

SYNTHESIS OF NOVEL 3D MICROENVIRONMENT
USING ECM-ENHANCED COLLAGEN-PEG-A
HYDROGEL

BY

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List of Abbreviations

^1H NMR: ^1H Nuclear Magnetic Resonance

AC: Acellular Construct

ADC: Adipose Derived Stem-Like Cells

CB-Buffer: Carbonate-Bicarbonate-Buffer

CEPA: Collagen:ECM:PEG-Acrylate

Col: Collagen

CPA: Collagen:PEG-Acrylate

cPEG-A: Cellularized PEG-Acrylate

CS: Compartment Syndrome

DMEM Low: Dulbecco's Modified Eagle Medium with Low Glucose

ECM: Extracellular Matrix

ECM-MA: Extracellular Matrix - Methacrylate

FBS: Fetal Bovine Serum

GC-MS: Gas Chromatography-Mass Spectroscopy

H&E: Hematoxylin and Eosin

MLR: Multiple linear regression

NMMP: Nonmyogenic mesenchymal progenitors

PEG-A: Polyethylene Glycol – Acrylate

PEG-DA: Polyethylene Glycol Di-Acrylate

PFA: Paraformaldehyde

GAG: Glycosaminoglycan

SMA: Smooth Muscle Actin

TNBS: 2,4,6-Trinitrobenzenesulfonic acid

UV: Ultraviolet

Abstract

The causes of the cellular dysfunction that impedes tissue regeneration are not well understood and are often multi-factorial but are associated with major tissue trauma such as in volumetric muscle loss (VML). Outcomes vary in severity; however, these conditions can lead to loss of limb function, deformity, and disability. There are relatively few reliable models to research such a disorder and fewer treatments available to overcome loss of function. Bioengineering has several promising avenues for treatments through tissue specific biomaterial development such as incorporating key micro-environmental and mechanical properties necessary for normal cellular function. In this thesis we developed a hydrogel augmented with extracellular matrix and seeded with adipose derived stem-like cells (ADCs) to provide the groundwork for a regenerative medicine approach utilizing a cell type found in the periphery of skeletal muscles. We hypothesize that incorporation of decellularized extracellular matrix (ECM) into collagen-poly (ethylene-glycol)-acrylate (CPA) hydrogels will provide a stable and survivable environment for ADCs.

CPA, and Collagen-Peg-A-ECM (CEPA) hydrogels were assessed via rheology and exhibited varying levels of structural rigidity and storage modulus was modeled using a multiple linear regression (MLR) model and an additive model. ADCs encapsulated in both CPA and CEPA hydrogels showed differences in cell viability.

In conclusion, we successfully incorporated methacrylated decellularized skeletal muscle ECM into a 4-arm PEG-A based bioink. Cellularization of the created hydrogels indicates further research into this novel hydrogel may be beneficial for creation of a more accurate mimic of the *in vivo* microenvironment *in vitro*. However, further testing is required to increase hydrogel stability during processing to provide an increase in image clarity post-staining.

Specific Aims

Specific Aim 1: Create a stable hydrogel formulation that incorporates skeletal muscle ECM into a Collagen:PEGAcrylate (CPA) Hydrogel.

- a) Preparation of ECM for incorporation into CPA hydrogel via decellularization, lyophilization, and methacrylation.
 - i) Visual analysis of solid construct boundaries compared to local environment of growth media.
- b) Analyze the mechanical properties of Collagen:ECM:PEG-Acrylate (CEPA) constructs compared to the Collagen:Peg-Acrylate (CPA).
- c) Test the printability of the hydrogel using a microvalve-based printer.

Specific Aim 2: Determine the effects of skeletal muscle ECM on adipose cell viability and differentiation.

- a) Measure cellular viability within the CEPA hydrogels
- b) Analyze adipogenic differentiation of ADCs within CEPA hydrogels
- c) Compare changes in cell counts, viability, and gene expression of ADCs in CEPA constructs to CPA controls via immunohistochemistry and immunofluorescence.

Specific Aim 3: Create predictive mathematical models to determine storage moduli based on concentration changes in solution of hydrogel components.

- a) Develop an equal contribution model (additive model) with parameters for both the stock solution concentrations and concentration in solution.
- b) Develop a multiple linear regression (MLR) model of storage moduli based off observed rheology data.

Introduction

Acute Compartment Syndrome (CS)

Tissue damage can be caused by a multitude of external and internal factors. One such condition is CS, a condition in which the fluid or inflammation inside of a tissue compartment, typically the anterior calf compartment, builds up¹. With no more room to push outward, we begin to see the intracompartmental space of afflicted areas increase in pressure. When intracompartmental pressure rises occur, regional arteries and veins can become occluded, resulting in a decrease or cessation of oxygenation of the local tissues such as skeletal muscle and nerves, ultimately leading to necrosis². Development of this disease predominantly occurs in areas which have high muscle density such as the legs, however, it has been noted to occur in the arms, hands and fingers³. In a clinical setting, any intracompartmental pressure that exceeds the diastolic blood pressure by 30 mmHg is considered an indicator of CS. However, even when treatment is expeditious for CS, complications can occur and result in permanent damage or even death⁴. VML is one such example of a complication; it is a condition in which skeletal muscle is permanently lost in either function or density **Figure 1**.



Figure 1. Volumetric Muscle Loss

Credit Dr. P Marazzi / Science Photo Library

Crush injury scarring to leg. Scarring from old crush injury, and scar from decompression surgery for compartment syndrome on the back of the calf of a 46-year-old man's leg. Compartment syndrome be caused by crush injuries requiring surgery to open up the affected fascial compartment (sections of the arms and legs) to relieve (decompress) the pressure on the muscle and nerves (Property release not required).

VML primarily affects individuals who serve in the military. While there are civilian VML injuries, these result mostly from compound fractures and do not typically result in

permanent disability of the affected limb. Those who serve in some capacity for the military, hereby referred to as the warrior population, receive VML injuries due to a variety of traumatic events, primarily blunt force trauma to an extremity. During Operation Enduring Freedom and Operation Iraqi Freedom, 77% of the warrior population (n = 14500) evacuated due to medical reasons had musculoskeletal injuries or open soft tissue extremity wounds, which are precursors to VML⁵. If a warrior developed VML, the estimated life-time cost could be around \$440,000, which includes medical costs for interventions like delayed amputation or muscle massaging to prevent deep vein thrombosis.

Prognosis is bleak for warriors with VML. Once function has begun to degrade, the ability to regenerate it diminishes rapidly. This leads to long-term disability due to limb non-functionality, and permanently renders the warrior unable to complete a wide variety of daily tasks which are limb dependent. While there has been research into treatment of VML, there is no standard treatment which is capable of complete regeneration of form and function for the affected tissue⁶⁻⁹. This affected tissue, however, has many peripheral cell types that it interacts with which may be used to influence the growth of novel tissue post-trauma.

Regenerative Medicine Approach to Muscle Tissue Repair

There is no current technology on the market which can successfully treat volumetric muscle loss. If a drug or co-administration therapy is created that can stimulate muscle growth, then the form and function may be restored in either condition.

This may be done by stimulating muscle growth via peripheral signaling, or by inducing growth through the usage of multipotent or pluripotent stem cells that do not form teratomas¹⁰. Additionally, muscle regeneration using autologous transplantation of live skeletal muscle has had limited success^{6,11}. As such, there is a need for a transplantable biomaterial seeded with cells from the host, which can both integrate in situ with currently present muscle and influence the growth of novel muscle. Such biomaterials that can support cells, known as hydrogels, are ubiquitous in the field for regenerative medicine. There are many ways to create hydrogels today, and even more hydrogel formulations to choose from¹². Some constructs are designed to be 3D printed, and may be seeded with cells such as human primary muscle progenitor cells with the purposes of enhancing regenerative capacity of a human body¹³. Other constructs in the regenerative medicine field focus on regeneration of functional capacity of limbs via hydrogels seeded with neuronal cells¹⁴.

Skeletal muscle is considered a complex heterogenous component of the body which is not easily replicable. Many components can be supplemented into hydrogels to mimic the microenvironment, such as fibroblast growth factor, Human growth hormone, or vascular endothelial growth factor¹⁵. Further, usage of a variety of componentry for hydrogels to attempt to improve biocompatibility has been attempted using agarose, alginate, gelatin, and many other natural materials. These each present with unique properties which, when formed into a cellularized hydrogel, may be capable of incorporation for the purpose of muscular regeneration. The incorporation of endothelial

cells within a hydrogel also provides an interface which is present *in vivo* and has been noted that angiogenesis has a marked upregulation post-trauma ¹⁵.

Other papers have explored the incorporation of ECM proteins such as laminin into scaffolds in order to increase muscle cell proliferation and function ¹⁶. These showed promising results due to the incorporation of two extra degrees of freedom: tensile and electrical stimulation, which were not used for this research. Incorporation of a tensile component may provide the ADCs used in this study a more analogous environment to peripheral skeletal muscle, promoting the release of growth factors beneficial for cellular proliferation. Further, since electrical stimulation is constantly present via neurological functions, this may also influence the ADCs *in vitro* and would further contribute to a mimicked *in vivo* microenvironment.

Our skeletal muscle ECM infused hydrogel aims to mimic the local microenvironment, increasing biocompatibility and attempting to further refine the results to better match those found *in vivo*. This hydrogel aims to completely integrate the ECM inside it using a process which permanently crosslinks the radical ends of the ECM to the Collagen-Methacrylate and PEG-A radical ends, thereby augmenting the hydrogel with ECM. ECM substitutes have been used in the past, such as alginate-based hydrogels¹⁷, and some ligands have been augmented in hydrogels¹⁸, however less research is present in the area of methacrylate ECM hydrogels. This is a path of research which may yield a customizable hydrogel for autologous augmentation of ECM, in a clinical setting, derived from regionally important ECM. Both the incorporation of collagen itself and the addition of ECM to a hydrogel are not new ideas, however research into methacrylate ECM

incorporation into collagen hydrogels is limited¹⁹. Autologous transplantation by using ECM harvested from a patient may avoid antibody specific reaction, improving biocompatibility, and allowing for treatment using hydrogels without the necessity for immunosuppressants²⁰. These hydrogels, implanted in-situ near an injury, might be able to help repair recent traumatic injuries to muscle. If large scale in-situ implantation near an injury is required, synthesizing a hydrogel with a similar storage modulus to the local microenvironment within the tissue can aid with phenotypic expression regulation^{21–24}.

Adipose derived stem-like cells (ADCs) are strong candidates for research and translation. ADCs can play an important role in tissue engineering or regenerative medicine for the development of novel treatments both in-situ and in-vitro. ADCs have been used for the treatment of diabetic foot ulcers, osteoarthritis, and peripheral neuropathy among others^{25–27}. ADCs are ubiquitous in the human body, and directly integrate into the human body in a plethora of regions which contain a multitude of tissue types. This makes them good candidates for use in regenerative medicine because of their abundance in the body. By using ADCs which can both differentiate and potentially send signals to peripheral cells that influence peripheral cell genotypic expression, we may be able to stimulate cells to begin proliferating again. On a macro scale, this would result in the complete regeneration of tissue post-trauma, as well as restoration of functionality. These cells are capable of attaching to hydrogels they are encapsulated in via the same mechanisms found *in vivo*. Cells utilize proteins such as laminin $\alpha 4$, integrin $\alpha 7$, collagen, and others to attach to the extracellular membrane. The usage of an ECM-augmented hydrogel may infuse the hydrogel with some of the ligands integrin binds to thereby stimulating the

attachment of cells to the hydrogels²⁸. Utilization of ADCs embedded within ECM augmented hydrogel may increase biocompatibility and integration capability of the hydrogel into the native tissue.

Here we propose a potential solution in which ADCs can be grown in a 3D environment similar to the *in vivo* environment, including ECM signaling and storage modulus. This mimicked microenvironment will provide the framework for inducing cellular signaling from ADCs which can create positive feedback loops in cells which have a more direct interaction with skeletal muscle with the goal of inducing novel tissue growth through paracrine signaling.

In previous literature, the influence of ECM has been both theorized and studied to have a large importance on cell mediation and tissue-specific gene expression^{29,30}. Part of the influence ECM has is due to the collagen component which provides cells a rigid substrate for adhesion³¹. Large-scale physiological responses to adipose tissue implantation and its attenuation of metabolic dysregulation has also been studied in mice³². With ECM influencing ADCs, which in turn may influence the surrounding area, it is possible that the proper signaling to incite a regenerative response may be possible in recent trauma afflicted areas.

The usage of 3D constructs for cell culture is done to bridge the gap between 2D *in vitro* modeling and in situ or *in vivo* studies. 3D constructs have been well studied over the last decade, however, there has not been a definitive mixture created which is a true accurate representation of areas of an organism. Since the local microenvironment differs per

organ in organisms, the incorporation of ECM is only the first step in creating an accurate *in vitro* model. However, this paper serves as a basis for the creation of a novel hydrogel which can support the proliferation, differentiation, and viability of ADCs.

This research aims to create a novel hydrogel that incorporates a photo-crosslinkable decellularized skeletal muscle ECM to better improve hydrogel stability and mimic *in vivo* conditions *in vitro* as well as determine the influence of the chemically modified ECM has on cells encapsulated within the novel biomaterial. Our results showed that there were significant differences in the storage modulus dependent on the composition of the constructs, as well as notable decreases in long-term cell viability in constructs containing ECM. Taken together, we conclude that a composition is required which can match the storage modulus found in skeletal muscle tissue that are biocompatible with ADCs.

Methods

Cell Culture

Adipose Derived Cells (ADCs) which were obtained from a primary culture line at Wake Forest Institute for Regenerative Medicine and cultured with Dulbecco's Modified Eagle Medium with Low Glucose (DMEM Low) supplemented with 10% Fetal Bovine Serum (FBS) and 1% anti-biotic/anti-mycotic. Media was changed every 2 days. When cells became confluent, they were trypsinized using .25% Trypsin and expanded using standard cell culture protocols³³.

Cell Counting

100 µL of suspended cell media was placed into a NucleoCounter® cartridge, and a NucleoCounter® NC-200™ was used to read the cell count. The total cells in solution were then calculated **Equation 1**. A minimum of 30 million cells was necessary for the experiments performed.

$$C_{\tau} = C_l \cdot S$$

Equation 1. Cell count equation

Total cell count in solution where C_{τ} is the total cell count in the solution, C_l is the cell count via the NucleoCounter®, and S is the total cellularized solution size in mL.

Differentiation

For differentiation of ADCs *in vitro*, StemPro™ Adipogenesis Differentiation Kit (Catalogue # A1007001) was added to the DMEM Low to reach a final concentration of 2

mM of GlutaMAX™ 5 µg/mL of Gentamicin reagent, and 10% FBS, and the media was refreshed every two days in culture for up to a week.

Decellularization and ECM Preparation

Rat skeletal muscle was harvested from the quadriceps region as well as the dorsal thoracic region. Once the tissue was separated from the bone, it was cut into 1mm³ pieces left to rinse overnight in 1 liter of DI at 4°C. Decellularization was achieved using 1L of DI water with 1% Triton-X 100 and 0.1% ammonium hydroxide in solution. Muscle tissue was placed into a new container and 1 liter of decellularization solution was added to the skeletal muscle and placed on a shaker at 150 RPM and 23°C. The solution was changed daily for 5-7 days, or until the tissue appeared white and opaque to translucent with a DNA content under 50 ng/mL **Figure 2**.

Decellularized tissue was moved into a 50mL centrifuge tube and spun at 220 g for 5 minutes, and the supernatant was removed. A solution of pepsin was made using 100 units/mL in .37M HCl with DI water. The tissue was submerged in pepsin and placed on a rotary spinner at 60 rpm in a warmed refrigerator walk in, kept at 37 C, for 48 hours. The tissue was then spun at 5000 RPM for 5 minutes and the supernatant was moved to a new centrifuge tube and spun down again at the same speed and time interval. The supernatant was then moved to a new weighed centrifuge tube, ensuring not filling beyond halfway to avoid boil-over during lyophilization.

The tissue was then placed in a pre-weighed centrifuge tube and moved into the -80°C overnight to fully freeze it. The cap of the centrifuge tube was removed and

replaced with a Kimwipe fastened using a rubber band. The centrifuge tube was placed into the lyophilization flask and lyophilized for 2-3 days. The tube was recapped with a centrifuge cap and placed in the -80°C freezer until ready for methacrylation.

ECM Methacrylation

The lyophilized ECM was weighed out using a scale, and then resuspended in 1% acetic acid at a concentration of 10 mg/mL. A one-pot method was used to methacrylate this ECM solution by vortexing until the ECM was fully solubilized in solution³⁷. A Carbonate-Bicarbonate-Buffer (CB Buffer) was prepared using 53 mg/mL Na_2CO_3 and 40 mg/mL of solution of NaHCO_3 added into a flask with DI water. The CB buffer was combined with the acetic acid ECM solution at a 1:1 ratio. The combined solution was then placed on a hot plate and stirred using a stir bar until the components were fully solubilized.

Once fully solubilized, Methacrylic Anhydride (MA) was added dropwise at 10 $\mu\text{L}/\text{mL}$. The solution was then capped with a ventilated stopper and left to stir at 500 RPM at 50°C for an hour. PBS was then added at 50 $\mu\text{L}/\text{mL}$ to stop the reaction. If dialysis was not immediately performed, then the mixture was moved to the -20°C freezer until further use.

A large container was filled with around 10 L of DI water. HCl was added to the methacrylated solution until the pH was 3. 12-14 kDa dialysis tubing was cut into a long section, submerged quickly in water, and the lower half was tied off. The methacrylate solution was then pipetted into the tubing, ensuring only half the tubing was filled, and

the top half was then tied off. This tube, or tubes, was then placed into the large container and tied off or clamped to the top of the container to prevent it from moving. A large stir bar was placed in the bottom of the DI water, and the entire container was put on a hot plate at 37°C, stirring at 150 RPM. The solution was dialyzed for 3 days with DI water changes daily. The dialysis bag was removed from the DI water and the top was cut off. The purified solution was pipetted from the tubing into a pre-weighed 15 mL or 50 mL centrifuge tube to less than halfway. In the event precipitate was present, the solution was centrifuged at 5000 RPM for 5 minutes to isolate the supernatant, which was transferred into a new pre-weighed centrifuge tube. The post-lyophilization weight was then recorded for use in resuspension of ECM. For resuspension, 2% acetic acid was used, and the resulting solution was a methacrylated ECM (ECM-MA).

¹H Nuclear Magnetic Resonance

¹H Nuclear magnetic resonance (NMR) was performed on samples in order to qualitatively determine the presence of MA in the ECM component used for construct synthesis. A human derived dermal ECM (Huma) non-methacrylate and Huma-Methacrylate were both used to show a positive and negative control compared to Methacrylic Anhydride. In a clean glass tube, 2 mg/mL of each sample was diluted in 1 mL of D₂O. Samples were placed on a 4-way shaker at 4°C for 1 hour to aid in solubilization. 800 µL of each sample was transferred to a Bruker SampleJet NMR tube (Bruker, Billerica, MA). ¹H NMR analysis was done at a frequency of 400 MHz.

ECM Augmented Hydrogel Preparation

Collagen was removed from a 4°C refrigerator and placed on ice. Peg-A and the solubilized ECM-MA were both removed from the -20°C freezer and thawed to room temperature. Collagen and ECM-MA were combined at set concentrations (dependent on hydrogel composition) in an Eppendorf tube. Then, PEG-A or cellularized PEG-A was added to the Eppendorf tube along with 86 μL of a neutralization solution (Advanced Biomatrix, Catalog #5153, Neutralization Solution only) and the solution was pipetted up and down 10 times while stirring to fully mix all components, and then left to equilibrate for 5 minutes in a sterile area which did not contain direct sunlight before pipetting to make hydrogels. For the control, a 1:1 ratio of ColMA to PEG-A was used (CPA hydrogel). Low collagen hydrogels used a 1:3:3 ratio of ColMA to ECM-MA to PEG-A. 100 $\mu\text{L}/\text{mL}$ of ColMA was added to a 1mL Eppendorf tube, along with 300 $\mu\text{L}/\text{mL}$ of ECM-MA. Low ECM constructs had 100 $\mu\text{L}/\text{mL}$ of ECM-MA and with 300 $\mu\text{L}/\text{mL}$ of ColMA in an Eppendorf tube. These ratios are shown below **Table 1**. All cellularized constructs had PEG-A added to a centrifuge tube containing ADCs at 10m cells/mL (cPEG-A). The cPEG-A was then micropipette into the ColMA:ECM mix at 300 $\mu\text{L}/\text{mL}$. Finally, neutralization solution was incorporated at 86 $\mu\text{L}/\text{mL}$ of ColMA in solution. This mixture was then pipetted repeatedly in 150 μL increments while also stirring with the tip of the pipette to ensure full mixing of the solution. Following mixing, the solution was left at room temperature for 5 minutes before using. The mixture was micropipette in 20 μL increments in a slow and controlled manner to allow the mixture to take a spherical shape in a non-tissue culture plastic 6 or 48-well plate. These constructs were then crosslinked for 5 seconds (.5 kJ) using a DYMAX

BlueWave. Acellular constructs were incubated at 37°C for 20 minutes. Following incubation, Phosphate-Buffered Saline (PBS) was pipetted into each well containing NCCs to maintain their integrity and prevent dehydration of the constructs.

Components	Control Hydrogel	Low PEG-A Hydrogel	Low ECM-MA Hydrogel	Low ColMA Hydrogel
ColMA	3 (300 µL)	3 (300 µL)	3 (300 µL)	1 (100 µL)
ECM-MA	3 (300 µL)	3 (300 µL)	1 (100 µL)	3 (300 µL)
PEG-A	3 (300 µL)	1 (100 µL)	3 (300 µL)	3 (300 µL)

Table 1: Ratio and volume of materials per composition

The ratio (shown as a whole number) and the volume amount (shown in parentheses) of the ratios used for each composition of the varying types of hydrogels.

Swelling Ratio

In order to test the swelling percentage of the constructs, the acellular constructs were created and crosslinked in a 6-well plate. After incubating for 30 minutes, the constructs were imaged using a bright-field microscope and the computer program LAS-X (Leica Microsystems). A ruler was placed under the 6-well plate to determine diameter of each construct, and the constructs were then placed into PBS for 24 hours and incubated at 37 C. The constructs were imaged again sequentially and the difference in diameter was taken. There was no notable height difference between constructs, and therefore only diameter needed to be measured.

Rheology

Rheological assessment of variable composition constructs was done to determine the change in storage modulus of constructs based on individual components using a Discovery HR-2 Hybrid Rheometer (TA Instruments). All tested constructs were

made in wells, which had 8 mm silicon cut-outs in them. The constructs were cross-linked for 5 seconds and immediately moved to the rheometer for testing. An 8 mm probe was installed on the rheometer, and sandpaper was glued to the base of the probe and tester to prevent movement of the constructs during testing. For all tests, the crossheads oscillations were set at 10 Hz, and the normal force applied to the sample, initially, was 1 N. Strain was increased from 0% to 100% by 10% increments. Due to the high loss modulus of the samples, only an initial normal force was measured.

Cellular Viability Assays

CellTiter Glo 3D™

Cell Viability Assay kit from ProMega (Catalog # G9681) was removed from the –20°C freezer and left to thaw at room temperature for 30 minutes to an hour. During this cooling time, a plate containing the hydrogels was removed from the incubator and allowed to cool to room temperature. The hydrogels were then moved to individual wells of a 48 well plate with 100 µL of room temperature media. 100 µL of the assay solution was then aliquoted into each well, and the plate was gently agitated on an oscillating platform for 30 minutes to induce cell lysis and diffusion of ATP throughout the full volume of the well. 100 µL of the ATP solution from each well was transferred into a new white non-transparent 96 well plate, and the luminescence was recorded via a plate reader.

Live Dead Staining

To visualize changes in cell viability over time, a live/dead kit (Thermo Fisher, catalog #L3224) was used. Green-fluorescent Calcein-AM a fluorescent marker for intracellular esterase activity and red-fluorescent Ethidium Homodimer-1, a marker for lack of cell membrane stability, were thawed from -20°C to room temperature and vortexed for 30 seconds. Then, 1 μL of Calcein-AM was added to an Eppendorf tube, along with 1.8 μL of Ethidium Homodimer-1. This was then filled with 1 mL of PBS. The 3D constructs were then moved to a new 48-well plate and washed with PBS twice to clear any media residue. Then the samples were immersed in the live dead solution and incubated for an hour. After an hour, the plate was taken out of the incubator and imaged via fluorescent microscopy using an Olympus IX-83.

Live/dead images were quantified utilizing a customized image J script. The files were exported from the Olympus IX-83 as burned-in info .tif files. These files were then opened in “(Fiji Is Just) ImageJ” and a macro was used to convert the images to grayscale and perform quantification of the constructs ***Supplementary Information 1, Supplementary Information Figure 2***. Regions of images which presented areas with physiologically erroneous imaging artifacts due to overexposure from high fluorophore accumulation that resulted in aberrant pixel saturation, when possible, were not used or counted toward the live dead analysis. This data was then input into Microsoft Excel and a percent total of living cells was taken.

Immunohistochemistry

Constructs were placed in 4% paraformaldehyde (PFA) in a 15mL centrifuge tube for 48 hours in a 4°C refrigerator for fixation. Constructs were removed from PFA and transferred to a histology cassette inside an enclosed piece of lens paper. The cassettes were then left in a sealed container submerged in 70% ethanol.

The constructs were dehydrated and via stepwise ethanol gradient and cleared utilizing Clear-Rite 3 instead of xylene. The constructs were then removed to a paraffin bath. Post-paraffinization the constructs were embedded and blocked using standard protocols. The blocks were then sectioned in 5 µm increments using a Leica RM2265 and subsequently dried.

Hematoxylin and Eosin Stain

A 95% alcohol solution was prepared using 5 mL of deionized water and 95 mL of 100% Ethanol. The slides were deparaffinized to water and stained in Harris Hematoxylin Solution for 2.5 minutes. They were then rinsed in running tap water and dipped twice in a differentiation solution. The slides were rinsed in running tap water again, and then placed in Blue in Scott's Tap Water Substitute for 30 seconds, and then dehydrated in the 95% alcohol for 30 seconds. Then, the slides were counterstained with Eosin Y Solution for 30 seconds and dehydrated and cleared to xylene³⁴.

Herovici's Young and Old Collagen Stain

The slides were deparaffinized and hydrated to water. They were placed in deionized water until ready for staining. The nuclei were stained using Weigert's Iron

Hematoxylin for 10 minutes and rinsed in running tap water for 10 minutes. The slides were placed into a .05% Methyl Blue, 20% Glycerol, and 10% Lithium Carbonate solution. They were then washed in 1% acetic acid for 2 minutes, and then dehydrated and cleared to xylene and cover-slipped following the acetic acid wash³⁵.

Alcian Blue Stain

Sections were deparaffinized and hydrated to deionized water and placed in Weigert's Iron Hematoxylin for 10 minutes. The sections were then rinsed for 10 minutes in running tap water. Each slide was dipped into acid methanol solution 3 times, and then placed in running tap water again for 5 minutes, then rinsed once with deionized water. The slides were then placed in Alcian Blue for 10 minutes, and then rinsed in running tap water for 5 minutes, following by a rinse in deionized water. The slides were then placed in a phosphomolybdic acid solution for 2 minutes and rinsed in deionized water for 1 minute. Then, the slides were stained with picosirius red solution for 1 hour, and then rinsed in 70% ethanol for 45 seconds. The slides were drained and immediately dehydrated and cleared to xylene, then coverslipped³⁶.

Immunofluorescence Staining

Slides were deparaffinized and cleared to water prior to immunofluorescent staining. Slides were loaded into a LabSat (Lunaphore Technologies) with a distributor chip placed on top. The machine was primed with all reagents, and an antigen retrieval solution (pH 6) was placed on the slides for 5 minutes. Triton X 0.2% was then used to permeabilize the contents of the slide for 1 minute, followed by a Serum-Free Ready-To-

Use Protein Block (Dako) for 30 seconds. The slide was washed with TBST, and then the slide was incubated with the primary antibody, either anti-Smooth Muscle Actin (catalog #MA5-11547, Thermo Fisher), or anti-Vimentin (catalog#EPR3776, abcam), for 4 minutes. Slides were washed again with TBST, and the secondary antibody, a **388 nm** goat anti-rabbit or **588 nm** goat anti-mouse was incubated on the slide for 4 minutes, followed by a final wash with TBST. Slides were then imaged using an inverted fluorescent microscope (Olympus IX-83).

Results

Muscle Tissue Decellularization

Decellularization of the muscle ECM was an important step in ensuring that the environment that the cells were placed into was a controlled environment. Quantitative data which validates the success of the decellularization also serves the purpose of showing that minimal external DNA content will be present in the gels once the solutions are made. The initial step of the CEPA synthesis is to isolate skeletal muscle ECM by removal of cellular material. Muscle tissue was decellularized utilizing a triton x-100 buffer solution after gross sectioning. Hematoxylin and eosin (H&E) stain was performed to confirm decellularization. We compared the decellularized ECM to normal muscle tissue as a control and found there were no nuclei seen in the processed skeletal ECM after staining indicating the decellularization procedure was effective, and all the cells were removed from the ECM **Figure 2A, Figure 2B**. We also found there was less than 50 ng/mg of DNA within the material further reaffirming that most cellular constituents had been removed **Figure 2 inset**.

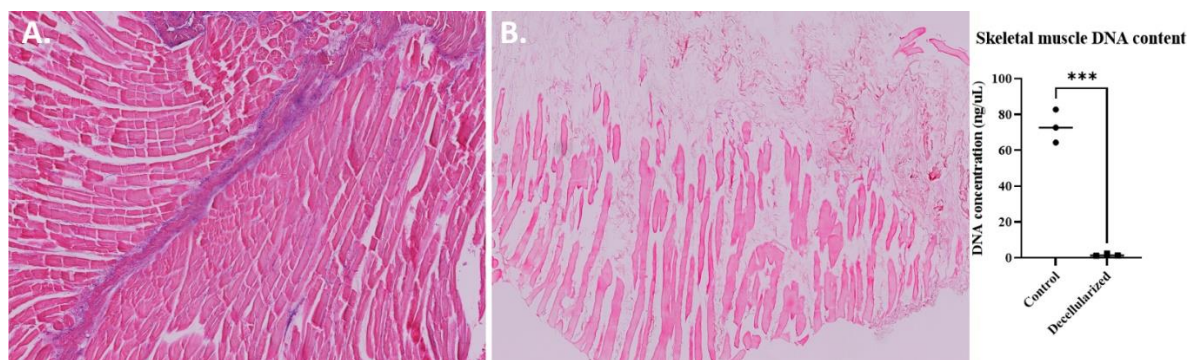


Figure 2. H&E Stain of Decellularized Tissue

2A) A control section of the skeletal muscle ECM which still contained some cells, indicated by nucleic staining in purple and cytoplasmic staining in pink, in an H&E stain. 2B) A decellularized section of the skeletal muscle ECM. No nuclei were seen during visualization of the sectioned decellularized tissue in under Brightfield microscopy, and there is substantially less purple staining which is indicative of nuclei in H&E. 2C) DNA quantification showed less than 50 ng/ μ L of DNA in the decellularized tissue (graph of the right).

Verification of ECM Methacrylation

To stably incorporate decellularized ECM into the hydrogel we first proceeded to methacrylate the ECM using the so called one-pot method, which modifies certain hydroxyl and amine groups of the ECM via methacryloyl substitution thus rendering the ECM photo-crosslinkable³⁷. While the one-pot methacrylation method is a previously studied procedure, it has never been scaled down to 1/10th the size for materials used³⁷. Therefore, testing of the tissue via ¹H NMR allowed for validation of scalability of this method, as well as confirmation of methacrylation of the tissue used for incorporation. We then compared the methacrylated skeletal ECM against a similar, albeit non-skeletal, material to qualitatively determine success of methacryloyl substitution resulting in ECM-

methacrylate (ECM-MA). The control used here is a HumaMatrix Dermal ECM, derived from isolated human tissue retrieved from the American Association of Tissue Banks³⁸. This Dermal ECM was only used for verification of methacrylation via ^1H NMR and is not present in any other experiments in this thesis.

Analysis of ^1H NMR shows a peak at around 1.9 ppm on HumaMatrix Dermal ECM-MA that is consistent with the peak for Methacrylic Anhydride **Figure 3B**. This peak is absent on the HumaMatrix Dermal ECM which is not methacrylated **Figure 3A**. The skeletal muscle ECM-MA presented with a peak at 1.9 ppm that is indicative that the Methacrylic Anhydride was capable of binding to the ECM during the one-pot methacrylation method **Figure 3C**. A parallel control ECM with a methacrylation comparison was used in lieu of a pre-methacrylate skeletal muscle ECM control. Methacrylic Anhydride also presents a double peak in the 5-6 ppm range; however, this was not focused on due to interference with peak presentation from D_2O .

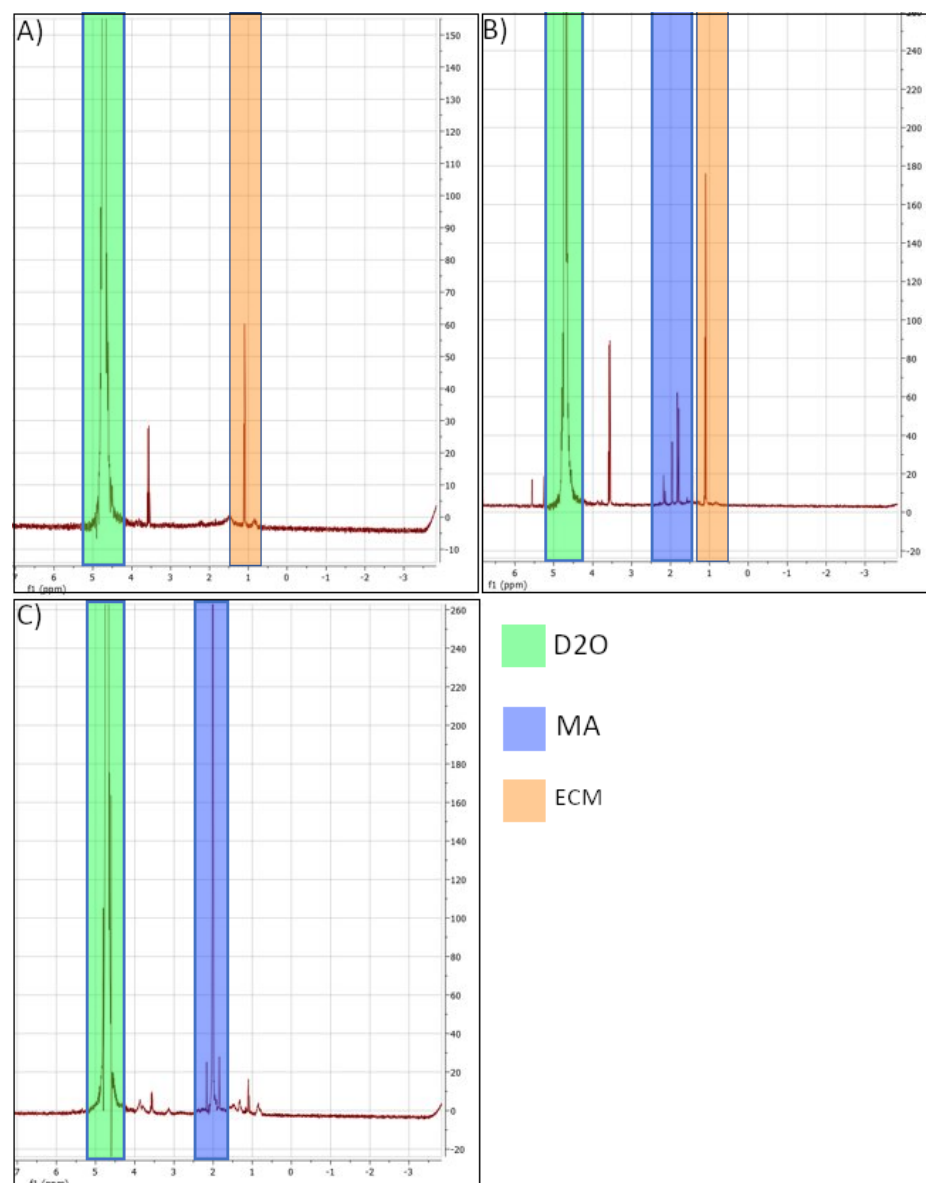


Figure 3. ^1H NMR

3A) HumaMatrix Dermal ECM has peaks at 1 ppm for the ECM component and D₂O at 5 ppm. 3B) HumaMatrix Dermal ECM-MA, has a methacrylate peak at 1.9 ppm. 3C) Skeletal Muscle ECM-MA also presented a peak at 1.9 ppm. This is indicative that the one-pot method of methacrylation was successful on the HumaMatrix Dermal ECM and potentially successful on the skeletal muscle ECM. A peak that is specific to the HumaMatrix Dermal ECM, but is not identified, is present in at 3.5 ppm.

Preparation of ECM Augmented Hydrogel

After methacrylation, the ECM-MA needed to be incorporated into the CPA gels. To achieve higher concentrations of ECM-MA and *in vivo* like stiffness a stiffening agent which could help achieve both was required. We developed a procedure utilizing PEG-A for this purpose. Briefly, different ratios of polyethylene glycol di-acrylate (PEGDA), collagen methacrylate (ColMA), and ECM-MA, and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) were mixed to ascertain the effect on construct stability of each of hydrogel constituents. All stock solutions remained the same: 50 mg/mL of PEG-A, 10 mg/mL of ECM-MA, and 6 mg/mL of Collagen. These were then added in a ratio of Collagen:PEG-A:ECM-MA, decreasing the final concentration in solution.

In the original synthesis of a CEPA construct a 2-arm 5 kDa PEG-A was used but it did not have the crosslinking capacity required, resulting in unstable constructs that easily dissolved in PBS. Thus, to create stronger hydrogels we substituted with a 5 kDa, 4-arm PEG-A at the same concentration which provided better stability when used in conjunction with the ECM-MA. However, with the incorporation of the ECM the structural stability of the entire construct decreased substantially. The PEG-A was changed from a 2-arm 5 kDa to a 4-arm 5 kDa PEG-Acrylate to increase structural stability and reach physiological storage and loss moduli **Figure 4**. If the introduction of new components into a hydrogel were to change stability, changes to the kDa of the PEG-A can be used to adjust the constructs back to *in vivo* metrics for storage and loss moduli.

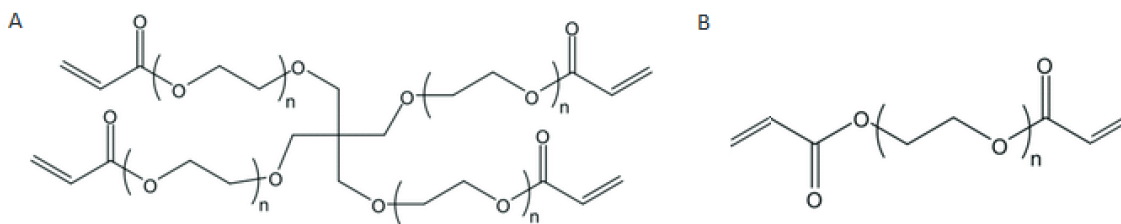


Figure 4. 4-Arm PEG-A Chemical Formula Differences

A) 4-arm PEG-A with a terminal alkene and carbonyl present at 4 different end points. B) PEGDA, a 2-arm precursor to the 4-arm PEG-A, also has terminal alkene and carbonyl groups present. The doubled amount of crosslinking points in PEG-A compared to the initially used PEGDA allows for a substantial increase in the structural rigidity of constructs. The group $(CH_2CH_2O)_n$ present in each arm is increased or decreased during synthesis to change the overall kDa of the compounds.

Beyond chemical composition, physical characteristics are also an important part of the success of a hydrogel. If a hydrogel is not uniform in shape, there may be an issue in the chemical composition which will lead to an early degeneration of the gel. In this situation, breakdown of the hydrogels is disadvantageous to the research, and as such a focus in the longevity of the hydrogels was preferred. To do this, we created different ratios of Collagen:PEG-A:ECM-MA from the stock solutions. We initially used brightfield microscopy to determine the viability of different construct compositions. At lower concentrations and longer kDa lengths, deformities such as incomplete crosslinking or tearing in the hydrogel began to occur **Figure 5**. The incorporation of a short-chain (5 kDa) 4-arm PEG-A created a more spherical shape compared to longer-chain (20 kDa) created hydrogels. This is due to the changes in rigidity which occur with manipulation of chain length. Imperfections such as tearing in the construct, improper formation of a sphere, or dissolving of the construct, were labeled with an orange arrow as “unstable border,”

which indicated the mixture was not capable of maintaining structure. The disparity in stiffness of the hydrogels comes from the difference in molar weight at the same concentrations for varying chain lengths of the PEG-A. A short chain will have a larger molar concentration due to decreased molecular weight, increasing stiffness.

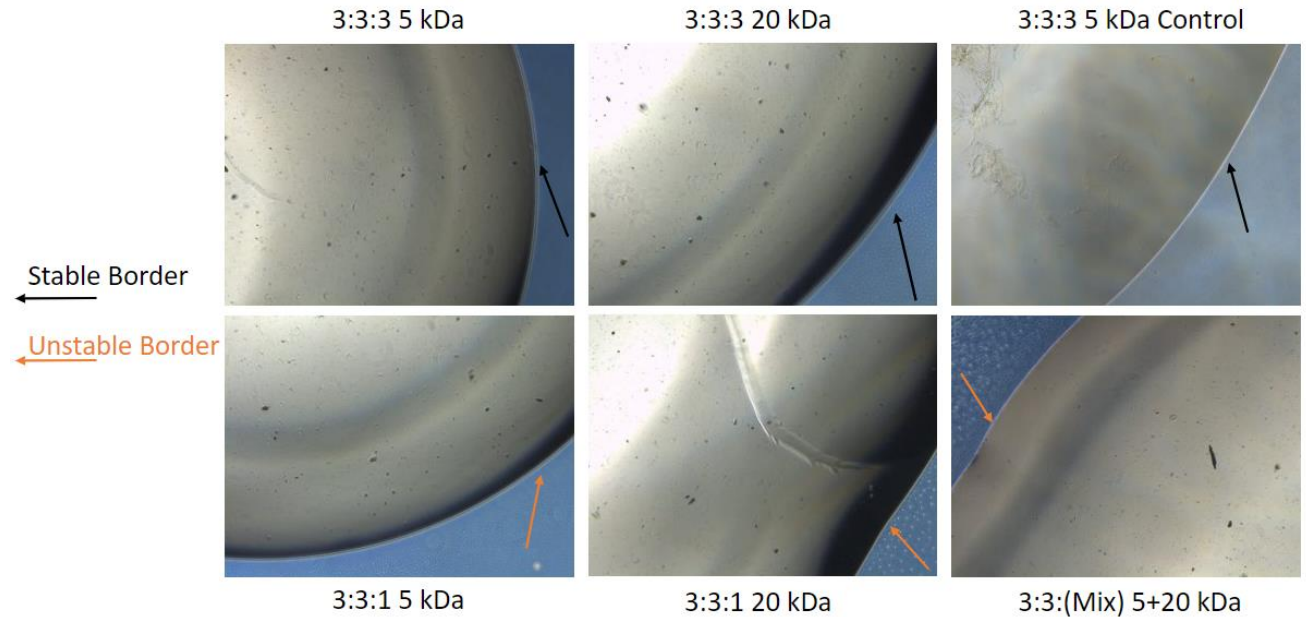


Figure 5. Brightfield Microscopy of Hydrogels

Hydrogels were divided into subgroups based on the ratio of Collagen:ECM:PEG-A present in the solutions used to make them. Hydrogels were visualized using 4x magnification bright-field imaging on a Leica DMI-8 microscope. The control, as well as three of the mixtures which had an equivalent ratio of PEG-A to ECM, maintained a spherical shape for up to 3 days. Comparatively, lower PEG-acrylate mixtures did not maintain their shape and would eventually dissolve over the 3-day period.

Swelling Test of Acellular Constructs

The CEPA hydrogels also needed to be compared to their CPA counterpart regarding influx or efflux of water. A swelling test, in which the hydrogels are submerged

in a neutral liquid for 24 hours, provides information about how much of a change in size the hydrogels will undergo while in media. This also tells us if there is any significant difference due to changes in composition between the CPA and CEPA hydrogels. The constructs were measured using a ruler underneath either the CPA control or CEPA hydrogel **Figure 6**. For the CPA control (n=5), an average swelling ratio of 6.43% was measured using *Equation 2*. The CEPA hydrogel (n=5) had an average swelling ratio of 5.93%, which was not statistically significantly different from the CPA control **Figure 7**. A positive percentage is indicative that the material is hypotonic and is absorbing water from the surrounding liquid. The swelling ratio was calculated by taking the summed values (n) from subtracting the initial diameter (W_f) from the initial diameter (W_s), divided by W_s , and dividing by the total sum (n) **Equation 2**.

$$\text{Average Swelling Ratio}(\%) = \frac{\sum_{i=1}^n \left(\frac{(W_{f_i} - W_{s_i})}{W_{s_i}} \right)}{n}$$

Equation 2. Swelling Ratio Equation

The average swelling ratio is the summed average of all final diameters (W_f) of constructs in PBS for 24 hours, minus the average of all initial measurements (W_s), per construct, divided by the average measurement of the initial measurements, and this final valuation was converted to a percent. A positive value is indicative of swelling, or influx, whereas a negative value shows efflux of liquid.

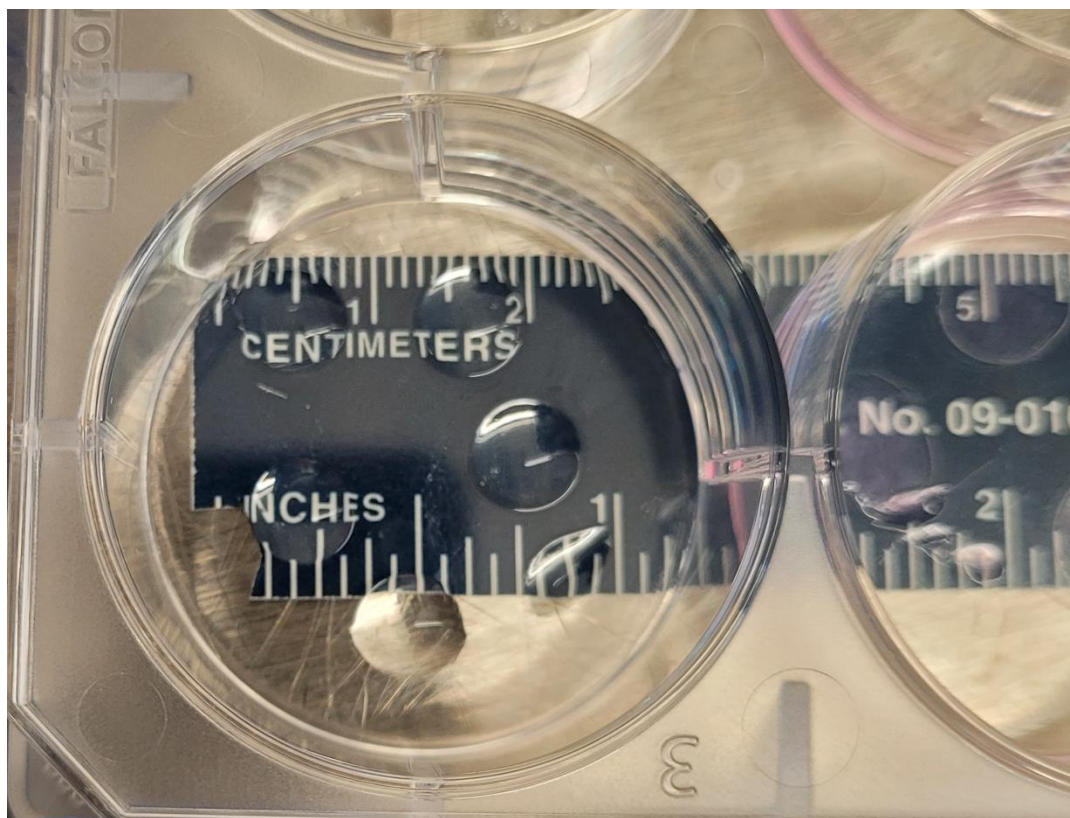


Figure 6. Ruler Positioning for Swelling Test

Position of the ruler for measuring constructs, measured by the diameter of the construct in centimeters.

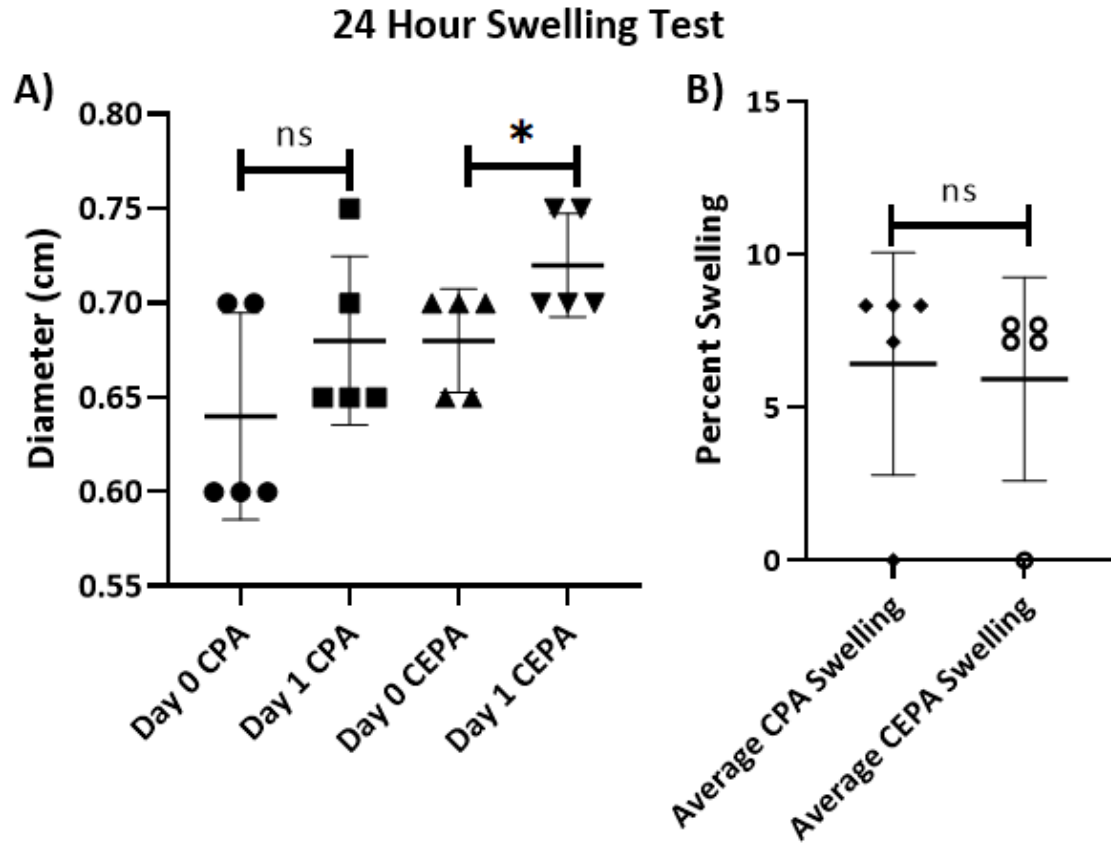


Figure 7. Swelling Test Data

A) A significant ($p < .05$) difference was found between day 1 and day 0 diameter for the CEPA hydrogel. B) The average percentage swelling of the CPA and CEPA hydrogels had no significant difference.

Rheological Assessment of Storage Moduli

These models allow for quick evaluation of the impact each component of a hydrogel has on the storage modulus. In future development of the model, a maximum should be taken for a PEG-A hydrogel to determine the threshold at a given solution concentration. This, along with data points from CEPA gels of varying concentrations, will provide boundaries to work in and aid to refine the estimates from the model to decrease Variance. The framework for this mathematical model may apply to different

formulations provided the assumptions are adjusted. In the MLR model, high residuals that are left over are indicative that this is not the best fit regression model for this gel type. However, the low residual present at around 2500 storage modulus shows this model may trend toward an increase in accuracy for higher storage moduli. Neither the additive model nor the MLR model are an exact fit for mathematical model projections, and further data collection is needed to refine these models. Similar models have been created for predicting Young's Modulus, which is a measure of elasticity, however no mathematical models were found which were created using a multi-component hydrogel solution for storage modulus^{39–41}.

For regenerative medicine purposes, this mathematical model provides the ability to estimate a given CEPA hydrogel formulation based on given parameters, allowing for better matching of *in vivo* storage modulus of a given tissue **Figure 8**. This is especially beneficial for *in vitro* modeling of skeletal tissue using CEPA hydrogels, where the storage modulus can be matched to skeletal tissue's (10 kPa) by adjusting concentrations, allowing for a more accurately mimicked *in vivo* microenvironment *in vitro*⁴².

We performed rheological testing which assessed storage and loss moduli with the different hydrogel formulations for use in a mathematical model. Storage and loss moduli are measured on a curve, however data from the 10% strain was chosen for usage in the mathematical models since they are not under load in culture. The introduction of PEG-A at varying concentration yielded a direct correlation between PEG-A concentration and storage modulus **Figure 8, Figure 9**. Furthermore, the usage of higher concentrations of Collagen and ECM-MA trends toward inverse relationships with storage modulus.

There was a significant difference between all four tested hydrogel types, with the lowest recorded storage moduli (<500 Pa) being the +Low PEG-A hydrogels, and the highest storage moduli (>2 kPa) in the +Low Col hydrogel.

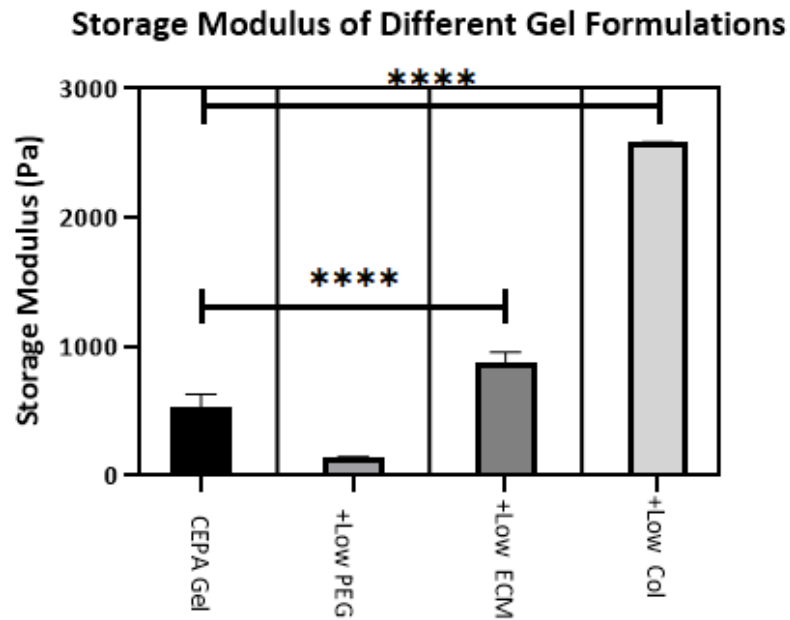


Figure 8. Storage Moduli Differences Between Hydrogels

CEPA hydrogels have varying storage modulus that is dependent on composition. In the presence of lower concentrations of PEG-A, the hydrogels were unable to hold any discernable shape. A decrease in the percent of ECM and Collagen (+Low ECM, +Low Col respectively) presented with significant ($p < .0001$ for both) increases in storage modulus.

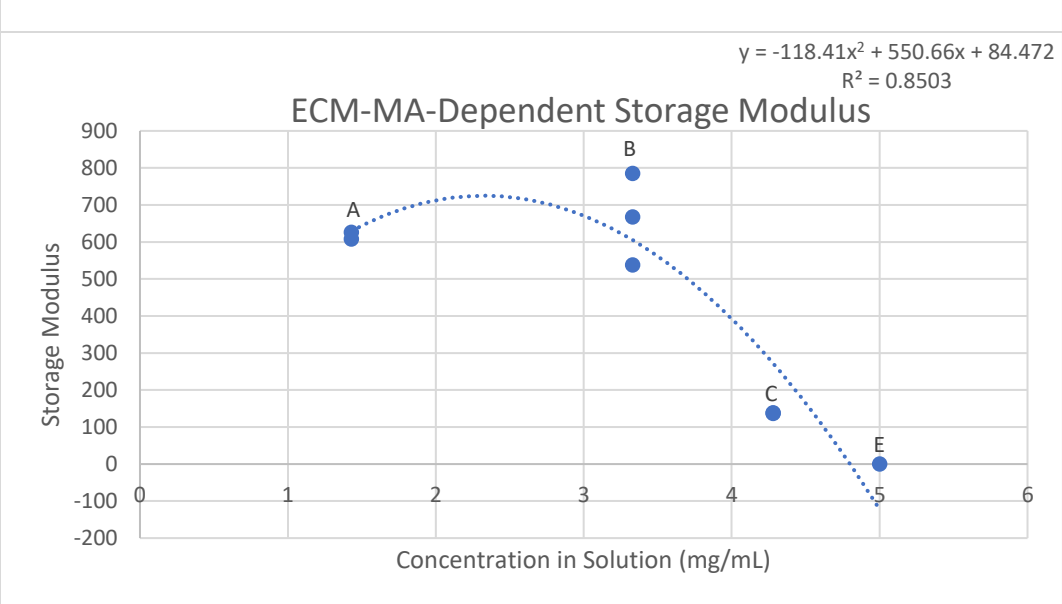
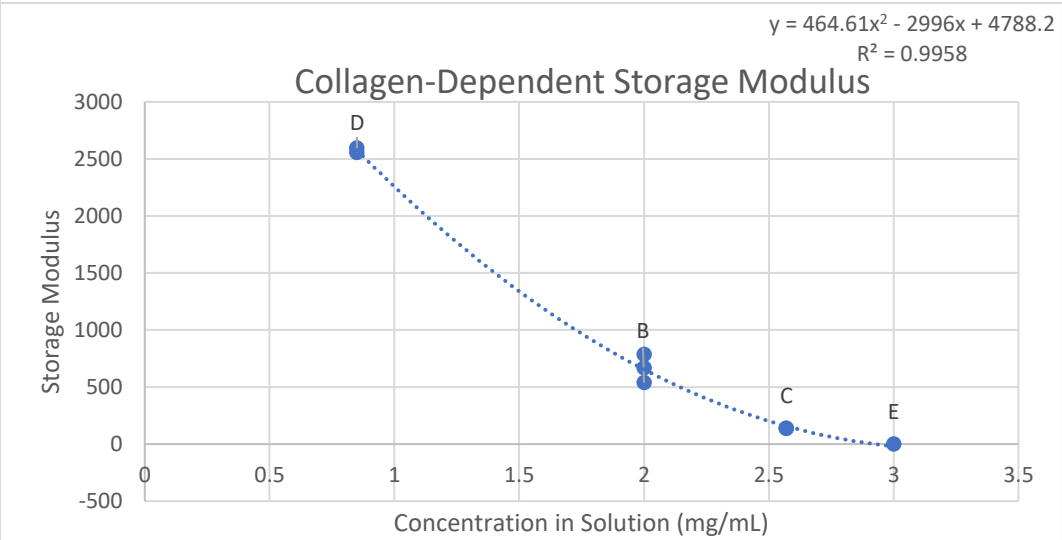
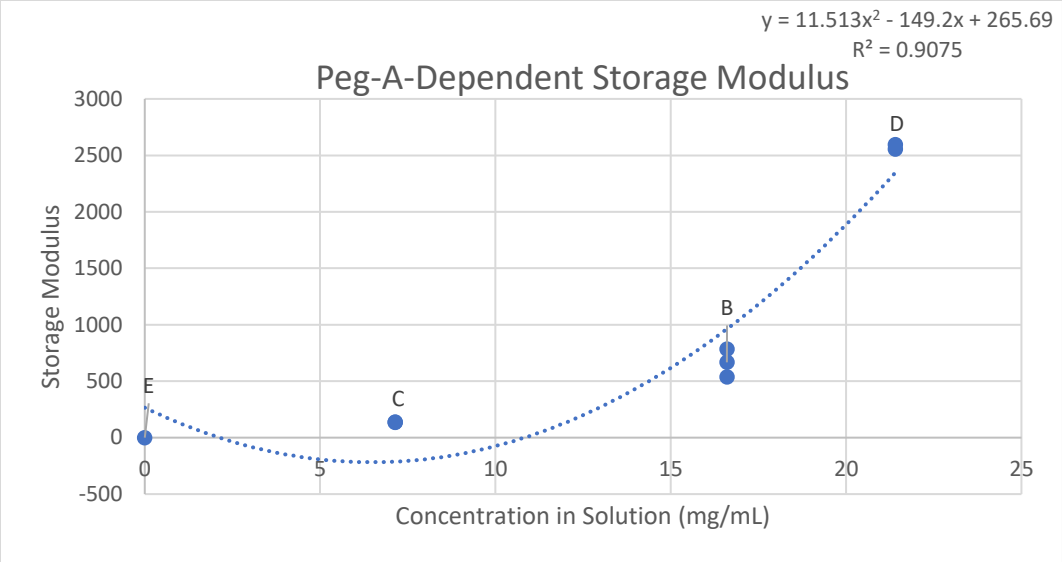


Figure 9. Graphical Representations of Storage Modulus per Formulation

Graphs showing the 10% strain storage moduli of 4-arm PEG-A, ECM-Methacrylate, and Collagen at varying concentrations in solution. At 5 mg/mL and 3 mg/mL, respectively, the solution is comprised only of ECM-methacrylate and collagen, and no PEG-A is present per assumption (2). Here (A) refers to the Low-ECM (1/7th ECM-MA, 3/7th Collagen, 3/7th PEG-A) group, (B) refers to the CPA control, (C) is the Low PEG group (1/7th PEG-A, 3/7th Collagen, 3/7th ECM-MA), (D) is the Low Collagen group (1/7th Collagen, 3/7th PEG-A, 3/7th ECM-MA), and E is a theoretical point based off assumption (2). Negative storage moduli, present in the PEG-A graph, are shown for completeness of a trendline, however are indicative of a hydrogel synthesis which is less-than or near non-viable.

Storage Modulus Analysis of Each Hydrogel Component

We developed two models for the purpose of predictive modeling of storage moduli at variations in concentration and solution. The goal of these models is to show the influence of each component of the hydrogel on the storage modulus. We define the coefficients of influence in this model as the contribution the component has on average to the structural rigidity of the hydrogels (Model 1: x_{var}/s_{var} , Model 2: β). In order to create this mathematical model, a few assumptions were put in place. 1) All reactive groups present have crosslinked with one another, and there are an equal number of crosslinks between constructs within a formulation. 2) As PEG-A concentration trends toward 0 storage modulus also trends toward zero, therefore the storage modulus of a formulation without PEG-A is 0. 3) UV light intensity for crosslinking is the same across all constructs. For the first model, a 4th assumption was made: each equation contributes linearly to the final storage modulus. This model was created using 2nd order polynomial trend lines from data at 10% strain. The final equation was then coded under varying parameters for each

of the conditions and graphed using MATLAB R2020b **Supplementary Information 2**. The second model was made using a multiple linear regression (MLR) in GraphPad Prism 8.4.3 using all storage modulus data and assumptions 1-3.

Additive Model

The equation G is a measurement of storage modulus in a 50 μL hydrogel using Peg-A, Collagen, and ECM at 10% strain *Equation 3*. (G_c) is the contribution to change in storage modulus, C , due to change in final concentration (x_c) of collagen. The 2nd order polynomial is divided by (S_c/x_c) , where (S_c) is the stock solution concentration of Collagen. This is the percentage of contribution that G_c provides to the storage modulus in solution. (G_p) is the contribution of change in the storage modulus of the Peg-A (P). S_p is the stock solution concentration of PEG-acrylate, and x_p is the concentration of Peg-A in solution. (G_e) is the contribution of change in storage modulus of the ECM-Methacrylate to the final solution. S_e is the concentration of the stock solution, and x_e is the concentration of the ECM in solution. The 2nd degree polynomial trend lines had R^2 values of 0.8503 (ECM-Methacrylate), .9075 (4-Arm PEG-A), and .9958 (Collagen-Methacrylate). This equation has been modeled for projections from 1 mg/mL of PEG-A to 25 mg/mL of PEG-A with a 6% variance compared to rheological data **Figure 10**. In order to adjust the overall storage modulus of the CEPA gels to match this, a projected graph using an adjusted PEG-A concentration gradient was made which further expands upon the previous graph **Figure 16**. There is a potential limiting factor involving the maximal storage modulus of the 4-arm 5 kDa PEG-A, and future work should expand upon the mathematical model to include changes in both the number of radical arms on PEG which are capable of binding,

as well as kDa used. A storage modulus of 10 kPa is not an unreasonable amount to achieve. Previous research has shown that using higher percentages of PEG-A can achieve storage moduli up to 20.6 kPa, albeit with different components than used in these experiments **Figure 11**⁴³.

$$G_C + G_P + G_E = G$$

$$G_C = (464.61x_C^2 - 2996x_C + 4788.2) \frac{S_C}{x_C}$$

$$G_P = (11.513x_P^2 - 149.2x_P + 265.69) \frac{S_P}{x_P}$$

$$G_E = (-118.41x_E^2 + 550.66x_E + 84.872) \frac{S_E}{x_E}$$

$$x_C, x_P, x_E \neq 0$$

Equation 3. Additive Model for Storage Modulus Prediction

This additive model is the summation of storage moduli G_C , G_P and G_E for final storage modulus (G) at 10% strain. This initial value can give an estimate of the storage modulus for a given solution prior to creating constructs, providing information about the ability of constructs to retain elastic potential energy in an

elastic manner. A higher storage modulus is indicative of an increase in degree of crosslinking, allowing for increased retention of energy.

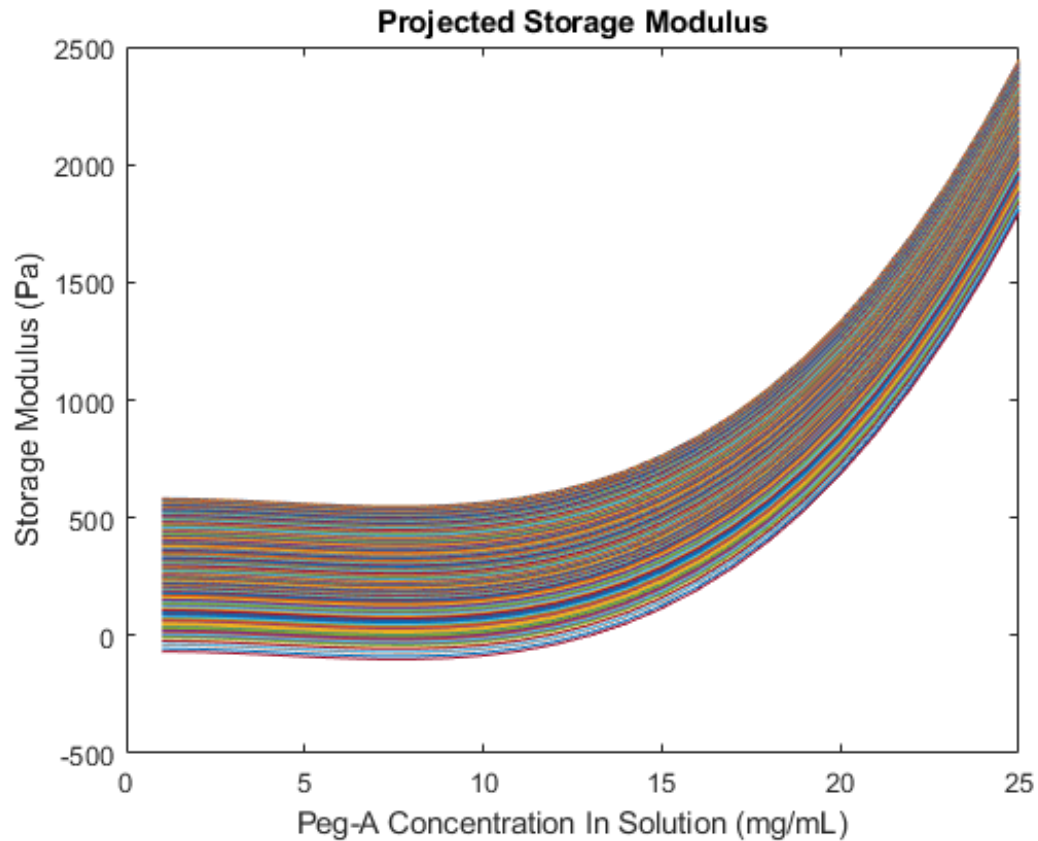


Figure 10. Projected Storage Modulus

The storage modulus increases in all solutions as PEG-A concentration increases, up to 25 mg/mL. The variation in colors indicates different potential concentrations that can be tried, and the expected storage modulus of each one. Sub-zero storage moduli are equivalent to zero and are indicative of a non-viable formulation. In vitro, most formulations under 1,000 Pa will not maintain shape for more than 24 hours. The mean at 1 mg/mL is 247.43 Pa, and at 25 mg/mL is 2117.67 Pa, with a 2.27% change in standard deviation as concentration increases.

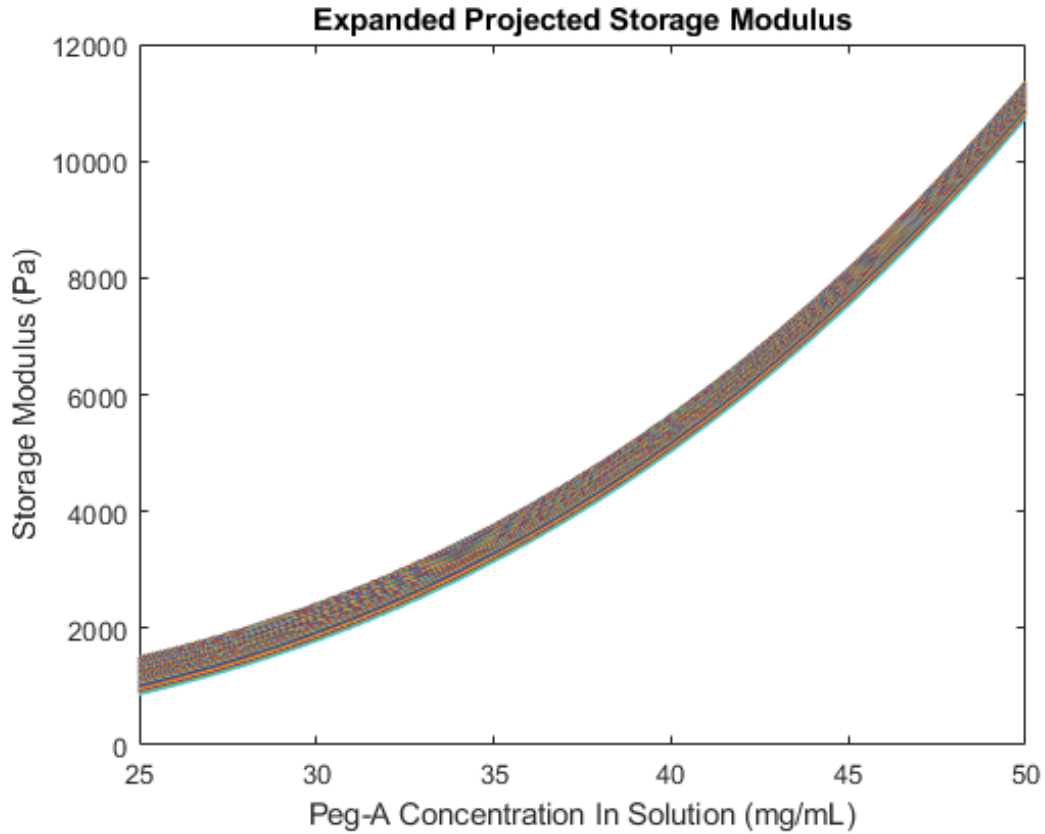


Figure 11. Projected Storage Modulus, Expanded

The projected CEPA hydrogel reaches 10 kPa between 46 mg/mL of PEG-A and 48 mg/mL of PEG-A in solution. This equation will be adjustable based on difference concentrations and 2nd order polynomial functions derived from rheological data at 10% strain. The addition of novel materials and adjustment of stock solution concentrations as well as "in solution ratios" allows for expedited adjustment and analysis of potentially optimal concentrations for usage in solution.

Multiple Linear Regression Model

The multiple linear regression equation is used to analyze the effect of all three components on the storage modulus based on their individual contributions *Equation 4*. While a relatively high standard error was present in β_0 and β_2 (81.75 and 41.87 respectively), this equation appears to have more accuracy for the low PEG group

compared to equation 3. For β_{1-3} , the standard error ranged from 5.024 to 41.87, and the R^2 for multicollinearity was $<.3$ for all variables, and therefore no components are perfectly correlated. P is the concentration of PEG-A used, C is the concentration of collagen used, and E is the concentration of ECM used.

$$G = \beta_1(P) + \beta_2(C) + \beta_3(E)$$

$$\beta_1 = 95.39$$

$$\beta_2 = -630.3$$

$$\beta_3 = 218.8$$

$$P, C, E \neq 0$$

Equation 4. MLR Equation

A multiple linear regression equation which can be used to predict storage modulus of a CEPA hydrogel. This equation appears to have higher degrees of accuracy at higher storage moduli. Further data points could refine this analysis and decrease the standard error present in the equation.

The multiple linear regression model provides more accuracy in both the lower and upper bounds of assumptions, however, deviates greatly around 1000 Pa **Figure 12**. This model may also be accurate for these projected measurements due to an R^2 of .9319, meaning it is highly correlated to the three variables.

Actual vs Predicted Plot of Storage Modulus

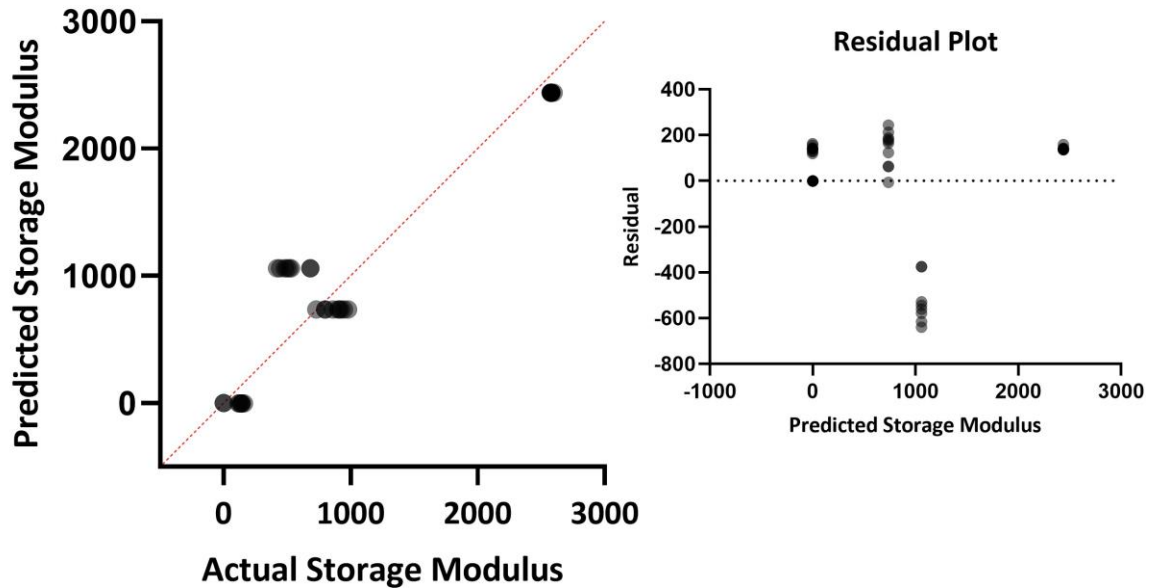


Figure 12. Actual vs Predicted Storage Modulus – MLR

The predicted storage modulus is only accurate for some of the actual storage modulus. The outlier group present in the 700-900 actual storage modulus range is the “+Low ECM” group, which has a residual of up to -700 , *inset 1*. These outliers do not pass any normality of residuals test, indicating that an alternative prediction method may be better suited to modeling this data.

Parameter	Variable	Estimate	Standard error	95% CI	P value
β_0	Intercept	0.1489	81.75	-164.6 to 164.9	0.9986
β_1	PEG-A	95.39	5.024	85.26 to 105.5	<0.0001
β_2	Col	-630.3	41.87	-714.7 to -545.9	<0.0001
β_3	ECM-MA	218.8	25.16	168.1 to 269.5	<0.0001

Table 2: Parameters, Standard error and 95% Confidence Interval of MLR Model

The parameters, β_0 to β_3 , each of the corresponding variables, standard errors, and 95% confidence intervals which were used to create the equation.

To show a clearer comparison, the concentration for collagen was reduced from a maximum of 3 mg/mL to 2 mg/mL, and ECM’s minimum was increased from 1 mg/mL to

3 mg/mL, removing some of the lower bounds of the lines **Figure 13**. The blue MLR line and red Additive Model line were compared against the average storage moduli collected at 10% strain, and neither line connects with all four points. Therefore, we surmise that while both models have high fidelity within a small scope, neither one is accurate across all changes in formulations. Further testing is required to refine these models and create one which is more accurate at current concentrations for usage in projections of storage modulus for novel formulations.

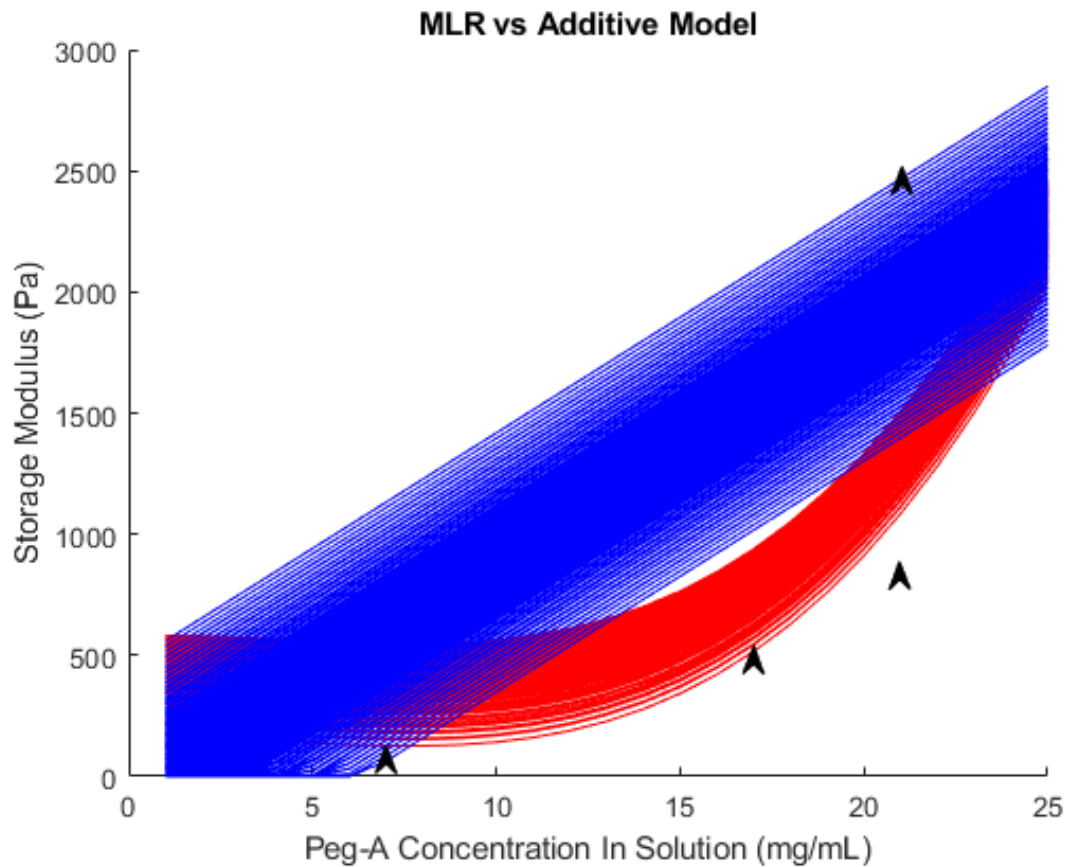


Figure 13. MLR vs Additive Model Graph

The MLR line and additive model line compared to the average of the observed data points (black arrows).

The concentration of PEG-A is the same in both the low collagen and low ECM groups, resulting in two data

points at 21.42 mg/mL. The primary hardening agent, PEG-A, is shown on the x-axis as it is the most influential component on stiffness of the hydrogel.

Cell Viability in CEPA Constructs

To compare the effects of CEPA on cell viability vs CPA we performed a series of live/dead analysis to determine the difference in viability over time and between construct types. Briefly, cells were encapsulated in PEG-A at 10 million cells/mL and then combined with the neutralized Collagen and ECM-MA solution. This solution was then mixed and pipetted in 20 μ L increments onto a non-tissue culture plate and submerged in DMEM Low media. Constructs were stained with live/dead kit and then analyzed by immunofluorescence microscopy. Images were then analyzed by specialized counting macro on ImageJ. Results show that the CPA constructs showed a statistically significant ($p < .01$) change in viability from days 1 to 7 **Figure 14A**. Both the 3:3:1 Collagen:PEG-A:ECM-MA (Low ECM) and 1:3:3 Collagen:PEG-A:ECM-MA (Low Col) hydrogels showed no significant change from days 1 to 4, however had a sharp decline in viability on day 7 **Figure 14B, Figure 14C**. Low Collagen CEPA hydrogels had a significant ($p < .01$) difference from Low ECM CEPA hydrogels on day 7 **Figure 14D**.

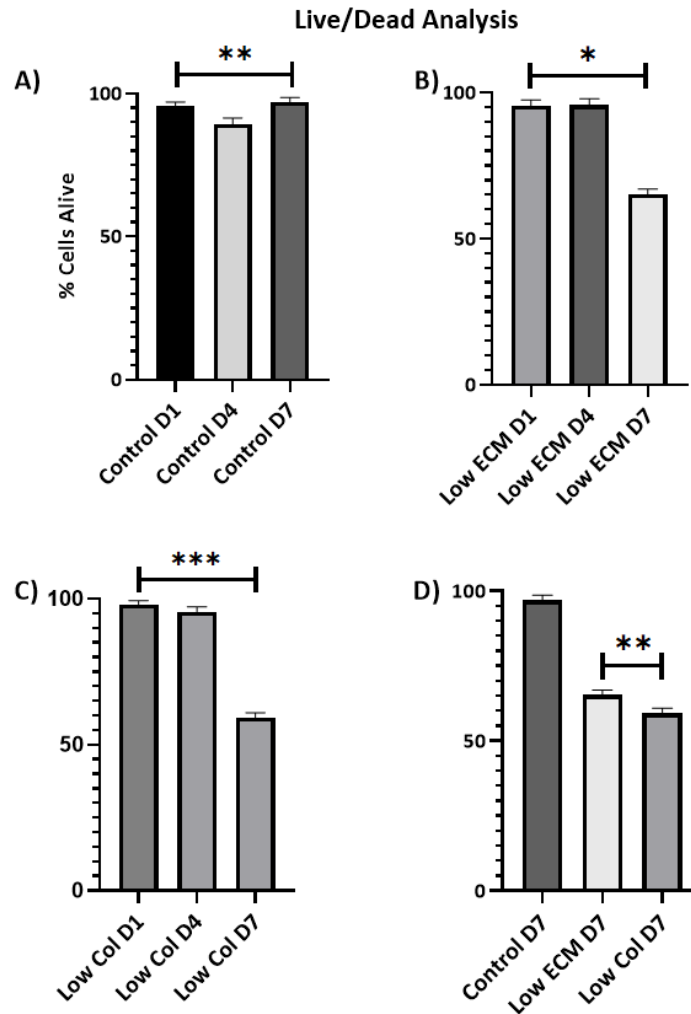


Figure 14. Live/Dead Analysis

A) CPA hydrogel live/dead analysis for days 1-7 showed a significant ($p < .01$) change in % cell viability, with a decrease from days 1-4 and an overall increase from days 1 to 7. B) Low ECM CEPA hydrogels showed a sharp decline between days 4 and 7 for cell viability and had a significant ($p < .05$) change in cell viability between days 1 to 7. C) Cell viability significantly dropped at day 7 in cultures made with low collagen. D) Low ECM had a statistically significantly ($p < .01$) higher cell viability compared to the Low Collagen CEPA hydrogel.

Histological Assessment of Cellularized Constructs

Briefly, ADC cells were encapsulated within three different hydrogel compositions; Peg-A:ColMA (control), Low ECM, and Low Col and left to culture for a period of 7 days. We characterized aspects of the cellularized constructs such as cell distribution, GAG content, and collagen synthesis using several different histological tissue stains. We attempted to stain the constructs with hematoxylin and eosin to analyze cell distribution and morphology. However, The Low Col CEPA hydrogels underwent deformation due to excess dehydration during the required processing procedure prior to embedding in paraffin. This resulted in a spindle or snake-like appearance between days 1 and 7 across all processed constructs (n = 3). In the hematoxylin and eosin stain the nuclei of cells, shown in dark blue, and cytoplasm pink. The constructs are non-specifically stained light purple due to the composition having collagen and extracellular matrix **Figure 15**. All images were taken at 10x magnification.

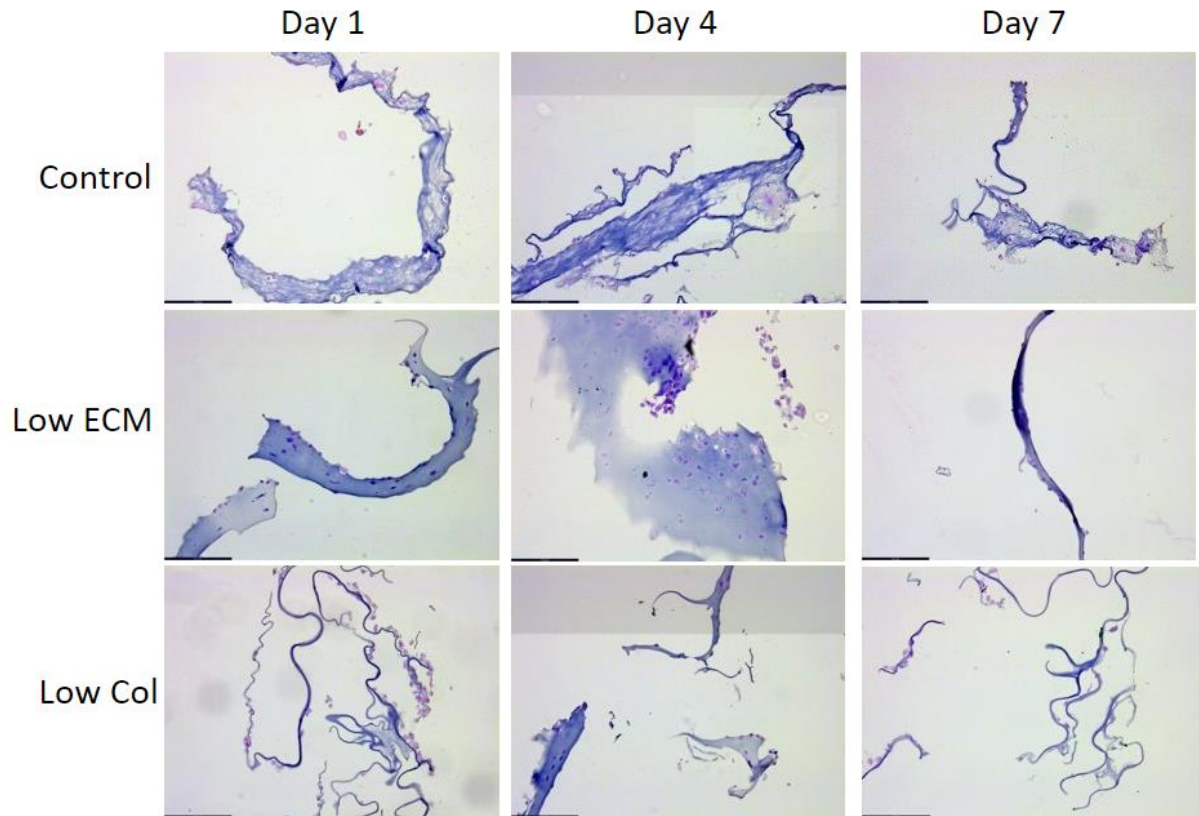


Figure 15. H&E Stain

Hematoxylin and Eosin (H&E) stain for nuclei (dark blue) and cytoplasm (pink). Cells are present in all three times points across all three groups, with no apparent differences in density noted.

Herovici's Stain for young and old Collagen was used to assess if the ADC were actively remodeling the constructs. The stain interacted heavily with each of the hydrogel compositions, creating a much darker hydrogel in non-ECM containing hydrogel, and a yellow-tinted color in the ECM-containing hydrogels **Figure 16**. Cells, stained dark blue by Weigert's Hematoxylin, are present in both timepoints and the experimental and control groups. The stain appears to have interacted heavily with the Low Col CEPA hydrogel and

stained the entire surface of the hydrogel yellow on Day 1, as well smaller snake-like sections on day 7.

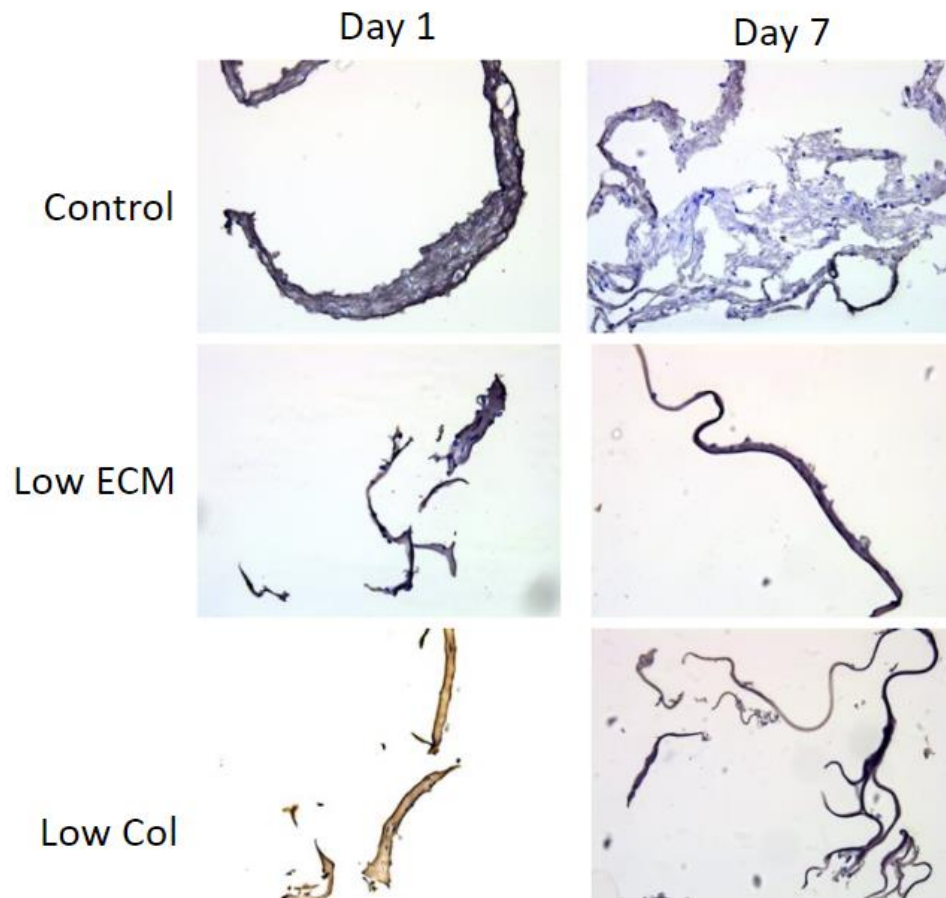


Figure 16. Herovici's Stain

Herovici's Stain for Young and Old Collagen did not uniformly stain the constructs, and instead heavily stained the ECM in the Low ECM and Low Col CEPA hydrogels yellow. Overall dehydration of the constructs can be noted in both the Low ECM and Low Col CEPA hydrogels.

To assess the constructs for GAG content we stained the constructs with an Alcian Blue Stain. This stain showed a change in color saturation from days 1 to 7 in all 3 groups

Figure 17. There is a distinct lightening between days 4 and 7 in the Low ECM and Low Col CEPA hydrogels which matches the control. This may be an indicator of glycoprotein production in hydrogels.

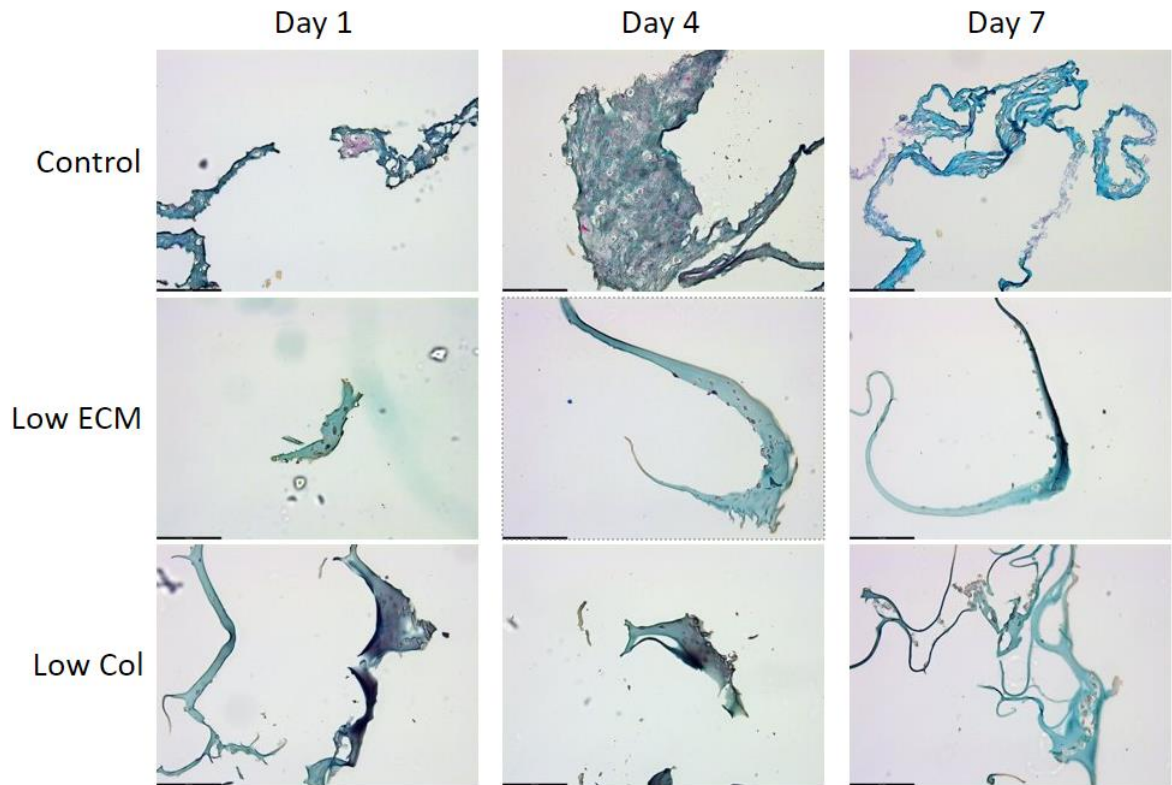


Figure 17. Alcian Blue Stain

Alcian Blue Stain shows stained cell nuclei and cytoplasm (pink), and a large amount of blue staining which is traditionally indicative of acidic glycoproteins. This blue staining appears to lighten from days 1 to 7 in both the Low ECM and Low Col CEPA hydrogels, indicating a potential increase in glycoproteins.

To assess the effect of CPA and CEPA hydrogels on the ADCs phenotype we stained two protein markers, alpha-smooth muscle actin and vimentin with fluorescent immunohistochemistry. by^{44,45}. Collagen is known to present high levels of auto fluorescence from around 380 nm to 450 nm⁴⁶ **Figure 18.** This coincides with the

immunohistochemistry data which showed unusual properties compared to the expected staining presentation.

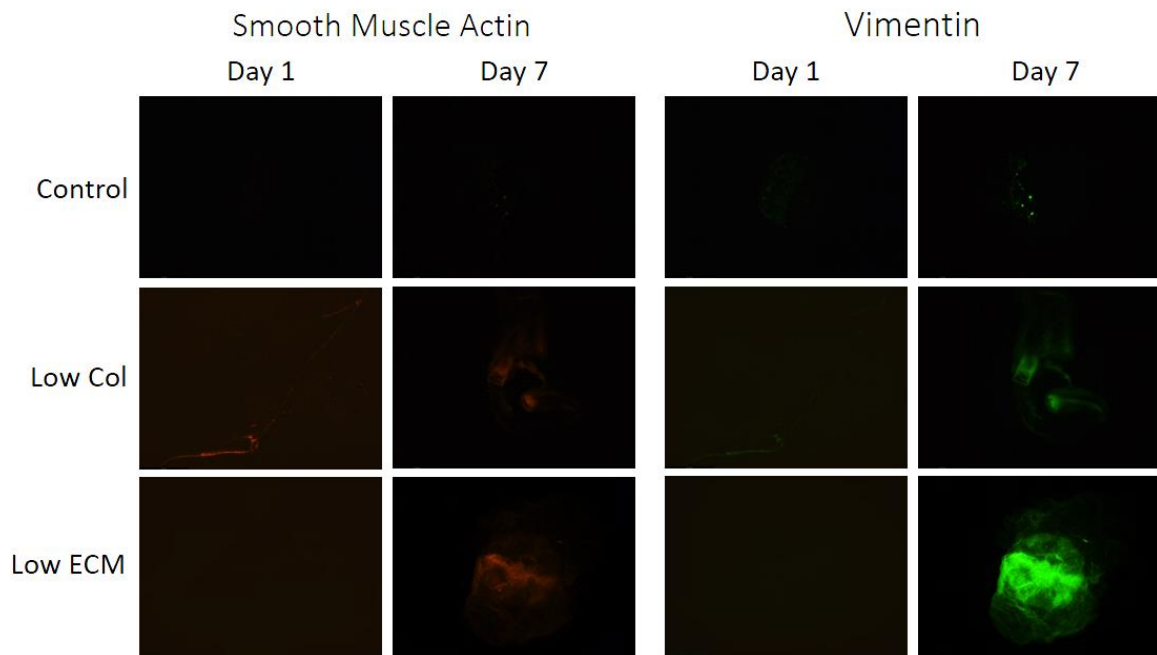


Figure 18. Immunofluorescence

Immunofluorescent stains for Smooth Muscle Actin (SMA) and Vimentin both showed increased fluorescence at day 7 compared to day 1. This may be due to autofluorescence of the materials used or due to presentation in the cells. Here a control (Collagen and PEG-A seeded with ADCs) hydrogel was used to compare against the Low Col and Low ECM groups. There is no significant indication of SMA present in any group for the day 1 or day 7 groups. There is very little to no presentation of Vimentin in day 1 groups, however the day 7 Low ECM and Low Col groups show a significant increase in fluorescence which may match the fluorescent morphology of Vimentin (n=2).

Discussion

In this thesis we synthesized a novel hydrogel incorporating decellularized skeletal muscle ECM that had been processed via methacrylation that allowed for a stable and photo-crosslinkable hydrogel. We then tested the hydrogels mechanical properties and additionally investigated the influence of the hydrogel on adipose derived cells in terms of viability, proliferation, and some functionality. This hydrogel uses PEG-A as the base of the hydrogel, which was added due to its chemical properties making it act as a stiffening agent for the hydrogels. By modulating the concentration we are able to change the storage modulus of the constructs to match *in situ* muscle tissue⁴². PEG-A is a good contender for usage in hydrogels due to being bioinert and readily chemically modifiable⁴⁷⁻⁴⁹. The adjustment of ratios of incorporation for each component allowed for determining concentrations which were necessary for hydrogels to be stable over extended periods of time. Collagen supplementation into the hydrogels aids as a substrate for the ADCs to adhere to and allows for replacement of any collagen which may have been lost due to nonspecific digestion via pepsin of the ECM. Finally, the decellularized skeletal muscle ECM was chemically processed to produce ECM-MA that contains a litany of growth factors and biomolecular cues found *in vivo* and more accurately recapitulates the microenvironment of the skeletal muscle tissue as compared to collagen or PEG-A alone⁵⁰⁻⁵².

The methacrylation of the lyophilized ECM was required to stably incorporate the material into the PEG-A whereas it is possible non-methacrylated ECM may precipitate out of a hydrogel. We found the presence of the methacrylation peak in the skeletal

muscle ^1H NMR is qualitative data which supports the notion that the one-pot method of methacrylation in small scale batches was successful. However, it is difficult to properly ascertain the degree of methacrylation from ^1H with a novel material. For future work, a 2,4,6-Trinitrobenzenesulfonic acid (TNBS) assay against a Gelatin-Methacrylate control would provide quantitative proof that the methacrylation was a success⁵³. In lieu of these options, the stability of the hydrogels over extended periods of time is indicative that there is cross-linking occurring, and that they are stable due to the insignificant change in diameter during the 24-hour swelling test.

As hydrogel stability is an important part of the *in vitro* experimentation process. In previous attempts using PEG-Di-Acrylate, the CEPA hydrogels were in culture for up to 48 hours before either dissolving into the media or degrading into non-salvageable pieces. Our initial makeup of the CEPA hydrogel was not stable and had very low stiffness (data not shown). This poor outcome led us to the use of an increased molar concentration of PEG-A, which increases the crosslinking capability of the overall hydrogel. This allowed for an increase in both the concentration and volume of ECM in working solutions. A good qualitative indicator of hydrogel stability is how the borders look under Brightfield microscopy immediately after crosslinking **Figure 5**. When the constructs have poor boundaries, it is probably indicative of either a composition issue or a homogeneity issue. Visualization and identification of positive and negative borders on hydrogels significantly decreases troubleshooting lag time. Qualitative scaling can also be performed to home in on the concentration boundaries between viable and non-viable hydrogels. Stability analysis by storage modulus is an easy test to determine the likelihood of a hydrogel

degrading over time under physiological conditions⁵⁴. CEPA constructs with very low storage moduli (< 1 kPa), as observed, will have difficulty maintaining form beyond 48 hours. As cells respond to changes in the mechanical properties of the tissue substrate achieving a similar stiffness to in situ tissue achieving physiological normal stiffness is an important aspect of a hydrogel's property⁵⁵⁻⁵⁷. In this study we found that our hydrogel had a maximal overall storage modulus of approximately 2500 Pascals, which is below the threshold for skeletal muscle storage modulus of approximately 10,000 Pascals⁴².

Additionally, the lack of significant difference in the swelling test, as well as the observed boundaries, are indicative that the ECM component may have been properly methacrylated and significantly binding with the construct⁵⁸. To confirm this, analysis of the crosslinking density may be possible. This has been done in rubber by determining the molecular weight of the monomers and dividing by the cross-links per molecule, followed by dividing by the polymer density⁵⁹. Analyzing the hydrogels in a similar fashion may be possible given further research.

After testing the mechanical properties, we set out to test the effect of the CEPA hydrogel on adipose derived cells. Adipose tissue itself is a reservoir of mesenchymal stem cells (MSC) which are relatively easy to isolate while maintaining good yields^{60,61}. As adipose derived MSCs can be differentiated into a variety of cell types their culture including skeletal muscle, their use is well warranted^{62,63}. We found that the CEPA hydrogels have maintained the adipose derived cells for up to 7 days. Overall, the viability of the cells indicates that the construct is not overtly a cytotoxic environment. There was

some decrease in viability from days 4-7, but this could be due to lack of diffusion in constructs due to the volume.

To investigate the makeup of the hydrogel as well as cell morphology and functionality constructs were fixed, processed, and sectioned. We attempted an H&E staining and found that in one of the groups that was well sectioned (Figure 9, Low Col, day 4) we do have a distribution of cells throughout the construct. Glycosaminoglycans (GAG) are important ECM proteins that are found within muscle tissue, which can be stripped by decellularization⁶⁴. We attempted to ascertain the presence of GAG's within the construct, but were unsuccessful in this task, further refinement of the Alcian Blue staining will need to be made in order to ascertain GAG content of this hydrogel.

We also investigated the cellular distribution, remodeling, as well as collagen content via Herovici's stain and H&E staining. None of the hydrogels had any notable remodeling of the ECM occur within the 7-day period. Herovici's stain resulted in erroneous colorations in the low collagen formulation, and as such an alternative staining method may be beneficial for clearer visualization of potential collagen remodeling when using alternative cells, such as skeletal muscle cells.

Alternative methods of processing such as cryo-freezing may help with visualization of lipid droplets, or new staining methods may be required to decrease the amount of non-specific staining which occurs. Vimentin is an intermediate fiber which is found in mesenchymal stem cells, and is a marker for epithelial-mesenchymal transition⁶⁵. The presence of Vimentin *in-vitro* is an indication of adipogenic differentiation, making it a good candidate for use during the differentiation experiment⁶⁶. Smooth Muscle Actin is

a marker which when present indicates that ADCs are capable of adipogenic differentiation⁶⁷. ADCs will present with vimentin during adipogenic differentiation, and SMA prior to differentiation^{66,67}. This makes these two markers notable for use during the entire differentiation process, as well as for determining if the ECM augmented groups have an increased propensity for differentiation.

Further testing is required to determine the presence and expression of genetic signals that the cells are releasing in order to fine-tune the composition of the hydrogel for clinical needs. An auto-fluorescent quencher may be used to reduce the amount of fluorescent bloom which was present due to the auto-fluorescent nature of collagen and could allow for increased clarity in immunofluorescent imaging. As the data collected is refined, this hydrogel can be further stiffened using the PEG-A and an increased concentration of the ECM-MA allowing for further mimicking of the microenvironment in periphery to skeletal tissue.

Mathematical models allow for quick evaluation of the impact each component of a hydrogel has on the storage modulus. In future development of the model, a maximum should be taken for a PEG-A hydrogel to determine the threshold at a given solution concentration. This, along with data points from CEPA gels of varying concentrations, will provide boundaries to work in and aid to refine the estimates from the model to decrease variance. The framework for this mathematical model may apply to different formulations provided the assumptions are adjusted. In the MLR model, high residuals that are left over are indicative that this is not the best fit regression model for this gel type. However, the low residual present at around 2500 storage modulus shows this model may trend toward

an increase in accuracy for higher storage moduli. Neither the additive model nor the MLR model are an exact fit for mathematical model projections, and further data collection is needed to refine these models.

For regenerative medicine purposes, this mathematical model provides the ability to estimate a given CEPA hydrogel formulation based on given parameters, allowing for better matching of *in vivo* storage modulus of a given tissue **Figure 16**. This is especially beneficial for *in vitro* modeling of skeletal tissue using CEPA hydrogels, where the storage modulus can be matched to skeletal tissue's (10 kPa) by adjusting concentrations, allowing for a more accurately mimicked *in vivo* microenvironment *in vitro*⁴².

Conclusions

The creation of a novel ECM-containing hydrogel provides the foundations for the creation of autologous constructs. In a clinical setting, the ability to use ECM from a patient inside of a hydrogel may increase biocompatibility when implanted *in situ*. However, prior to that more analysis of both the materials and cellularized constructs must be done to determine optimal ratios for incorporation, as well as refine the mathematical formulas to a high degree of fidelity. Immunofluorescence should be performed on more ADC specific markers related to both differentiation and proliferation under a variety of conditions so that media compositions and growth factor incorporations can be tailored to individuals with the purpose of regeneration of muscular mass.

In this thesis we synthesized a novel, biocompatible hydrogel which incorporated skeletal muscle ECM that had been decellularized, lyophilized and methacrylated. The PEG-A which was added acts as a hardening agent for the hydrogels, and by modulating the concentration we can change the storage modulus of the constructs. The adjustment of ratios of incorporation for each component allowed for determining concentrations which were necessary for hydrogels to be stable over extended periods of time. Collagen supplementation into the hydrogels aids as a substrate for the ADCs to adhere to and allows for replacement of any collagen which may have been lost due to nonspecific digestion via pepsin of the ECM. Furthermore, while there are issues with cell viability present in the current CEPA hydrogel, it is possible that modification of the formulation used may result in a change to cell viability and provide a better 3D microenvironment

for ADCs to proliferate and differentiate in. Finally, ECM-MA being present in the 3D microenvironment that cells are growing in begins to create a microenvironment which more accurately mimics *in vivo* conditions *in vitro*.

Future Work

With further testing, the CEPA hydrogel is capable of becoming a versatile platform for expeditious autologous augmentation of ECM, followed by implantation in individuals suffering from tissue loss. After determining the cause of cell viability decreases which occurred on day 7, as well as refining of storage moduli prediction equations, it is possible for this hydrogel to become a powerful regenerative medicine asset. Future research should resolve both the mathematical equations, as well as the issues with biocompatibility, and then focus on the regenerative medicine aspect in regard to muscle regeneration in rats, using ADCs from rats. Doing so will aid in determining the influence these hydrogels have on the local microenvironment, as well as determining how bioinert the hydrogels are without using materials from different species. Further testing may also be beneficial using different cell types, such as rat muscle progenitor cells or non-myogenic mesenchymal progenitors (NMMPs).

NMMPs have been shown to influence the growth of skeletal muscle cells and be the origin of fatty and fibrous tissue⁶⁸. NMMPs influence satellite cell up and down regulation. In mice, if satellite cells are damaged or removed via some form of trauma then muscles can lose the entirety of their muscle's regenerative ability. It is therefore

probable that a lack of proper signaling from NMMPs would result in dysregulation of satellite cells and the same end pathway may occur over time.

Muscle regeneration via NMMP signaling is a vital part of the muscle repair process. This stimulation for proliferation of cells is vital for regeneration to occur, and without this permanent degradation of muscle can occur. In muscle tissue it has been hypothesized that autocrine signaling of cytokines may play an important physiological role in muscle tissue⁶⁹. It is possible providing an increase in signaling to NMMPs, as well as proper levels of specific cytokines via *in situ* implantation of ADC hydrogels, may increase the regeneration of muscle cells post-trauma.

When dysregulation occurs, these progenitors are the source of growth within skeletal muscle which can contribute to diseases such as volumetric muscle loss. The dysregulation of specific cells is an issue which can result in drastic changes in the local microenvironment. ADCs are cells which are found throughout the body and have clear stromal markers that can be identified⁷⁰. If the ADCs used in a skeletal muscle ECM-based hydrogel exhibit stromal markers consistent with those present peripheral to skeletal muscle *in vivo*, then it is possible that these cells would influence the growth of novel skeletal tissue. This is, as such, a step closer to a model *in vitro* which mimic *in vivo* more accurately.

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Supplementary Information

- 1) Code accessed from <https://www.allevi3d.com/livedead-assay-quantification-fiji/>, December 15th, 2021. Coding language is Java. This was used on combined red and green channel images **Supplementary Information 5**.

```
//// this macro calculates live and dead cells
////and % of live cells from live/dead assay using single RGB image.
//clears previous results
run("Clear Results")
if (isOpen("Results")) {
selectWindow("Results");
run("Close");
}
//open the file
run("Open...");
close("\\Others");
//set initial number of live and dead cells to 0
liveno=0;
deadno=0;
originalTitle = getTitle();
run("Split Channels");
selectWindow(originalTitle + " (blue)");
run("Close");
////red channel////
selectWindow(originalTitle + " (red)");
run("8-bit");
// min and max values adjust brightness+contrast red channel
setMinAndMax(5, 150);
run("Apply LUT");
setAutoThreshold("Moments dark");
run("Convert to Mask");
run("Watershed");
selectWindow(originalTitle + " (red)");
//adjust size of the particles to detect in the red channel below
run("Analyze Particles...", "size=0.0-Infinity circularity=0.00-1.00
show=Nothing include add_in_situ");
deadno=roiManager("count");
if (deadno>0) {
roiManager("Show All with labels");
roiManager("Show All");
array1 = newArray("0");
for (i=1;i<roiManager("count");i++){
array1 = Array.concat(array1,i);
}
roiManager("select", array1);
roiManager("Delete");
}
////green channel////
selectWindow(originalTitle + " (green)");
run("8-bit");
// min and max values adjust brightness+contrast green channel
setMinAndMax(5, 150);
setAutoThreshold("Huang dark");
run("Convert to Mask");
run("Watershed");
selectWindow(originalTitle + " (green)");
//adjust size detected in the green channel
run("Analyze Particles...", "size=0.0-Infinity circularity=0.00-1.00
show=Nothing include add_in_situ");
liveno=roiManager("count");
roiManager("Show All with labels");
roiManager("Show All");
array1 = newArray("0");
for (i=1;i<roiManager("count");i++){
array1 = Array.concat(array1,i);
}
roiManager("select", array1);
roiManager("Delete");
//prints results
print("live: ", liveno , "; dead: ", deadno , "; total: ",
liveno+deadno, "; %live: ", liveno/(liveno+deadno));
```

2) MATLAB R2020b code for storage modulus projection via equation. Written 1/3/2022

by Seth Mischo.

```
%Defined arrays
y1 = []; %Collagen
y2 = []; %Peg
y3 = []; %ECM
fg = []; %Final eq
for x = 0:1:50
    y1(x+1,1) = (11.513*x^2)-(149.2*x)+265.69;
end

for x = 0:1:3
    y2(x+1,1) = ((464.61*x^2)-(2996*x)+4788.2);
end
for x = 0:1:5
    y3(x+1,1) = (-118.41*x^2)+(550.66*x)+84.472;
end
figure
% plot(y1)
% plot(y2)
% hold on
% plot(y3)
xlim([0,5])
for x = 0:1:5
    y3(x+1,1) = (-118.41*x^2)+(550.66*x)+84.472;
end

%This is for control gel with bounds 1-50 mg/mL PEG
% 1-3 mg/mL with bounds collagen
% 1-5 mg/mL with bounds ECM:
%both via linspace m, n respectively
x = 1:1:25
for x_index = 1:length(x)
    c = 0;
    for y = 1:.1:3;
        for z = 1:.1:5;
            c = c + 1;
            fg(x_index,c) = (((11.513*((x_index)^2))-(149.2*
(x_index))+265.69)*((x_index)/(50)))+(((464.61*((y)^2))-(2996*
(y))+4788.2)*((y)/6))+(((118.41*((z)^2)))+(550.66*(z))+84.472)*
((z)/10));
        end
    end
end
hold off
%Graph fg
figure
plot(y1)
plot(y2)
plot(y3)
plot(fg)
xlim('auto')
ylim('auto')
xlabel('Peg-A Concentration In Solution (mg/mL)')
ylabel('Storage Modulus (Pa)')
title('Projected Storage Modulus')
```

3) Adjusted MATLAB code for projected storage modulus. Written 1/7/2022, Seth Mischo.

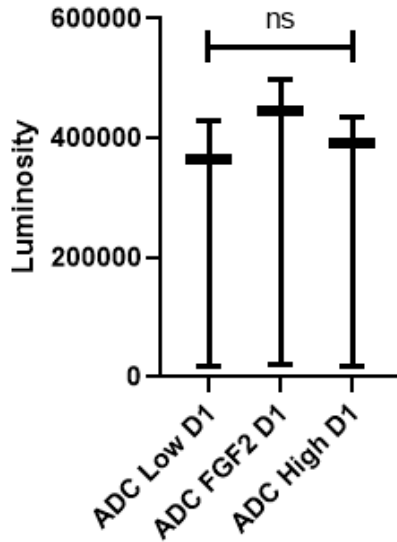
```
x = 25:1:100
Sp = 100
Sc = 6
Se = 10
for x_index = 25:1:50;
    c = 0;
    for y = 0:.1:3;
        for z = 0:.1:5;
            c = c + 1;
            fg(x_index,c) = (((11.513*((x_index)^2))-
(149.2*(x_index))+265.69)*((x_index)/(Sp)))+(((464.61*
((y)^2))-(2996*(y))+4788.2)*((y)/Sc))+((( -118.41*
((z)^2)))+(550.66*(z))+84.472)*((z)/Se));
            % if fg(x_index,c) < 0
            %     fg(x_index,c) = 0
            %end
        end
    end
end
hold off
%Graph fg
figure
plot(y1)
plot(y2)
plot(y3)
plot(fg)
xlim([25,50])
ylim('auto')
xlabel('Peg-A Concentration In Solution (mg/mL)')
ylabel('Storage Modulus (Pa)')
title('Expanded Projected Storage Modulus')
```

2D FGF-2 ADC Viability Assay

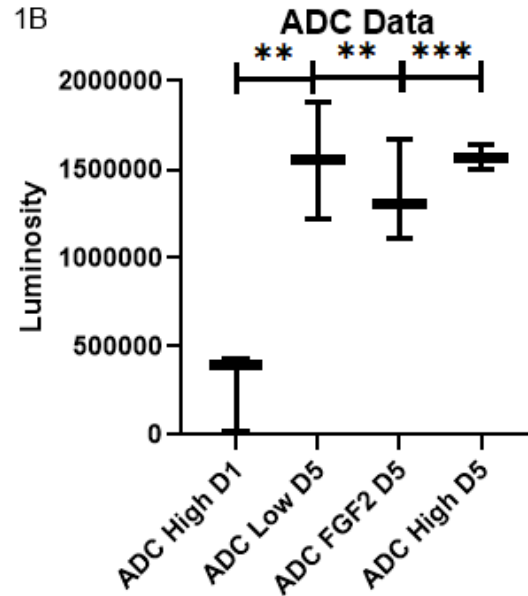
10×10^4 ADCs were seeded into a 6 well plate ($n = 6$). The ADC Low group was given DMEM Low-Glucose (1 g/L) with 1% FBS and 1% A/A (Cytiva, SH30021.01). The ADC FGF-2 group was given DMEM Low-Glucose with 1% FBS, 1% A/A and 10 ng/mL of FGF-2. The ADC High group was given DMEM Low-Glucose with 10% FBS and 1% A/A. Media changes were performed every 2 days. The media was aspirated from the well and 200 μ L of CellTiter Glo 3D was added to the well. An equivalent volume of media was added, and the plate was shaken at room temperature for 5 minutes. Once removed from the shaker, the plate was left to equalize at room temperature for 25 minutes, and then it was transferred to a white solid-backed 96-well plate and read using a Veritas Microplate Luminometer from Turner Biosystems.

We tested FGF-2 to determine if the anti-apoptotic signaling it provides is significant under challenge conditions (1% FBS) to explore potential ways to reduce the amount of cell death within hydrogels. There is no significant difference in the luminosity present between any of the day 5 groups using a one-way ANOVA (analysis of variance) test **Figure 7**. This is possibly due to the short timeframe that was used for this experiment; however, it is also possible that the concentration of FBS is not a major impactor in the overall growth of ADCs. While the average growth of each group may not have a significant difference, there was a substantially larger standard deviation present in the Low FBS and the Low FBS+FGF-2 groups compared high FBS

1A Day 1 FGF-2 Experiment



1B



Supplementary Figure 19. ADC FGF-2 Experiment

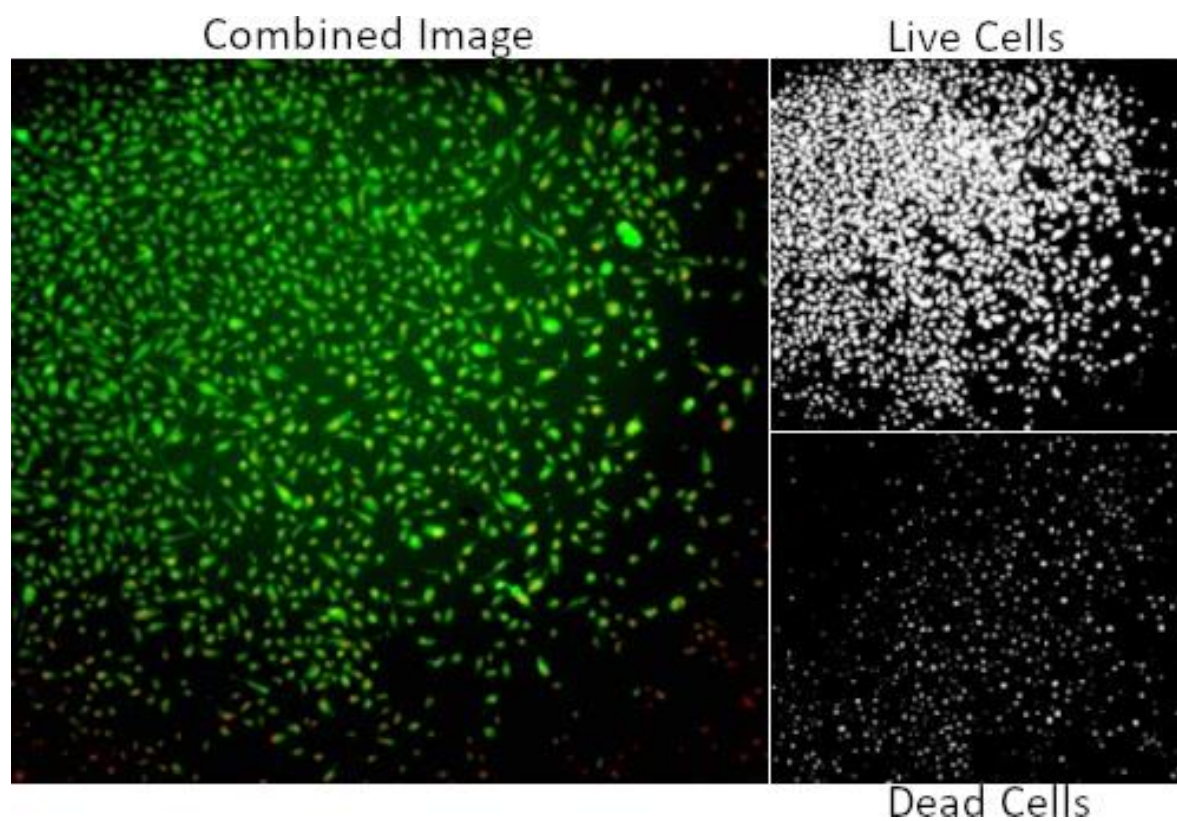
1A) Day 1 ATP Analysis to ensure proper seeding densities for each group. 1B) No significant difference was found between the Low FBS, high FBS, and low-FBS+FGF-2 groups after 5 days of growth in a 24 well plate. This is indicative that the FGF-2 did not have a significant impact on proliferation or anti-apoptotic pathways in the low FBS groups during this time period.

The lack of significant difference in ATP production between low FBS with FGF-2, low FBS without FGF-2, and high FBS without FGF-2 indicates that ADCs may not increase proliferation in response to FGF-2. This may be because ADCs are resistant to apoptosis *in vitro* in low FBS conditions, in which case a more powerful assay must be used to determine the efficacy of FGF-2 on ADCs. While there is a lot of supporting data for the benefits of FGF-2, there was no marked reason to test this growth factor on ADCs in the CPA or CEPA hydrogels. Further testing of both this growth factor and others may benefit in determining the best anti-apoptotic, proliferation, and differentiation growth factors

for use on ADCs in conjunction with an ECM incorporated hydrogel. Similarly, FBS may impact some regulatory pathways within ADCs, but a lack of adequate concentration may cause dysregulation *in vitro*.

Live/Dead Assay

10x magnification images, from a 20 μ L control CPA gel seeded with 10 million cells/mL of ADCs after 4 days of growth, containing the combined image and the separated green and red channels, representing live and dead cells, respectively.



Supplementary Figure 20. Day 4 CPA Hydrogel Live/Dead

The original combined image taken on an Olympus IX-83 and the green channel (live cells), and red channel (dead cells) separated for processing using (Fiji is just) ImageJ.

Biosketch

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MS. Thesis Aug. 2020-May 2022 Primary Advisor: Shay Soker

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- Study of 4-arm Polyethylene Glycol – Acrylate and ECM augmented collagen-based hydrogels, and their influence on tissue cell growth and proliferation.
- Research on rat muscle progenitor cells and disadvantageous and advantageous conditions for them in both 2D and 3D environments.

United States Army

December 2012 to August 2017

Airborne Infantry

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- Airborne Infantryman in the 1st Battalion 143rd Infantry Regiment.
- Long term maintenance and knowledge for assembly and disassembly of all pieces of equipment, as well as proper communication for accurate relaying of information.
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Education

Rhode Island College

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