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Tissue-engineered pediatric patches: bioprinting structured collagen to mimic the mechanical properties of native blood vessels

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Thesis

TISSUE-ENGINEERED PEDIATRIC PATCHES: BIOPRINTING STRUCTURED COLLAGEN TO MIMIC THE MECHANICAL PROPERTIES OF NATIVE BLOOD VESSELS

by

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TISSUE-ENGINEERED PEDIATRIC PATCHES:

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ABSTRACT

Congenital heart defects are the most common category of birth defects, mostly affecting the blood vessels, walls, or valves of the heart. For example, pulmonary atresia occurs when the connection between the right ventricle to the main pulmonary artery is not fully formed. A heart defect such as pulmonary atresia may need surgery to close up any malformations in walls and blood vessels, and unfortunately, because the patients are infants, they will need to undergo several surgeries in their lifetime to accommodate a heart patch that will fit the size of their hearts at each stage of their life. A better solution would be to create a biomimetic vascular patch that could be anatomically accepted by the patient's body as its own, allowing it to grow with the patient without the residue of scar tissue. Instead of propagating scar tissue in the area, it would propagate healthy cells that would integrate into the surrounding tissue. For this to become a reality, one strategy for a biomimetic vascular patch would be to build it like a blood vessel in layers, beginning with the tunica adventitia. The goal of this thesis is to engineer and design the foundation for a biomimetic vascular patch with a crimped, collagen-integrated scaffold, focusing on optimizing the mechanical properties of the hybrid structure. The crimped structure, using sine waves generated from Python code and fabricated with bioprinting technology, mimics the natural formation of collagen fibers in native blood vessels. Additionally,

testing the scaffolds on the Instron allows for characterization of the mechanical behaviors of an optimal and repeatable foundation for a tissue-engineered tunica adventitia.

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BACKGROUND AND SIGNIFICANCE

Congenital heart defects are the most common category of birth defects, mostly affecting the blood vessels, walls, or valves of the heart. For example, pulmonary atresia occurs when the connection between the right ventricle to the main pulmonary artery is not fully formed. In the United States, about 1 in every 7,100 babies are born with this condition each year ("Congenital Heart Defects - Facts about Pulmonary Atresia," 2019). A heart defect such as pulmonary atresia may need surgery to repair any malformations in walls and blood vessels. Unfortunately, because the patients are infants, they will need to undergo several surgeries in their lifetime to accommodate a heart patch that will fit the size of their heart at each stage of their life ("Congenital Heart Defect - Corrective Surgery," 2020).

Although there have been extensive efforts to develop tissue-engineered blood vessels and improvements are continually being made, they are not yet common replacements for congenital heart defects such as pulmonary atresia (Shinoka, et al., 2008). Currently, one solution pediatric surgeons use to address these defects is the exGraft by PECA Labs (Loneker, et al., 2018). It is composed of polytetrafluoroethylene (ePTFE), and it expands like an accordion to imitate the natural mechanical function of a blood vessel. Unfortunately, the material is synthetic and will not grow with the patient. This requires multiple surgeries to replace it to accommodate the growing heart, leaving behind an increasing amount of scar tissue ("Congenital Heart Defect - Corrective Surgery," 2020).

A better solution would be to create a biomimetic vascular patch that could be

anatomically accepted by the patient's body as its own, allowing it to grow with the patient without the residue of scar tissue. Instead of propagating scar tissue in the area, it would propagate healthy cells that would integrate into the surrounding tissue. This is not a new concept. In the 1980s a group of researchers at MIT were the first to show that this was possible by suspending bovine endothelial, vascular smooth muscle, and fibroblasts cells in a tubular collagen gel and gradually forming in culture to result in viable tissue. Technology has since rapidly advanced, narrowing in on obtaining more control of the process and desired product (Niklason, et al., 2020). One example is three-dimensional bioprinting technology, specifically extrusion-based bioprinting, in which a machine extrudes a biocompatible material through a needle while following an inputted print path. The print path is repeated as layers until the 3D construct, or scaffold, is finished (Kačarević, et al., 2018).

There are countless approaches to bioprinting a blood vessel from start to finish, but the overarching route that this thesis follows is to bioprint the tissue-engineered blood vessel to mimic the structural organization of a native blood vessel, i.e., layer by layer, as seen in **Figure 1**.

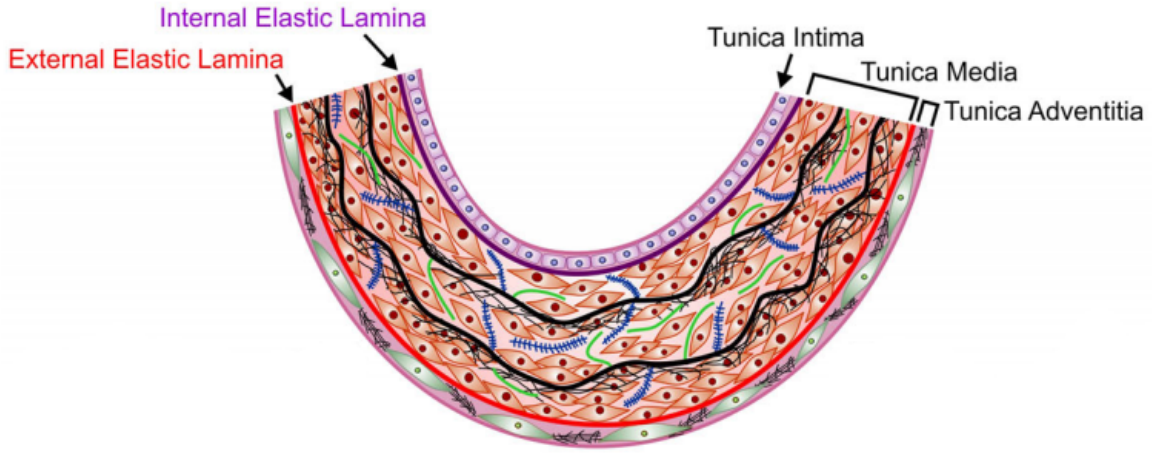

Figure 1: The cross section of the blood vessel wall shows the three different layers, the tunica adventitia, the tunica media, and the tunica intima. These layers are separated by elastic lamina (Jana, et al., 2019). This is an oversimplified visual of the components of a blood vessel; components include immune cells, progenitor cells, enzymes, and proteins (Stenmark, et al., 2013).

Blood vessels consist of three main layers: the tunica adventitia, the tunica media, and the tunica intima being the innermost layer. The layers have distinct functions, properties, and components, and can be "built" in the lab as separate layers with bioprinting technology. The tunica adventitia provides tensile strength to the vessel due to the mechanical properties of collagen, fibroblasts, and elastic fibers (Jana, et al., 2019) as well as the dynamic contribution of progenitor cells – the cells that determine vessel size, structure, maintenance, and repair (Majesky, et al., 2011). It is important to note the adventitia is a dynamic tissue; however, this thesis focuses on the structure of the tunica adventitia, specifically the stress-strain relationship of the structure and the ability to recreate this relationship through bioprinting.

PROJECT AIM

The main goal of this thesis is to demonstrate that the behavior of natively formed collagen can be mimicked artificially using a bioprinter. The long-term goal is to engineer and design a foundation for a biomimetic vascular patch with a crimped collagen-integrated scaffold, focusing on optimizing the mechanical properties of the hybrid structure. The crimped collagen structure, using sine waves generated from Python code, mimics the natural formation of collagen fibers in native blood vessels. Collagen is difficult to bioprint due to its low viscosity, so to resolve this, the collagen is often embedded in a hydrogel, specifically alginate, when it is bioprinted. The explicit goal of this thesis is to bioprint a scaffold, or support structure, with a collagen-alginate bioink.

The project aim is broken down into the following tasks:

1. Print alginate in a sine wave pattern and test the tensile strength of the resulting structure.
2. Print alginate-collagen mixture in a sine wave pattern and test the tensile strength of the resulting structure.

RATIONALE

The anticipated mechanical property of collagen in a blood vessel is characterized by a "j-shaped" stress-strain curve from the literature. The first section of the curve is the toe region as seen in **Figure 2**. The collagen uncoils as if it were a spring, aligning the fibers without stretching them. The collagen then passes through the heel region, where some fibers begin to be stretched. By the time the collagen reaches the third section, the linear region, all fibers have been straightened out and are stretching together until they reach failure (Doehring, et al., 2005).

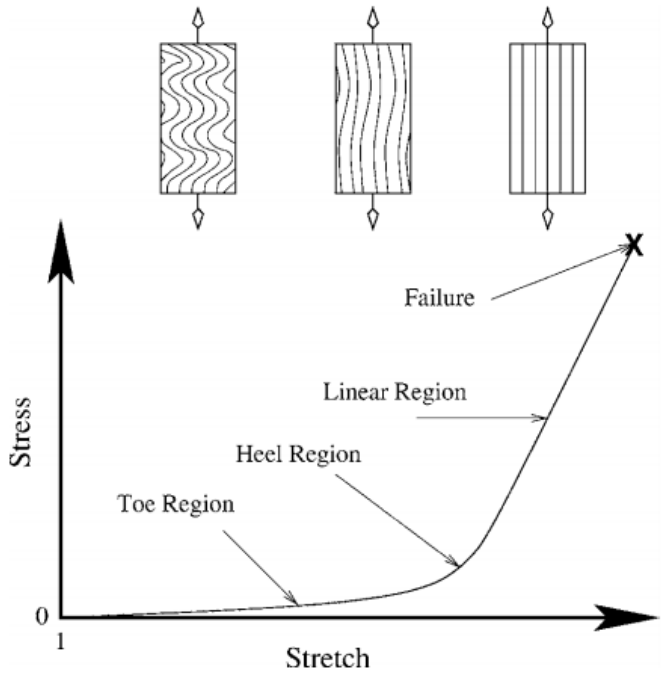

Figure 2: The response of collagenous tissue as it is being stretched (Doehring, et al., 2005).

Prior research indicates that collagen types I and III in adventitial tissue naturally form in a crimped shape, which allows for the uncoiling in the first section of the j-shaped curve. In **Figure 3**, the crimped collagen exhibits this phenomenon of the

fibers straightening out as the strain increases (Chow, et al., 2014). The alginate – collagen patterns explored in this thesis models the crimped shape and is aligned in several parallel lines for tensile testing. A specific range of strain or stress was not targeted because it has been shown that tissue without cells and all ECM components have less extensibility and a higher tensile modulus (Williams, et al., 2009).

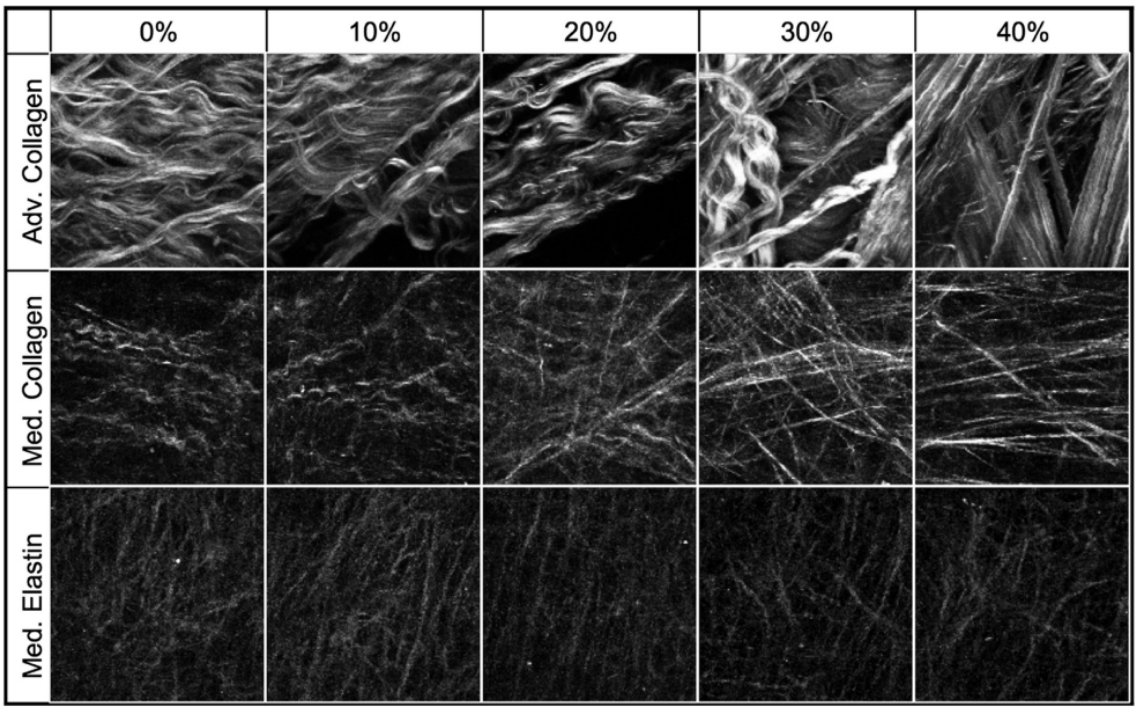

Figure 3: Collagen and elastin uncoil as it is being stretched biaxially. Porcine thoracic aortas cut in 30mm sized samples so that the edges were square with the longitudinal and the circumferential directions (Chow, et al., 2014).

This thesis used alginate to model the crimped shape of collagen. Alginate hydrogels exhibit a minimal j-shaped stress-strain curve. Previous testing of alginate samples in conventional dogbone-shaped specimen shows this minimal toe region with a significant linear region (Drury, et al., 2004) in **Figure 4**.

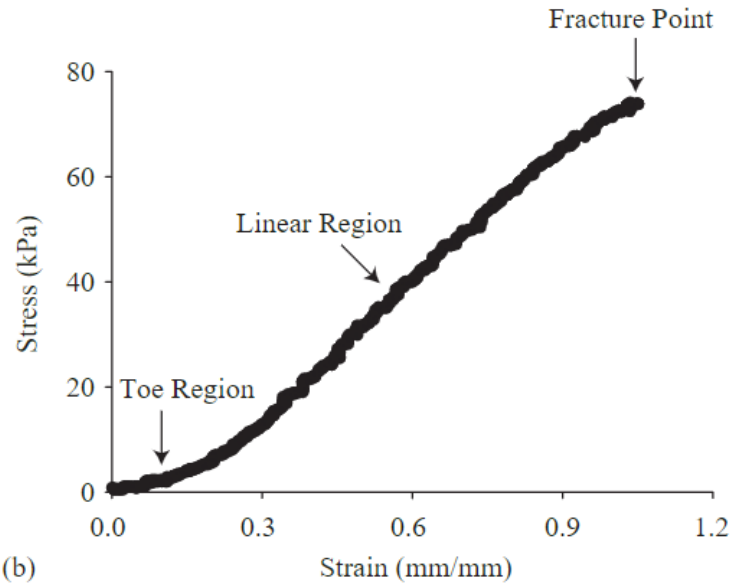

Figure 4: Stress-strain relationship of alginate shows a minimal toe region and prominent linear region. A 2% alginate sample gelled in a calcium sulfate slurry tested after resting in calcium containing Dulbecco's Modified Eagle Medium (DMEM) with sodium bicarbonate and penicillin-streptomycin (Drury, et al., 2004).

The shape of the stress-strain curve can be modulated by printing a stiff material in a pattern, as shown in previous studies. For example, while the strain is similar for polyimide printed as triangles, honeycombs, and kagome waves, the shape of the curves reveal differences between the patterns (Jang, et al., 2015). Specifically, the triangle pattern (**Figure 5**) follows a j-shaped curve and can be stretched a greater distance at a relatively lower force than the other two patterns. This study further motivated the idea of bioprinting collagen in sine waves in this thesis to modulate the linear region of its stress-strain curve.

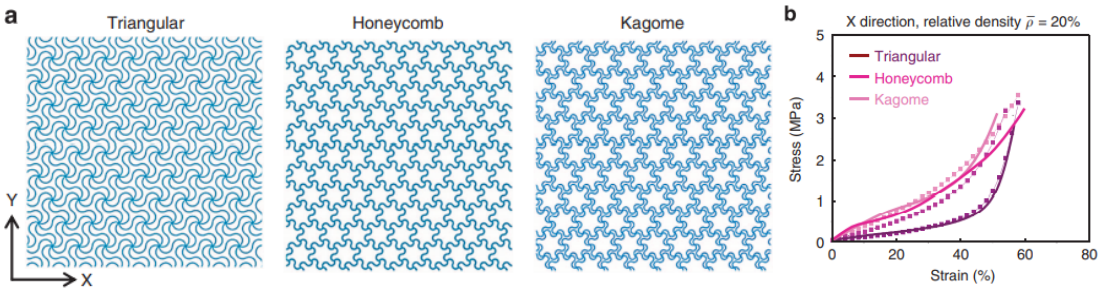

Figure 5: Examples of possible patterns and associated stress-strain relationships. Comparing the mechanical properties (right) of three distinct patterns (left) of polyimide designed with photolithography (Jang, et al., 2015).

MATERIALS AND METHODS

Materials used for this project were alginic acid (CAS: 9005-38-3) (Sigma-Aldrich, St. Louis MO); calcium chloride (Fisher, Waltham MA), Type I collagen (Corning, Tewksbury MA); sodium hydroxide (Sigma-Aldrich). Materials were bioprinted with a Bio X printer (Cellink, Boston MA) with a pneumatic printhead with 22-gauge tapered needles (0.41 mm inner diameter) (Nordson EFD, East Providence RI).

To bioprint the alginate and alginate-collagen scaffolds, it was necessary to simplify the natural 3D helical shape to a bioprintable structure, namely 2D-unidirectional sine waves. Different ratios and concentrations of alginate and collagen were tested in order to compare the resulting mechanical properties. The printing parameters were varied with the aim of attaining good mechanical properties as tested on an Instron (5944 Micro-tester, Instron®, Norwood, MA).

Alginate was chosen for its biocompatibility characteristics. Moreover, it will be a good base for cell seeding in future steps. In addition, it has a good viscosity value for printing.

Mixtures

The alginate gel was an 8% mixture of alginic acid in de-ionized water. It was mixed on a stir plate at a temperature of 35°C overnight and stored at room temperature. Two alginate-collagen mixtures were used—a 4:1 volumetric ratio and a 3:1 volumetric ratio—and were mixed overnight on a tilting laboratory shaker at 4°C. 1.5 M sodium hydroxide (NaOH) was used to neutralize the mixtures before printing. Both alginate-collagen mixtures were stored at 4°C. Immediately after the prints

were finished, they were immersed in 5 mL of 50 mM calcium chloride for 14 hours, as seen in **Figure 6**.

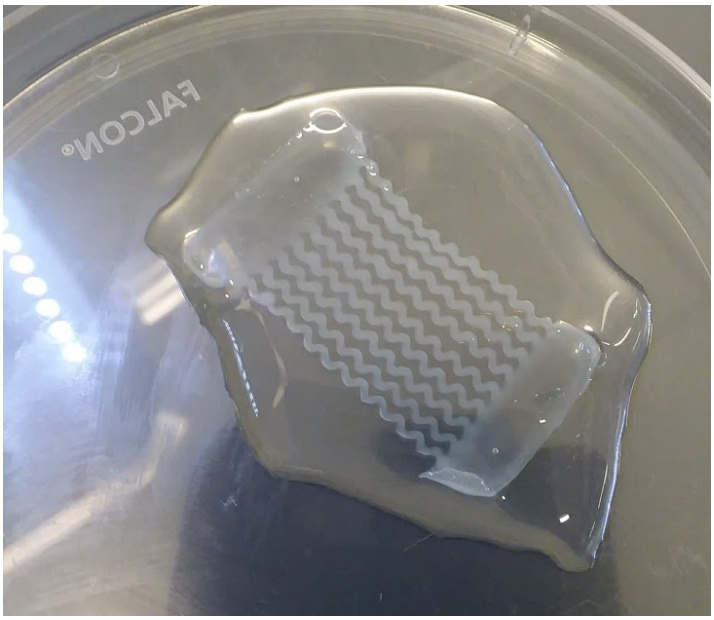

Figure 6: An example of an 8% alginate, printed sample crosslinking in 50mM calcium chloride solution.

Bioprinting

A Cellink Bio X bioprinter and its pneumatic printhead were used for this thesis. A custom gcode file, as seen in **Figure 7**, was written for these print samples with overall dimensions of 20mm by 40mm. The sine wave area is 20 mm by 30 mm with tabs on each side for mechanical testing. The tabs are each 5 mm wide. After preliminary testing, the optimal parameters were found to be a travel speed of 3.8 mm/s at an extrusion pressure of 87 kPa.

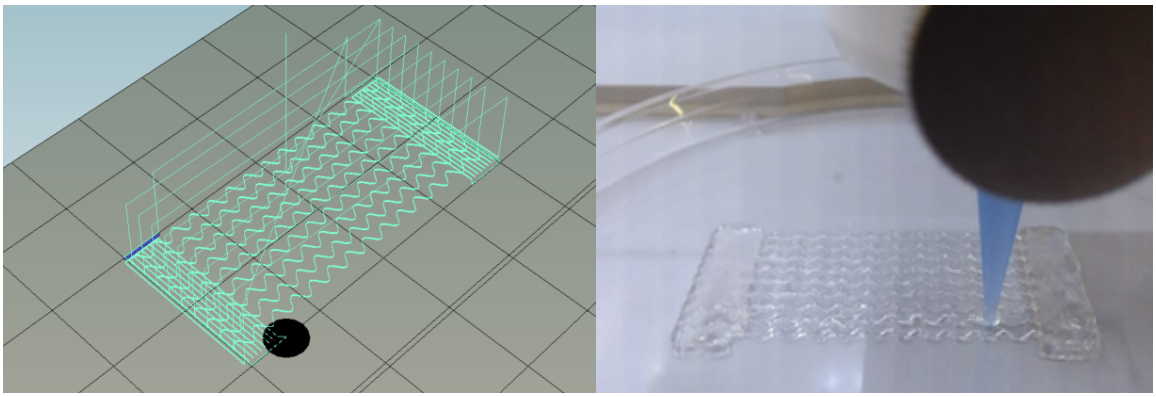

Figure 7: Visualization of the gcode (left) and a sample being printed with 8% alginate (right). The areas on the sides are tabs for the purpose of mechanical testing.

The sine wave (**Eqns. 1**) that was coded and printed is graphed in **Figure 8** for visualization. The sine wave is approximated by point-to-point vectors.

$$y = abs(0.7 \sin\left(\left(\frac{2\pi}{2.8}\right)(x + 0.7)\right) - 0.7) \qquad \text{(Equation 1)}$$

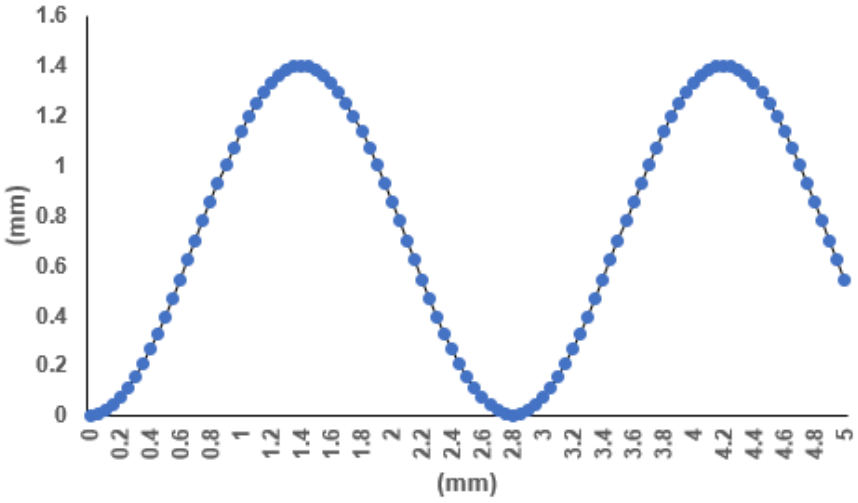

Figure 8: The point-to-point vectors as defined by the code that approximates the sine wave.

Mechanical Testing

After the samples were crosslinked in the calcium chloride solution for 14 hours, they were removed for mechanical testing. An Instron® 5944 Micro-tester was used to test the tensile strength of the printed samples at a displacement rate of 1 mm/s. The Instron® sample holder clips were modified to better fit the bioprinted samples. Binder clips, balanced between a set of paperclips, were used for a stronger grip. Folded pieces of notecard were used to more evenly distribute the pinch of the binder clip to prevent tearing in the sample tab. **Figure 9** shows the complete setup.

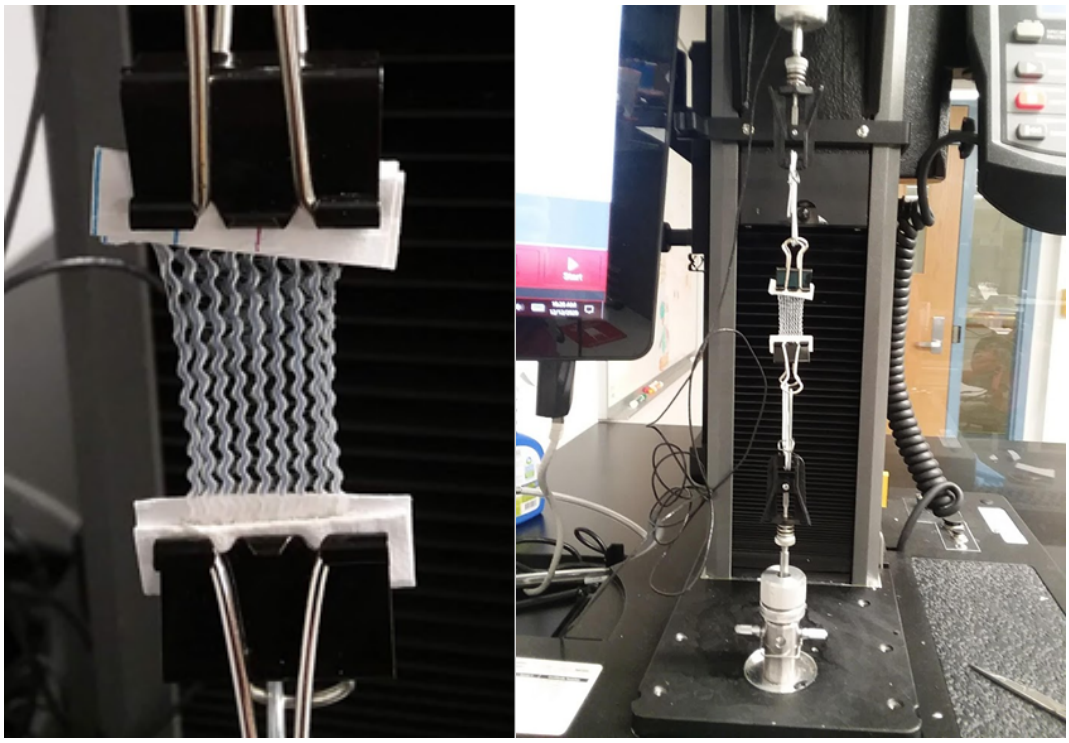

Figure 9: The modified set-up on the Instron® illustrating modifications to the sample holder clips.

Data Analysis

The printed samples are assumed to be incompressible. Moreover, although we are mostly interested in the unfolding of the sine waves, sample deformation must be considered. Stretch can be expressed as the Instron®-pulled end-to-end sample length divided by the original end-to-end length. Because incompressibility is assumed, the stretched sample volume equals the original volume. With the definition of volume and rearranging the equations (**Eqns. 2 – 6**), the Cauchy stress can be expressed as the product of force and stretch divided by the original area.

$$stretch = \lambda = \frac{L}{L_o} \qquad \text{(Equation 2)}$$

$$V = V_o \qquad \text{(Equation 3)}$$

$$AL = A_o L_o \qquad \text{(Equation 4)}$$

$$A = \frac{A_o L_o}{L} = \frac{A_o}{\lambda} \qquad \text{(Equation 5)}$$

$$Cauchy\ stress = \sigma = \frac{F}{A} = \frac{F\lambda}{A_o} \qquad \text{(Equation 6)}$$

RESULTS

A time-lapse of a uniaxial stress-stretch test of a crimped alginate sample being in the Instron® is shown in **Figure 10**. To mimic the crimped shape behavior of natural collagen, we anticipated the alginate sine waves would first straighten in unison into parallel vertical lines and then all extend together until failure. This would correspond to the "j-shape" stress-strain curve (**Figure 2**) with an initial prominent low slope, and it hypothetically should not require much force to straighten the sine waves. However, when the sine waves were stretched on the Instron®, (**Figure 10**), the sine waves did not straighten in unison.

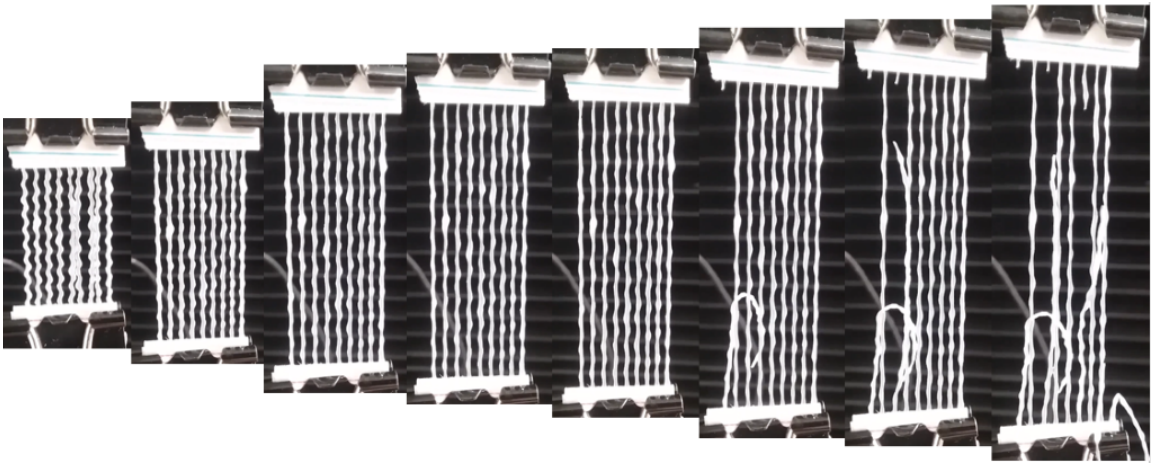

Figure 10: A time-lapse series of a sine wave alginate sample being tested on the Instron®.

When compared with samples printed with straight lines under identical printing parameters, the sine wave samples stretched further (**Figure 11**). This is not surprising because while the straight lines and sine waves sample lengths are 30 mm, the sine waves provide almost 50% more (43.86 mm) for the same end-to-end distance.

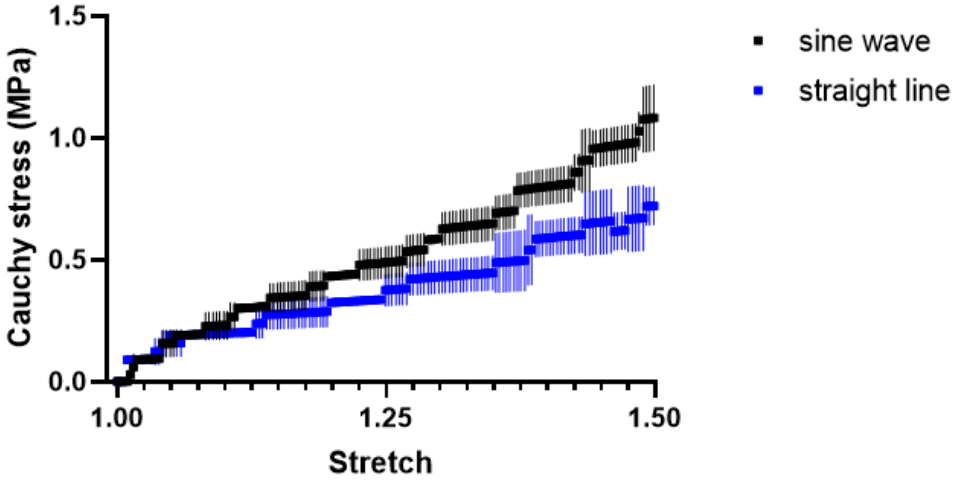

Figure 11: Averaged tensile properties of sine wave compared to straight line alginate samples (N=3, error bars signify standard deviation).

Two alginate-collagen samples were printed: a 4:1 and 3:1 volumetric ratio. However, the 3:1 alginate-collagen samples were too delicate to secure in the Instron®, and only the 4:1 samples could be tested. **Figure 12** shows a side-by-side comparison of the resulting printed mixtures.

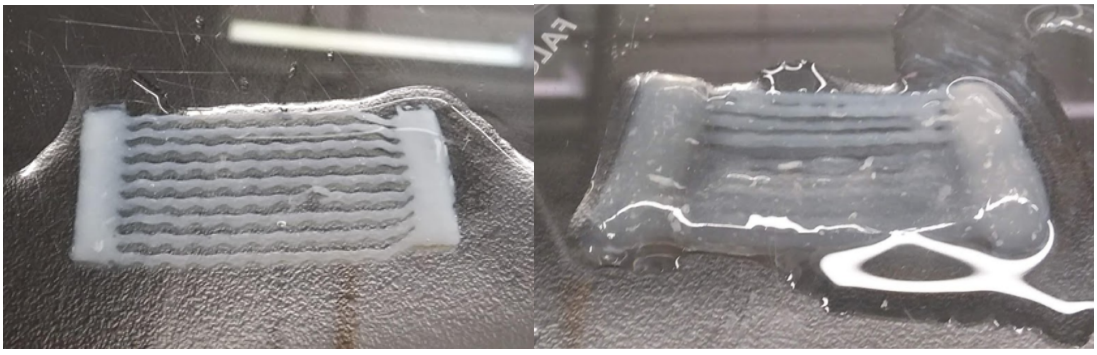

Figure 12: A 4:1 alginate-collagen sample (left) and a 3:1 alginate-collagen sample (right).

When the 4:1 alginate-collagen were compared to pure alginate sine wave samples, the distance stretched remained unchanged. However, the alginate-collagen samples required more force to reach the same distance as the alginate samples (**Figure 13**).

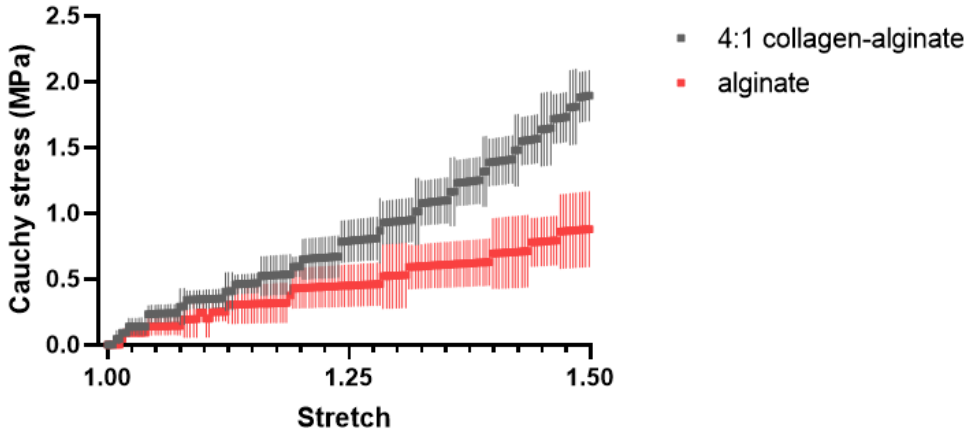

Figure 13: Averaged tensile properties of 4:1 alginate-collagen compared to pure alginate sine wave samples (N=2, error bars signify standard deviation)

DISCUSSION

Bioprinting of collagen materials has proven to be challenging due to its low viscosity when it is dissolved in acid and its high viscosity and tendency to aggregate when it is neutralized. The high viscosity and aggregation also limit the diameter of the needle used for extrusion. Collagen is a stiff material, but we hypothesized the stiffness can be manipulated into a j-shaped stress-strain curve by printing it in a wavy pattern. We tested this hypothesis with 8% alginate, a material characterized with a mostly linear stress-strain behavior (Drury, et al., 2004), by printing samples with straight lines and samples with sine waves. After testing, both patterned samples resulted in a j-shaped stress-strain curve, however, the sine waves required more force for the same distance stretched because of the uneven distribution of extruded material along the wave, i.e., more material was extruded on the waves' crests and troughs. This is consistent with the density increase of points at the peaks in the sine wave (**Figure 8**). Additionally, the increase in stress-to-reach-failure is most likely due to these areas with excess material. Preventing deposition of the unexpected excess material will be addressed in future studies. Adding collagen to the 8% alginate further increased the stiffness (**Figure 13**). This is consistent with other studies as collagen has been added to other materials, such as elastin, to increase the modulus of the stress-strain curve (**Figure 14**) (Black, et al., 2008).

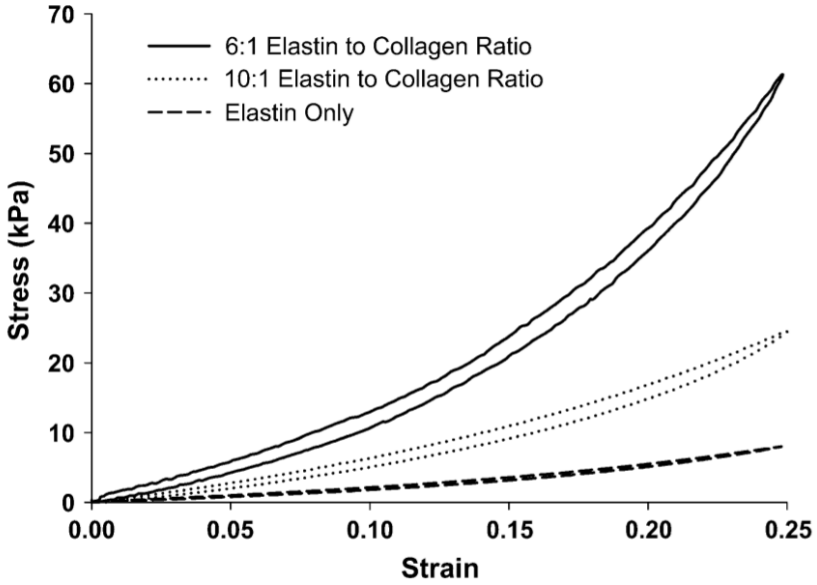

Figure 14: The mechanical properties of elastin and elastin with collagen (Black, et al., 2008).

We attempted to modulate the linear region of the stiff mixture with a sine wave pattern and can compare our study to previous studies of materials printed in specific patterns (**Figure 5**). The sine waves modulated alginate's mostly linear stress-strain curve to exhibit a j-shape curve. The stress-strain curve of the collagen-alginate samples exhibited a stiffness greater than the alginate samples, however, the details of the waves in the pattern can further alter the stress-strain behavior. From experiments in which wave angle and print thickness were varied, higher degrees of "waviness" and thinner patterns allowed more stretch with less force or stress (**Figure 15**).

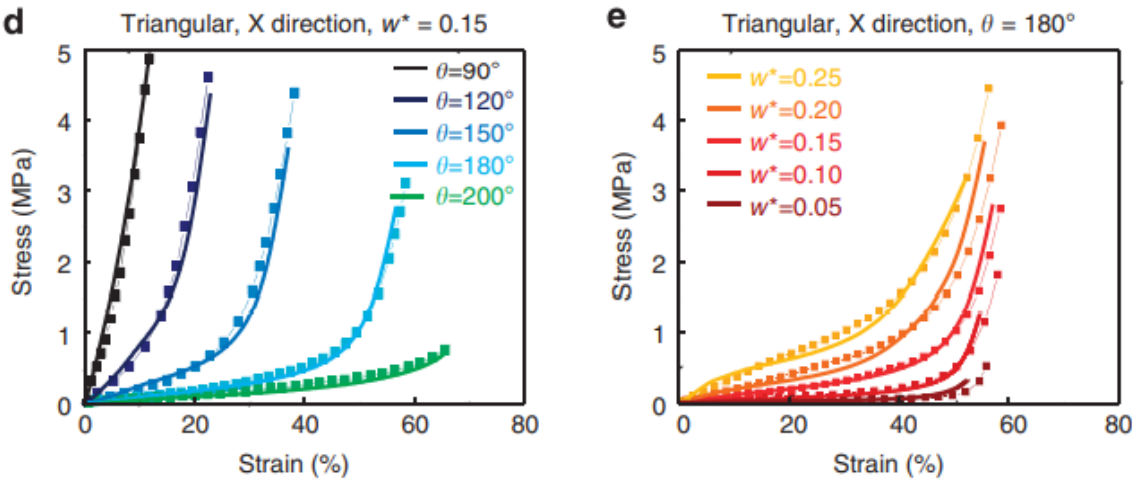

Figure 15: As the angle of the arc in the wave varies (left) and as the thickness of the print varies (right), the strain-stress curve changes (Jang, et al., 2015).

The ultimate goal of creating a biomimetic blood vessel will need to consist of more than a collagen-hydrogel scaffold. For example, decellularized tissue is a prominent component of the aimed, tissue-engineered hybrid blood vessel. However, decellularization does not preserve the mechanical properties of native tissue. Specifically, **Figure 16** shows native tissue is significantly less stiff and more extensible than decellularized tissue (Williams, et al., 2009). From previous research and from the results of this thesis, it is clear that the pattern in which the material is printed can modulate the stress-strain behavior.

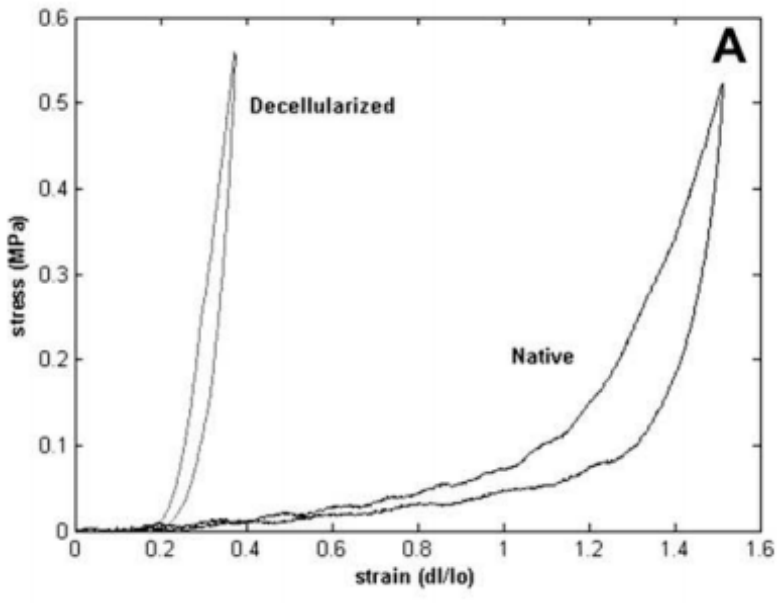

Figure 16: The strain-stress relationship of decellularized tissue compared to native tissue (Williams, et al., 2009).

CONCLUSIONS AND FUTURE DIRECTIONS

Pediatric patches constructed through the use of bioprinting technology holds promise. Overall, the bioprinting process is reproducible and a good first step of creating adventitial tissue, but there is a lot of room for improvement and refinement. Firstly, sine waves may not be the best pattern. The wavy triangular pattern (**Figure 5**) with a large angle in the alternating arcs is a next logical step. Independent of the pattern, the print should be compact, with various orientations, and printed with thin lines. The samples should also be designed for biaxial testing, as both circumferential and longitudinal mechanical properties are more would more closely mimic the mechanical load conditions of actual blood vessels.

Secondly, the sample rest time in calcium chloride should be re-evaluated. A resting time of 14 hours was chosen out of time constraints, and long crosslinking times most likely do not contribute positively to the mechanical properties. Time trials of 4 and 8 hours are suggested as a starting point. Other hydrogels such as gelatin can be tested.

Additionally, bioprinting collagen is not limited to embedding the collagen in alginate. Different options for collagen bioinks and techniques include (1) a collagen solution with riboflavin photocrosslinking (Diamantides, et al., 2017), (2) a collagen-Matrigel mixture (Nerger, et al., 2019), and (3) a collagen bioink printed in freeform reversible embedding of a suspended hydrogel (FRESH v2.0) (Lee, et al., 2019).

Thirdly, the adventitial scaffold can be seeded with fibroblast cells in one of two ways: (1) embedding the cells in the bioink before printing and (2) seeding the

scaffolds after they are printed. The method that proves better cell viability should be continued through the project. Seeding the scaffold with the fibroblast cells will promote synthesis and production of relevant extracellular matrix components of adventitial tissue (Tomasina, et al., 2019).

Lastly, the scaffolds can be crosslinked to elastin lamella using droplets of genipin (Chow, et al., 2011) distributed uniformly throughout the wave pattern to further the advancement of the structure of the adventitial tissue. The summarized future steps of this project are illustrated in **Figure 16**.

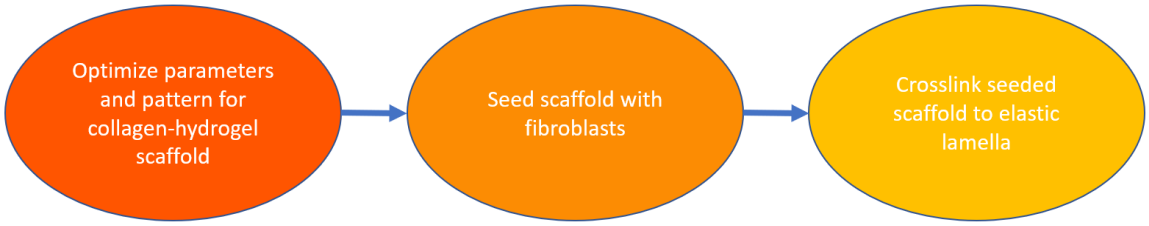

Figure 16: A visual, chronical summary of the future steps of this project.

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