SB^{3}C2021
Summer Biomechanics, Bioengineering and Biotransport Conference
June 14-18, Virtual

MECHANICAL TESTING OF TYPE II COLLAGEN NETWORKS: TOWARDS MODULATING MECHANICS, IDENTIFYING SINGLE-FIBER PROPERTIES, AND ESTABLISHING MULTISCALE MODELS

Phoebe Szarek (1), David M. Pierce (1,2)

(1) Department of Biomedical Engineering
University of Connecticut
Storrs, CT, USA

(2) Department of Mechanical Engineering
University of Connecticut
Storrs, CT, USA

INTRODUCTION

Articular cartilage and other collagenous soft tissues rely heavily on their internal networks of collagen for mechanical strength. When placed in tension, e.g. due to external loading or osmotic swelling, collagen fibers contribute to the mechanical response and stiffness of these tissues. Collagen networks also provide a foundation for many applications in tissue engineering. We seek to establish a robust methodology and platform for testing isolated collagen networks to aid researchers in investigating the effects of mechanical loading (e.g. repeated, damage-inducing, rate-dependence) and biochemical modifications (e.g. temperature, +/- crosslinking, cell products) on this essential mechanical constituent within tissues and biomaterials. Such understanding may aid in the development and testing of therapeutics aiming to heal damaged tissues, specifically those developed to treat diseases of articular cartilage, i.e. osteoarthritis. Furthermore, isolating collagen networks from the tensile forces generated by osmotically induced pretension may aid in understanding intra-constituent interactions and their roles in tissue biomechanics. Testing of isolated networks of collagen fibers may lead to understanding of the mechanics of individual fibers that can inform multiscale mechanical models.

METHODS

We sequentially focused on sourcing, preparing, and testing networks of type II collagen and sought to optimize each step. We first established a method for isolating collagen networks from bovine articular cartilage. Additionally, cartilage contains a collagen network with through-thickness heterogeneity, a factor that we can include as a variable or control for with specific protocols. We extracted full-thickness cartilage from the patellofemoral groove and femoral condyles attached to the subchondral bone in 1×2 cm pieces. We then sliced the cartilage pieces into 200 μm thick sheets (the thickness of the well-aligned superficial zone of cartilage [1]) using a microtome, before digesting out the proteoglycan (PG) constituents using trypsin [2]. We used the most superficial two or three sheets of cartilage to control for variability in the fiber alignment through the thickness of cartilage [3].

We determined the appropriate trypsin concentration and the duration of digestion using another set of specimens extracted from the tibial plateau and patella for 24 or 48 hours in 0.25, 0.5, 1.0, or 2.0 mg/mL enzyme in phosphate buffered saline (PBS). We quantified the PG remaining and the collagen loss using glycosaminoglycan and hydroxyproline assays. We chose the enzyme concentration and duration that removed the most PG with minimal loss of and damage to collagen, also aiming to minimize the digestion time and the amount of enzyme required. We also investigated the volumetric effects of digestion by imaging specimens before and after digestion and computed the deformation gradients associated with the digestion.

We then established the appropriate specimen dimensions. We used the ASTM D1708 standard dimensions for microtensile testing as a baseline and scaled these down to reduce the amount of tissue required for each test [4]. We performed finite element analyses to ensure that the geometry of the specimen we selected did not create problematic stress concentrations and that the stress distribution in the gauge region remained homogenous. We then varied the specimen thicknesses starting at a maximum of 200 μm, aiming to create sheets of collagen as thin as possible, but thick enough to manipulate during testing and while minimizing undesirable edge effects from damage sustained during specimen preparation. We selected the thinnest specimens that did not present obvious loss of stiffness in the bulk network.

We next determined the displacement rate for tensile testing to eliminate strain-rate dependence while still achieving a reasonable test duration. We started at 75 μm/min (previously determined to be quasi-static [5]) and increased the rate until we noted rate dependence in the

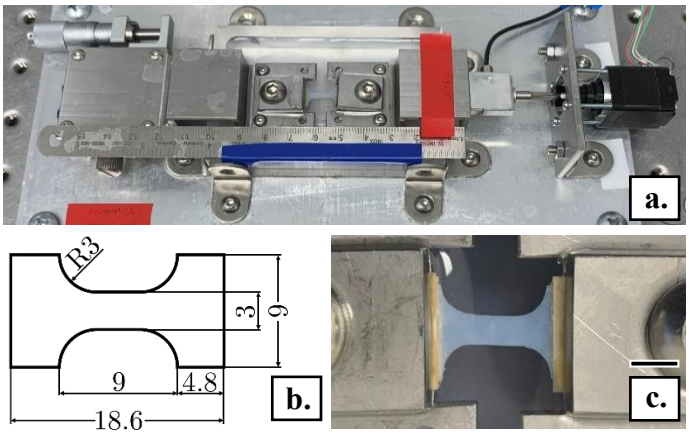

Figure 1. Mechanical testing of collagen networks: a. microtensile testing device; b. dimensions of our tensile specimen in mm; c. specimen mounted within the microtensile device, bar = 5 mm.

mechanical results. Aiming to create a test platform for efficient research, we chose the highest displacement rate that did not increase the apparent stiffness of the collagen network.

We performed all tensile tests on a custom-built microtensile device (**Fig. 1a**) with a displacement range of 12.5 mm (resolution: 2.5 µm) and force capacity of 20 N (resolution: 0.3 mN). We kept tissues hydrated in a PBS bath at 25°C and aligned specimens with the principal fiber orientation, but designed the device to fulfill requirements and constraints of future studies on collagen networks (e.g. visualizing fiber structures, quantifying damage during loading, studying temperature effects). We secured the specimens using cyanoacrylate glue and sandpaper (**Fig. 1c**) and monitored testing to ensure tissue failure occurred within the gauge region.

RESULTS

Table 1 shows the preliminary results of testing our protocols for enzymatic digestion. We will finalize a digestion protocol to reduce the PG in the tissue to less than 2% of the original PG mass with less than 1% collagen mass lost or damaged, while still aiming to reduce the cost and time needed for the specimen preparation. **Fig. 1b** shows the dimensions of our specimens that reduce the required tissue but still maintain a gauge region with homogenous stresses to simplify subsequent mathematical analyses of fiber networks under loading. Preliminary tests also show this level of digestion and PG removal does not cause considerable changes in specimen geometry, i.e. deformation that should be included in interpreting the resulting mechanical data.

Table 1. Mass Percent PG Remaining Post Digestion (Mean ± Standard Deviation, *n* = 6 per test group)

	Trypsin Concentration (mg/mL)				
Duration	0	0.25	0.5	1	2
24 hr	87.4 ± 4.8	2.2 ± 0.4	1.3 ± 0.8	1.1 ± 1.3	1.6 ± 0.5
48 hr	84.2 ± 4.9	1.2 ± 0.8	1.3 ± 0.6	1.5 ± 0.4	1.1 ± 0.4

Preliminary tests show that digested specimens have a stiffness less than that of intact specimens and that extreme digestion can over soften tissue by damaging the collagen network during removal of PG (**Fig. 2**). Preliminary results show loss of collagen (and thus network damage) at the higher concentrations shown in **Table 1**. Additionally, increasing the strain rate stiffens the tissue response due to the viscoelastic response of collagen fibers [6]. Finally, preliminary tests show that there is a minimum thickness (threshold) below which the stiffness of the tissue decreases due to edge effects from damage.

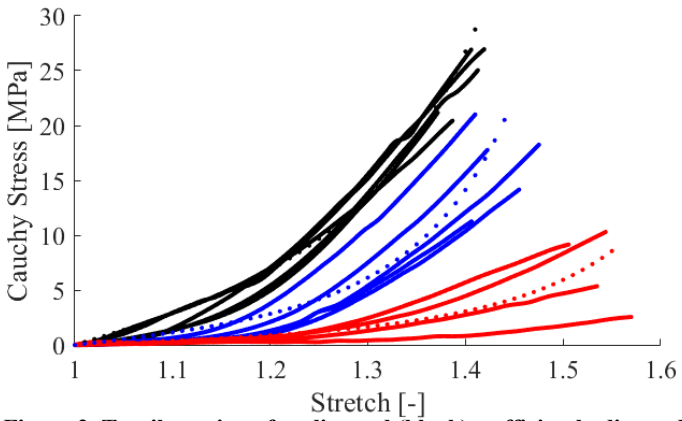

Figure 2. Tensile testing of undigested (black), sufficiently digested (blue), and overly digested (red) cartilage specimens as a source of type II collagen networks. The dashed lines show the prediction based on the average parameters fit from each test type (*n* = 5).

DISCUSSION

Variability within cartilage and collagen networks arises from many factors. With this protocol, we aim to create a process that is repeatable so that effects presenting in future tests reflect the independent variable of interest and not the innate variability between tissue specimens. We also aim to quantify intra-specimen variability to inform future research.

Preliminary results show that the test parameters we are investigating are relevant in altering the apparent mechanics of collagen networks, speaking to the effects of chemical and mechanical damage as well as the strain-dependent response of collagen networks and possibly fibers, yet we will also evaluate our testing protocol further. We plan to visualize potential damage to the collagen network using collagen hybridizing peptide (CHP) to ensure internal damage without loss of collagen is not present. We also plan on using digital image correlation (DIC) to confirm that strain is homogeneous within the gauge region and investigate the mechanism of failure.

While establishing a testing platform, we needed to make decisions to transform a theoretical test into a practical experiment. Conflicting constraints and aims limit the optimization of these methods, e.g. an ideally thin (<200 nm) sheet of a single layer of collagen fibers would be nearly impossible to handle and mount for tensile testing. We also established arbitrary (but practical) cutoffs for acceptable levels of remaining PG and lost/damaged collagen since we cannot reasonably perform a complete digestion of PG with no effect on the collagen.

Future work using this test platform will characterize the mechanical response of individual type II fibers by analyzing the network structure using a host of tools including second harmonic generation microscopy, transmission electron microscopy, and micro-digital image correlation. Mechanical characterization of individual collagen fibers and isolated collagen networks will also inform models aiming to include micro-mechanics into models of bulk tissues.

ACKNOWLEDGEMENTS

NSF 1662429, GAANN P200A160323, GE Fellowship. Christopher Lemay, Zachary Ohayon, Anthony Rivera.

REFERENCES

[1] Mow, V et al., *Basic orthopaedic biomechanics & mechanobiology*, 180-258, 2005. [2] Torzilli, P et al., *J. Biomech.,* 30:9, 1997. [3] Kempson, G et al., *Biochim. Biophys. Acta*, 297, 1973. [4] ASTM Intl., *D1708-18*, 2018. [5] Maier, F et al., *J. Mech. Behav. Biomed.,* 65, 2017. [6] Li, L.P. et al., *Biorheology,* 41, 2004.