Raman Monitoring of Neocartilage Growth for Different Hydrogel Scaffolds

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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 (sGAG) matrix (5-15% wet weight) that retains interstitial water (interstitial fluid load support, *IFLS*). These components act synergistically, bestowing the rheological and tribological material properties essential to cartilage function.[1] Cartilage tissue engineering (CTE) strategies typically utilize a resorbable scaffold, seeded with stem cells or chondrocytes that are cultivated in an appropriate milieu to generate a neo-cartilage suitable to repair focal chondral defects. These constructs attempt to recapitulate the composition, structure, and mechanical properties of hyaline cartilage by forming a GAG-rich extracellular matrix (ECM) embedded within a COL fibril network. Standard CTE workflow consists of 2–6-weeks *in vitro* cultivation to generate an ECM with a sufficient sGAG concentration to support physiologic loads[2], followed by surgical implantation at the defect where the neo-cartilage continues to evolve *in vivo* compositionally, structurally and mechanically. An impediment to CTE success is the lack of non-destructive *in vitro* and *in vivo* diagnostic platforms to longitudinally monitor construct composition, structure and material properties before and after implantation. Conventional *in vitro* biochemical assays and histologic assessments of construct composition and structure are destructive. *In vivo* monitoring via arthroscopic tissue grading systems (Outerbridge, ICRS) and/or MRI (T1rho, T2*) to evaluate neo-cartilage composition and morphology only moderately correlate with tissue biochemistry and material properties. Raman spectroscopy is an inelastic light scattering technique that reflects the vibrational modes of the biochemical building blocks (amides, sulfates, hydroxyls) of cartilage ECM constituents: sGAG, COL, H_2O. We developed a needle probe for Raman spectral analysis that specifies ECM-specific compositional biomarkers that account for the material properties of native and TE cartilage[3-6]. Here we demonstrate the ability of Raman spectroscopy-derived biomarkers to portray longitudinally the evolving composition of TE neo-cartilage developed on a range of scaffold materials: agarose, collagen, polyethylene glycol (PEG), and scaffold-free.

METHODS: *CTE constructs*: Immature bovine chondrocytes were isolated and seeded at a density of 30×10^6 cells/mL in hydrogel scaffolds of agarose (2% w/v; type VII), collagen (3mg/mL; rat tail type-I), or PEG (10% w/v; 4-arm PEG thiol + 4-arm PEG acrylate) to generate ∅4×2mm cylindrical plugs, or assembled in a scaffold-free pellet (4×10^6 chondrocytes centrifuged at 800g). All constructs were cultured in chondrogenic medium ± TGF-β3 (10ng/mL) for the initial 2 weeks[7], except for scaffold-free constructs (cultured entirely -TGF-β3) and PEG constructs (cultured entirely +TGF-β3). Constructs were removed weekly (n=6 per scaffold per TGF-β group) for Raman, mechanical, and biochemical endpoint analysis through day 56 for agarose and through day 28 for other scaffolds. *Raman spectral analysis* was performed with a fiber-optic Raman probe (∅10mm; RIP-RPB, OceanOptics) coupled to a NIR diode laser (ex=785nm, 125mW) and a spectrometer (QEPro, OceanOptics) (**Fig.1A**). The cartilage spectra (800-1800cm^{-1}) was fit to a multivariate linear regression model: $\mathbf{Construct_{spectra}}= sGAG_{score}*(\mathbf{sGAG_{REF}})+COL_{score}*(\mathbf{COL_{REF}})+H_2O_{score}*(\mathbf{H_2O_{REF}})+Scaffold_{score}*(\mathbf{Scaffold_{REF}})$, where $\mathbf{sGAG_{REF}}$, $\mathbf{COL_{REF}}$, $\mathbf{H_2O_{REF}}$, and $\mathbf{Scaffold_{REF}}$ are reference spectra of purified powder chemicals for each ECM constituent; the "scores" are the regression coefficients reflecting the relative contribution of each constituent (**Fig.1B-I**). The high-wavenumber range spectra (2700-3800cm^{-1}) was used to compute the area under the OH peak (OH_{area}), reflecting tissue hydration[5] (**Fig.1J**). $Scaffold_{REF}$ was omitted for collagen scaffolds owing to the inability of Raman spectral analysis to differentiate between COL-I scaffold and Col-II ECM. For scaffold-free constructs $Scaffold_{REF}$ was derived from a dried cell pellet. Spectra were collected with the probe focused ~10mm from the construct surface over a 30 second integration time. Constructs were analyzed for compressive Young's modulus (E_Y), gravimetric water content, and sGAG content (DMMB). An additional batch of agarose-scaffold constructs were cultivated ± TGF-β3 (n=6 per group) and subjected to repeated Raman spectroscopy under aseptic conditions at weekly intervals over 6 weeks.

RESULTS: The multivariate spectral regression model accounted for 86% ± 3% of the variation in the construct Raman spectra, demonstrating the ability of Raman spectroscopy to differentiate evolving neo-cartilage ECM from scaffold material (**Fig.1B-I**). For all scaffolds, Raman GAG and COL scores increased, while H_2O and scaffold scores decreased over time. Raman $sGAG_{score}$ accounted for 90%, 68%, 73%, and 69% variation of sGAG content in agarose, collagen, PEG, and scaffold-free, respectively. Raman OH_{area} accounted for 87%, 76%, and 80% variation of H_2O content in agarose, collagen, and PEG, respectively, but was not correlated with the scaffold-free constructs (p=0.17). Raman $sGAG_{score}$ accounted for 86% and 67% variation of E_Y for agarose and collagen constructs respectively. Multivariate linear regression combining $sGAG_{score}$ and OH_{area} proved valuable, improving predictions of the variation in E_Y for collagen constructs (67% to 78%) and predictions of the variation in sGAG content (69% to 83%) for scaffold-free constructs. Repeated-measure assessments of agarose-scaffold constructs illustrated a progressive increase in sGAG and COL, and decrease in H_2O and scaffold with time—this evolution was more pronounced for the +TGF-β group (**Fig.3**). At day 42, no difference in viability (not shown) or E_Y was observed between constructs subjected (726.6±86.9 kPa) or not subjected (527.5±45.9 kPa) to repeated Raman analysis, validating that the procedure was not detrimental to neo-cartilage development.

DISCUSSION: Raman spectral analysis accurately measured the composition (sGAG, H_2O) and predicted the material properties (E_Y) of developing neo-cartilage. ECM-specific biomarkers derived from the composite Raman spectra profile allowed for longitudinal assessment of newly deposited ECM developed on a range of scaffold materials with unique spectral signatures, including a natural carbohydrate (agarose), natural protein (collagen), and synthetic polymer (PEG) or no scaffold (scaffold-free). Combining the GAG_{score} and the hydration-associated OH_{area} improved predictions of E_Y and sGAG content. The Raman probe adopted in this study was ideal for *in vitro* monitoring of neocartilage owing to its high spectral collection efficiency and integration with a long-distance lens that did not require specimen contact. Previously, we demonstrated that a Raman needle probe (∅2mm) configured for arthroscopic assessments of cartilage can achieve similarly accurate measurements of agarose-scaffold derived neocartilage composition and material properties (R^2=0.72-0.88)[5,6].

SIGNIFICANCE: This study demonstrates that Raman spectroscopy, using this platform can monitor the evolution of neo-cartilage composition germane to its material properties *in vitro* and *in vivo*, providing non-destructive objective assessments of the efficacy of chondroregenerative therapies.

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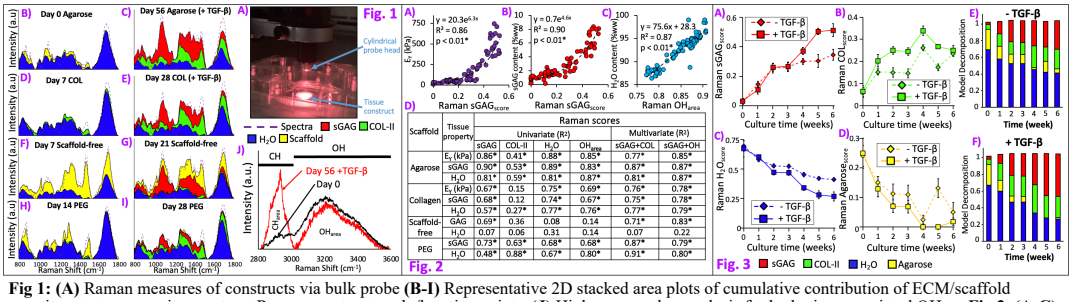

Fig 1: (A) Raman measures of constructs via bulk probe **(B-I)** Representative 2D stacked area plots of cumulative contribution of ECM/scaffold constituents to composite construct Raman spectra at early/late timepoints. **(J)** Highwavenumber analysis for hydration-associated OH_{area}. **Fig 2**: **(A-C)** Regression correlations between Raman $sGAG_{score}$ and **(A)** sGAG content and **(B)** E_Y, and **(C)** OH_{area} and water content for all analyzed agarose-scaffold-derived constructs. **(D)** Univariate and multivariate correlation coefficients between Raman biomarkers and construct properties of all constructs analyzed for each scaffold material, *p<0.01. **Fig 3: (A-D)** Evolution of Raman biomarker scores over time for repeated measure Raman acquisitions. **(E-F)** Raman biomarker distributions for constructs cultivated **(E)** -TGF-β3 and **(F)** +TGF-β3 over time.