Noninvasive Assessment of Bound and Unbound Water Content in the Intervertebral Disc using Raman Spectroscopy

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Disclosures: The authors have nothing to disclose.

INTRODUCTION: The intervertebral disc is a highly hydrated material that acts to provide spinal support, stability, and mobility. Despite differences in composition and structure of the nucleus pulposus (NP) and annulus fibrosus (AF), major biochemical constituents include water, proteoglycans, and collagen fibers [1]. Previous studies examined disc composition by collecting specimens from the several locations within the disc, such as the NP and AF. However, this traditional approach for assessing of biochemical composition is highly time-consuming and destructive. Hence, in recent years, several spectroscopy and imaging tools have emerged as promising instrumental techniques for non-destructive assessment of biochemical composition. Raman spectroscopy has some advantages over other techniques, as it can collect real-time data without the use of dyes or other contrast-enhancing agents. Raman has been previously used to analyze biochemical composition of cartilage [2], bone [3], skin [4], and brain [5], but there has been little to no work in applying this technique for studying spatial variations in biochemical composition of the intervertebral disc. Therefore, the objective of this study was to use Raman spectroscopy to nondestructively measure water content throughout the intervertebral disc. With this technique we were able to assess bound and unbound water molecules in the tissue.

METHODS: All Raman measurements were collected using a Horiba Jobin Yvon Labram spectrometer equipped with confocal microscope (Olympus BX41) and a 632.8 nm laser as the excitation source. Spectra of bovine caudal discs (n = 3) were first measured in the NP and AF over a wide frequency range (300 – 3800 cm^{-1}). All spectra were normalized to have the same signal intensity at 2945 cm^{-1}, which corresponded to the strongest CH vibration line. Representative spectra for each tissue region were obtained by taking the average of twelve spectra collected from each region. The wavelengths representing the OH and CH bands were determined from the representative spectra of the two anatomical regions.

Based on the results from the representative spectra, water content distribution was assessed in the 2800 – 3800 cm^{-1} range for an additional seven bovine discs. For each disc, measurements were taken at the inner NP (i-NP), outer NP (o-NP), inner AF (i-AF), and outer AF (o-AF). Discs were then freeze-dried for 72 hours, and measurements were repeated on dry samples. Water content of wet and dry samples was determined from the spectra by integrating peak areas of OH and CH bands (3020 – 3800 cm^{-1} and 2800 – 3020 cm^{-1}, respectively) and calculating the ratio of the two areas (OH:CH), which was proportional to the ratio between water content and protein/lipid content. These water:protein ratios were compared to data collected from destructive biochemical assays (*i.e.*, water content/(collagen + gag content) with respect to tissue wet weight) [6]. The percentage of the bound water in the tissue was estimated by finding the ratios of water:protein ratios obtained from the dry and wet samples. Finally, the water spectrum was deconvoluted into five Gaussian components to determine the contribution of each peak, as previously reported [7,8].

RESULTS: Similar to other biological tissues, the Raman spectra of the NP and AF were dominated by the bands from amino acids and lipids (Fig. 1A). There were similarities in NP and AF spectra, with distinct peaks at 863, 940, 1252, 1455 and 1661 cm^{-1} in the low-frequency zone (Fig. 1A - <1800 cm^{-1}). Bands located at 2800 – 3020 cm^{-1} were associated with stretching modes of CH_2 and CH_3, while bands at 3020 – 3800 cm^{-1} were associated with water molecules. There was a linear decrease in the water:protein ratio from the disc center to the outer periphery (Fig. 1B & 1C). Moreover, the water:protein ratio obtained from the Raman spectra agreed well with the measurements collected from traditional biochemical analysis (Fig. 1C – $p = 0.17$). There was no significant difference in the amount of bound water measured in different tissue regions (one-way ANOVA; $p > 0.5$), but bound water accounted for 2.5 ± 1.4 % and 10.5 ± 4.7 % of the total water content at inner NP and outer AF, respectively (Fig. 1D). The Raman spectra of water had a complex structure, which was well described by five Gaussian-shaped peaks centered at 3073, 3214, 3386, 3499 and 3619 cm^{-1}.

DISCUSSION: In this study we presented novel measurements of bound and unbound water molecules and water content distribution throughout the intervertebral disc. This is the first study to measure and report NP and AF Raman spectra. Spectra observed here were similar to observations in cartilage and skin [2, 4, 9], as well as for bulk water and hydrogels [7,8]. Peaks at 1252 and 1661 cm^{-1} have been associated with amide bonds in collagen fibers and other proteins (*i.e.*, amide III and amide I, respectively) [10]. Previous studies associated peaks at 3073, 3214, 3386 cm^{-1} with water molecules that have strong hydrogen bonds, either with other water molecules or proteins, whereas the last two peaks (*i.e.*, ~3499 and 3619 cm^{-1}) have been associated with weak hydrogen bonds or free water molecules [3, 7]. A further analysis of the complex water spectra can provide insight into the structure of water molecules in the tissue. The decrease in the signal intensity of water spectra after freeze-drying is likely due to the removal of free water in the tissue, as the water spectra of wet tissue have contributions from both bound and unbound water molecules. These findings suggest that the percentage of bound water changes with respect to spatial location (*i.e.*, NP versus AF), and the percentage of bound water molecules in the NP agreed well with values reported for cartilage [9, 11]. Although degeneration is known to decrease total water content, there is not much known about changes in water structure due to degeneration. Thus, future work will focus on evaluating the effects of degeneration and aging on the spatial distribution and structure of water.

SIGNIFICANCE: This study applied nondestructive measurement techniques using Raman spectroscopy to evaluate spatial variations in bound and unbound water content in the intervertebral disc.

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ACKNOWLEDGEMENTS: This work was supported by NSF CAREER Grant (1751212).

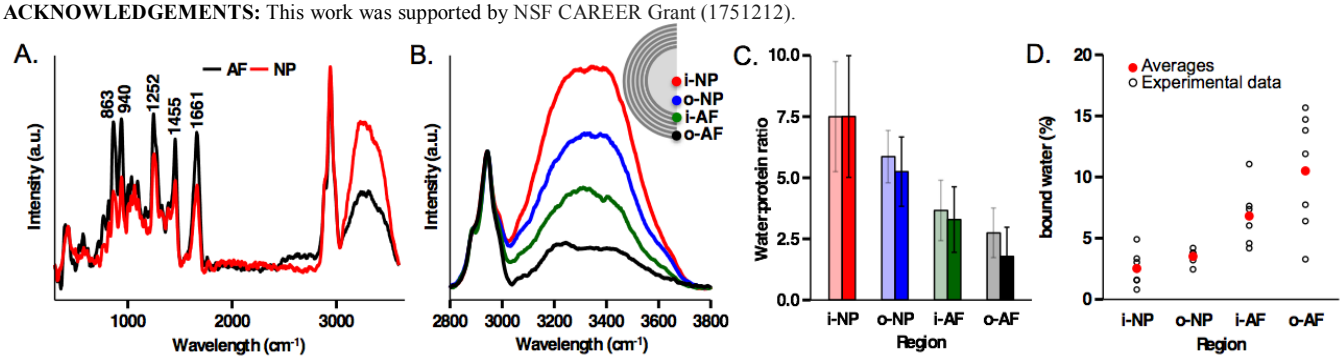

Figure 1: A) Representative spectra for the AF and NP, B) spectra collected from four regions within the disc, C) comparison of water:protein ratios collected from Raman spectroscopy (dark bars) and biochemical analysis (light bars) [6], and D) bound water content associated with four regions.