

A Conductivity Approach to Measuring Fixed Charge Density in Intervertebral Disc Tissue

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Abstract—A new method for measuring the fixed charge density (FCD) in intervertebral disc (IVD) tissues employing a two-point electrical conductivity approach was developed. In this technique, the tissue is first confined and equilibrated in a potassium chloride (KCl) solution, and the tissue conductivity is then measured. This is then repeated with a second concentration of KCl solution. The FCD can be determined from the conductivity measurements. Using this method, the FCD values of bovine annulus fibrosus (AF) and nucleus pulposus (NP) tissues were determined to be 0.060 ± 0.027 mEq/g wet tissue and 0.19 ± 0.039 mEq/g wet tissue, respectively. The FCD of AF was significantly lower than that of NP tissue, similar to results in the literature for human IVD tissues. In order to verify the accuracy of the new method, the glycosaminoglycan (GAG) contents of the tissues were measured and used to estimate the tissue FCD. A strong correlation ($R^2 = 0.84$ – 0.87) was found to exist between FCD values measured and those estimated from GAG contents, indicating that the conductivity approach is a reliable technique for measuring the FCD of IVD tissues.

Keywords—Annulus fibrosus, Nucleus pulposus, Proteoglycan, Glycosaminoglycan, Electrical conductivity.

INTRODUCTION

Low back pain is a prevalent ailment in the United States, afflicting approximately 15–45% of the population annually, and more than 70% of all individuals at some point in their lifetime.⁵¹ The annual expense of low back pain is upward of \$100 billion, including both direct and indirect costs, making it a significant economic concern. Despite this, the exact cause of low back pain remains unknown. However, many believe that degeneration of the intervertebral discs (IVD) of

the spine is a primary etiologic factor contributing to the onset of low back pain.^{4,9,33,63}

The IVD consists of the nucleus pulposus (NP), the annulus fibrosus (AF) and cartilaginous end-plate, each of which has a unique composition and structure.^{21,41} In this study, the NP and AF are investigated (see Fig. 1a). The central NP is made of randomly oriented collagen fibers enmeshed in a proteoglycan (PG) gel. The AF surrounds the NP and is made up of a series of concentric lamellae having a highly organized structure of collagen fiber bundles.^{25,42} The disc consists primarily of water, comprising 60–90% of the wet weight, and also has significant quantities of collagen (10–50% dry weight) and PG (15–45% dry weight).^{9,16,17,24,31,35,53,54} The composition of the AF varies from that of the NP, with the AF having a higher collagen content, and lower water and PG contents compared with NP tissue.

The IVD is characterized as a charged, hydrated soft tissue. The negatively charged nature of the disc results from the charged groups on glycosaminoglycan (GAG) molecules on the PGs found in the extracellular matrix of the disc (see Fig. 1b). The fixed charge density (FCD) is a measure of the negative charges (per unit volume) attached to the disc matrix. The electrostatic interactions between fixed charges and mobile free ions result in physiochemical and electrokinetic effects, including Donnan osmotic pressure and swelling, streaming current and potential, and electro-osmosis effects.^{8,14,17,18,36,60,61} Therefore, both the transport of solutes and fluid and the swelling behavior in the disc are dependent upon many physiochemical factors, including FCD.^{13,16,18,19,60} Consequently, it is necessary to determine the FCD of IVD tissue in order to quantify the transport behavior in the disc, as well as provide insight into the mechanical behavior of the tissue.

In previous studies, both destructive and nondestructive techniques for measuring FCD in cartilaginous and IVD tissues have been developed, including

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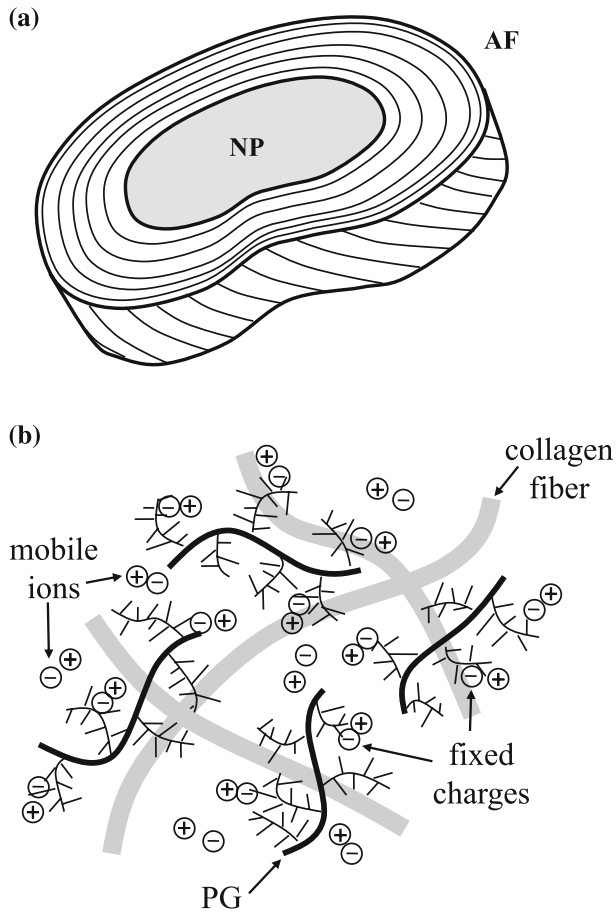


FIGURE 1. (a) Schematic showing the structure of the IVD; samples were taken from both the AF and NP regions of the disc. (b) Schematic diagram showing the charged extracellular matrix of IVD tissue. PG proteoglycan.

tracer cation methods, electrokinetic or streaming potential techniques, imaging methods (e.g., MRI, NMR spectroscopy, and micro-CT), and indentation testing.^{1,3,6,7,10,11,14,27,37–40,43,44,46,47,49,50,52,56,58,59,62} The tracer cation technique, developed by Maroudas and Thomas⁴⁷ has generally been considered the “gold standard” for measuring the FCD of cartilaginous tissues. However, due to the potential hazard of using radio-labeled ions, this method has not been commonly used in regular laboratories. Therefore, there is a need for a new method for measuring tissue FCD that is safe, reliable and easy to perform at a low cost. Hasegawa *et al.* previously correlated conductivity with FCD for articular cartilage.²² However, to our knowledge, no previous study has used electrical conductivity to measure tissue FCD. Therefore, the main objective of this study was to develop a new technique for measuring the FCD of IVD tissue. This new approach employed a two-point conductivity method. The technique was verified by measuring the GAG contents of the tissue specimens, and correlating results

with the values of FCD calculated. This study is important in further understanding the transport and mechanical behavior of IVD tissues, as both are dependent upon the tissue FCD.

THEORETICAL BACKGROUND

The electrical conductivity (χ) of a tissue under zero fluid flow conditions is given by Helfferich²³:

$$\chi = \frac{F_c^2 \phi^w}{RT} \sum_{i=1}^n z_i^2 c_i D_i \quad (1)$$

where F_c is the Faraday constant, R is the gas constant, T is the absolute temperature, ϕ^w is the water volume fraction, z is the valence of a particular ion, c is the concentration of the ion, and D is the diffusion coefficient of the ion. For potassium chloride (KCl), where the value of z for K^+ is +1 and that for Cl^- is -1, the equation reduces to:

$$\chi = \frac{F_c^2 \phi^w}{RT} (c^+ D^+ + c^- D^-). \quad (2)$$

For negatively charged tissues, such as IVD and articular cartilage, the ion concentrations are related through the electroneutrality condition^{32,36}:

$$c^+ = c^- + c^F \quad (3)$$

where c^F is the absolute value of the negative FCD. The ion concentrations within the tissue can be calculated from the ideal Donnan equation^{44,45}:

$$c^+ = \frac{c^F + \sqrt{(c^F)^2 + 4(c^*)^2}}{2} \quad (4)$$

$$c^- = \frac{-c^F + \sqrt{(c^F)^2 + 4(c^*)^2}}{2} \quad (5)$$

where c^* is the concentration of the bathing solution.

In this study, a new method was proposed for determining the FCD in IVD tissue using an electrical conductivity approach. This measurement can be achieved by equilibrating the tissue specimens in two concentrations of solution and measuring the corresponding value of electrical conductivity of the tissue. For KCl solution, because the Stokes' radii (r_s) of K^+ and Cl^- are approximately equal (for K^+ , $r_s = 0.137$ nm and for Cl^- , $r_s = 0.142$ nm^{20,34}), the values of their diffusion coefficients can also be approximated as equal.²⁰ Therefore, Eq. (2) can be reduced to:

$$\chi = \frac{F_c^2 \phi^w}{RT} D \sqrt{(c^F)^2 + 4(c^*)^2}. \quad (6)$$

Because the values for electrical conductivity, χ , water content, ϕ^w , and temperature, T , can be

measured, and those for concentration of bathing solution, c^* , Faraday's constant, F_c , and the gas constant, R , are known, only two unknowns remain in Eq. (6): FCD (c^F) and ion diffusivity (D). Assuming that ion diffusivity is independent of bathing solution concentration, the values for FCD and diffusivity can be evaluated using simultaneous equations. That is, by measuring the conductivity of a tissue equilibrated in two different known concentrations of KCl bathing solution (i.e., a two-point method), the FCD and ion diffusivity in a given tissue can be determined. Because the value of FCD for a tissue specimen remains constant, given that no swelling occurs (i.e., water content remains constant), we can show that:

$$\left(\frac{\chi RT}{F_c^2 \phi^w}\right)^2 \Big|_{c_1^*} - 4D^2(c_1^*)^2 = \left(\frac{\chi RT}{F_c^2 \phi^w}\right)^2 \Big|_{c_2^*} - 4D^2(c_2^*)^2 \quad (7)$$

where c_1^* and c_2^* are the two bathing solution concentrations. Therefore, tissue FCD can be calculated by:

$$c^F = 2 \sqrt{\frac{\chi^2|_{c_1^*} \cdot [(c_1^*)^2 - (c_2^*)^2]}{[\chi^2|_{c_1^*} - \chi^2|_{c_2^*}]}} - (c_1^*)^2, \quad (8)$$

where $\chi^2|_{c_1^*}$ and $\chi^2|_{c_2^*}$ are the values for electrical conductivity for a tissue specimen equilibrated in bathing solutions with concentrations c_1^* and c_2^* , respectively, at the same temperature. Here, the tissue FCD is determined in units of moles of charge per fluid volume (m^3) in the tissue. Thus, by measuring the electrical conductivity of a tissue specimen equilibrated in two different concentrations of KCl solution, the FCD of the tissue can be determined.

Additionally, as was previously noted, this method also yields the value for K^+ or Cl^- ion diffusivity in

tissue. The value of ion diffusivity can be determined from the equation:

$$D = \frac{RT}{2F_c^2 \phi^w} \sqrt{\frac{\chi^2|_{c_1^*} - \chi^2|_{c_2^*}}{(c_1^*)^2 - (c_2^*)^2}}. \quad (9)$$

This again assumes that the conductivity measurements are taken at the same temperature, and that the value of ion diffusivity is independent of the concentration of bathing solution.

MATERIALS AND METHODS

Specimen Preparation

Three lumbar discs (L3-4, L5-6, and L6-7) were harvested from two bovine lumbar spines (~3-6 months) obtained from Animal Technologies, Inc. (Tyler, TX). Cylindrical specimens having a 5 mm diameter and a 3 mm thickness were prepared from both AF and NP tissues using a stainless steel corneal trephine (Biomedical Research Instruments, Inc., Silver Spring, MD) and sledge microtome (Model SM2400, Leica Instruments, Nussloch, Germany) with freezing stage (Model BFS-30, Physitemp Instruments Inc., Clifton, NJ, USA). All specimens were prepared with axial orientation. Two groups of specimens were tested in this study: AF ($n = 15$) and NP ($n = 15$).

Specimen Equilibration

Following preparation, specimens were confined both radially, in an acrylic chamber, and axially, via two sintered stainless steel filters (316L stainless steel, 40 μm pore size, 50% open area, Small Parts, Inc., Miami Lakes, FL, USA; see Fig. 2). To minimize

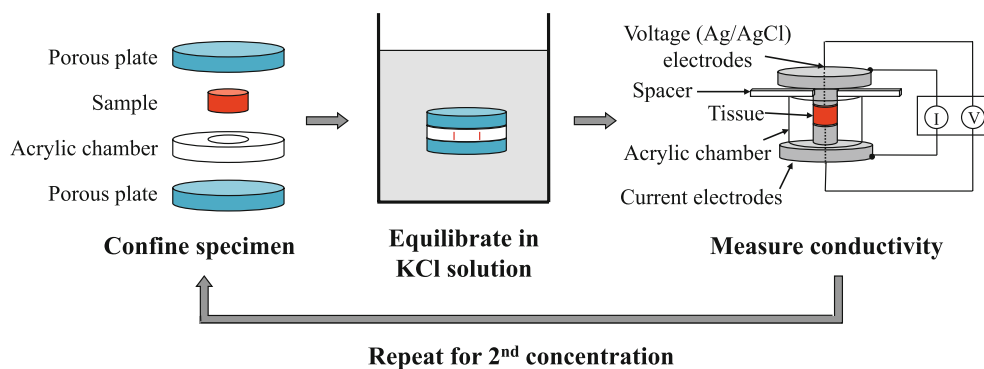


FIGURE 2. Flowchart detailing the experimental protocol of the two-point conductivity method for measuring FCD. The sample was first confined in an acrylic chamber between two porous plates and was then immersed in KCl solution for equilibration. Following equilibration, the sample was moved to the conductivity apparatus, where the resistance across the specimen was measured using a 4-wire sense-current method. The procedure was repeated with a second concentration of KCl solution.

swelling and PG leaching, the tissue specimens were confined while equilibrating in the bathing solution. The confined specimens were then placed in a beaker filled with KCl solution with the desired concentration, either 0.2 M or 0.02 M. These concentrations were chosen based on the results of a preliminary study using agarose gels, which showed that these were the optimal conditions for this experimental method (data not shown). The beaker was sealed with parafilm. Each equilibration period lasted a minimum of 24 h at 4 °C.

Conductivity Measurements

A previously developed conductivity apparatus^{12,13,28} was used to measure the electrical conductivity of IVD samples at zero fluid flow conditions (see Fig. 2). The apparatus consisted of two stainless steel current electrodes coaxial to two Teflon-coated Ag/AgCl voltage electrodes, a spacer, and a nonconductive acrylic chamber. Using a Keithley SourceMeter (Model 2400, Keithley Instruments Inc., Cleveland, OH), a four-wire sense current method was applied, and the resistance (Ω) across the specimen was measured at a very low, constant current of 10 μ A. The electrical conductivity, χ , of the specimen was calculated from:

$$\chi = \frac{h}{\Omega A}, \quad (10)$$

where h is the thickness of the specimen and A is the cross-sectional area.

Conductivity values were determined for specimens equilibrated in two different concentrations of KCl solution. Following equilibration, each specimen was transferred from the confining apparatus to the conductivity chamber for resistance measurement. Then, the specimen was again confined for equilibration in a new solution. Following the two resistance measurements, the water volume fraction of each sample was measured.

Water Content Measurement

The water content, or porosity, of each sample was measured using a buoyancy method published in the literature.^{15,16} Briefly, the tissue specimen was weighed in air (W_{air}) and in PBS solution (W_{PBS}), and then lyophilized to obtain the specimen dry weight (W_{dry}). The water content was then calculated by:

$$\phi^w = \frac{W_{\text{air}} - W_{\text{dry}}}{W_{\text{air}} - W_{\text{PBS}}} \frac{\rho_{\text{PBS}}}{\rho_w}, \quad (11)$$

where ρ_{PBS} and ρ_w are the mass densities of PBS and water, respectively.

TABLE 1. Results for volume fraction of water (ϕ^w), FCD, and GAG content for bovine lumbar AF and NP tissues.

	AF	NP
ϕ^w	0.81 \pm 0.054	0.93 \pm 0.022
FCD (mEq/g wet tissue)	0.060 \pm 0.027	0.19 \pm 0.039
GAG per dry weight (μ g/mg)	54 \pm 41	490 \pm 64

Values were determined at room temperature (23.8 \pm 0.24 °C). For each group, $n = 15$. For all measures, AF < NP ($p < 0.05$).

Measurement of GAG Content

Samples were analyzed for GAG content using a 1,9-dimethylmethylene blue (DMMB) binding assay.¹⁰ Prior to lyophilizing the AF samples, the tissue was chopped into small pieces to facilitate tissue digestion; preliminary studies showed that this was not necessary for the NP samples. The dry tissue specimens were solubilized using 0.5 mg/mL proteinase-K (Sigma–Aldrich, St. Louis, MO) at 65 °C for 18–24 h. The DMMB assay was applied to the digested sample and the absorbance of light at 525 nm was measured spectrophotometrically (SmartSpec Plus Spectrophotometer, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The tissue GAG content was determined using a bovine chondroitin sulfate type A standard. Values were normalized by tissue dry weights.

RESULTS

The results for FCD, GAG content, and water content in bovine lumbar AF and NP tissues are shown in Table 1. All values were determined at room temperature (23.8 \pm 0.24 °C) and are shown in mean \pm standard deviation. The units for tissue FCD were converted from equivalent moles per m³ to milliequivalent moles (mEq) per gram wet tissue using water content measurements; this conversion allowed for comparison of results with those in the literature.^{58,59} A Student's t -test was performed using Excel Spreadsheet software (Microsoft Corp., Seattle, WA, USA) to determine if the differences between AF and NP groups were statistically significant for each of the measured parameters. The significance level was set at $p < 0.05$. Analysis showed that significant differences existed between AF and NP tissues for FCD, GAG content, and water content.

DISCUSSION

In this study, a new technique for measuring the FCD of IVD tissue was developed, employing a two-point conductivity approach. Using this technique, the FCD of both AF and NP tissues from bovine lumbar

discs was measured; the results of this study, ranging from 0.026 to 0.261 mEq/g wet tissue, were similar to those found in the literature for human IVD specimens (~0.08–0.28 mEq/g wet tissue).^{58,59} It was also determined that the FCD in AF tissue is significantly lower than that in NP tissue for bovine lumbar IVD, which is in agreement with results for human IVD in the literature.^{58,59}

To calculate the FCD with this new method, the electrical conductivity of the tissue specimens in two different concentrations of KCl bathing solutions (i.e., two-point method) was measured. The results for measured conductivity in AF and NP tissues at two concentrations are shown in Fig. 3. For both concentrations, the conductivity of AF tissue was significantly lower than that of NP tissue (*t*-test, $p < 0.05$), which is in agreement with previous studies in the literature using bovine and human IVD tissues.^{28,29} Furthermore, for both AF and NP tissues, the conductivity values for tissue equilibrated in 0.02 M KCl solution were significantly lower than those equilibrated in 0.2 M KCl (*t*-test, $p < 0.05$). This is in agreement with a previous study showing that the conductivity of articular cartilage decreased significantly with decreasing bathing solution concentration.⁴³

In order to determine if the new method was able to reliably measure the FCD of IVD tissue, the GAG content of tissues was also measured for comparison. The values for the GAG content of bovine lumbar IVD tissues measured in this study, shown in Table 1, were similar to values found in the literature.^{2,26,55} The GAG content of AF tissues measured here is slightly

lower than those found in the literature for calf lumbar spine.² However, it should be noted that previous studies indicated that the GAG content increases moving from outer to inner AF.²⁶ While the region of specimens was not specifically noted in this study, most samples were taken from the outer area of the AF; this may account for the lower value for GAG content for AF tissue found here.

The FCD of a tissue may be estimated from the GAG content within the tissue using the relationship between the molecular weight of a chondroitin sulfate (CS) disaccharide and the quantity of charge on each disaccharide unit. By assuming 2 moles of charge per mole of GAG in the tissue and a molecular weight of 502.5 g/mole GAG, the FCD (in mEq/g wet tissue) can be calculated using the following equation^{1,38}:

$$c^F = \left(\frac{2 \text{ mol charges}}{502.5 \text{ g GAG}} \right) \frac{W_{\text{GAG}}}{W_{\text{air}}} \quad (12)$$

where W_{GAG} is the GAG content (in grams) in the sample, and W_{air} is the weight of the wet tissue in air (from Eq. 11). The results for GAG contents in AF and NP tissues were used to estimate the FCD for comparison with the results from conductivity testing. For AF tissue, the average FCD calculated from Eq. (12) was 0.043 ± 0.023 mEq/g wet tissue, while that in NP was 0.215 ± 0.065 mEq/g wet tissue. A graph showing the measured FCD vs. the estimated FCD is shown in Fig. 4. Two linear curve-fits were performed, one with the constraint of passing through the origin, and another without this constraint. Both show significant correlations ($R^2 = 0.84$

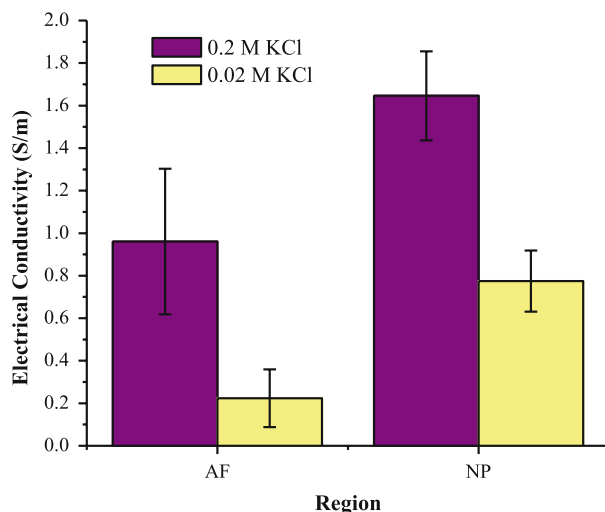


FIGURE 3. Results for electrical conductivity in AF and NP tissues equilibrated in 0.2 M KCl and 0.02 M KCl. For each group, $n = 15$. For conductivity in both solutions, $\text{AF} < \text{NP}$ (*t*-test, $p < 0.05$). For conductivity in both tissues, 0.02 M KCl < 0.2 M KCl (*t*-test, $p < 0.05$).

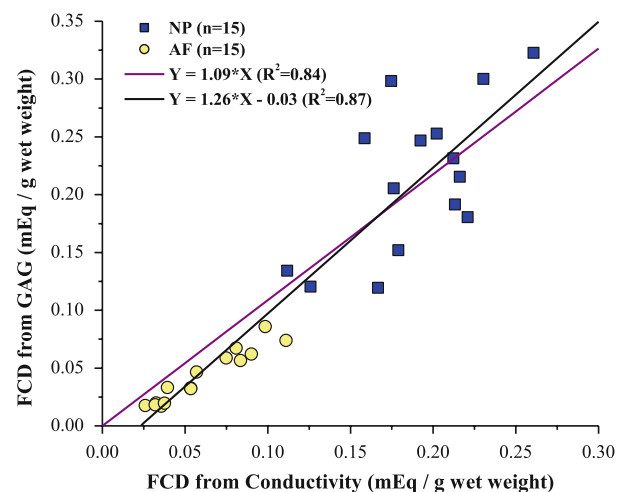


FIGURE 4. Correlation between FCD values measured using conductivity method and those estimated from GAG content and Eq. (12). For both AF and NP, $n = 15$. The theoretical value of the slope is 1. For both linear curve-fits (passing through the origin and not), there is a strong correlation between measured and estimated values ($R^2 = 0.84$ and $R^2 = 0.87$, respectively).

and $R^2 = 0.87$, respectively) between the measured and estimated values for the tissue FCD, indicating that our method is able to reliably measure the FCD of IVD tissues. Moreover, the slope of the curve-fitted line constrained to pass through the origin (slope = 1.09) is close to the theoretical value of 1, further demonstrating that the FCD of IVD tissues may be measured using the electrical conductivity approach.

It should be noted that, while the estimation of FCD from GAG content using Eq. (12) assumes that there are 2 moles of charge per mole of GAG, there are actually two types of disaccharides in IVD tissues contributing to the FCD: CS, which carries two negative charges per disaccharide, and keratan sulfate (KS), which has only one charged group per disaccharide. Previous studies have shown that disc PGs contain greater quantities of KS than those in articular cartilage.⁵⁷ Furthermore, it has been shown that, for human IVD tissue, the ratio of KS to CS varies across the disc.^{58,59} Consequently, in order to accurately calculate the FCD from GAG content, the ratio of KS to CS in the tissue must be known and accounted for in Eq. (12). This difference likely contributed to the discrepancy in the measured and estimated values of FCD shown in Fig. 4.

In addition to calculating values for FCD in IVD tissues, the two-point conductivity method is also able to estimate values for the diffusivity of Cl^- (or K^+) ions in the disc tissue. For AF tissue, the value was determined to be $7.56 \pm 2.12 \times 10^{-10} \text{ m}^2/\text{s}$ ($n = 15$), which corresponds to a relative diffusivity (i.e., the ratio of the diffusivity in tissue to that in aqueous solution) of 0.47 ± 0.13 . The Cl^- ion diffusivity in NP tissues was $1.03 \pm 0.16 \times 10^{-9} \text{ m}^2/\text{s}$ ($n = 15$), corresponding to a relative diffusivity of 0.64 ± 0.10 . Previous studies have shown that, for small solutes, diffusivity in cartilaginous tissues and IVD is approximately 35–60% of that in aqueous solution.^{5,20,29,30,48,58} The results found here are in agreement with those in the literature for diffusivity of ions in IVD tissues.^{20,29} The slightly higher value for NP tissues is likely due to the higher water content of the tissue, as diffusivity has been shown to be dependent upon tissue hydration.^{20,48}

As noted previously, several methods can be found in the literature for measuring the FCD of cartilaginous tissues. The tracer cation method,⁴⁷ which has been considered a “gold standard,” has not been readily adopted in regular laboratories, due to the use of potentially hazardous radiochemicals. Furthermore, several of the alternative techniques reported require the use of expensive equipment for obtaining FCD measurements. The method presented in this study is not only safe and reliable, but it is also easy to perform

without special equipment. Therefore, we believe the two-point electrical conductivity approach represents a viable alternative technique for measuring FCD of cartilaginous tissues.

In summary, a new technique for measuring the FCD of IVD tissue using a two-point conductivity approach has been developed. Using this new method, it was determined that the FCD of AF tissue is significantly lower than that in NP tissue, which is in agreement with previous studies. Furthermore, the analysis of GAG content, and its strong correlation with FCD measurements, indicates that the new technique is able to measure FCD reliably in IVD tissues. This study provides a dependable and straightforward alternative to current methods for measuring the FCD in disc tissue. Better understanding of the FCD in IVD tissues is important for elucidation of tissue transport and mechanical behaviors.

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