NDnano Undergraduate Research Fellowship (NURF)
2013 Project Summary

1) **Student name:** Michael Hunckler
2) **Faculty mentor name:** Dr. Jenni Tilley and Dr. Ryan Roeder
3) **Project title:** Feasibility of Gel Electrophoresis to Determine the Permeability Properties of Tendons

4) **Briefly describe any new skills you acquired during your summer research:**
Over the course of the summer, I have learned to optimize many facets of the gel electrophoresis method. I have optimized the construction of a low cost electrophoresis chamber, various tendon cutting techniques, run times, voltage levels, electrode material, and gel analysis techniques. I have also been taught to optimize efficiency and organization when reading dozens of literature articles, enabling easy reference throughout the project.

5) **Please briefly share a practical application/end use of your research:**
Though it may not be feasible to apply voltage to a tendon *in vivo*, the gel electrophoresis can reveal many structural components and composition of the tendon tissue. Gel electrophoresis reveals relative mobilities of various sized dyes through various regions of the tendon. From the gel electrophoresis tests, the compressive region of the tendon and the parallel orientation of the collagen fibers yielded faster mobilities. Such knowledge would have important implications for the feasibility of gold nanoparticles (AuNPs) as contrast agents for imaging tendon degeneration.

Project Summary:

 Prevalence of spontaneous tendon rupture has increased dramatically in recent decades, and is typically an end-state manifestation of degenerative changes within tendons. A prominent pathological change associated with tendon degeneration is mucoid degeneration (increased concentrations of proteoglycans (PG) and glycosaminoglycans (GAG)) between the collagen fibers. The ability to non-invasively detect changes in PG and GAG concentrations will therefore facilitate monitoring of disease progression. Cationic functionalized gold nanoparticles (AuNPs) have been proposed as an X-ray contrast agent that will bind to the negatively charged PGs and GAGs. To be successful, these contrast agents must diffuse throughout the tendon tissue. However, a thorough literature review has revealed that little is known regarding the diffusive properties of tendon. Furthermore, preliminary *in vitro* incubation experiments have failed to observe significant penetration of AuNPs, or other dye molecules, into tendon tissue in the absence of a significant driving force. The objective of this research is to determine the feasibility of gel electrophoresis to determine the permeability properties of tendon.

 Slices of bovine foot tendons were embedded in agarose gel. Various samples were cut parallel and perpendicular to the collagen fibers within both the compressive and tensile regions of the tendon. Using gel electrophoresis, the cationic dye Pyronin Y was passed through the agarose gel in the absence and presence of different tendon slices. The intensity profile of the dye path was measured using ImageJ and a Gaussian fit was performed to determine the distance 50% of the dye had traveled. The electrophoretic mobility (cm^2/Vs) of the dye through tendon was then calculated by the following equation: rate of migration/electric field. The results

revealed that in the tensile region of the tendon, the electrophoretic mobility was higher in the parallel direction than in the perpendicular direction ($p<0.05$). However, there was no significant difference between the electrophoretic mobility in the perpendicular and parallel directions within the compressive region ($p>0.05$). These results are in agreement with the structure and composition of the various tendon regions, and suggest that gel electrophoresis may be a feasible method with which to measure the permeability and diffusive properties of tendon tissue.
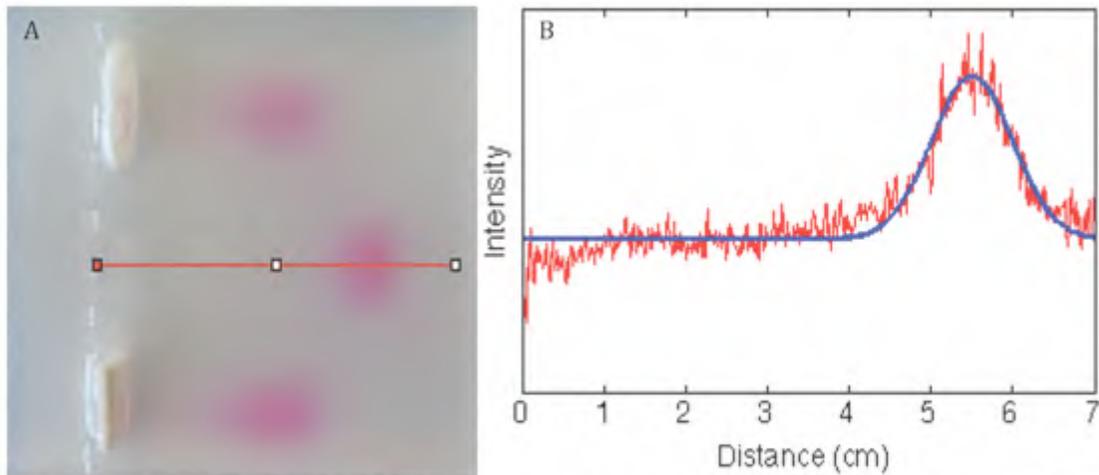
A) Agarose gel with embedded tendon slices and the Pyronin dye moving through the gel. The intensity of the line in the middle lane is analyzed (B). A Gaussian fit is used to find the distance 50% of the dye traveled.

Publications (Poster and Paper)

A poster was presented at the 2013 Summer Undergraduate Research Symposium. A research paper was also written to summarize my research (begins on the following page).

Feasibility of Gel Electrophoresis to Determine the Permeability Properties of Tendons

Michael D. Hunckler, Dr. Jenni Tilley, Dr. Ryan Roeder

University of Notre Dame, Notre Dame, IN 46556

Introduction

- Prevalence of spontaneous tendon rupture has increased dramatically in recent decades. Rupture is typically an end-state manifestation of degenerative changes within the tendon tissue.[1,2]
- Mucoid degeneration is a prominent pathological change associated with tendon degeneration. This is characterized by increased concentrations of proteoglycans (PG) and glycosaminoglycans (GAG) between collagen fibers[3].
- The ability to non-invasively detect changes in PG and GAG concentrations will therefore facilitate monitoring of disease progression.[4]
- Gold nanoparticles (AuNPs) with cationic functional groups have been proposed as an X-ray contrast agent that will bind to the negatively charged PGs and GAGs, thus enabling detection.
- To date, there is no literature on permeability and diffusive properties of tendons. Furthermore, preliminary *in vitro* incubation experiments have failed to observe significant penetration of AuNPs, or other dye molecules, into the tendon tissue, presumably due to the absence of a significant driving force.
- **The objective of this feasibility project is to determine if gel electrophoresis is a viable method to measure the permeability of various dyes through the tendon tissue.**
- We hypothesize that electrophoretic mobility will be influenced by fiber orientation within different tendon samples (e.g. tensile=uniaxial fibers, compressive= biaxial fibers) and dye molecular weight (M_w).

Materials/Methods

- Frozen bovine foot tendons were sliced parallel and perpendicular to the collagen fibers within the compressive and tensile regions.
- The samples were defrosted in Tris/Borate/EDTA (TBE) Buffer (1X) for 12-24 h before being embedded in 1% agarose.
- Each gel was placed in a TBE bath with electrodes at either end and 0.05 %(w/v) of either Pyronin Y or Safranin O (cationic dyes) were loaded into the wells (Figure 1).
- The gel was run for either 2h (Pyronin Y) or 3h (Safranin O) at 80V. The gels were then imaged, and the intensity of the dye path was measured using a macro written using ImageJ.
- A Gaussian fit was performed on the intensity profile using Matlab, and the maximum point (50%) was recorded as the distance traveled by the dye (Figure 2B).
- The rate of migration of the dye through the tendon was calculated using Eqn. 1, where w = thickness of tendon slice (cm), t = time elapsed (s), s = total distance travelled by dye in sample lane (cm), and c = total distance travelled by dye in control lane (cm). Electrophoretic mobility (EM) was then calculated using Eqn. 2.
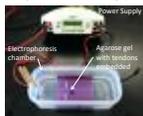

Figure 1: Setup of gel electrophoresis chamber

$$\text{Rate of migration} = \frac{w}{t\left(1-\frac{s-w}{c}\right)} \quad (1)$$

$$\text{EM (cm}^2\text{/Vs)} = \frac{\text{Rate of migration}}{\text{Electric field}} \quad (2)$$
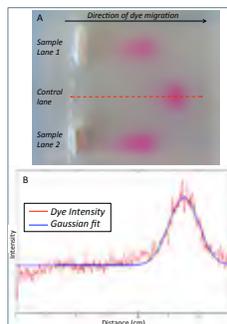

Figure 2: Analysis of distance travelled by dye. (A) Agarose gel with tendon slices embedded. Cationic dye travels to the right through the tendon. The line along which an intensity profile was extracted is represented by a dashed line. (B) Corresponding intensity profile along the dashed line, and the Gaussian fit used to determine the distance travelled by 50% of the dye.

Results and Discussion

- In tensile samples, EM was higher in the direction parallel to collagen fiber orientation compared with perpendicular to collagen fiber orientation, regardless of which dye was used (Figure 4, 5). This suggests that fiber orientation influences dye mobility.
- Within the compressive region there was no difference between EM in the direction parallel and perpendicular to fiber orientation (Figure 4), possibly reflecting the biaxial orientation of fibers in these samples.
- Dye M_w also affected electrophoretic mobility, with the larger dye, Safranin O (M_w= 350.8 g/mol), exhibiting a decreased EM compared with the smaller dye, Pyronin Y (M_w= 302.8 g/mol) (Figure 5).
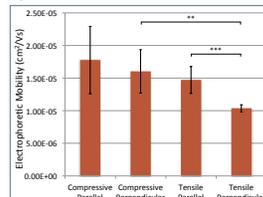

Figure 4: EM of Pyronin Y perpendicular and parallel to collagen fiber orientation in the compressive and tensile region of tendon. Results are shown as mean ± SD. Statistical significance (t-test) is indicated using standard notation (*=p<0.05, ** =p<0.01, ***=p<0.001).
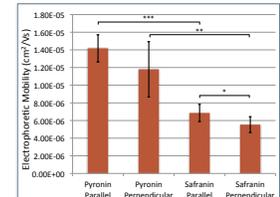
Figure 5: EM perpendicular and parallel to collagen fibers orientation of Pyronin Y (302.8 g/mol) and Safranin O (350.8 g/mol) in tensile samples. Results are shown as mean ± SD. Statistical significance (t-test) is indicated using standard notation (*=p<0.05, ** =p<0.01, ***=p<0.001).

Conclusions

- In agreement with our hypothesis, these results indicate that EM is influenced by fiber orientation and dye M_w. Specifically, EM is greater parallel to the collagen fibers and for smaller dyes.
- These results demonstrate that gel electrophoresis is a highly suitable technique for measurement of the permeability and diffusive properties of tendon tissue.

Future Work

- Additional tests with various sized and charged dyes should now be performed to obtain a greater understanding of tendon permeability.
- The effect of trypsin degradation of tendon tissue on the permeability properties should also be investigated in order to elucidate the influence of PG and GAG concentrations.

References

1. Kannus, P. *et al.* (1991) J Bone Joint Surg 73(10):1507-25.
2. Järvinen, M. *et al* (1997) Scand J Med Sci Spor 7(2):86-95.
3. Bacic, G. *et al* (1997) Magn Reson Med 37(5):764-8.
4. Palmer, A. *et al* (2006) Proc Natl Acad Sci 103(51): 19255-66.

Acknowledgements

Feasibility of gel electrophoresis to determine the permeability properties of tendons

Michael Hunckler, Dr. Jenni Tilley, Dr. Ryan Roeder
Department of Aerospace and Mechanical Engineering, University of Notre Dame

INTRODUCTION

The prevalence of spontaneous tendon rupture has increased dramatically during recent decades.[1] Clinically, this condition is typically an end-state manifestation of degenerative changes within the tendon tissue, indicated by pathological changes observed in *ex vivo* characterization of the tendons. One prominent pathological change is mucoid degeneration, wherein large vacuoles composed of proteoglycans (PG) and glycosaminoglycans (GAG) appear between the collagen fibers.[2]

While PG content can be assessed with histological analysis of biopsy samples, such assays cannot monitor progressive changes in tendon tissue.[3] Therefore, the ability to noninvasively detect changes in PG and GAG concentrations could provide an indication to the integrity of the tendon and potentially monitor disease progression.[4]

Previous attempts have been made to correlate PG content with MRI characteristics (T_1, T_2, and proton density), but no significant changes have been observed with the depletion of PG.[5] Radiography is a viable option for imaging tendons because it is more widely accessible, less expensive, enables rapid reconstruction of the tissue, has improved spatial resolution, and faster image acquisition times compared with MRI.[6] Owing to the low X-ray attenuation of tendon tissue, X-ray imaging of tendon would require the use of a contrast agent. It has been proposed that a positively charged contrast agent, specifically gold nanoparticles (AuNPs) with cationic functional groups, would bind to the negatively charged PGs and GAGs, making them detectable with X-ray imaging, thus facilitating monitoring of tissue degeneration.

For AuNP contrast agents to be successful, they must diffuse through the tendon and bind to the PGs and GAGs. However, a thorough search of the literature has revealed that, to date, there have been no studies of the permeability or diffusivity of molecules through tendon tissue.

In contrast, there is a substantial amount of literature pertaining to diffusion and permeability through articular cartilage. While the structures of tendon and articular cartilage tissues are different (Figure 1), the components are very similar, although in different proportions (Table 1). Cartilage and tendons are also both relatively avascular, relying primarily on diffusion to transport nutrients and waste products.[7]

Table 1. Dry tissue weight composition articular cartilage and tendon tissue.[8,9]

	Tendon	Articular Cartilage
Collagen	86%	65%
Proteoglycans	1-5%	25%

The main structural difference between tendons and cartilage is the orientation of the collagen fiber network. In cartilage, the collagen fibers have a random orientation with proteoglycans interspersed throughout (Figure 1a).[10] In tendons, the collagen fibers run parallel to the tendon long-axis, and proteoglycans form a matrix between the fibers (Figure 1b).[11] Knowledge of cartilage diffusion properties may therefore shed some light on the expected permeability and diffusion behavior of tendon tissue.

Numerous studies have investigated the diffusion and permeability properties of articular cartilage, and found them to be strongly dependent on the structure and composition of the tissue. For example, Kantor et al found bovine nasal cartilage to be permeable to thirteen cationic dyes and impermeable to all but one of fourteen anionic dyes they tested, and suggested that this was probably due to the fixed anionic charge associated with the negatively charged PG and GAG content.[12] Furthermore, Kulmala et al suggested that the widely spaced collagen fibrils within cartilage hinder the diffusion of large molecules (40-500 kDa) while proteoglycans are the primary cause of hindered diffusion of small molecules (3 kDa).[13,14] Several studies have found that diffusion rate through cartilage is inversely proportional to the size of a solute and charge.[9,14,15] Based on these studies, we propose that small (<<3 kDa) positively charged dye molecules, such as Pyronin Y (302.8 Da) and Safranin O (350.8
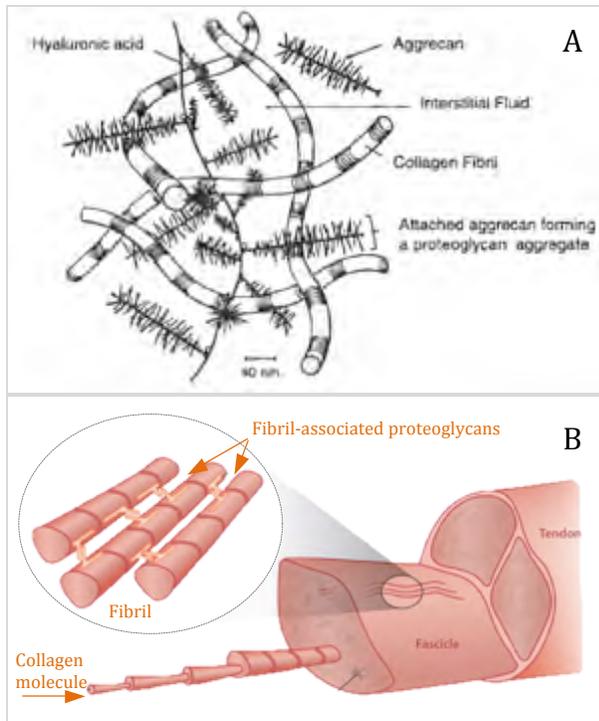
Figure 1. Articular cartilage structure, comprising of randomly oriented collagen fibers and proteoglycans (A).[10] Tendon structure, comprising of parallel collagen fibers and proteoglycans (B).[11]

Da), may be the most suitable molecules for measurement of diffusion through the tendon.

Given the long-term objective of diffusing AuNPs into tendon in order to visualize tissue degeneration using X-rays, the goal of this study is to measure the diffusive and permeability properties of tendons. Preliminary *in vitro* incubation experiments measuring the diffusion through tendons failed to observe significant penetration of the dye in the absence of a driving force. Thus, gel electrophoresis has been proposed as a means of driving the charged dye through the tendon. The objective of this feasibility project is to determine if gel electrophoresis is a viable alternative to measuring the permeability of the tendon from different locations within the tendon (compressive versus tensile) and in different orientations (parallel and perpendicular to the collagen fiber axis) to different sized cationic dyes (Pyronin Y (M_w=302.8 Da) and Safronin O (M_w=350.8 Da)). Based on the previous findings for cartilage studies, we hypothesize that these small (<<3 kDa) dye molecules will readily penetrate the tendon

tissue, but that origin and orientation of the tendon samples will affect the overall mobility of the dyes within the tissue.

MATERIALS AND METHODS
Preparation of solutions
Tris/Borate/EDTA (TBE) buffer (5x) was prepared using the Cold Spring Harbor Protocols[16]. 0.5 M EDTA was prepared by adding 186.1 g disodium EDTA•2H$_2$O (99.0-101.0%, Sigma-Aldrich, St. Louis, MO) to 1 L of de-ionized (DI) water, and the pH was adjusted to 8.0 using NaOH (Amresco, Solon, Ohio) while stirring. A 5X stock solution of TBE was prepared in 1 L of DI H$_2$O with the following: 54 g Trizma base (≥99.9%, Sigma-Aldrich, St. Louis, MO), 27.5 g boric acid (≥99.5%, Sigma-Aldrich, St. Louis, MO), and 20 mL of 0.5 M EDTA (pH 8.0). The stock was passed through a Corning 0.22-µm filter (Sigma-Aldrich, St. Louis, MO) to delay the formation of precipitates.

The two dyes used were Pyronin Y (50%, Sigma-Aldrich, St. Louis, MO) and Safranin O (Amresco, Solon, Ohio). 5 mg of dye was added to 3 g sucrose (certified ACS, Fisher Scientific, Waltham, MA) and 7 mL of TBE buffer (1X).

Electrophoresis chamber construction
A gel electrophoresis chamber was constructed with a 1.2 liter Rubbermaid container (11.99 x 24.10 x 7.31 cm). 0.07 mm stainless steel wire attached to the bottom with hot glue and routed over the edge of the container served as a cathode, while replaceable electrodes were used for the anode. The replaceable anodes were constructed out of plastic tabs (7.2 x 2.9 cm) with 0.07 mm stainless steel wire wrapped twice around the bottom and extending to the top. A clothespin was used to hold the anode tab in place (Figure 2).

Tendon samples and preparation
Frozen bovine foot tendons were cut into 2±0.2 mm thick sections with a #22 scalpel blade (Miltex, York, PA). Various samples were cut parallel and perpendicular to the collagen fibers within the compressive and tensile regions of the tendon. The samples were stored in TBE buffer (1X) for 12-24 hours before being placed in the pre-solidified agarose gel.

Casting the agarose gels

1 g agarose (Fisher Scientific, Waltham, MA) was added to 100 mL TBE Buffer (1X). The solution was microwaved for ~2.5 minutes until the agarose was completely dissolved. The solution was removed and let to slightly cool for 5 minutes. The ends of the casting tray (8.95 x 10.55 cm base) were sealed with electrical tape. The agarose solution was poured into the tray and the bottom of the comb was placed ~3 mm from the bottom. The tendon samples were inserted into the liquid gel, flush against the comb. The middle lane was left open as a control lane. The agarose was let to solidify for 1 h at room temperature (~20˚ C).

Running the gels

Once cast, the agarose gel was placed in the electrophoresis chamber, and the chamber was filled with TBE buffer (1X) to a depth of approximately 2 mm above the gel (~350 ml TBE). 10 µl of the dye mixture was pipetted into each well. The electrodes were attached to the BioRad PowerPac HC and a constant voltage was set at 80 volts (Figure 2). The gel was run for either 2 hours (Pyronin Y) or 3 hours (Safranin O). Each anode corroded within 45 minutes and was immediately replaced.

Imaging the gels

After each run, the gel was removed from the tray and placed on a glass slide. The slide was placed on white paper, and a picture was taken using an iPhone 4 (Apple, Inc.). The intensity profile of the dye path was analyzed on ImageJ (v10.2, National Institutes of Health) and a Gaussian plot fit of the intensity profile was created on Matlab (v8.1.0.604).

Analysis of the gels

An ImageJ macro, written by the author, analyzed the intensity of the dye in the lane along a given line. This is used to measure the distance traveled by 50% of the dye in order to determine the electrophoretic mobility of the dye. In Figure 3a, the dashed line is drawn from the well (initial point) to beyond the dye band. The intensity along this line was analyzed with the macro, Intensity_Line.txt (Figure 3b). A Gaussian fit was performed on the intensity profile using Matlab,
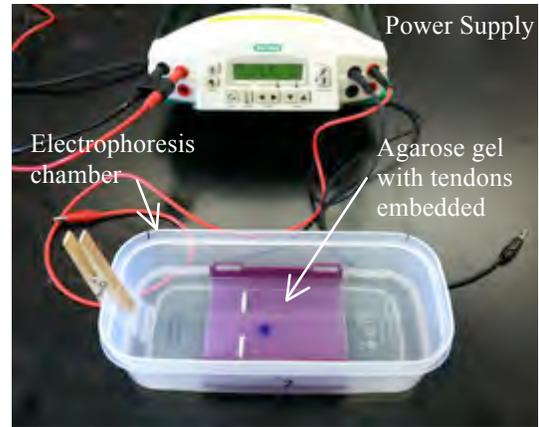

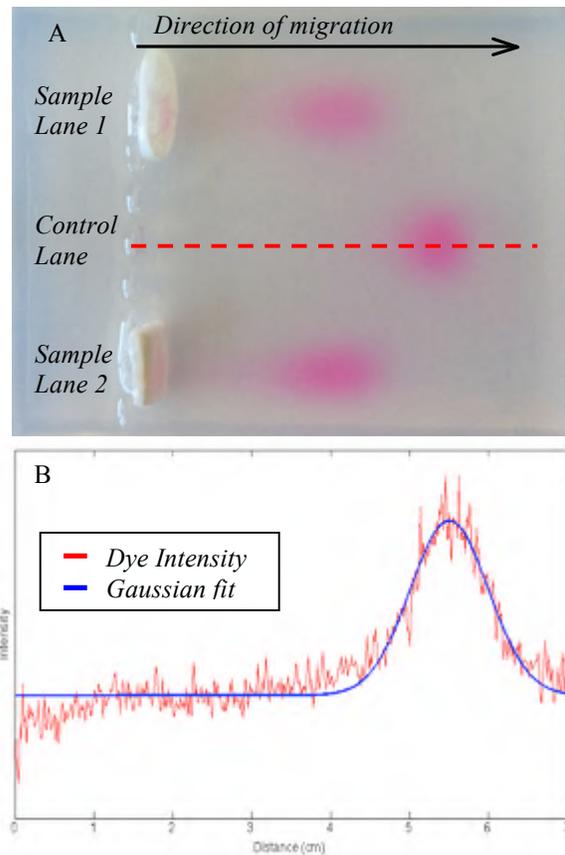**Figure 2.** Setup of gel electrophoresis chamber with agarose gel running.

Figure 3. Analysis of distance travelled by dye. (A) Agarose gel with tendon slices embedded. The cationic dye travels to the right through the tendon. The line along which an intensity profile was extracted is represented by a dashed line. (B) Corresponding intensity profile along the dashed line, and the Gaussian fit used to determine the distance travelled by 50% of the dye.

and the maximum point (50%) was recorded as the distance traveled by the dye.

The rate of migration of the dye through the tendon was calculated using Eqn. 1:

$$\text{Rate of migration (cm/s)} = \frac{w}{t\left(1 - \frac{s - w}{c}\right)} \quad (1)$$

where w = thickness of tendon slice (cm), t = time elapsed (s), s = total distance travelled by dye in the sample lane (cm), and c = total distance travelled by dye in control lane (cm). The electrophoretic mobility (EM) of the dye through the tendon was then calculated using Eqn. 2:

$$EM = \frac{\text{Rate of migration (cm/s)}}{\text{Electric field strength (V/cm)}} \quad (2)$$

Data was presented for each sample group as mean ± SD. A t-test was performed between each group, and significance was identified at p<0.05.

RESULTS AND DISCUSSION
In the tensile samples, EM was higher (t-test, p= 4.32×10^{-4}) in the direction parallel to the collagen fiber orientation compared with perpendicular to

collagen fiber orientation, regardless of which dye was used (Figure 4, 5). Since the tensile region consists of uniaxial collagen fibers, this suggests that fiber orientation influences dye mobility.

Within the compressive region, there was no significant difference between EM in the direction parallel and perpendicular to the fiber orientation (t-test, p=0.263) (Figure 4). The compressive region of the tendon, also known as fibrocartilage, consists of an interwoven network of collagen fibers similar to a basketweave arrangement.[17] This partial biaxial orientation of the fibers yields less distinction between the parallel and perpendicular orientations of the tendon samples; thus, the EM was not significantly different between the two orientations within the compressive region.

An increased dye molecular weight (M_w) decreased the EM, with the larger dye, Safranin O (M_w=350.8 g/mol) exhibiting a decreased EM compared with the smaller dye, Pyronin Y (M_w=302.8 g/mol) (Figure 5). A two-fold increase in EM was observed with the decrease of the dye M_w by 48 g/mol. This is consistent with Maroudas, in which permeability through articular cartilage increases with decreasing molecular weight.[15]
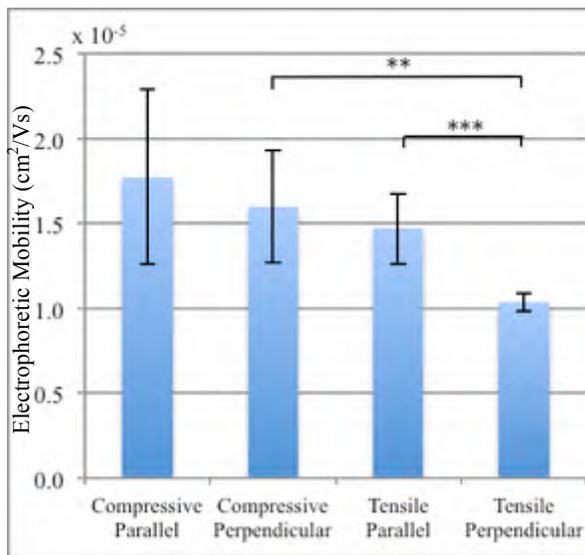

Figure 4. EM of Pyronin Y perpendicular and parallel to collagen fiber orientation in the compressive and tensile region of the tendon. Results are shown as mean ± SD. Statistical significance (t-test) is indicated using standard notation (*=p<0.05, **=p<0.01, ***=p<0.001).
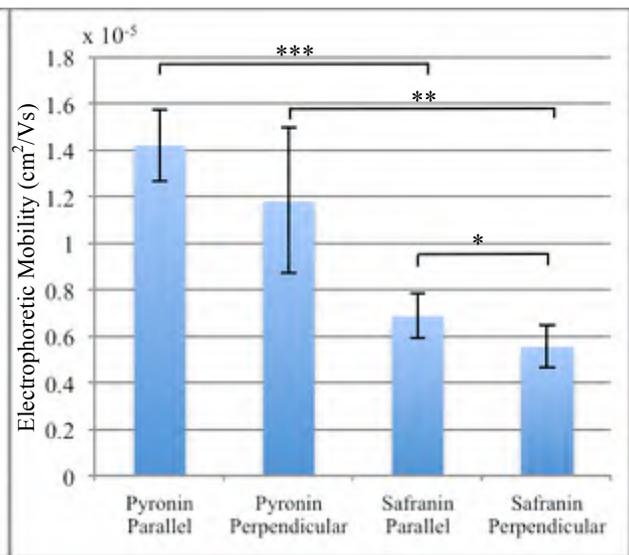
Figure 5. EM perpendicular and parallel to collagen fiber's orientation of Pyronin Y (302.8 g/mol) and Safranin O (350.8 g/mol) in tensile samples. Results are shown as mean ± SD. Statistical significance (t-test) is indicated using standard notation (*=p<0.05, **=p<0.01, ***=p<0.001).

CONCLUSION/FUTURE WORK

In agreement with previous findings for articular cartilage and with our hypothesis, the results presented here indicate that EM of cationic dyes through the tendon tissue is influenced by the origin and orientation of the tissue as well as the dye M_w. Specifically, EM is greater parallel to the collagen fibers and for smaller dyes. These results demonstrate that gel electrophoresis is a highly suitable technique for measurement of the permeability and diffusive properties of tendon tissue.

In the future, additional tests with various sized and charged dyes should be performed to obtain a greater understanding of tendon tissue. Furthermore, since Maroudas' findings also suggest that permeability in articular cartilage increases a hundredfold with a threefold decrease in PG and GAG content from trypsin degradation[15], the effect of trypsin degradation of tendon tissue on the dye EM should also be investigated in order to elucidate the role of PG and GAG concentration on the permeability and diffusive properties of tendon. Such knowledge would have important implications for the feasibility of AuNPs as contrast agents for imaging tendon degeneration.

REFERENCES

1. Kannus P, Józsa L. Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. Journal of Bone and Joint Surgery, 1991. 73(10): 1507-25.
2. Järvinen M, Józsa L, Kannus P et al. Histopathological findings in chronic tendon disorders. Scandinavian Journal of Medicine and Science in Sports, 1997. 7(2):86-95.
3. Bacic G, Liu KJ, Goda F et al. MRI Contrast Enhanced Study of Cartilage Proteoglycan Degradation in the Rabbit Knee. Magnetic Resonance in Medicine, 1997. 37(5):764-8.
4. Palmer AW, Guldberg RE, Levenston ME. Analysis of cartilage matrix fixed charge density and three-dimensional morphology via contrast-enhanced microcomputed tomography. Proceedings of the National Academy of Sciences, 2006. 103(51):19255-66.
5. Paul PK, O'Byrne E, Blancuzzi V et al. Magnetic resonance imaging reflects cartilage proteoglycan degradation in the rabbit knee. Skeletal Radiology, 1991. 20(1):31-6.
6. Joshi NS, Bansal PN, Stewart RC et al. Effect of Contrast Agent Charge on Visualization of Articular Cartilage Using Computed Tomography: Exploiting Electrostatic Interactions for Improved Sensitivity. Journal of American Chemical Society, 2009. 131(37):13234-5.
7. Burstein D, Gray ML, Hartman AL et al. Diffusion of small solutes in cartilage as measured by nuclear magnetic resonance (NMR) spectroscopy and imaging. Journal of Orthopaedic Research, 1993. 11(4):465-78.
8. Zhang L, Szeri AZ. Transport of neutral solute in articular cartilage: effects of loading and particle size. Proceedings of the Royal Society A, 2005. 461(2059):2021-42.
9. Józsa L, Kannus P. Human Tendons: Anatomy, Physiology, and Pathology. Champaign, IL: Human Kinetics, 1997.
10. Cohen NP, Foster RJ, Mow VC. Composition and dynamics of articular cartilage: structure, function, and maintaining healthy state. Journal of Orthopaedic and Sports Physical Therapy, 1998. 28(4):203-15.
11. Connizzo BK, Yannascoli SM, Soslowski LJ. Structure-function relationships of postnatal tendon development: A parallel to healing. Matrix Biology, 2013. 32(2):106-16.
12. Kantor TG, Schubert M. The difference in permeability of cartilage to cationic and anionic dyes. Journal of Histochemistry and Cytochemistry, 1957. 5(28):28-32.
13. Kulmala KAM, Korhonen RK, Julkunen P et al. Diffusion coefficients of articular cartilage for different CT and MRI contrast agents. Medical Engineering and Physics, 2010. 32(8):878-82.
14. Torzilli PA, Arduino JM, Gregory JD, Bansal M. Effect of proteoglycan removal on solute mobility in articular cartilage. Journal of Biomechanics, 1997. 30(9):895-902.

15. Maroudas A. Transport of solutes through cartilage: permeability to large molecules. Journal of Anatomy, 1976. 122(2):335-47.
16. Cold Spring Harbor Protocols. 2006. doi:10.1101/pdb.rec8458.
17. Benjamin M, Ralphs JR. Fibrocartilage in tendons and ligaments – an adaptation to compressive load. Journal of Anatomy, 1998. 193(4):481-94.