Determining the Fixed Charge Density of Nucleus Pulposus Tissue via CA4+ Staining and LA-ICP-MS

Jordin T. Marshall[1], Jonathon Hammond[1], Anthony D. Lutton[2], Katherine Hohl[3], Mark Grinstaff[3], John W. Olesik[2], Benjamin A. Walter[1]
[1]Biomedical Engineering, The Ohio State University; [2]School of Earth Sciences, The Ohio State University; [3]Biomedical Engineering, Boston University,
marshall.1147@buckeyemail.osu.edu

Disclosures: Nothing to disclose

INTRODUCTION: Daily cycles in the intervertebral disc's (**IVD**) height engender diurnal fluctuations in the disc's fixed charge density (**FCD**) and corresponding osmotic environment, which serve as an important mechanobiological signal regulating cellular metabolism [1–3]. However, understanding how these osmotic signals serve as a biophysical signal requires understanding what the cells experience within the pericellular matrix (**PCM**), a specialized extracellular matrix (**ECM**) that surrounds NP cells. Prior work has qualitatively demonstrated via correlations with staining intensity that the PCM has a higher FCD than the bulk ECM [3]. We have further demonstrated that the PCM has a higher sodium content than the surrounding ECM using laser ablation-inductively coupled plasma-mass spectrometry (**LA-ICP-MS**), a technique used for elemental analysis at the micron scale, to measure sodium content in cryo-sectioned IVD tissue [4]. Collectively, this suggests cells from the central nucleus pulposus (**NP**) region experience exaggerated osmotic cycles compared to the ECM. However, direct assessment of PCM FCD, which is an important input into constitutive models, was prevented by the physiologic sodium content of the bathing solution [5]. This study aims to determine whether LA-ICP-MS measurements of the iodine after staining tissue sections with a cationic hexa-iodinated contrast agent (**CA4+**), which has previously been shown to accumulate in areas of high GAG concentration [6], can be used to quantify FCD.

METHODS: Two human lumbar spines (1 F, 1 unknown) were obtained from the Cooperative Human Tissue Network at The Ohio State University. NP tissue from 7 IVDs (n=7) was isolated and divided into 5 pieces. The tissue pieces from each IVD were weighed and underwent equilibrium dialysis in one of five solutions for 72hrs; 5, 10, 15 or 20% PEG w/v in 0.015M NaCl or 10% PEG in 0.15M NaCl to manipulate FCD. Agarose gels (2% w/v) were equilibrated in each solution as negative controls. At 0.015M NaCl the internal sodium directly corresponds to the FCD avoiding additional sodium present within the tissue due at a higher physiological 0.15M NaCl concentration [5]. Following equilibration, tissue was weighed and separated into 3 pieces to assess: (1) GAG content via DMMB, (2) intra-tissue sodium via inductively coupled plasma optical emission spectroscopy (**ICP-OES**) and, (3) iodine via LA-ICP-MS on CA4+ stained cryosections. Tissue taken for GAG and ICP-OES were lyophilized and used to determine water content. DMMB and ICP-OES were conducted as previously described [4,7]. *LA-ICP-MS Validation:* 10µm thick cryo-sections from four IVDs (N=4/PEG%) were stained with CA4+ (24mI/mL for 10min) and dried overnight prior to ablation. Iodine content was assessed from the average of 36 spots, each 10µm x10µm, organized in a 6x6 grid. Measurements on the remaining 3 IVDs are ongoing. Statistics: A two-way ANOVA was used to assess the effects of osmotic pressure on FCD and Iodine content between tissue and agarose controls. A linear correlation was used to determine agreement between OES sodium and GAG content and between iodine content and FCD. Student's t-test was used to assess the effect of bath concentration on FCD, GAG and iodine concentration.

RESULTS: *FCD:* Intra-tissue sodium content/FCD significantly increased with increasing osmotic pressure/PEG solution (Fig 1A). Sodium content remained constant in agarose controls across applied osmotic pressures. *GAG Content:* A strong correlation was observed between FCD and GAG/wet weight (Fig 1B). There was no difference in GAG/dry weight between samples and no GAG was present in agarose controls. *LA-ICP-MS Validation:* Iodine concentration significantly increased with increasing PEG/osmotic pressure (Fig 1C) and a linear correlation was observed between FCD and iodine concentration (Fig 1D). *Effect of Bath Concentration:* In tissue equilibrated in 10% PEG, there was a significant difference in intra-tissue sodium content between the 0.015M and 0.15M NaCl equilibration baths. No difference was observed between similarly equilibrated agarose samples. There was no difference in tissue iodine measurements between 0.015M and 0.15M NaCl equilibrated samples (Table 1). The theoretical FCD of 0.15M NaCl samples was similar to FCD of 0.015M NaCl equilibrated samples determined via ICP-OES.

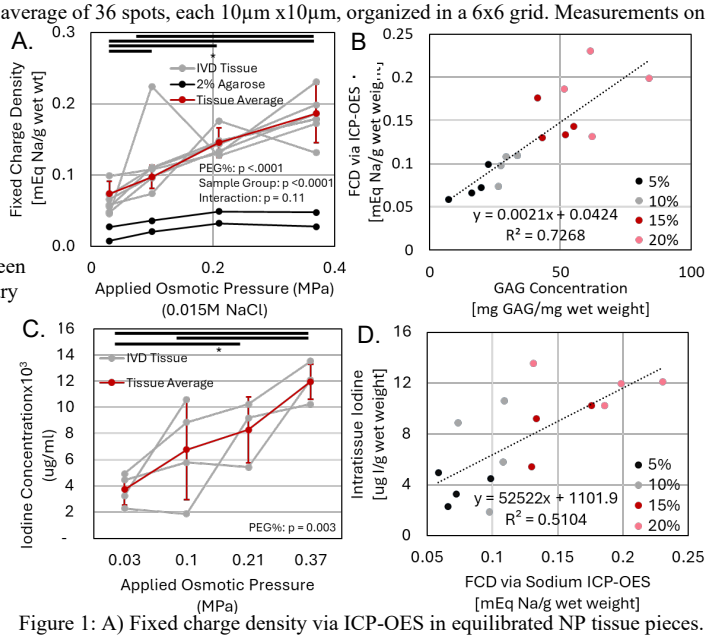

Figure 1: A) Fixed charge density via ICP-OES in equilibrated NP tissue pieces. B) Correlation between sodium via LA-ICP-MS and GAG concentration via DMMB. C) Intra-tissue iodine concentration via LA-ICP-MS in equilibrated NP tissue pieces. D) Correlation between intra-tissue iodine via LA-ICP-MS and FCD via ICP-OES.

Table 1

Measurement		10% PEG 0.015M NaCl	10% PEG 0.15M NaCl
ICP-OES Sodium (mEq/g wet wt)	Agarose	0.03±0.01	0.01±0.05
	IVD Tissue	0.12±0.05*	0.25±0.03*
LA-ICP-MS Iodine (ug/g wet wt)	IVD Tissue	6,787±3,834	7,229±1,655

DISCUSSION: Agarose was used as a negative control because their wet weight was similarly modified by equilibrium dialysis, decreasing with increased bath PEG, but no change in internal sodium content was observed. This together with the significant increase in intra-tissue sodium with increasing applied osmotic pressure together demonstrates that intra-tissue sodium measurements are reflective of FCD. Additionally, the strong correlation between GAG/wet weight and intra-tissue sodium further supports that ICP-OES measurements of sodium are due to increased FCD. Changes in tissue iodine with PEG% mirrored those as FCD, increasing with PEG and there was a linear correlation between FCD and intratissue iodine. There is greater variability than expected in iodine measurements, and efforts to correct for variations in tissue thickness are ongoing. Comparing bath concentrations, the osmotic pressure exerted by both solutions (i.e., 10% PEG+0.015M and 10% PEG+0.15M) are essentially equivalent [5], therefore the samples would be expected to have the same FCD at equilibrium. This expectation aligns with the finding that there was no significant differences in the iodine content between groups (Table 1), GAG/wet weight concentrations between groups (0.15M: 35.4±4.9 mg/mg; 0.015M:43.1±20.1 mg/mg) or between measured FCD in the 0.015M group (0.12±0.05 mEq/g wet wt) and theoretical FCD in the 0.15M group (0.16±0.05 mEq/wet wt., p=0.25). However, significant differences in intra-tissue sodium between samples would be expected based on Gibbs-Donnan theory and were observed (Table 1). CA4+ has been previously used to assess GAG content of cartilage in microCT studies due to the charge attraction between the positively charged iodinated molecule and the negatively charged GAGs within the tissue [6]. Overall, our results suggest that LA-ICP-MS measurements of iodine from tissue sections stained with CA4+ are reflective of FCD and may provide a new approach to quantitatively assess spatial variations in FCD on the cellular scale. i.e., facilitate measurements of FCD at the pericellular scale. These measurements could theoretically occur in tandem with histological staining for cell identification, which inherently adds sodium to the tissue.

SIGNIFICANCE/CLINICAL RELEVANCE: Overall, this study demonstrates: (1) that LA-ICP-MS measurements of iodine on the micron scale correlate with fixed charge density and (2) the potential of this novel technique to allow quantitative measurements of the pericellular matrix's FCD.

REFERENCES: [1] Zelenski +2015, [2] Hayes +2021, [3] Korhonen +2011, [4] Marshall +2025, [5] Urban +1979, [6] Lakin +2013, [7] Krull +2022

ACKNOWLEDGEMENTS: This work was funded by NSF CAREER 2143779.