Piezoelectricity in the Intervertebral disc


A B S T R A C T

Lower back pain is a major global health challenge that can often be caused by degeneration of the Intervertebral Disc (IVD). While IVD biomechanics are a key factor in the degenerative cycle, many mechanotransduction pathways remain unknown, in particular the electro-mechanical coupling in the loaded tissue. However, despite evidence for a role in the mechanically-induced remodelling of similar tissue, piezoelectricity has been overlooked in the IVD. In this study, we investigate the piezoelectric properties of the Annulus Fibrosus (AF) and the Nucleus Pulposus (NP) by measuring the direct piezoelectric effect of mechanically-induced electrical potential change. To verify these findings, we conducted Piezoresponse Force Microscopy (PFM) to measure the inverse effect of electrically-induced deformation. We demonstrate that, for the first time, piezoelectricity is generated throughout the IVD. Piezoelectric effects were greater in the AF than the NP, owing to the organised collagen networks present. However, the piezoresponse found in the NP indicates piezoelectric properties of non-collagenous proteins that have not yet been studied. The voltage generated by longitudinal piezoelectricity in-vivo has been calculated to be ~1 nV locally, indicating that piezoelectric effects may directly affect cell alignment in the AF and may work in conjunction with streaming potentials throughout the IVD. In summary, we have highlighted an intricate electro-mechanical coupling that appears to have distinct physiological roles in the AF and NP. Further study is required to elucidate the cell response and determine the potential role of piezoelectric effects in regeneration and preventative measures from degeneration.

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1. Introduction

Lower back pain (LBP) is the most debilitating disease worldwide. It has remained the leading cause of years lived with disability since at least 1990 (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018), and costs the EU economy up to €300 billion each year (Baker et al., 2010). Degeneration of the Intervertebral Disc (IVD) is a major factor in LBP, being involved in at least 40% of cases (Luoma et al., 2000). The response of the IVD under load is thought to be a primary factor in the degenerative cycle (Vergroesen et al., 2015). As such, the biomechanics of the IVD is intricately linked with a major global health problem that will affect 60–70% of people in their lifetime (Duthey, 2013).

However, a major challenge in the prevention and treatment of LBP is that the biomechanics of the IVD remains poorly understood. IVD cells are sensitive to the forces applied, with the response of cells from the Nucleus Pulposus (NP) and Annulus Fibrosus (AF) being catabolic or anabolic subject to the type, magnitude, frequency and duration of the applied force (Korecki et al., 2009; Neidlinger-Wilke et al., 2006). The ability of NP cells, for instance, to produce aggrecan, the primary proteoglycan responsible for retaining water content under load, is crucial in maintaining tissue structural integrity, but the mechanotransduction pathways largely remain to be elucidated (Li et al., 2015). Moreover, cells of the IVD may change phenotype in degeneration (Tang et al., 2012), while the tissue composition of the ECM is also altered (Bendtsen et al., 2016) leading to a change in forces experienced (Iatridis et al., 2013).

While many mechanisms of IVD mechanotransduction have been studied, one area that has been overlooked until recently is the cell response to electrical potentials. In bone, evidence suggests that stress-induced remodelling is mediated by local changes in the electrical potential (Friedenberg and Brighton, 1966; Sing and Saha, 1984; Zigman et al., 2013). Leveraging this, electrical stimulation of bone has consistently produced matrix (Brighton et al., 1985; Griffin et al., 2013; Huang et al., 2008; Korenstein et al., 2004). In cartilage, voltage-gated ion channels mediate part of
the chondrocyte response to dynamic compression (Mouw et al., 2007), cyclic tensile strain (Tanaka et al., 2005) and shear strain (Srinivasan et al., 2015). Such evidence indicates that chondrocytes also respond to forces through sensing electrical potential changes. Recently, studies investigating the electrical stimulation of IVD cells have produced similar regenerative results (Miller et al., 2016; Shin et al., 2019; Wang et al., 2017).

Mechanisms that generate load-induced electrical potentials in the IVD include streaming potentials (Iatridis et al., 2009) and diffusion potentials (Lai et al., 2000). Both are a delayed response to loading, caused by constrained relative motion of positively-charged ions past negatively-charged surfaces of ECM molecules, and so requiring fluid or ion transport to occur. But experimental and numerical investigations have demonstrated a direct coupling between stress and ionic concentration change in both a gel and cartilage tissue (Jin and Grodzinsky, 2001; Roos et al., 2013). Piezoelectricity (Shamos and Lavine, 1967) may be another mechanism contributing to the intricate electro-mechanical coupling in hydrated tissues.

Piezoelectricity is the linear conversion between mechanical energy and electrical energy (Guerin et al., 2018). The direct conversion from mechanical to electrical energy takes the form:

$$\{D\} = \{d\} \{T\} + \{s\}' \{E\}$$

(1)

While the inverse conversion of electrical to mechanical energy follows:

$$\{S\} = \{s\}' \{T\} + \{d\}' \{E\}$$

(2)

where $D$ is electrical displacement, $\{d\}$ is the direct piezoelectric effect matrix, $T$ is a constant stress field, $\{s\}'$ is the transpose of the permittivity matrix, $E$ is electric field strength, $S$ is strain, $\{s\}'$ is compliance in a constant electrical field, and $\{d\}'$ is the matrix for the inverse piezoelectric effect.

This linear electromechanical coupling occurs only in non-centrosymmetric structures. As many biological molecules and proteins lack a centre of symmetry, an applied stress can induce dipole moments to generate a net polarisation at the surface (Stapleton et al., 2017). Many biological structures demonstrate this piezoelectric effect, such as amino acids (Guerin et al., 2018), DNA (Ando and Fukada, 1976), collagen (Marino et al., 1980) and bone (Tofail et al., 2011). Indeed, the direct piezoelectric effect in bone is thought to act in conjunction with streaming potentials to mediate the remodelling process (Ahn and Grodzinsky, 2009).

The aim of this study is to, for the first time, investigate piezoelectricity in the IVD as a source of strain-induced electrical potentials. Both the direct and inverse piezoelectric effect is examined in the bovine caudal disc separated into AF and NP. For simplicity, only the $d_{33}$ tensor in relation to the macro-scale tissue sample is examined; that is, vertical out-of-plane piezoelectricity when the tissue is loaded axially. Samples are tested initially in the dehydrated state to isolate the effects of piezoelectricity from electrokinetic effects, while hydrated samples are tested for an indication of in-vivo relevance. By calculating the physiological piezoresponse, the relevance of these electrical potential changes to IVD cell mechanotransduction is inferred.

2. Methods and materials

2.1. Sample preparation

Bovine tails were harvested from 18 to 24 month-old animals in a local abattoir. Tails were dissected in a sterile environment and the caudal discs of interest isolated within 3 h of slaughter. Discs from Cd1, Cd2 and Cd3 levels were separated into AF (posterior) and NP while carefully avoiding the transition zone and trimming away outer ligamentous tissue. For whole-disc tests, lower levels of caudal discs (-Cd5) were isolated. Samples were washed three times in PBS supplemented with 2X antibiotic-antimycotic (Gibco) prior to testing or further preparation.

2.2. Demonstrating longitudinal piezoelectricity through the direct piezoelectric effect

The direct piezoelectric effect was investigated in the AF and NP by subjecting isolated samples to a stress and measuring the induced electrical potential. Samples were dehydrated to: (1) isolate piezoelectric effects from electrokinetic effects; (2) allow for stress application without large tissue deformation, and; (3) test the isolated NP, which was difficult to grip in the test equipment and was otherwise largely destroyed by the stress applied. Following washes, AF and NP samples were fixed in 4% paraformaldehyde (ThermoFisher) for 48 h to preserve the tissue structure. Samples were then dehydrated through immersion in graded ethanol washes and chemically dried using hexamethyldisilazane (HMDS; Sigma-Aldrich). Immersions took place in steps of varying ethanol dilutions: 10 mins each of 30%, 50%, 70%, 80%, 90% and 95% EtOH, followed by two immersions for 20 mins in 100% EtOH. Samples were dried by immersion in 50–50 EtOH-HMDS solution and two immersions in 100% HMDS for 20 mins each. Tissue was finally allowed to air-dry in a fume hood overnight (Fig. 1(a) and (b)).

Test equipment limited the application of stress to compression. Preliminary tests on AF samples indicated that compression in the circumferential direction had a similar piezoelectric behaviour but a lesser magnitude than compression in the longitudinal direction, and so, for simplicity, the stress applied was compression in the longitudinal axis for NP and AF samples. Samples were tested using a PiezoMeter system (PM300R; PiezoTest). After gripping between electrode clamps to a force of 10 N, samples were subject to an oscillating force of 0.25 N rms, during which the resultant longitudinal charge ($d_{33}$) was measured (Fig. 1(c)). Segments of posterior AF and full NP were tested from the Cd1, Cd2 and Cd3 levels of three bovine tails ($n=9$). Each sample was turned from an upright to inverted orientation five times to measure the repeatability of charge direction change. Testing of paper was expected to produce zero $d_{33}$ values as it is a non-piezoelectric material.

Fig. 1. Measurement of the direct piezoelectric effect in dehydrated samples. (a) Dehydrated AF sample. Scale bar = 5 mm. (b) Dehydrated NP sample. Scale bar = 5 mm. (c) Samples during testing in the Piezometer system which was used to apply a compressive force to the samples and record the resultant longitudinal charge generated.
material. Paper was used as a negative control sample for piezometer calibration.

2.3. Verification of piezoelectricity through the inverse piezoelectric effect

To verify the piezoelectric properties of the IVD, the inverse piezoelectric effect was investigated, whereby the deformation induced by electrical potential change is measured. This was measured by Piezoresponse Force Microscopy (PFM), a derivative of AFM. A voltage was applied at a conductive probe tip in contact with the sample surface and the resultant deformation measured by a photodiode system (further information in Supplementary Material).

Testing on dehydrated samples failed as the surface roughness was too irregular, even after trimming with a scalpel. Therefore, tissue samples were sectioned using a cryostat (CM1860; Leica Biosystems) to ensure a smooth surface. Following washes, AF and NP sections were similarly fixed in 4% paraformaldehyde prior to immersion in 30% sucrose at 4 °C overnight for cryoprotection.

Samples were then frozen using liquid nitrogen in Frozen Section Medium (Neg50; ThermoFisher) and through 2-methylbutane to control the rate of freezing. Samples were stored at −80 °C for <1 month before cryosectioning to 20 μm-thick sections adherent to glass slides (Superfrost Plus; Thermo Scientific). Sections were stored at −80 °C until 1 h before testing, during which samples were allowed to thaw and air-dry. To ensure an electrical current is passed through the sample and therefore grounding the equipment, sections were sputtered-coated with a layer of gold particles ~32 nm in thickness.

After attaching a copper electrode grounding pin, the sample was placed in the PFM equipment (NTegra Spectra; NT-MDT Spectrum Instruments). Semi-contact mode was used to generate a topographical image of a selected area. A contact mode scan of the same area produced measurements of vertical out-of-plane piezoelectricity. Relating areas of piezoresponse to topographical features on the semi-contact mode scan, point-curves were taken by sweeping the voltage between 0 and 40 V.

2.4. Preliminary testing of piezoelectricity in the physiological state

To determine the piezoelectric properties of physiological IVD tissue, AF and whole-disc samples were tested using the piezometer, similar to dehydrated samples. After washes, samples were stored in tightly-fastened plastic tubes to limit water evaporation and tested within 2 h of dissection. For AF tissue, segments of posterior AF were measured from the Cd1 and Cd2 levels of five bovine tails (n = 9). To allow gripping of the NP, the central NP portion of whole-disc samples were measured. Electrokinetic effects produced initially high $d_{33}$ values under the 10 N clamping load, but these were negated by only taking measurements once the charge decayed to a steady state. AF tissue was inverted five times, while disc samples were inverted only three times to prevent excessive NP tissue damage.

2.5. Data analysis and statistics

Piezometer results were ordered into upright/inverted values and averaged from five repetitions. This created groups of AF (dehydrated) (n = 27), NP (dehydrated) (n = 27), AF (hydrated) (n = 9) and NP (hydrated) (n = 6), each with upright and inverted $d_{33}$ values (averaged). Statistical analysis was performed using SPSS software (IBM). 95% Confidence Intervals (CI) were used to determine if upright/inverted charges are significantly positive/negative, respectively. To determine the average $d_{33}$ constant, the magnitudes of upright and inverted charge values were further averaged. As these $d_{33}$ values were therefore always positive, AF and NP values were skewed to the right, so the Mann-Whitney U test was used to determine differences between $d_{33}$ values of the AF and NP. The Kruskal-Wallis H test was used to investigate any differences in $d_{33}$ magnitude across disc levels. In PFM, representative curves of $d_{33}$ from local features were taken for analysis of the inverse piezoelectric effect to demonstrate the property (further data in Supplementary Material).

The slope of these point-curves was taken to calculate the piezoelectric coefficient:

$$d_{33}^{eff} \left( \frac{pm}{V} \right) = \frac{\text{Slope of piezoresponse curve}}{\text{Gain} \times \text{Input} \times \text{IOS} \times 1000} \quad (3)$$

where $d_{33}^{eff}$ is the effective piezoelectric coefficient and IOS (inverse optical sensitivity) is a conversion factor relating the recorded deformation to the desired unit of quantification (calculated as 0.05 in this study). Current/voltage curves were converted to deflection/voltage curves using eq. (3). To relate measurements to the piezoelectric voltage constant, and thus the voltage generated under load, the following equation was used (Guerin et al., 2019):

$$g_{33} \left( \frac{Vn}{N} \right) = \frac{d_{33} \left( \frac{pm}{V} \right)}{e_{33} \left( \frac{C}{m} \right)} \quad (4)$$

where $g_{33}$ is the piezoelectric voltage constant, $d_{33}$ is the piezoelectric strain constant and $e_{33}$ is the dielectric constant (calculated as 61.1 F/m at 110 Hz (Gabriel, 1996)), all in the longitudinal direction.

3. Results

3.1. The direct piezoelectric effect in dehydrated samples

Piezometer results on dehydrated sections demonstrate a weak but significant direct piezoelectric response in both AF and NP tissue (Fig. 2). AF upright piezoresponses are significantly likely to be positive (95% CI lower bound = 0.058 pC/N), while AF inverted piezoresponses are significantly negative (95% CI upper bound = −0.047 pC/N). This demonstrates a clear switching of charge direction upon inversion and therefore piezoelectric behaviour. NP tissue demonstrated similar behaviour. Regarding average $d_{33}$ values for each tissue type, AF tissue demonstrated a median $d_{33}$ magnitude of 0.073 pC/N, significantly greater than the 0.034 pC/N shown by NP tissue (p-value = 0.03). There was no difference in magnitude of $d_{33}$ for AF or NP tissue across the three levels of tissue tested (p-value >0.05).

3.2. The inverse piezoelectric effect

Semi-contact scanning of the AF revealed a highly-organised collagen matrix (Fig. 3(a)). Contact scanning revealed the high areas of piezoresponse across this area, which, after relating these areas to the image, corresponded to a sectioned collagen fibre end (longitudinal to fibre) as well as across the side of a fibre running parallel to the surface (transverse to fibre). Point-measurements were made at these features and representative curves shown (Fig. 3(c)). Semi-contact scanning of the NP revealed only a granular, unorganised matrix of ECM proteins (Fig. 3(b)). Contact scanning still highlighted areas of piezoresponse, however, for which point-measurements were similarly taken and current converted to deflection (Fig. 3(d)). Using the slope of current graphs with eq. (3), the $d_{33}^{eff}$ constant was found to be 1.38 pC/N longitudinal to the collagen in the AF, 0.87 pC/N transverse to the collagen in the AF, and 0.59 pC/N at an ECM protein in the NP.
3.3. Indications of the piezoresponse magnitude in-vivo

The $d_{33}$ found in hydrated samples was more variable than that of dehydrated sections (Fig. 4), with one orientation tending to centre towards zero. AF tissue generated a mean $d_{33}$ constant of 0.65 pC/N with a bias for charge direction to be positive. As such, while mean values of $d_{33}$ constant were 0.87 pC/N and −0.04 pC/N in the upright and inverted positions, respectively, the inverted values were not significantly likely to be negative (95% CI upper bound = 0.36 pC/N). NP tissue generated a mean $d_{33}$ of 1 pC/N, with a similar bias towards negative charge direction. Mean $d_{33}$ values were 0.19 pC/N and −1.23 pC/N in the upright and inverted positions, but upright values, in this case, were not significantly positive (95% CI lower bound = −0.98 pC/N). Only a third of AF samples (AF3, AF5 and AF6) and half of NP samples (NP1, NP4 and NP6) demonstrated true piezoelectric behaviour (consistent switching of charge direction upon inversion).

3.4. Determining in-vivo voltages generated by longitudinal piezoelectricity

To relate the piezoresponse found in tests, Eq. (4) was used to calculate the voltage generated as a function of applied load. For dehydrated samples, low magnitudes of 1.3 fVm/N and 0.74 fVm/N were generated in the AF and NP, respectively. But PFM measurements of the inverse piezoelectric effect yielded values as high as 22.6 fVm/N in the AF and 9.7 fVm/N in the NP. Hydrated testing suggests a similar piezoelectric voltage in the range of 12.7 fVm/N in the AF and 20 fVm/N in the NP. This agreement indicates that longitudinal piezoelectricity could be responsible for generating voltages in the range of 0.38–1.5 nV locally through the bovine caudal IVD.

4. Discussion

While biomechanics are thought to be a major factor in IVD degeneration, many parameters surrounding the cell response to physiological forces remain to be elucidated. In this study, we have demonstrated that piezoelectricity is generated through the loaded IVD, with greater voltages generated in the AF than in the NP, suggesting that this mechanism may incite a specific cell response. By dehydrating tissue, piezoelectric effects were effectively isolated from electrokinetic effects and subsequently demonstrated. However, removing the water content disrupted the native tissue structure by shrinkage, particularly in the NP which is composed of 85% water (Urban and McMullin, 1988). This limitation was overcome by demonstrating the inverse effect through PFM, whereby shrinkage of tissue upon drying was limited by covalently-bonding to the slide surface. These results indicate that components of both AF and NP tissue are piezoelectric, while the AF demonstrates a greater piezoresponse.

With regards testing on hydrated samples, the 10 N clamping load subjected the unconfined tissue to large deformations, thus disrupting the native structure upon which piezoelectricity is dependent (Denning et al., 2014). Furthermore, macro-scale measurements are a summation of local piezoresponses, shown by a 10-fold difference in $d_{33}$ values between nano-scale PFM and macro-scale piezometer measurements (Denning et al., 2017). These constraints may account for the insignificant switching of charge direction upon inversion. However, the magnitude of $d_{33}$ remained similar between all samples and selected samples, and also aligned with PFM measurements. As such, testing hydrated samples served to support the range of physiological magnitudes suggested for $d_{33}$ generated in-vivo.

Biological piezoelectricity has traditionally been thought to be confined to well-organised structures of fibrous proteins (Shamos 1988).
and Lavine, 1967), while collagen appears to be the primary constituent responsible (Minar-y-Jolandan and Yu, 2009; Williams, 1982). In agreement with this, the AF exhibited a greater piezoelectric effect than the NP. The AF is composed of lamellae of parallel collagen fibres running +/-60° to the vertical, while NP is composed of disorganised collagen fibres running through a proteoglycan-rich matrix (Urban and Roberts, 2003). The $d_{33}$ found here in the AF aligns well with similar measurements on tendon: 0.073 pC/N in AF versus 0.086 pC/N in tendon at the macro-scale, and 0.87 pC/N in AF versus 0.89 pC/N in tendon at the nano-scale (Denning et al., 2017). This supports the evidence of collagen-generated piezoelectricity in such tissues.

The recent discovery of piezoelectricity in globular proteins (Stapleton et al., 2017) suggests that non-collagenous proteins in the NP are responsible for piezoelectricity. However, the lower magnitudes found and the high native water content suggests that streaming potentials may dominate the electro-mechano coupling. While piezoelectricity may be more physiologically-relevant in the AF, the presence of a significant NP piezoresponse warrants study of the piezoelectric properties of aggrecan, laminin and other non-collagenous proteins.

IVD cells sense and respond to local electrical potential changes (Shin et al., 2019; Wang et al., 2017). Large voltages of ~3.5 μV/N, or ~1.4 mV, were found in the loaded disc in-vivo (Iatridis et al., 2009). However, longitudinal piezoelectricity found here only accounts for ~1 nV. But as argued by Ahn and Grodzinsky (2009), piezoelectricity may act in conjunction with streaming potentials by altering the zeta potential at the surface and influencing the charge generated. A response which may be more directly attributable to piezoelectricity is cell alignment. Cell alignment with collagen fibrils is related to cell polarization (Friedrichs et al., 2007; Li et al., 2014), whereby the polarised cell extends processes preferentially in the direction of the collagen axis. The cause of cell polarisation, itself, is less well understood, but Denning et al. (2012) argue that piezoelectricity may be responsible. The greater magnitude of piezoresponse in the AF owing to its structured collagen network supports this; AF cells typically align parallel to collagen fibrils, while NP cells are rounded and randomly-distributed (Horner et al., 2002). Further study is needed to elucidate the effects of electrical stimulation on IVD cell morphology, but this initial evidence infers the potential of incorporating such stimulation in regenerative efforts.

While large intra- and inter-sample variability was found across piezometer results, as evident by the range of data, this is a well-documented issue in macro-scale measurements caused by the
inhomogeneity of biological tissue (Williams, 1982). The cause of such discrepancies may be attributed to tissue shrinkage during dehydration, variation in sample size, surface area, location within posterior AF and number of lamellae. However, dehydrated tests demonstrate a significant direct piezoelectric effect, verified by PFM investigations.

In conclusion, this study demonstrates piezoelectricity across the IVD, with a greater effect in the AF. While the magnitude of voltage generated is small, only one tensor element of longitudinal piezoelectricity was studied. Physiologically, a range of piezoelectric coefficients could work in conjunction with other electrokinetic effects to influence the local electrical potential. Further study is required to investigate shear piezoelectricity, piezoelectric properties of non-collagenous proteins, and investigate the cell response. We anticipate that this work will open the field of electro-mechanical coupling to IVD cell mechanobiology to better inform regenerative and preventative treatment strategies from degeneration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiomech.2020.109622.

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