Raman compositional biomarkers.

SIGNIFICANCE: This work supports the use of Raman spectroscopy acquired through needle probes as a ubiquitous research tool. It serves to perform comprehensive diagnostics of the composition, structure, and functional properties of articular cartilage in health and disease. Moreover, it acts as a non-destructive tool to monitor the tissue response to chondroprotective and chondroregenerative therapies at hierarchical scales *in-vitro*, *ex-vivo*, *in-vivo* and clinically using Raman arthroscopy.

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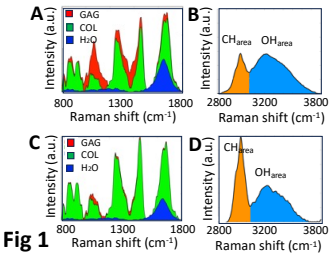

Fig 1

Tissue property	Raman Scores				
	Univariate correlations			Multivariate correlations	
	GAG	COL	OH	GAG+OH	COL+OH
E_{eq}	0.76*	0.64*	0.17*	0.81*	0.65*
E_{dyn}	0.38*	0.18*	0.23*	0.38*	0.18*
θ	0.45*	0.45*	0.23*	0.55*	0.51*
k	0.25*	0.16*	0.01	0.25*	0.16*
GAG	0.82*	0.78*	0.07	0.81*	0.78*
COL	0.54*	0.55*	0.14*	0.57*	0.61*
PI	0.50*	0.59*	0.07	0.58*	0.62*

Fig 2

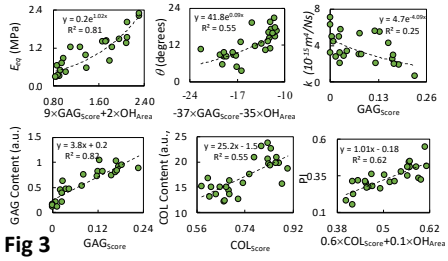

Fig 3

Fig 1: (A,C) Representative 2D stacked area plot of cumulative contribution of GAG, COL, H_2O to measured Raman cartilage spectra GAG replete **(A)**, and GAG depleted **(C)** specimen. **(B,D)** Representative CH_{area} and OH_{area} in highwavenumber region for **(B)** high H_2O and **(D)** low H_2O content specimens. **Fig 2:** Correlation coefficients for univariate and multivariate correlations between probe-dervied Raman scores and measured tissue properties. **Fig 3:** Univariate and multivariate correlations between probe-dervied Raman scores and measured tissue properties.