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Molecular transport in collagenous tissues measured by gel electrophoresis

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ABSTRACT

Molecular transport in tissues is important for drug delivery, nutrient supply, waste removal, cell signaling, and detecting tissue degeneration. Therefore, the objective of this study was to investigate gel electrophoresis as a simple method to measure molecular transport in collagenous tissues. The electrophoretic mobility of charged molecules in tissue samples was measured from relative differences in the velocity of a cationic dye passing through an agarose gel in the absence and presence of a tissue section embedded within the gel. Differences in electrophoretic mobility were measured for the transport of a molecule through different tissues and tissue anisotropy, or the transport of different sized molecules through the same tissue. Tissue samples included tendon and fibrocartilage from the proximal (tensile) and distal (compressive) regions of the bovine flexor tendon, respectively, and bovine articular cartilage. The measured electrophoretic mobility was greatest in the compressive region of the tendon (fibrocartilage), followed by the tensile region of tendon, and lowest in articular cartilage, reflecting differences in the composition and organization of the tissues. The anisotropy of tendon was measured by greater electrophoretic mobility parallel compared with perpendicular to the predominate collagen fiber orientation. Electrophoretic mobility also decreased with increased molecular size, as expected. Therefore, the results of this study suggest that gel electrophoresis may be a useful method to measure differences in molecular transport within various tissues, including the effects of tissue type, tissue anisotropy, and molecular size.

1. Introduction

Molecular transport in collagenous tissues is important for drug delivery (Bajpayee et al., 2014; Prausnitz and Noonan, 1998), contrast agent administration (Bansal et al., 2011; Kulmala et al., 2010), detecting tissue degeneration via changes in tissue architecture or composition (e.g., proteoglycan content in cartilage) (Bansal et al., 2011; Torzilli et al., 1997), cellular nutrient supply and waste removal (Knothe Tate et al., 1998; Maroudas et al., 1988), and cellular signaling (Knothe Tate et al., 2000; Swartz and Fleury, 2007). General mechanisms for molecular transport in tissues include mass transport due to a pressure gradient (i.e., fluid flow), concentration gradient (i.e., diffusion), or electric potential gradient (i.e., electrophoresis). Molecular transport within collagenous tissues is primarily impeded by cell networks (e.g., endothelium) (Mehta and Malik, 2006) and the macromolecular organization and composition of the extracellular matrix (Swartz and Fleury, 2007).

Forced fluid flow, or mass transport due to a pressure gradient, has been used to measure the permeability of collagenous tissues and tissue scaffolds (Pennella et al., 2013). A pressure gradient is induced across a tissue sample using either gravity feeding (O'Brien et al., 2007) or a piston/pump (Maroudas, 1968; Naumann et al., 1999). Darcy's law is used to calculate permeability from the flow rate, fluid pressure, and sample dimensions (Mow et al., 1984; Pennella et al., 2013). Challenges and limitations of this approach include a priori knowledge of the fluid properties (e.g., viscosity), non-uniform deformation of the specimen due to the fluid drag force, the application of high pressures, and sealing/clamping specimens (Maroudas, 1968; Mow et al., 1984; Pennella et al., 2013).

Molecular diffusion within collagenous tissues has been investigated by measuring the time-dependent transport of a solute or dye, often from a bath, in specimens due to a concentration gradient. Molecular concentrations within the tissue can be measured from cross-sectional profiles or sequential sectioning using colorimetric intensity (Brower et al., 1962; Kantor and Schubert, 1957), fluorescence (Decker et al., 2013; Knothe Tate et al., 1998; Lee et al., 2011), scintillation counting (Torzilli et al., 1997; 1998), X-ray

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attenuation in computed tomography (Arbabi et al., 2015; Bansal et al., 2011; Kulmala et al., 2010; Palmer et al., 2006), or magnetic resonance imaging (Bacic et al., 1997; Kulmala et al., 2010). Site-specific molecular diffusion and relative anisotropy have also been measured using fluorescence recovery after photobleaching (FRAP) and related techniques (Leddy and Guilak, 2003; Travascio et al., 2009). A limitation of these methods is that the diffusion of large molecules (e.g., >500 Da) can exceed the available time window for in vivo imaging prior to washout (removal) of the contrast agent (Burstein et al., 2001; Tiderius et al., 2003). Moreover, long incubation times for diffusion in excised tissue specimens can be prohibitive and possibly lead to erroneous measurements due to tissue degradation (Kantor and Schubert, 1957; Maroudas, 1970; 1976). Finally, the use of a bath requires a large volume of potentially costly solute or dye molecules and can lead to build-up at the tissue interface (Arbabi et al., 2015).

Electrophoresis can be used as an alternative and/or complementary method for measuring molecular transport in collagenous tissues to overcome limitations of the above methods. For example, the independent effects of fluid flow, diffusion, and electrophoresis on molecular transport in cartilage were previously investigated using an apparatus with electrodes added to a diffusion chamber (Minerva Garcia et al., 1996). Gel electrophoresis is commonly used to separate proteins of different size and charge for a variety of biochemical assays (Haas et al., 1994; Pluen et al., 1999; Rill et al., 2002). Thus, the necessary apparatus is readily available in most labs and relatively inexpensive. However, gel electrophoresis has not been previously used to measure molecular transport in collagenous tissues.

Therefore, the objective of this study was to investigate gel electrophoresis as a simple method to measure molecular transport in collagenous tissues. The electrophoretic mobility of charged molecules in tissue samples was measured from relative differences in the velocity of a cationic dye passing through an agarose gel in the absence and the presence of a tissue section embedded within the gel. Differences in electrophoretic mobility were measured for the transport of a molecule through different tissues and tissue anisotropy, or the transport of different sized molecules through the same tissue.

2. Materials and methods

2.1. Preparation of buffer and dye solutions

Tris/borate/ethylenediaminetetraacetic acid (TBE) buffer (5X) was prepared in deionized (DI) water with 54 g Trizma base ($\geq$99.9%, Sigma-Aldrich, St. Louis, MO), 27.5 g boric acid ($\geq$99.5%, Sigma-Aldrich), and 20 mL of 0.5 M ethylenediaminetetraacetic acid (EDTA). The EDTA solution was prepared by adding disodium EDTA$\cdot 2H_2O$ (99.0–101.0%, Sigma-Aldrich) to DI water under stirring and adjusting the pH to 8.0 using NaOH (Amresco, Solon, OH). As-prepared buffer solutions were passed through a 0.22 μm vacuum filter (Corning®, Sigma-Aldrich) to delay the formation of precipitates. Two cationic dyes were investigated: pyronin Y (302.8 g/mol, Sigma-Aldrich) and safranin O (350.8 g/mol, Amresco). Dye solutions were prepared by adding 5 mg of dye and 3 g sucrose (certified ACS, Fisher Scientific, Waltham, MA) to 7 mL of 1X TBE buffer.

2.2. Tissue samples and preparation

Fresh-frozen bovine flexor tendons were thawed and cut into 2.0 ± 0.2 mm thick sections either parallel or perpendicular to the collagen fibers within the proximal and distal regions which are known to experience distinct tensile and compressive forces, respectively, and exhibit corresponding histological characteristics resembling tendon and fibrocartilage, respectively (Benjamin and Ralphs, 1998; Koob and Vogel, 1987; Vogel and Evanko, 1987). Fresh-frozen bovine articular cartilage from the patella was also thawed and cut into 2.0 ± 0.2 mm thick sections parallel to the patellar surface. The planar area of all tendon and cartilage tissue sections was at least 1 cm^2. All tissue samples were stored in 1X TBE buffer for 12–24 h before embedding in agarose gels.

2.3. Gel electrophoresis

In order to accommodate tissue samples, a custom gel electrophoresis chamber was constructed using a $\sim 12\times 24$ cm^2 tray with a 0.07 mm diameter stainless steel wire cathode and replaceable anodes connected to an electrical power supply (BioRad PowerPac HC, Hercules, CA). The replaceable anodes were constructed out of plastic tabs (3×7 cm^2) with 0.07 mm diameter stainless steel wire wrapped twice around the bottom and extending to the top. Anodes were preemptively replaced after 45 min of use to prevent failure by corrosion.

The gel was prepared by adding 1 g agarose (molecular biology grade, Thermo Scientific, Rockford, IL) to 100 mL TBE buffer (1X). The solution was heated in a microwave oven for $\sim$2.5 min to dissolve the agarose, removed, and cooled for 5 min. The agarose solution was poured into a tray (8.95×10.55 cm^2 base) and the bottom of the comb was placed $\sim$3 mm above the base of the tray. Tendon and cartilage tissue samples were inserted into the warm liquid gel, directly against the comb. The middle lane of each gel was left open as a control lane. The agarose was solidified for 1 h at room temperature ($\sim$20°C). The solidified agarose gel was placed in the electrophoresis chamber which was filled with 1X TBE buffer to a depth of approximately 2 mm above the gel ($\sim$350 mL TBE). Dye solutions were pipetted in 10 μL aliquots into each well.

Each gel was run between 2 and 3 h at 80 V. After each run, the gel was removed from the tray, placed on a glass slide, and photographed at a resolution of $\sim$100 pixels/cm. The intensity profile of the dye in each lane was measured using ImageJ (v10.2, National Institutes of Health) and fit to a Gaussian distribution (Fig. 1) to measure the mean distance traveled by the dye. The pooled mean coefficient of determination (R^2) for all samples was 0.865. The rate of dye migration through the tissue sample, v (cm/s), was derived (Appendix A) as,

$$v=\frac{w}{t\left(1-\frac{d_i-w}{d_o}\right)} \quad (1)$$

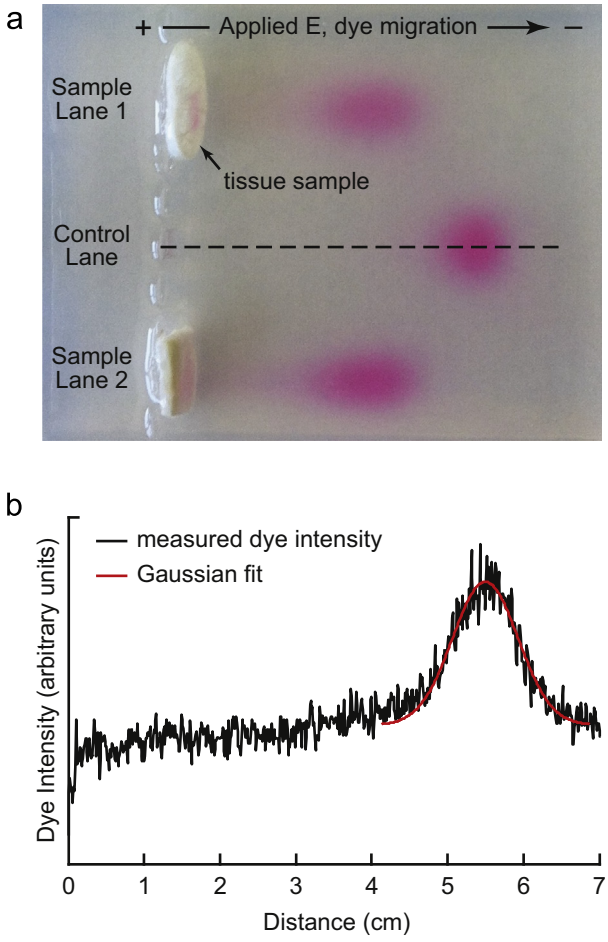

Fig. 1. Electrophoretic mobility in response to an applied electric field (E) was measured in an (a) agarose gel by the delayed migration of a cationic dye through embedded tissue samples (sample lanes) compared to the gel alone (control lane) as quantified by (b) the mean distance of migration from a Gaussian fit of the line intensity profile of each lane.

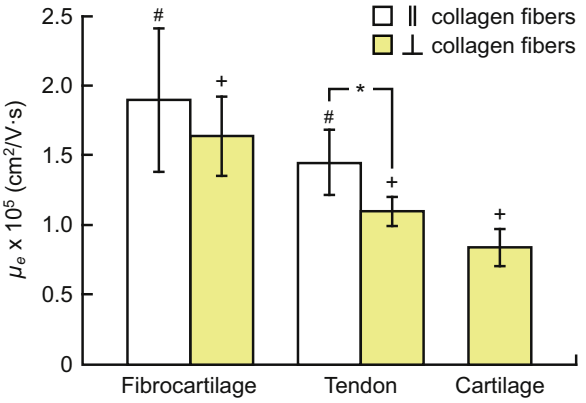

Fig. 2. The electrophoretic mobility, μ_e, of pyronin Y (302.8 Da) in fibrocartilage, tendon, and cartilage tissue samples either parallel (∥) or perpendicular (⊥) to the predominate collagen fiber orientation. Error bars show one standard deviation of the mean. The measured electrophoretic mobility was greatest in the compressive region of the tendon (fibrocartilage), followed by the tensile region of tendon, and lowest in cartilage ($^{+}p < 0.05$, t-test, ⊥; $^{\#}p = 0.05$, t-test, ∥). Tendon was anisotropic with greater electrophoretic mobility parallel compared with perpendicular to the predominate collagen fiber orientation ($^{*}p < 0.05$, t-test).

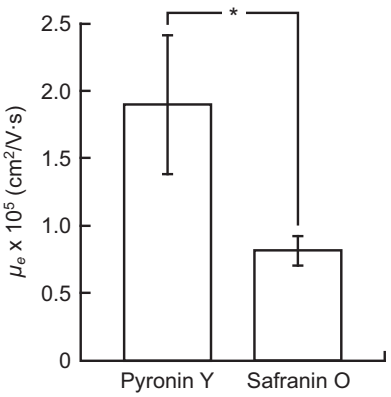

Fig. 3. The electrophoretic mobility, μ_e, of pyronin Y (302. Da) and safranin O (350.8 Da) in fibrocartilage tissue samples parallel to the predominate collagen fiber orientation. Error bars show one standard deviation of the mean. The electrophoretic mobility was significantly decreased for the larger molecule ($^{*}p < 0.0001$, t-test), as expected.

where w is the tissue sample thickness (cm), t is the elapsed time (s), d_i is the mean distance traveled by the dye in the sample lane (cm), and d_o is the mean distance traveled by the dye in the control lane (cm). The electrophoretic mobility, μ_e (cm^2/V s), for passing the dye through the tissue sample was then calculated as,

$$\mu_e = \frac{v}{E} \tag{2}$$

where v is the rate of migration through the tissue sample (Eq. (1)) and E is the applied electric field strength (V/cm).

2.4. Statistical methods

Electrophoretic mobility was reported as the mean (±standard deviation) of tissue sample replicates in each experimental group (n=5–9/group). Differences between groups were compared using one- and two-way analysis of variance (ANOVA; JMP 11, SAS Institute, Cary, NC). Planned pairwise comparisons were performed using unpaired Student's t-tests. The level of significance for all tests was set at $p < 0.05$.

3. Results

The electrophoretic mobility of pyronin Y was measured in tendon, fibrocartilage, and cartilage tissue specimens either parallel or perpendicular to the predominate collagen fiber orientation. The measured electrophoretic mobility was greatest in the compressive region of the tendon (fibrocartilage), followed by the tensile region of tendon, and lowest in the cartilage ($p < 0.05$, t-test) (Fig. 2). Tendon exhibited anisotropy with greater electrophoretic mobility parallel compared with perpendicular to the predominate collagen fiber orientation ($p < 0.05$, t-test) (Fig. 2). The anisotropy exhibited by fibrocartilage was not statistically significant ($p = 0.35$, t-test).

The electrophoretic mobility of pyronin Y (302.8 Da) and safranin O (350.8 Da) was compared in fibrocartilage tissue samples parallel to the predominate collagen fiber orientation. The electrophoretic mobility was significantly decreased ($p < 0.0001$, t-test) with increased molecule size (Fig. 3), as expected.

4. Discussion

Gel electrophoresis was able to measure differences in molecular transport within different collagenous tissues, including cartilage, fibrocartilage, and tendon (Fig. 2). Note that molecular transport in cartilage has been widely studied, but comparable measurements for fibrocartilage and tendon, including anisotropy, did not previously exist. Tendon and fibrocartilage specimens were harvested from the tensile and compressive regions, respectively, of bovine flexor tendons and thus also represented differences in anatomic location within the same tissue. Gel electrophoresis was also able to measure differences in molecular transport due to the effects of tissue anisotropy in tendon (Fig. 2). These measured differences in molecular transport, or electrophoretic mobility, reflected differences in the composition and organization of the tissues.

Cartilage exhibited the lowest overall electrophoretic mobility for the cationic dye amongst the tissues (Fig. 2). Articular cartilage comprises ~25 wt% (dry) anionic proteoglycan aggregates (Buckwalter and Mankin, 1997; Zhang and Szeri, 2005), compared with ~3.5 wt% proteoglycans in fibrocartilage (Vogel and Heinegard, 1989) and ~0.2–0.5 wt% proteoglycans in tendon (Vogel and Heinegard, 1985). Moreover, articular cartilage comprises primarily type II collagen fibers (Buckwalter and Mankin, 1997; Zhang and Szeri, 2005), which are primarily oriented parallel to the articular surface in superficial regions and perpendicular to the articular surface in deep zones below the surface (Fig. 4) (Mow et al., 1974). However, the measurements in this study neglected zonal differences (Arbabi et al., 2015), and the relatively small thickness (~2 mm) of articular cartilage prevented measurements of electrophoretic mobility, and thus anisotropy, normal to the tissue thickness. Thus, the lowest overall molecular transport measured in articular cartilage primarily reflected the fixed charge associated with a substantially greater anionic proteoglycan content, as proteoglycans are ~7X and ~50–125X more abundant in articular cartilage than fibrocartilage and tendon, respectively (Fig. 4) (Buckwalter and Mankin, 1997; Vogel and Heinegard, 1985, 1989; Zhang and Szeri, 2005).

Fibrocartilage from the distal region of bovine flexor tendon exhibited the greatest overall electrophoretic mobility for the cationic dye amongst the tissues and less, if any, anisotropy compared with tendon (Fig. 2). The distal region of the bovine flexor tendon is adapted to primarily compressive loading such that fibrocartilage exhibits greater cellularity, more loosely packed and disorganized collagen fibers exhibiting an interwoven organization, greater amounts of anionic glycosaminoglycans (GAGs), including elevated levels of large (~220 kDa) aggrecan proteoglycans, and thus greater water retention compared with tendon (Fig. 4) (Benjamin and Ralphs, 1998; Koob and Vogel, 1987; Vogel and Evanko, 1987). Tendon exhibited lower overall electrophoretic mobility for the cationic dye compared with fibrocartilage, and distinct anisotropy (Fig. 2). The proximal region of the bovine flexor tendon is adapted to primarily tensile loading

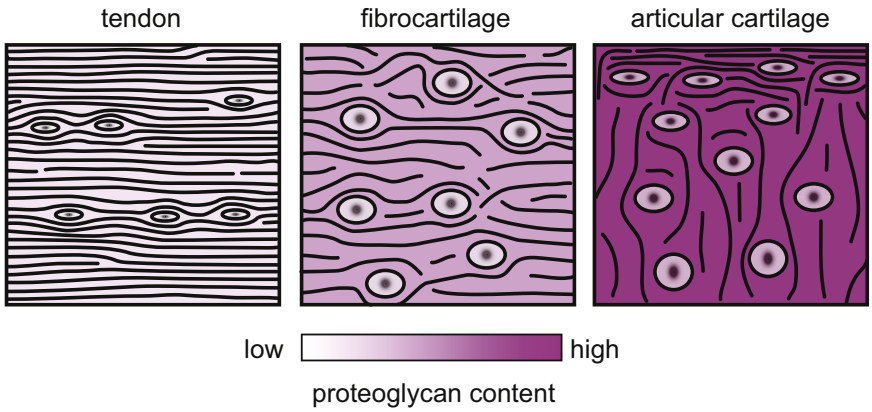

Fig. 4. Schematic diagram showing key differences in the composition (e.g., proteoglycan content and cellularity) and organization (e.g., collagen fiber density and orientation) of the collagenous tissues investigated in this study, including tendon, fibrocartilage, and articular cartilage, which governed differences in the electrophoretic mobility or molecular transport of cationic dyes measured using gel electrophoresis.

Table 1
Mean (± standard deviation) diffusion coefficients, D, calculated using the Nernst–Einstein equation (Eq. (3)) from the electrophoretic mobility, μ_e, measured by gel electrophoresis of pyronin Y (302.8 Da) and safranin O (350.8 Da) in fibrocartilage, tendon, and cartilage tissue samples either parallel or perpendicular to the predominate collagen fiber orientation. Note that fibrocartilage and tendon samples were taken from the compressive and tensile regions, respectively, of the bovine flexor tendon.

Tissue	Direction	Dye	$\mu_e \times 10^5$ (cm^2/V s)	D (μm^2/s)
Fibrocartilage	parallel	pyronin Y	1.89 (0.51)	48.2 (13.1)
	parallel	safronin O	0.79 (0.11)	20.0 (2.9)
	perpendicular	pyronin Y	1.64 (0.28)	41.6 (7.2)
Tendon	parallel	pyronin Y	1.45 (0.23)	36.8 (6.0)
	perpendicular	pyronin Y	1.10 (0.10)	27.9 (2.7)
Articular cartilage	perpendicular	pyronin Y	0.84 (0.13)	21.3 (3.4)

such that tendon exhibits lower cellularity, more densely packed and uniaxially aligned type I collagen fibers, lower amounts of anionic GAG content comprising primarily small dermatan sulfate proteoglycans, and thus lower water content compared with fibrocartilage (Fig. 4) (Benjamin and Ralphs, 1998; Connizzo et al., 2013; Koob and Vogel, 1987; Vogel and Evanko, 1987). Thus, the measured differences in molecular transport between tendon and fibrocartilage primarily reflected known differences in the collagen fiber density and organization. Moreover, the results of this study may suggest that the effects of collagen fiber density and organization were more influential than the effects of composition (proteoglycan content) which would have been expected to produce opposite differences in molecular transport.

The Nernst–Einstein equation was used to convert measurements of electrophoretic mobility into a diffusion coefficient as,

$$D = \frac{k_B T}{Q_{eff}} \mu_e \tag{3}$$

where D is the diffusion coefficient (m^2/s), k_B is Boltzmann's constant (J/K), T is the absolute temperature (K), Q_{eff} is the molecule charge, and μ_e is the electrophoretic mobility (m^2/V s). The calculated diffusion coefficients spanned ~20–50 μm^2/s and exhibited the same trends as electrophoretic mobility (Table 1). Numerous previous measurements for diffusion coefficients in articular cartilage using other methods have spanned ~10–600 μm^2/s for similarly sized molecules ranging 100–500 Da in molecular weight (Evans and Quinn, 2006; Jackson and Gu, 2009; Kulmala et al., 2010; Lee et al., 2011; Maroudas, 1968, 1970, 1976; Torzilli et al., 1987, 1997, 1998). The diffusion coefficient measured in this study for positively-charged pyronin Y in articular cartilage was ~20 μm^2/s which was within the lower range of previous measurements likely because these previous measurements primarily reflected uncharged molecules. Therefore, this general agreement and the wide range of previous measurements supports the use of gel electrophoresis as a simple, repeatable method for assessing molecular transport in collagenous tissues.

Gel electrophoresis was also able to detect differences in molecular transport due to a small change in molecular weight of less than 50 Da between two cationic dyes (Fig. 3). Molecular transport measured by electrophoretic mobility, or diffusivity, was inversely related to the molecular size, as expected. Numerous previous studies have reported a similar relationship, but typically for much larger differences in molecular size (Jackson and Gu, 2009; Kulmala et al., 2010; Maroudas, 1970, 1976; Torzilli et al., 1987, 1997, 1998).

The resistance (or permeability) of collageneous tissues to molecular transport is governed by the macromolecular organization and composition of the extracellular matrix (Swartz and Fleury, 2007) regardless of whether transport is driven by a pressure gradient (i.e., fluid flow), concentration gradient (i.e., diffusion), or electric potential gradient (i.e., electrophoresis). A modification of the Nernst–Planck equation can be used to relate or separate the effects of each driving force on molecular transport (Minerva Garcia et al., 1996). The Nernst–Einstein equation (Eq. (3)) was used in this study to determine a diffusion coefficient from the electrophoretic mobility, assuming negligible diffusion independent of electrophoresis under the conditions of this study, but could also be modified to also account for diffusion independent of electrophoresis and/or electro-osmosis. The contribution of electro-osmotic flow or mobility could be measured for a neutral molecule of equivalent size to a charged molecule. Moreover, capillary electrophoresis has been used to measure membrane permeability (Örnskov et al., 2005). Therefore, gel electrophoresis could be used as an alternative method investigating tissue permeability when methods for measuring hydraulic permeability or diffusion are not practical.

Despite the aforementioned advantages, gel electrophoresis is not without some limitations for measuring molecular transport within tissues. Molecules must be charged under physiologic conditions for investigating transport through tissues. Measurements for larger charged molecules may become impractical, similar to diffusion measurements, but the enhanced transport in electrophoresis would be expected to be more favorable. Tissue samples must be prepared by sectioning which may result in altered composition or organization. However, a previous study measured that less than 5% of GAGs were lost during the application of an electric field to sectioned cartilage specimens over a similar duration in time as this study (Minerva Garcia et al., 1996).

The tissue section thickness could be varied to tailor the rate of transport for investigating different sized molecules within a reasonable time frame. The results of this study also identified avenues for further investigation of the measured differences in molecular transport between cartilage, fibrocartilage, and tendon. The relative influence of tissue composition (proteoglycan content) versus organization (collagen fiber density and orientation) could be elucidated by measurements using dyes of varying size and charge, and by specimen-specific characterization of proteoglycan content and collagen density.

In summary, gel electrophoresis was demonstrated as a simple method able to measure differences in molecular transport within collagenous tissues, including differences due to tissue type (cartilage, fibrocartilage, and tendon), anatomic location, anisotropy, and molecular size. Gel electrophoresis utilized simple, low-cost, and widely-available instrumentation compared with methods for measuring molecular diffusion using fluorescence, nuclear imaging, computed tomography, magnetic resonance imaging, or hydraulic permeability. Molecular transport was inhibited by greater proteoglycan content, greater collagen fiber density, directions perpendicular to aligned collagen fibers (anisotropy), and greater molecular size, in agreement with previous measurements by other techniques. However, the vast majority of previous studies were focused on measurements of molecular transport in cartilage and the results of this study also provided new, previously unavailable data for molecular transport within fibrocartilage and tendon, including anisotropy.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

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Appendix A. Derivation of the dye velocity in the tissue sample during gel electrophoresis

The dye velocity within the tissue sample in the sample lane was derived from the measured dye velocity in the control lane and sample lane assuming: (1) constant dye velocity with time (no acceleration), (2) constant dye velocity in the agarose gel in either the control or sample lane, (3) no effect of the agarose–tissue interface on the dye velocity, and (4) the total elapsed time in the sample lane, t, was equal to the sum of the elapsed time in the tissue sample, t_{tissue}, and agarose gel, $t_{agarose}$, as,

$$t = t_{tissue} + t_{agarose} \tag{4}$$

The dye velocity in the agarose gel of the sample lane was set equal to the control lane as,

$$\frac{d_i - w}{t_{agarose}} = \frac{d_o}{t} \tag{5}$$

where w is the tissue sample thickness, d_i is the distance traveled by the dye in the sample lane, and d_o is the distance traveled by the dye in the control lane (cm). Eq. (5) was substituted into Eq. (4) as,

$$\frac{d_i - w}{d_o} t = t_{agarose} = t - t_{tissue} \tag{6}$$

which simplifies to,

$$t_{tissue} = t\left(1 - \frac{d_i - w}{d_o}\right) \tag{7}$$

Therefore, the dye velocity in the tissue sample, v, is,

$$v = \frac{w}{t\left(1 - \frac{d_i - w}{d_o}\right)} \tag{8}$$

Assumption (3) was verified by inserting 2 mm thick agarose samples prepared at 2–5% into the agarose gel in place of tissue samples and measuring no change in the dye velocity relative to the control lane. An additional control experiment was conducted by inserting similarly sized impermeable plastic specimens into the gel to verify that dye migration was blocked and the dye was unable to migrate around the impermeable specimen.

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