Effects of Varying Thrombin and Calcium Concentration on the Structure, Mechanical Properties, and Cellular Compatibility of Fibrin Gels

An Honors Thesis for the
Department of Biomedical Engineering

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Tufts University 2012

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Abstract

The creation and subsequent implantation of engineered heart tissue (EHT) serves as a promising treatment of complications resulting from cardiovascular disease (CVD). Although fibrin gels have previously been used as scaffolds for EHT, little is known about the effects of changing concentrations of reagents during their formation on their ultimate usability. Ultimately, we wish to develop a formulation that accurately mimics the mechanical properties of native heart tissue and allows for optimal attachment, proliferation, and extracellular matrix (ECM) deposition of cardiac cells. To work towards this long-term goal, this study aimed at measuring the effects of varying reagent concentrations, such as thrombin and calcium, on the structure, mechanical properties, and cellular compatibility on fibrin gels. In this study, independent experiments were conducted to determine the effects of varying reagent concentration on each of these parameters. We determined that increasing the calcium concentration within the gel led to an increase in fiber diameter and a decrease in individual fiber density, while increasing in thrombin concentration led to a decrease in fiber diameter and an increase in individual fiber density. In addition, we found that formulations leading to intermediate fiber diameters also gave rise to gels that were the most mechanically robust. Finally, although cells were able to adhere to and proliferate on fibrin gels created under all formulations, we determined that cells exhibited the greatest collagen deposition when grown on gels with small fiber diameters and high individual fiber densities.

Acknowledgements

I would like to thank my graduate student mentor, Kathy Ye, for helping me find direction to my project and for being there to give me advice during every step along the way. I would like to express gratitude towards my advisor, Dr. Lauren Black, for his guidance throughout the process. I would like to thank Dr. Lauren Black and Dr. Corin Williams for designing the LabVIEW code for the tissue stretcher that I used to mechanically test my gels. Moreover, I would like to thank Kelly Sullivan, Rachel Twardowski, Joshua Gershlak, Katherine Tang, and Kyle Boutin for their assistance with day-to-day tasks in the laboratory. I would not have been able to complete this senior project without the guidance of my mentors and the generosity of the Tufts University Department of Biomedical Engineering.

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1. Introduction

1.1 Significance

Cardiovascular disease (CVD) is a growing epidemic in the United States and around the world. In addition to being the leading cause of death in America, CVD affects more than a quarter of Americans (American Heart Association). Because current treatments for CVD serve to slow its progression, rather than reverse its consequences, research is being conducted to explore new treatment methods for the disease. One such method is cell therapy, which aims at restoration of cardiac functionality through injection of stem cells into the site of infarct on the heart. Although cell therapy has achieved some successes in clinical trials (Collins et al., 2007; Chang et al., 2008), it suffers from limitations associated with the lack of integration (Terrovitis et al., 2010) and proper functionality (Choi et al., 2010) of injected stem cells, as well as a lack of longevity of effect (D'Alessandro and Micheler, 2010).

In response to the limitations of cell therapy, the creation of engineered heart tissue (EHT) has been explored as an alternative method of treatment for CVD patients. One important consideration in the design of engineered tissue is the choice of an appropriate biomaterial to serve as scaffold onto which to seed and grow cells. This scaffold must be capable of supporting cellular growth and extracellular matrix (ECM) deposition, and must be biodegradable and resorbable within the body after a desired period of time. In the past, both natural and synthetic biomaterials have been used for cardiac tissue engineering (Wang et al., 2003; Malafaya et al., 2007; Kenar et al., 2010; Bouten et al., 2011). Though collagen is the most common scaffold of choice, it suffers from limitations such as lack of ECM deposition by constituent cells and poor mechanical strength of resultant tissues (Thie et al., 1991; Grassl et al., 2003).

The usage of fibrin gels serves as a promising alternative to the usage of collagen. Previous studies have demonstrated an increased capacity of cells grown in fibrin gels to deposit their own ECM, compared to cells grown in collagen gels (Clark et al., 1995; Grassl et al., 2003; Williams et al., 2006). Fibrin is a protein that plays roles in blood coagulation and platelet aggregation within the human body. It travels within the bloodstream in its inactive form, fibrinogen, until its activity is required. Activation of fibrinogen to form networks of fibrin involves the cleavage of several peptides, which is catalyzed by thrombin. Calcium is a cofactor that plays a role in this activation. After this cleavage, fibrin proteins dimerize and subsequently aggregate to form complex three-dimensional structures.

Three reagents – fibrinogen, thrombin, and calcium – are necessary to initiate fibrin gel formation in an *in vitro* setting. However, altering the ratios of the reagents has been shown to change the resultant architecture of the gel (Carr and Hermans, 1978; Ryan et al., 1999). In addition, changes in the structures of fibrin gels have been correlated to changes in their mechanical properties (Roberts et al., 1974; Gerth et al., 1974; Ryan et al., 1999). Previous studies have demonstrated success in culturing cardiac cells on gels at a variety of initial fibrinogen concentrations (Jockenhoevel et al., 2001; Grassl et al., 2003; Williams et al., 2006; Linnes et al., 2007; Black et al., 2009). However, concentrations of other reagents involved formation, such as thrombin and calcium, have been selected without much explanation. The effects of these choices on the growth and functionality of cardiac cells have not been fully explored. Thus, there exists a need to conduct a study that correlates the structural and mechanical differences obtained in fibrin gels by varying concentrations of thrombin and calcium with the resultant fates of heart cells when grown in these gels.

1.2 Long Term Goals

The long-term goal of this project is to develop an optimized formulation of fibrin that is best suited to serve as a scaffold in the creation of engineered heart tissue. The successful formation of EHT is desirable as a possible treatment for cardiovascular disease, since it has the potential to replace areas of tissue in the heart that has been damaged by its long term consequences. By exploring the effects of changing thrombin and calcium concentration on the structure and mechanical properties of the resultant fibrin gel, and by determining the consequences of these changes on cellular compatibility, we will take steps towards the fulfillment of our long-term goal. Ultimately, we wish to come up with a formulation that contains a well-characterized structure and mechanical properties that closely mimic that those of native tissue. In addition, we would like this formulation to support cellular attachment and growth in the short term but undergo degradation by cells in the long term.

1.3 Specific Aims

The first aim of this project was to study the effects of varying the concentrations of thrombin and calcium in the formulation of a fibrin gel on its resultant architecture, as well as its gelation time. We hypothesized that changes in the amount of each reagent will cause direct, quantifiable changes in structural parameters, such as fiber diameter and density of each fiber, which would in turn lead to changes in the mechanical properties and cellular compatibility of the fibrin gel. To test the effects of these changes, we used a previously described light-scattering method (Carr and Hermans, 1978). We created fibrin gels created under a variety of calcium and thrombin concentrations and determined differences in their structural parameters by determining

absorbance as a function of wavelength. In addition, fibrin gels were observed under a microscope to characterize visible differences in architecture.

The second aim of this project was to study the effects of varying the concentrations of thrombin and calcium concentrations during creation of a fibrin gel on its mechanical properties. We hypothesized that changes in the amount of each reagent would cause changes in resultant mechanical properties, and that we would be able to correlate these mechanical changes to changes in structural parameters, as determined by the fulfillment of the first aim. To measure changes in mechanical properties, gels were created under various concentrations of thrombin and calcium. Young's modulus was determined through uniaxial stretching, while storage modulus and loss modulus were found through rheology.

The third and final aim of this project was to study the effects of varying the concentrations of thrombin and calcium concentrations during creation of a fibrin gel on its cellular compatibility. We hypothesized that changes in the amount of each reagent would lead to differences in the resultant gel's ability to promote the adhesion, survival, and proliferation of cardiac native cells. Furthermore, we hypothesized that cells growing on gels with different initial formulations would exhibit varying degrees of success in remodeling their surrounding environments and laying down their own extracellular matrix (ECM). To measure differences in cellular compatibility, cellular constructs were created by seeding native cells in fibrin gels created under different concentrations of calcium and thrombin and grown up for seven days. DNA assays and histological staining for myosin heavy chain (MHC) and connexin 43 (Cxn43) were used to test for differences in adhesion, survival, and proliferation of cells, while staining for collagen I (col I) was used to test for differences in ECM deposition.

2. Background

2.1 Incidence of Cardiovascular Disease

Cardiovascular disease (CVD) is a class of diseases affecting the heart and blood vessels. Currently, CVD is a growing global epidemic. In the year 2008, over 17 million deaths that occurred around the world were attributed to CVD (World Health Organization). In addition, CVD is still the leading cause of death for Americans, killing more than 810,000 people in the year 2007 alone (American Heart Association). Moreover, over one-quarter of Americans, or more than 57 million people, are afflicted with a form of CVD that impairs their quality of life on a daily basis (American Heart Association).

Usually, CVD develops in patients due to atherosclerosis, or the deposit of fat and cholesterol on the inner walls of blood vessels, which eventually leads to plaque formation and vessel occlusion. A common complication of CVD is myocardial infarction (MI). During an MI, a coronary artery blockage cuts off the supply of oxygenated blood to the heart, causing tissue death. Due to the limited regenerative properties of cardiac tissue, patients who survive myocardial infarction are left with non-functional regions of tissue. Although this damaged tissue undergoes a process of negative remodeling, it cannot be fully restored to its healthy state, leading to a decrease in cardiac performance. To compensate for this diminished functionality, the left ventricle undergoes pathological hypertrophy, which involves the thickening and the enlargement of the ventricle. In the short term, this enlargement allows for the ventricles to pump the same amount blood with smaller amounts of force. However, over the time, the body loses its ability to compensate and becomes susceptible to a number of fatal conditions. One condition is congestive heart failure (CHF), which occurs when the heart has accumulated so much damage that it is no longer adequately able to pump blood throughout the body.

2.2 Treatment of Cardiovascular Disease

Past methods implemented toward the treatment of complications resulting from CVD have been limited in effectiveness. While these therapies aimed to slow the progression of CVD, few were able to restore proper function of damaged tissue.

Two new methods have been developed to treat complications resulting CVD through replacement of wounded cardiac tissue. One method, cell therapy, involves the injection of stem cells into the heart at the site of injury, with the expectation that some of these cells will differentiate into healthy cardiomyocytes (Collins et al., 2007; Chang et al., 2008; Schachinger et al., 2009). Clinical trials have shown the ability of cell therapy to improve left ventricular ejection fraction in post-MI patients by around 8% (Collins et al., 2007). In addition, cell therapy has proven capable of improving left ventricular synchronous contraction (Chang et al., 2008). However, there are a number of limitations associated with cell therapy. Successful implantation of cells injected into the site of infarct has been shown to be as low as 5-10% (Terrovitis et al., 2010), and stem cells that do engraft often experience problems with proper differentiation and functionality (Choi et al., 2010). In addition, because studies have shown that the positive effects of cell therapy are often transient, cell therapy does not represent an adequate long-term treatment method for CVD (D'Alessandro and Micheler, 2010).

Another method, the *in vitro* creation of engineered heart tissue (EHT) through manipulation of culture conditions, has been developed to circumvent the disadvantages associated with cell therapy. The usage of EHT is advantageous, since retention and integration of cells upon implantation onto the heart is increased when cells are introduced as functional tissues, rather than in isolation (Martin et al., 2004). Like cell therapy, implantation of engineered cardiac patches has proven to be successful in restoring mechanical function to

damaged regions of the heart. Preliminary studies have shown that engineered tissue grafts are capable of increasing systolic and diastolic performance in post-MI rat hearts (Zimmerman et al., 2006). In addition, one study demonstrated the introduction of a tissue-engineered graft reduced stroke work in wounded areas of the heart by at least 50% (Kochupura et al., 2005).

2.3 Biomaterials in Cardiac Tissue Engineering

One important parameter to consider in the development of engineered tissue is the choice of biomaterial to serve as a scaffold on which to grow this tissue. Scaffolds provide initial points of attachment for cells and provide them with physical support during tissue formation. Because the scaffold is meant to serve as an intermediary to facilitate cellular communication during the process of and tissue formation, it must be able to degrade in the body after a desired amount of time. Due to its fate of implantation into the body, the scaffold material and its byproducts must be compatible within physiological conditions. Lastly, to support appropriate cell growth and behavior, scaffolds must mimic the mechanical properties of the tissues on which the adherent cells are normally found.

A number of biomaterials, both natural and synthetic, have been explored as potential scaffolds for cardiac tissue engineering. Because synthetic biomaterials are created in the laboratory, their chemical properties can be easily tailored to specific applications. Their degradation profiles are easier to control than those of natural biomaterials (Wang et al., 2003; Kenar et al., 2010). In addition, they can be endowed with characteristics not found in nature, such as thermosensitive properties that allow greater ease of control of gelation (Mano and Ribeiro e Silva, 2011). However, usage of synthetic biomaterials has its drawbacks. Because synthetic biomaterials are not naturally found in the body, they are often prone to issues of

biocompatibility. For example, many synthetic biomaterials contain acid degradation products, which cause adverse reactions in the body such as inflammation and fibrous encapsulation (Thomson et al., 1995). In addition, they suffer from poor mechanical strength and exhibit stiffnesses that do not match that of native tissue (Yang et al., 2001).

Usage of natural biomaterials in creating scaffolds is advantageous because natural biomaterials are highly biocompatible and biodegradable. Due to their biological origin, these materials are able to accurate replicate the conditions found within the extracellular matrix (ECM) (Yoneko et al., 2005). In addition, natural biomaterials are often equipped with peptide sequences to which cells can bind, facilitating cell adhesion during the initial stages of tissue formation (Bouten et al., 2011). Lastly, these materials are often able to promote the healing of wounded tissue through stimulation of angiogenesis (Leor et al., 2000; Allison and Grande-Allen, 2006) However, there are disadvantages associated with the usage of natural biomaterials. Although natural biomaterials are degradable within physiological temperature and pH conditions, their rates of degradation are often hard to control (Malafaya et al., 2007). In addition, because their sources are often not well defined, purification is difficult to achieve and contamination is always a concern (Malafaya et al., 2007).

Currently, collagen scaffolds serve as the "gold standard" in cardiac tissue engineering (Malafaya et al., 2007; Schwartz and Simone, 2008; Bouten et al., 2011). Usage of collagen is advantageous, since collagen I is a major constituent of cardiac ECM (Malafaya et al., 2007). However, some drawbacks to its usage include poor stiffness and mechanical strength within the engineered tissue (Grassl et al., 2003). This deficiency arises from insufficient ECM synthesis exhibited by cells entrapped within collagen gels (Thie et al., 1991; Grassl et al., 2003).

2.4 Fibrin Scaffolds

Recently, the usage of fibrin gels has arisen as an attractive alternative. Fibrin is a fibrillar protein that plays an important role in biological processes, such as blood coagulation and platelet aggregation. Due to its natural origin, fibrin can be autologously sourced from a patient's own blood, minimizing the potential for adverse immunological reactions (Jockenhoevel et al., 2001; Linnes et al., 2007). Like collagen, fibrin is capable of forming a fibrous network that cells can recognize and attach to (Grassl et al., 2003; Bouten et al., 2011). Once adhered, these cells can remodel their scaffold, causing compaction and alignment of fibers within the gel (Grassl et al., 2003; Williams et al., 2006). In addition, because fibrin is a bioactive molecule, it is capable of stimulating entrapped cells to replace the initial scaffold with their own ECM. Previous studies have demonstrated that cells grown in fibrin gels are capable of depositing ECM proteins to a greater extent than cells grown in collagen gels (Clark et al., 1995; Grassl et al., 2003; Williams et al., 2006; Black et al, 2009). Finally, degradation of fibrin can be controlled through addition of protease inhibitors, such as ε-aminocaproic acid (ACA), to the culture medium in which the tissue is being grown (Grassl et al., 2003).

2.5 Mechanism of Fibrin Clot Formation

Fibrin is found in the blood in its inactive form, fibrinogen. Fibrinogen is a 340 kDa protein that is composed of two identical halves that are each composed of three distinct peptide chains – the Aα, Bβ, and γ chains (Ryan et al., 1999). The protein is arranged into one central domain and two end domains, and the entire structure is held together by disulfide bonds (Weisel and Nagaswami, 1992; Ryan et al., 1999). Upon the conversion of fibrinogen to fibrin, the A and B peptides on fibrinogen are hydrolytically removed (Figure 1). This conversion occurs via the

catalytic activity of thrombin in the presence of calcium ion (Ryan et al., 1999; Linnes et al., 2007). This change reveals binding sites in the protein's central domain. These binding sites interact with complementary sites on the end domains of adjacent fibrin proteins, allowing polymerization into two-stranded protofibrils (Weisel and Nagaswami, 1992; Ryan et al., 1999). In turn, these protofibrils undergo lateral aggregation to form complex, three-dimensional fiber networks (Weisel and Nagaswami, 1992; Ryan et al., 1999).

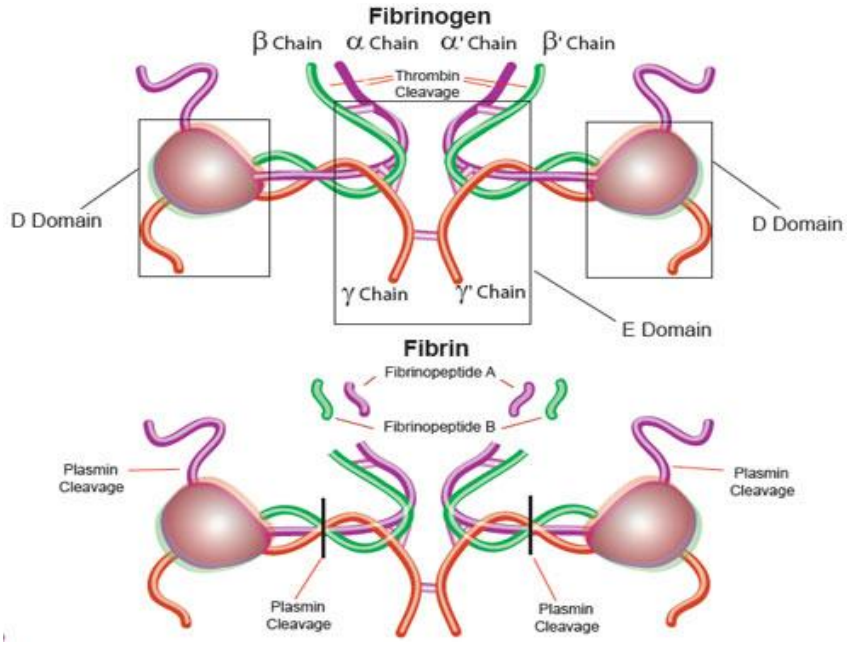


Figure 1: Cleavage of A and B peptides from fibrinogen molecule to form fibrin. This reaction is driven by the catalytic activity of thrombin (Sigma Aldrich, 2011).

Fibrin clots are dissolved by plasmin through a process known as fibrinolysis, and byproducts are eliminated through the liver and the kidneys (Linnes et al., 2007). This process can be slowed by the presence of protease inhibitors, such as tranexamic acid (AMCA) and ε-aminocaproic acid (ACA) (Andersson et al., 1965; Grassl et al., 2003).

2.6 Variations in Fibrin Gel Architecture

Though only three reagents are necessary for the formation of a fibrin gel, varying the ratios of these reagents has been shown to change the gel's architecture with respect to parameters such as fiber diameter and branchpoint density (Carr and Hermans, 1978; Ryan et al., 1999). In addition, changes in physical properties of the fibrin gel have been correlated to changes in its mechanical properties (Roberts et al., 1974; Gerth et al., 1974; Ryan et al., 1999). The structures of fibrin gels created under different initial conditions have been estimated through light-scattering and permeation methods (Carr and Hermans, 1978; Shah et al., 1985; Blomback et al., 1990; Dubey et al., 2001) and have been confirmed using electron microscopy (Weisel and Nagaswami, 1992; Ryan et al., 1999).

Previous experiments have demonstrated success in seeding and culturing cardiac cells on fibrin gels at various fibrin concentrations (Jockenhoevel et al., 2001; Grassl et al., 2003; Williams et al., 2006; Linnes et al., 2007; Black et al., 2009), which correlate directly to the concentration of fibrinogen used when making the gel. However, amounts of other reagents, such as thrombin and calcium, have often been used with little explanation about the motivation behind their selection. In addition, the implications of these choices on adhesion, survival, and proliferation of cardiac cells and the subsequent functionality of cardiac tissue have not been adequately explored. Although studies have explored optimal fibrin formulations for the creation of other engineered tissues, such as cartilage (Eyrich et al., 2007) and arteries (Grassl, et al., 2003), this type of investigation has yet to be conducted for creation of engineered heart tissue. Thus, there exists a need to conduct a comprehensive study that correlates the structural and mechanical differences obtained in fibrin gels by varying concentrations of thrombin and calcium with the resultant fates of heart cells when grown in these gels.

3. Methods

3.1 Determination of Structure

The goal of this experiment was to use light-scattering methods to characterize the architecture of fibrin gels formulated under the same fibrinogen concentration, which corresponded to the same final fibrin concentration, but different thrombin and calcium concentrations. Structural parameters, such as fiber diameter and density of each fiber, were measured by determining turbidity as a function of wavelength. Trends in these parameters due to changes of the concentrations of each reagent were characterized.

a. Creation of Fibrin Gels

To create fibrin gels, a modified version of an established protocol was used, which allowed for the creation of 1 mL of 3.3 mg/mL fibrin (Black et al., 2009; Ye, et al., 2011). F solution was created by adding 112 μL of fibrinogen stock solution (33 mg/mL fibrinogen in 20 mM HEPES buffered saline) to 558 μL of 20 mM HEPES buffered saline. The protocol called for T solution to be created by combining 17 μL of thrombin stock solution (25 U/mL thrombin in 0.81% NaCl solution), 1.3 μL of 2 N calcium chloride solution, and 152 μL phenol red-free Dulbecco's Modified Eagle Medium (DMEM). This would give rise to a gel containing final concentrations of 1.3 mM calcium and 0.425 U/mL thrombin. However, since our experiment involved modifying thrombin and calcium concentrations within fibrin gels, concentrations of reagents within our T solutions were modified depending on the condition being studied. Amounts of thrombin stock and calcium chloride solutions added to create each condition are indicated below (Table 1). Volumes of DMEM were modified to keep total T solution volumes consistent. Finally, the protocol called for C solution to be created by spinning down cells and

resuspending them to their desired seeding concentration in 170 μL of DMEM. However, since we wished to create acellular gels to prevent any exogenous interference with absorbance readings, our C solutions consisted only of phenol red-free DMEM.

Table 1: Amounts of thrombin stock and calcium chloride solutions added to T solutions. All possible combinations were tested, giving a total of 42 conditions. For each condition, volumes of DMEM were modified to keep total T solution volumes consistent at 170.3 μL.

Final Concentration of Calcium	Volume of Calcium Chloride Solution Added to T Solution	Final Concentration of Thrombin	Volume of Thrombin Stock Solution Added to T Solution
0.1 mM	0.1 μL	0.10 U/mL	4.0 μL
1.0 mM	1.0 μL	0.25 U/mL	10.0 μL
5.0 mM	5.0 μL	0.50 U/mL	20.0 μL
10.0 mM	10.0 μL	1.0 U/mL	40.0 μL
20.0 mM	20.0 μL	2.0 U/mL	80.0 μL
35.0 mM	35.0 μL	3.0 U/mL	120.0 μL
50.0 mM	50.0 μL		

To create gels for each of our conditions, F, T, and C solutions were combined in a 4:1:1 ratio and mixed thoroughly. Then, 100 μL of each mixture was added to each well of a black, clear bottom 96-well plate. Gels were given 30 minutes to solidify before measurements were taken. Previous work by our lab has demonstrated that 30 minutes was sufficient for gels created under a wide variety of calcium and thrombin concentrations to undergo at least 80 to 90% gelation (Wang 2012). We did not allow gels to sit for much longer to avoid the possibility of dehydration and subsequent loss of structural integrity.

b. Determination of Absorbance

To determine the change in absorbance as a function of wavelength, the absorbances of gels in each well of the 96-well plate were read using a spectrophotometer (SpectraMax M2, Molecular Devices). Measurements were taken over a range of 360 nm to 750 nm at intervals of 10 nm, with de-ionized water used as a blank.

c. Quantification of Physical Parameters

Previous literature has demonstrated that that the wavelength-dependent turbidity of a fibrin gel can be used to estimate various morphological parameters, such as fiber radius and mass-to-length ratio (Carr and Hermans, 1978; Dubey et al., 2001). It has been shown that the turbidity (τ) of a gel and the radius (r) and mass-to-length ratio (μ) of its constituent fibers can be described using the following relationship (Carr and Hermans, 1978):

$$c/(\tau\lambda^3) = A\mu^{-1} + B(r^2/\mu)\lambda^{-2}$$

Where A and B are constants defined by the following parameters:

$$A = [((88/15)\pi^3(dn/dc)^2)/N]^{-1}$$

$$B = (92\pi^2n^2/77)*A$$

In these equations, c is the initial concentration of fibrinogen in grams/cm^3, λ is the wavelength of light shone on the gel in cm, τ is the turbidity of the gel, n is the refractive index of fibrin, and N is Avogadro's number. Previous experiments performed using a refractometer have shown that, for fibrin, the value of n is 1.334, while the value of dn/dc is 0.178 cm^3/g (Dubey et al., 2001). The turbidity at each wavelength was determined by dividing absorbance by thickness and multiplying by a factor of ln 10 (Carr and Hermans, 1978).

Using the gathered data, a plot of $c/\tau\lambda^3$ vs. $1/\lambda^2$ was generated for gels created under each formulation. The fiber mass-to-length ratio in Daltons/cm was determined using the y-intercept, and the fiber radius in cm was determined using the slope. Fiber diameter in cm was determined directly from information about fiber radius, while density of each fiber in Daltons/cm^3 was calculated by dividing the mass-to-length ratio by the cross-sectional area. To assist with visualization of trends over all conditions, surface plots for diameters and densities of fibers within gels were generated using SigmaPlot (Systat Software).

d. Visualization of Fibrin Gels

To confirm these findings, fibrin gels with the following combinations of initial thrombin and calcium concentrations were created (Table 1, 2). After being given 30 minutes to solidify, gels were imaged using an Olympus IX70 Microscope.

Table 2: Concentrations of calcium and thrombin in fibrin gels created for imaging. For consistency, these formulations were carried through in later experiments.

	Low Thrombin	**Intermediate Thrombin**	**High Thrombin**
Low Calcium	1.0 mM Ca^{2+}, 0.25 U/mL Thr		1.0 mM Ca^{2+}, 2.0 U/mL Thr
High Calcium	20.0 mM Ca^{2+}, 0.25 U/mL Thr	20.0 mM Ca^{2+}, 1.0 U/mL Thr	20.0 mM Ca^{2+}, 2.0 U/mL Thr

3.2 Determination of Mechanical Properties

The purpose of this experiment was to correlate modifications in concentrations of thrombin and calcium in the formulation of a fibrin gel with resultant differences in their mechanical properties. To characterize these differences, two distinct tests were performed. Uniaxial stretching was performed to determine differences in Young's moduli of gels, while rheology was performed to characterize differences in viscoelastic properties of gels.

a. Uniaxial Stretching

The purpose of uniaxial stretching was to characterize differences in Young's moduli of gels created under different concentrations of thrombin and calcium. To accomplish this, gels were stretched quasi-statically along a single axis. The force generated by gels in response to application this stretch was recorded and analyzed.

i. Instrumentation

Mechanical testing was performed on gels by stretching them using a custom-made tissue stretcher (Figure 2). This device contains one fixed arm, which is held in place by a metal bar attached to device using two screws, and one mobile arm, which is hooked to the force transducer (Aurora Scientific Inc., Model 6350-358). Each arm contains a metal mount soldered to its end, onto which a metal clasp can slide. To mount samples, one metal clasp is super-glued to each end of the gel. After PBS is added to the trough in which the sample will sit, the clasps are slid onto their respective mounts, allowing the samples to be loaded to the machine.

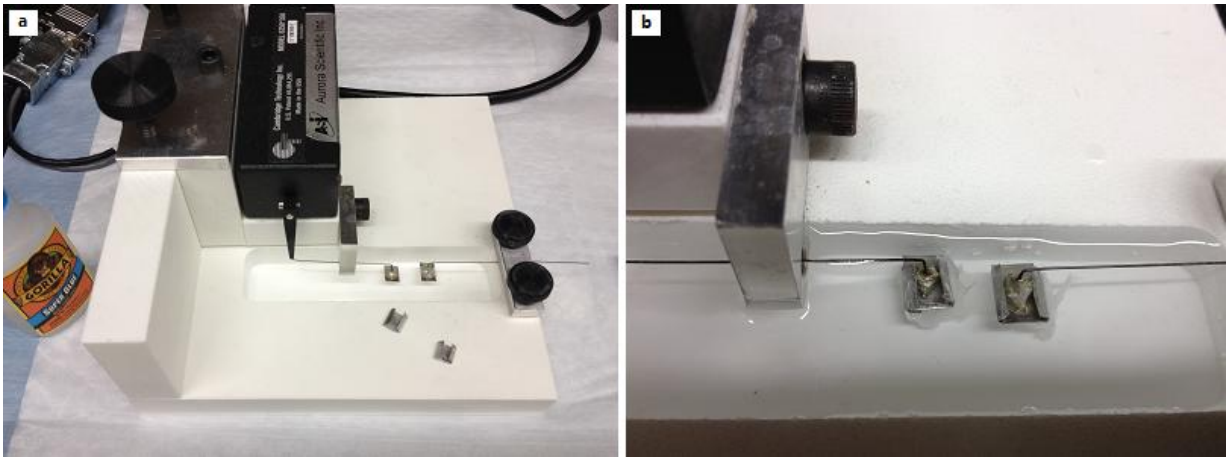


Figure 2: (a) Set-up of tissue stretching device, with fixed and mobile arms loaded. The clasps, which have yet to be glued to a sample, are resting on top of the machine. (b) Close-up of fibrin gel sample, which has been glued to the clasps and mounted to the machine.

The tissue stretcher was connected to a Dual-Mode Lever System (Aurora Scientific Inc.), which was in turn connected with a Data Acquisition (DAQ) Board (National Instruments). The DAQ board interfaced with LabVIEW (National Instruments).

ii. Labview Program

A custom, previously designed LabVIEW program was used to operate the mechanical stretcher. This program inputted a file containing the stretch profile that is to be delivered. In these experiments, a triangular waveform was selected as the stretch profile of interest. This profile was delivered to the tissue stretcher as electrical signal, with a calibration coefficient of 0.51 V/mm. The force transducer moved back and forth to stretch the sample. As the sample elongated and relaxed, the force associated with its changes in length was detected by the force transducer and outputted to LabVIEW as an electrical signal. This signal was then converted back to force, with a calibration coefficient of 98.80 mN/V.

iii. Stretching of Fibrin Gels

Fibrin gels for mechanical testing were prepared as previously described (Table 1, 2). After F, T, and C solutions were combined, 600 μL of the mixture was added to each well of a scored 6-well plate. After gels were given 30 minutes to solidify, one clasp was glued to each end. Cuts were made around the perimeter of gels using a scalpel, and gels were lifted from the 6-well plate and loaded to the tissue stretcher device. Dimensions of the gel were measured using a caliper. Gels were preconditioned by stretching at 25.0% for 5 cycles and then returned to rest in a relaxed state for 5 minutes. Then, gels were stretched again for 10 cycles at that strain level, and measurements for force and length were recorded.

iv. Determination of Young's Modulus

For each set of data, stress (σ) was calculated by dividing the force applied to the gel by the area to which this force was applied. Strain (ε) was calculated by dividing the change in length on the gel by its initial length. Plots of stress vs. strain were generated at 25.0% strain.

The Young's modulus was found at 5.0%, 10.0%, 15.0%, and 20.0% strain by determining the slope of the stress-strain curve at that percent strain.

b. Rheology

The purpose of rheology was to determine differences in viscoelastic properties of gels created under different concentrations of thrombin and calcium. One parameter was G', or storage modulus, which represents the energy stored during deformation and gives information about a material's elastic properties. Another parameter was G", or loss modulus, which represents the energy dissipated by a material during deformation and gives information about a material's viscous properties. Finally, the ratio of G" to G' was used calculate tan δ, or loss tangent, which provides information about the ratio of energy lost to energy stored during cyclic deformation and serves as a measure of a material's degree of viscoelasticity.

i. Instrumentation

Rheological properties of each fibrin gel were measured using an ARES Rheometer (Figure 3; TA Instruments). This machine consisted of an upper arm and a lower arm, both of which had plates attached to their ends. During loading, gels were placed on top of the lower plate, and the upper plate was vertically displaced until it rested snugly on the surface of the gel. The lower arm on the rheometer was attached to a motor, which allowed the arm to rotate to apply varying amounts of shear to the entrapped gel. The upper arm was attached to a force transducer, which measured the torque generated during testing and interfaced with the TA Orchestrator program (TA Instruments) to output rheological information. The ARES Rheometer was able to perform tests over ranges of parameters such as frequency and strain.

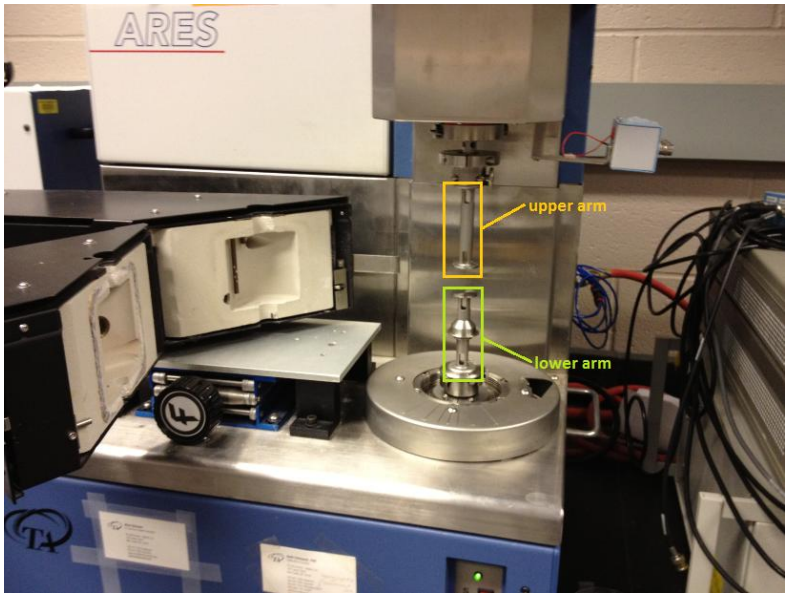


Figure 3: Image of rheometer used for rheological testing of fibrin gels. Gels are loaded between the upper and lower arms of the instrument, as indicated.

b. Determination of Rheological Parameters

Fibrin gels for rheological testing were prepared as previously described (Table 1, 2). After F, T, and C solutions were combined, 600 μL of the mixture was added to each well of a scored 6-well plate. After being given 30 minutes to solidify, cuts were made around the perimeter of gels using a scalpel, and gels were carefully lifted from the 6-well plate and loaded onto the plate on the lower arm of the rheometer. The upper arm was lowered onto the gel until the gap between the plates on the upper and lower arms was 0.800 mm. All gels were tested at 5.0% and 10.0% constant strain over angular frequencies spanning from 1 to 10 rad/sec. Information gathered by the rheometer was used to determine the G', G", and tan δ associated each gel as a function of frequency at each percent strain.

3.3 Determination of Cellular Compatibility

The goal of this experiment was correlate modifications in concentrations of thrombin and calcium in the formulation of a fibrin gel with resultant differences in cellular fate when grown in these gels. To determine these differences in cellular compatibility, native cells were isolated from neonatal rat pups and seeded into fibrin gels created under different formulations to create cellular constructs. Constructs were grown for 7 days, and cells were monitored closely for adhesion, proliferation, and survival during this time. On Day 7, a DNA assay was performed on these cellular constructs to measure total cell number. In addition, constructs were stained for collagen I (col I) deposition to measure the ability of cells to remodel their surrounding environments and for expression of myosin heavy chain (MHC) and connexin 43 (Cxn43) to probe for the presence of and communication between cardiomyocytes.

a. Isolation of Cardiomyocytes

To obtain cells for this experiment, native cells were isolated through a previously described method (Ye, et al., 2011). Briefly, hearts were isolated from 2 to 3 day old neonatal rat pups. These hearts were then placed in an ice-cold solution of phosphate-buffered saline (PBS) with 20 mM glucose inside a Petri dish. The hearts were then minced to pieces no larger than 1 mm^3. Next, tissue pieces were placed into conical and digested through sequential steps of addition of collagenase solution and tituration in order to break up the cardiac tissue. During each step, 7 mL of collagenase solution, consisting of 3,000 mg/mL of collagenase in PBS-glucose, was added. After gentle mixing on a shaker for 7 minutes, tissue pieces were agitated and allowed to settle back to the bottom of the conical, and as much supernatant as possible was aspirated away in preparation for addition of another round of collagenase solution. To ensure completely digestion, this step was performed a total of 7 times. Afterwards, cells were spun

down for 5 minutes and resuspended in 40 mL of DMEM. Finally, cell counting was performed using a hemocytometer to estimate the total number of cells obtained.

b. Creation of Cellular Constructs

To create cellular constructs, F solution was prepared as previously described, and T solutions were created individually for each condition being studied (Table 1, 2). C solution was made by adding DMEM to cells to achieve a final seeding concentration of 500,000 cells/mL. F, T, and C solutions were combined, and 500 μL of this mixture was added to each well of a scored 6-well plate, giving a total of 250,000 cells in each gel. Gels were given 30 minutes to solidify completely. Then, 6 mL of myocardial construct medium, consisting of DMEM with 2% fetal bovine serum (FBS), 10% horse serum, 1% penicillin/streptomycin, 2 μg/mL ε-aminocaproic acid, 50 μg/mL of ascorbic acid, and 2μg/ml of insulin (Ye at al., 2011), was added to each well to completely immerse the top of each hemisphere.

c. Growth of Cellular Constructs

Cells were cultured for one week, with medium changed every other day. For each medium change, 3 mL of myocardial construct medium was added to each well. During growth, constructs were imaged on Days 2, 4, and 7 using an Olympus IX70 Microscope.

d. DNA Assay

To quantify the number of cells growing in fibrin gels, a DNA assay was performed on Day 7. Constructs were carefully scraped from their wells and placed into Eppendorf tubes. Next, samples were digested in 0.5 mL of 0.5 mg/mL proteinase K in digestion buffer overnight inside an oven at 56°C. Digestion buffer was made up of 100mM tris(hydroxymethyl)aminomethane (Tris) and 50 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.4. The next day, Eppendorf

tubes removed from the oven. To create a standard curve, calf thymus DNA was diluted to concentrations of 6, 5, 4, 3, 2, 1, 0.5, and 0 μg/mL. 100 μL of each standard solution was added to one well of a black, clear bottom 96-well plate. Samples were diluted by 4x in TNE buffer, consisting of 10 mM Tris, 0.2 mM sodium chloride, and 1 mM EDTA at pH 7.4, and 100 μL of each diluted sample was added to each well of the 96-well plate. Next, 100 μL of 0.2 ng/mL Hoescht solution was added to each well containing sample.

Fluorescence measurements were taken using a spectrophotometer, with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Using measurements from the standards, in conjunction with their known DNA concentrations, a standard curve was generated. Using this plot, the amount of DNA in each construct was calculated, which used to determine the number of cells within that construct. Each cell was assumed to have 7.6 pg of DNA.

e. Histology

On Day 7, constructs were fixed in 4% paraformaldehyde for 3 hours and placed on PBS. While some constructs were left intact, some were embedded in an optimal cutting temperature (OCT) medium, flash-frozen, and sliced into 10 μm sections using a cryostat machine at -25°C. Sections were preserved on Superfrost Plus microscope slides.

Histological staining was performed both on sections and on constructs. Remaining OCT medium was washed off of microscope slides through a 30 minute soak in PBS. Then, all samples were permeated through exposure to 0.1% Triton X for 5 minutes for sections or 20 minutes for whole constructs. Samples were rinsed with PBS. Next, samples were blocked in 5% donkey serum and 0.1% BSA in PBS for 30 minutes for sections or 90 minutes for whole constructs. Next, primary antibodies were diluted in 0.1% BSA in PBS and then placed on samples for 1 hour for sections or 3 hours for whole constructs. Whole constructs were stained

for myosin heavy chain (MHC) and connexin 43 (Cxn 43) to determine the presence of and communication between cardiomyocytes growing within the fibrin gels. Tissues slices were stained for collagen I (Col I) to determine the ability of cells to lay down their own ECM. Cxn 43 and Col I antibodies were used at dilutions of 1:50, while MHC antibody was used at a dilution of 1:100. Next, samples were rinsed with PBS, and their appropriate fluorescent secondary antibodies were added for 1 hour for sections or 3 hours for whole constructs. All secondary antibodies were used at 1:400 dilutions in 0.1% BSA in PBS. Lastly, Hoescht stain was placed on all samples at 1:10,000 dilution to aid with visualization of nuclei. After a final PBS rinse, samples were visualized under various filters using an Olympus IX70 Microscope.

3.4 Statistical Analysis

All data is reported as mean ± standard deviation, with the number of samples indicated. To probe for differences between conditions, ANOVA tests were run using SigmaPlot. A p value of <0.05 was used to indicate statistical significance.

4. Results

4.1 Structure

a. Quantification of Physical Parameters

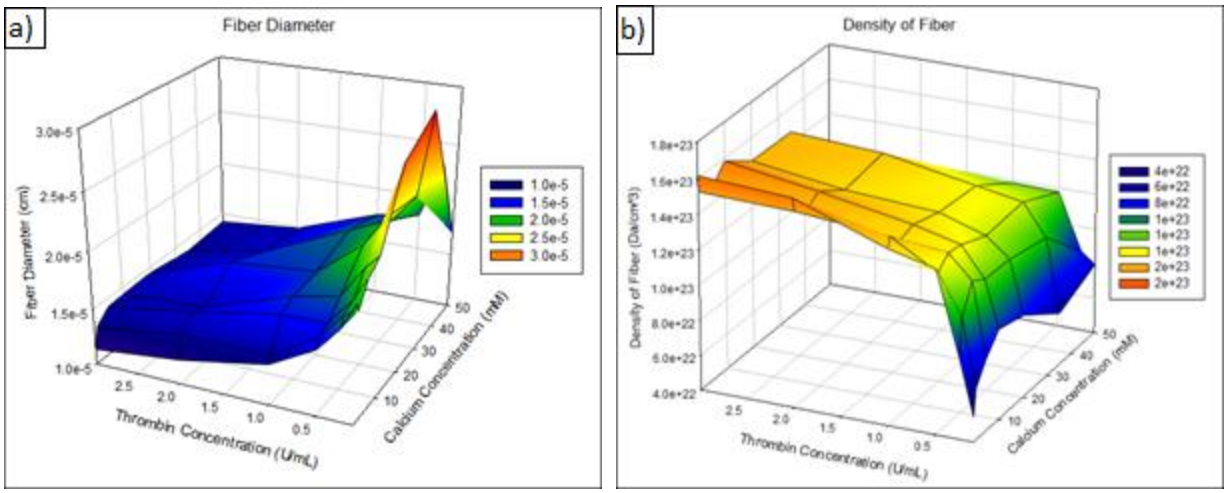
To calculate fiber diameter and density of each fiber in gels made under different formulations, a light-scattering experiment was performed as previously described. As the concentration of calcium within the gel was increased, fiber diameter increased and then decreased, reaching a peak at concentrations between 20.0 and 35.0 mM (Figure 4a,c). This trend was consistent between all thrombin concentrations, with some variation in the calcium concentration at which the maximum diameter was achieved. Fiber diameters of gels created under low calcium concentrations of 0.1 and 1.0 mM were significantly different from those of gels created under high calcium concentrations of 20.0 and 35.0 mM ($p \leq 0.018$).

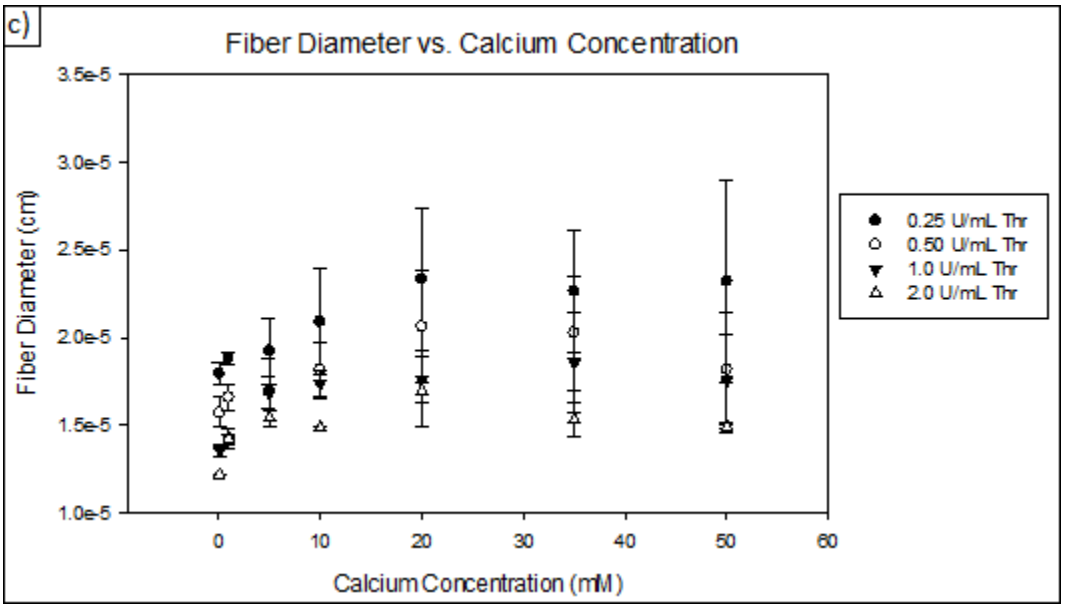
As the concentration of thrombin was increased, fiber diameter decreased and then leveled off at all calcium concentrations (Figure 4a,d). Fiber diameters of gels created under low thrombin concentrations of 0.10, 0.25, and 0.50 U/mL were for the most part significantly different from those of gels created under intermediate and high thrombin concentrations of 1.0, 2.0, and 3.0 U/mL ($p \leq 0.027$). One exception was the difference in fiber diameter in gels created under thrombin concentrations of 0.5 and 1.0 U/mL, which was not significant ($p=0.287$). In general, the smallest fiber diameters were achieved in gels formed under low thrombin and high calcium concentrations, while the largest fiber diameters were achieved in gels formed under high thrombin and low calcium concentrations.

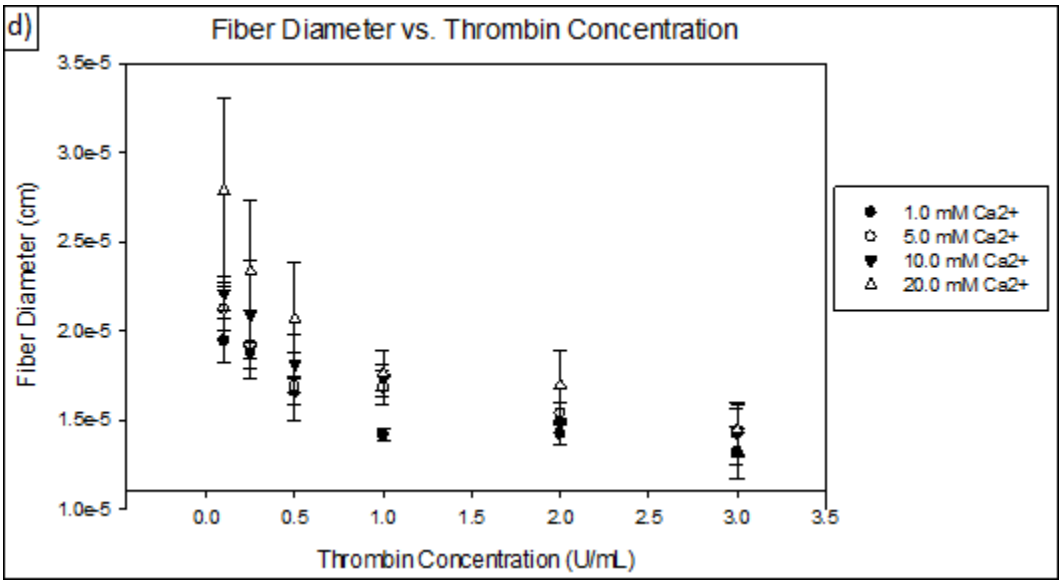
As the concentration of calcium was increased, the density of each fiber decreased (Figure 4b,e). While this decline as a function of calcium concentration was gradual at intermediate thrombin concentrations, it was more pronounced at low and high thrombin

concentrations. Between all cases, densities of fibers of gels created under intermediate calcium concentrations of 5.0 and 10.0 mM were significantly different from those of gels created under a high calcium concentration of 50.0 mM ($p \leq 0.021$).

As the concentration of thrombin was increased, the density of each fiber increased and then leveled off at all calcium concentrations (Figure 4b,f). Densities of fibers of gels created under low thrombin concentrations of 0.10, 0.25, and 0.50 U/mL were for the most part significantly different from those of gels created under intermediate and high thrombin concentrations of 1.0, 2.0, and 3.0 U/mL ($p \leq 0.007$). Again, one exception was the difference in fiber diameter in gels created under thrombin concentrations of 0.5 U/mL and 1.0 U/mL, which was not significant ($p=0.706$). In general, the smallest densities were achieved in gels made under low thrombin and high calcium concentrations, while the largest densities were achieved in gels formed under high thrombin and low calcium concentrations.


a)
Fiber Diameter
Fiber Diameter (cm)
3.0e-5
2.5e-5
2.0e-5
1.5e-5
1.0e-5
Thrombin Concentration (U/mL)
2.5
2.0
1.5
1.0
0.5
Calcium Concentration (mM)
10
20
30
40
50
1.0e-5
1.5e-5
2.0e-5
2.5e-5
3.0e-5
b)
Density of Fiber
Density of Fiber (Dalcm^3)
1.8e+23
1.6e+23
1.4e+23
1.2e+23
1.0e+23
8.0e+22
6.0e+22
4.0e+22
Thrombin Concentration (U/mL)
Calcium Concentration (mM)
4e+22
6e+22
8e+22
1e+23
1e+23
1e+23
2e+23
2e+23


c)
Fiber Diameter vs. Calcium Concentration
Fiber Diameter (cm)
3.5e-5
3.0e-5
2.5e-5
2.0e-5
1.5e-5
1.0e-5
0
10
20
30
40
50
60
Calcium Concentration (mM)
0.25 U/mL Thr
0.50 U/mL Thr
1.0 U/mL Thr
2.0 U/mL Thr


d)
Fiber Diameter vs. Thrombin Concentration
Fiber Diameter (cm)
3.5e-5
3.0e-5
2.5e-5
2.0e-5
1.5e-5
1.0e-5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Thrombin Concentration (U/mL)
1.0 mM Ca2+
5.0 mM Ca2+
10.0 mM Ca2+
20.0 mM Ca2+

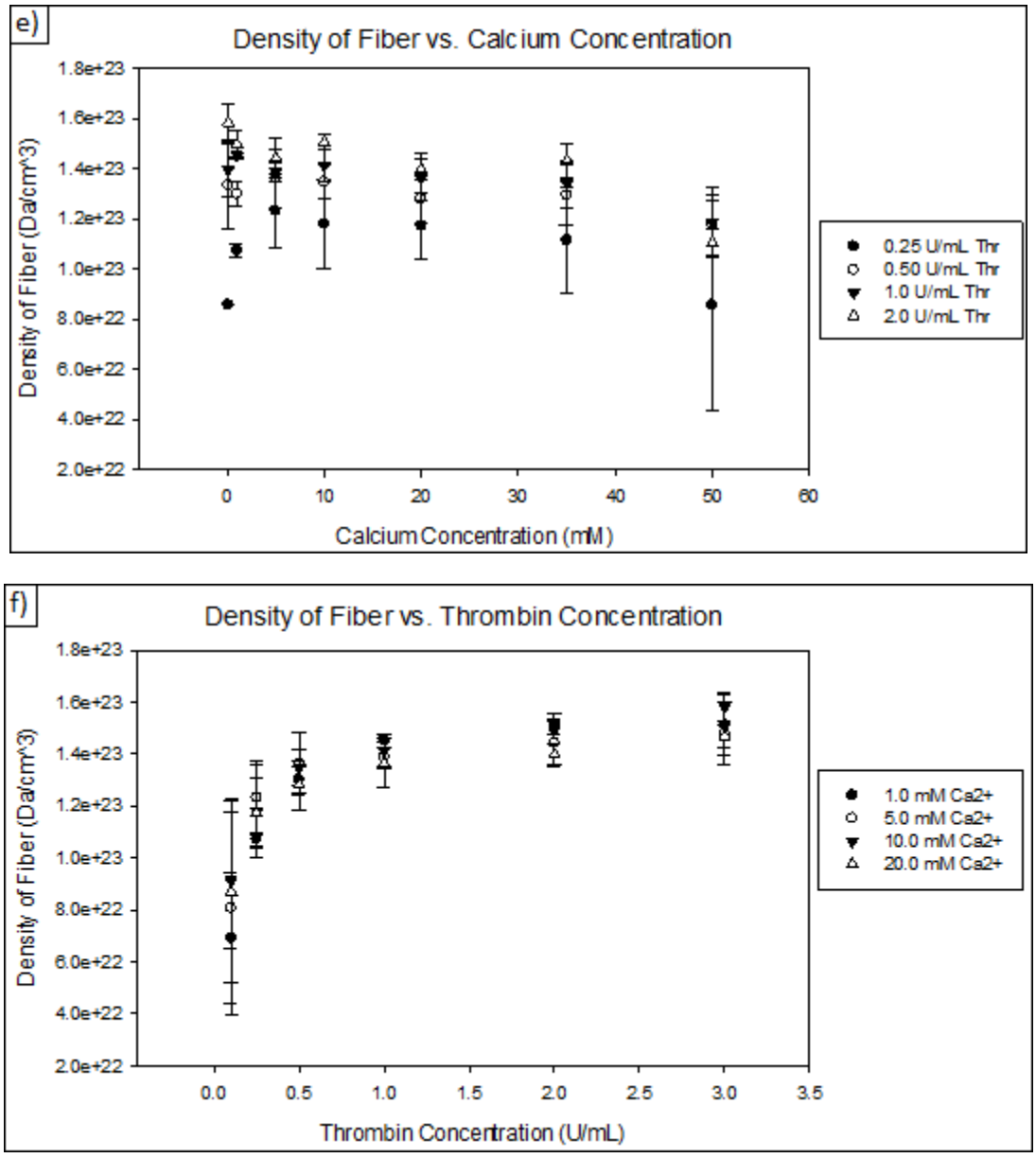
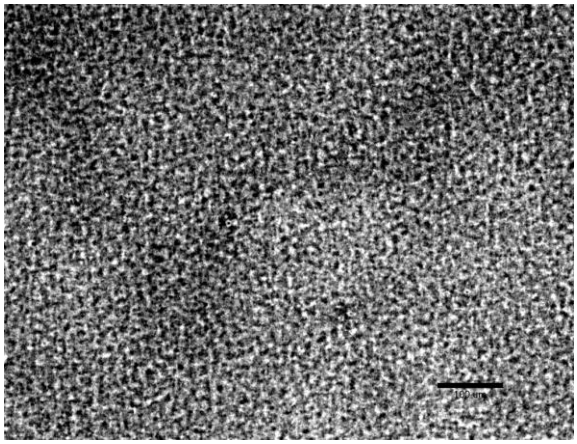


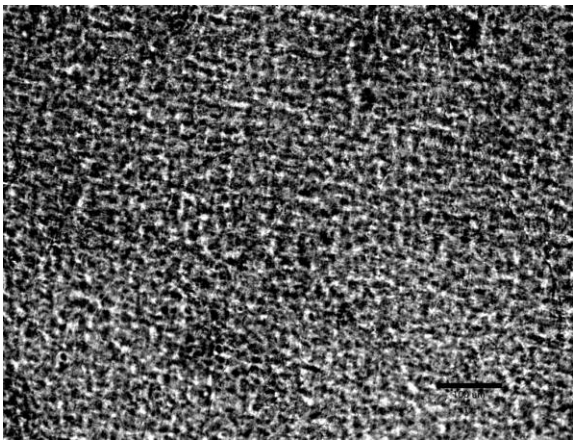
Figure 4: (a, b) Variations in (a) fiber diameter and (b) density of each fiber in gels formed under different concentrations of thrombin and calcium. (c, e) Variations in (c) fiber diameter and (e) density of each fiber as a function of calcium concentration at select thrombin concentrations. (d, f) Variations in (d) fiber diameter and (f) density of each fiber as a function of thrombin concentration at select calcium concentrations. For each formulation, turbidity was found by measuring absorbance over a range of 360-750 nm using a spectrophotometer. Mass-to-length ratio and fiber diameter were determined using regression lines of plots of $c/\tau\lambda^3$ vs. $1/\lambda^2$. The density of each fiber was calculated by dividing its mass-to-length ratio by its cross-sectional area. Data is reported as mean ± standard deviation with n=2.

b. Visualization of Fibrin Gels

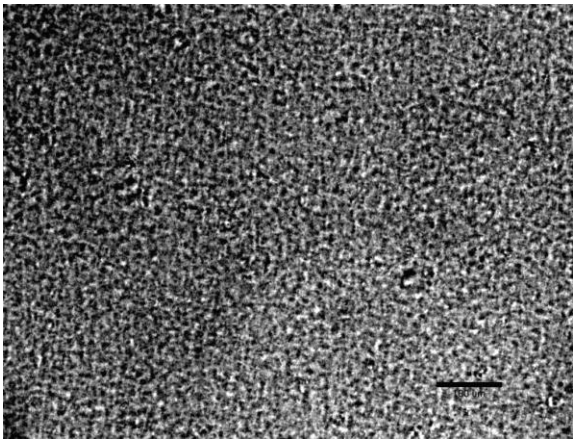
Images taken of fibrin gels created under different formulations were taken in order to verify trends that were obtained by light-scattering methods. Although actual fibers within the gel were not visible through conventional microscopy, visualization of porosity within these gels could be used to deduce fiber structure. Pores appeared smallest in gels created under high thrombin concentrations (Figure 5). This trend held true both at low and high calcium concentrations. On the other hand, pores appeared largest in gels created under high calcium and low thrombin concentrations. Pores in gels formed under low calcium and low concentrations were intermediate in size and did not appear noticeably different from pores within gels formed under high calcium and intermediate thrombin concentrations.



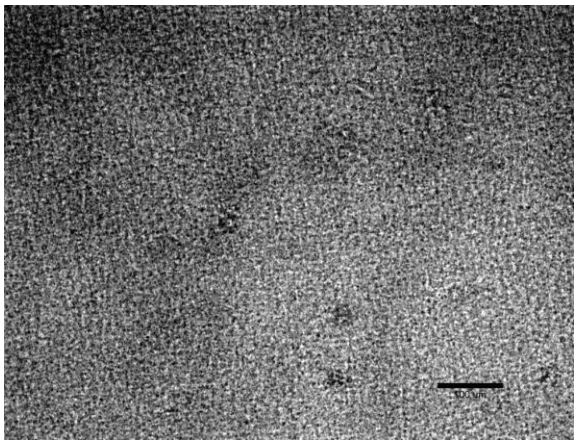
1.0 mM Ca^{2+}, 0.25 U/mL Thr



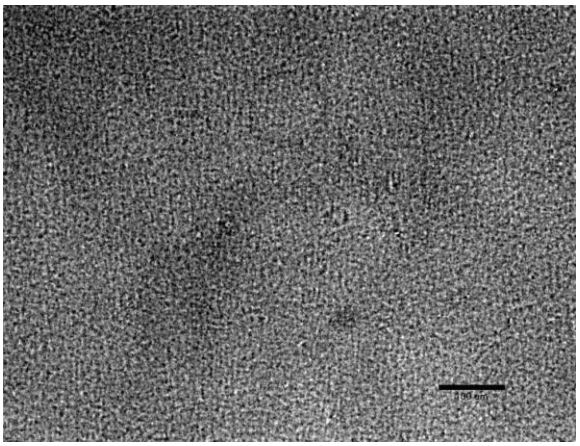
20.0 mM Ca^{2+}, 0.25 U/mL Thr



20.0 mM Ca^{2+}, 1.0 U/mL Thr



1.0 mM Ca^{2+}, 2.0 U/mL Thr



20.0 mM Ca^{2+}, 2.0 U/mL Thr

Figure 5: Fibrin gels made under various calcium and thrombin concentrations. After reagents were mixed, gels were given 30 minutes to solidify before imaging. Scale bars are set at 100 μm.

4.3 Mechanical Properties

a. Uniaxial Stretching

The mechanical properties of gels formed under different formulations were tested using tissue stretcher, as previously described. Gels made under high calcium and high thrombin conditions were stiffest, followed by gels made under high calcium and low thrombin conditions (Figure 6). The Young's moduli of both gels were significantly higher from those of other gels at all percent strains ranging from 5.0% to 20.0% ($p<0.001$).

Though gels made under conditions of low calcium and low thrombin, low calcium and high thrombin, and high calcium and intermediate thrombin had similar stiffnesses (Figure 6). Differences between these gels were more prominent as they were subjected to higher strains. At 5.0% strain, there were no significant differences in stiffness between any of these gels ($p\geq0.050$). At 10.0% and 15.0% strain, gels made under conditions of low calcium and low thrombin were significantly different from the other gels ($p\leq0.025$). At 20.0% strain, there were significant differences in stiffness between all of these gels ($p\leq0.049$).

The stiffnesses of gels increased as they were subjected to higher strains. Aside from an increase from 5.0 % to 10.0% strain, which had no significant effect on stiffness ($p=0.752$), all other increases in strain led to significant increases in Young's moduli measured at that percent strain ($p<0.001$). Furthermore, an increase in strain from 15.0% to 20.0% led to a greater change in stiffness than an increase from 10.0% to 15.0%. The increase in Young's modulus as a function of strain was not linear. Lastly, differences in mechanical properties were more prominent when testing was performed at higher percent strains.

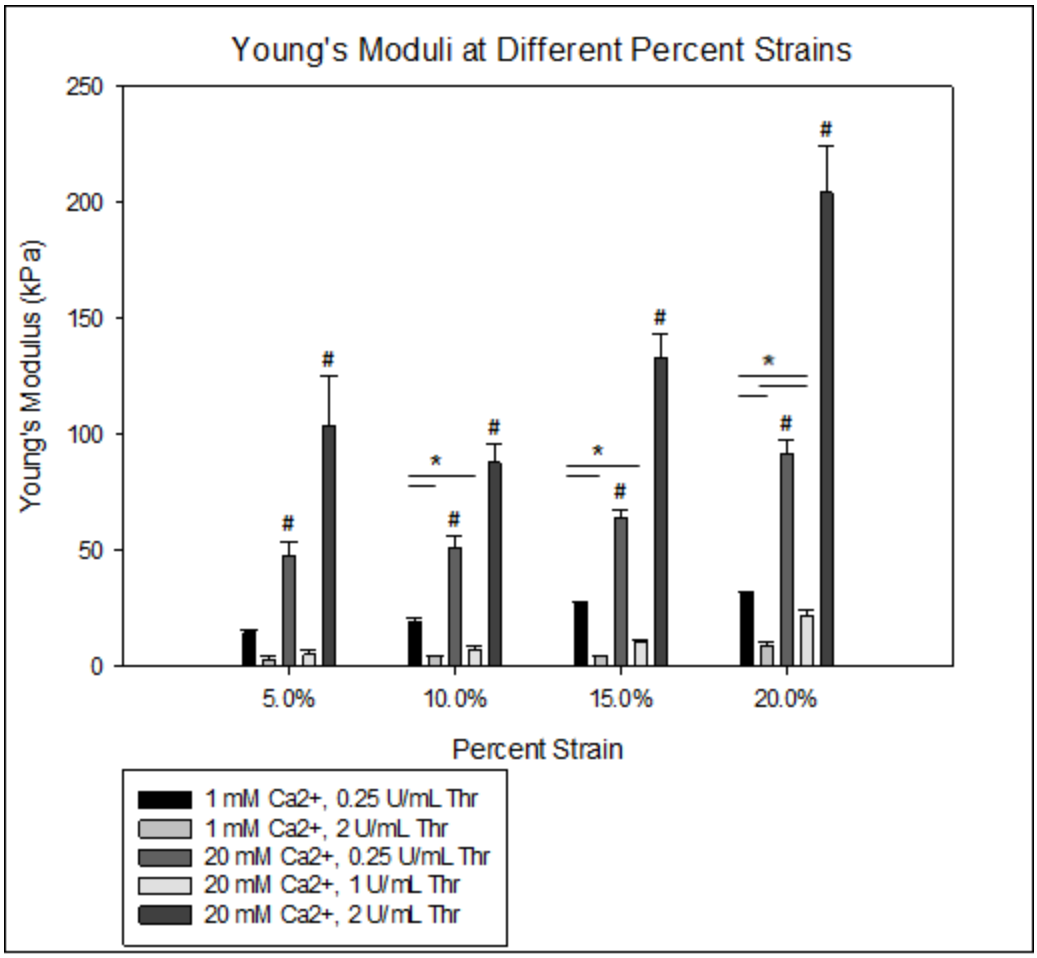


Figure 6: Young's moduli measured at different percent strains for fibrin gels formed under various calcium and thrombin concentrations. To determine the Young's modulus of each gel, it was loaded onto a tissue stretcher, preconditioned, and then stretched at 25.0% strain. Measurements were taken on ten cycles, and three cycles were selected at random to be analyzed. For each cycle, the slope of the stress-strain curve associated with strains of 5.0±1.0%, 10.0±1.0%, 15.0±1.0%, and 20.0±1.0% was found to determine the Young's modulus at that percent strain. Data is reported as mean ± standard deviation with n=1, where three trials were run on the same gel. (*) denotes a p-value of ≤0.05 between indicated conditions.

b. Rheology

Viscoelastic properties of gels formed under different formulations were measured using a rheometer, as previously described. Storage modulus (G') was smallest in gels created under high calcium and low thrombin concentrations (Figure 7). At 5.0% strain, G' was significantly smaller for these gels than for all other gels, except those created under low calcium and high

thrombin concentrations ($p \leq 0.014$). Once strain was raised to 10.0%, the difference in G' between this final pair of gels became significant as well ($p=0.035$). Gels made under low calcium and high thrombin concentrations had the next smallest values for G', though this difference was not significant at 5.0% or at 10.0% strain ($p \geq 0.163$). There were no obvious trends in G' as a function of calcium or thrombin concentration. But in general, gels that were created with a large amount of one reagent and a small amount of the other had smaller values for G' than gels that were better balanced in reagent concentration.

Loss modulus (G") was smallest in gels made under conditions of low calcium and low thrombin and conditions of low calcium and high thrombin (Figure 8). On the other hand, G" was greatest in gels made under conditions of high calcium and intermediate thrombin and conditions of high calcium and high thrombin. At both 5.0% and 10.0% strain, G" was significantly smaller for the first pair of gels than for the second pair ($p<0.001$). Lastly, gels made under high calcium and low thrombin concentrations contained intermediate values for G". At 5.0% strain, G" values of these gels were significantly larger that of the first set of gels ($p \leq 0.022$). On the other hand, at 10.0% strain, G" values of these gels were significantly smaller that for the second set of gels ($p \leq 0.044$). In general, increasing the concentration of calcium within the gel led to an increase in G", while increasing the concentration of thrombin led to an increase in G" only at high calcium concentrations.

Lastly, loss tangent (tan δ) was greatest in gels made under high calcium and low thrombin concentrations (Figure 9). The value for tan δ was significant smaller for these gels than for other gels at 5.0% strain ($p \leq 0.005$) and at 10.0% strain ($p<0.001$). At 5.0% strain, there were no differences between gels created under any of the other formulations. However, as strain was raised to 10.0%, differences began to emerge. At 10.0% strain, gels created under conditions

of high calcium and intermediate thrombin and under conditions of high calcium and high thrombin had larger tan δ values than gels created under low calcium and low thrombin concentrations ($p<0.001$) and gels created under low calcium and high thrombin concentrations ($p\leq0.005$). In general, increasing the concentration of calcium within the gel led to an increase in tan δ, while increasing the concentration of thrombin led to a decrease in tan δ.

As a general rule, values for G' and G" of gels decreased as percent strain was increased from 5.0% to 10.0%, while values for tan δ remained relatively constant. Differences in all viscoelastic properties became more prominent as percent strain was increased. Finally, although values for G', G", and tan δ varied slightly as a function of shear frequency from 1 to 10 rad/s for all conditions, there was no obvious trend to this variation.

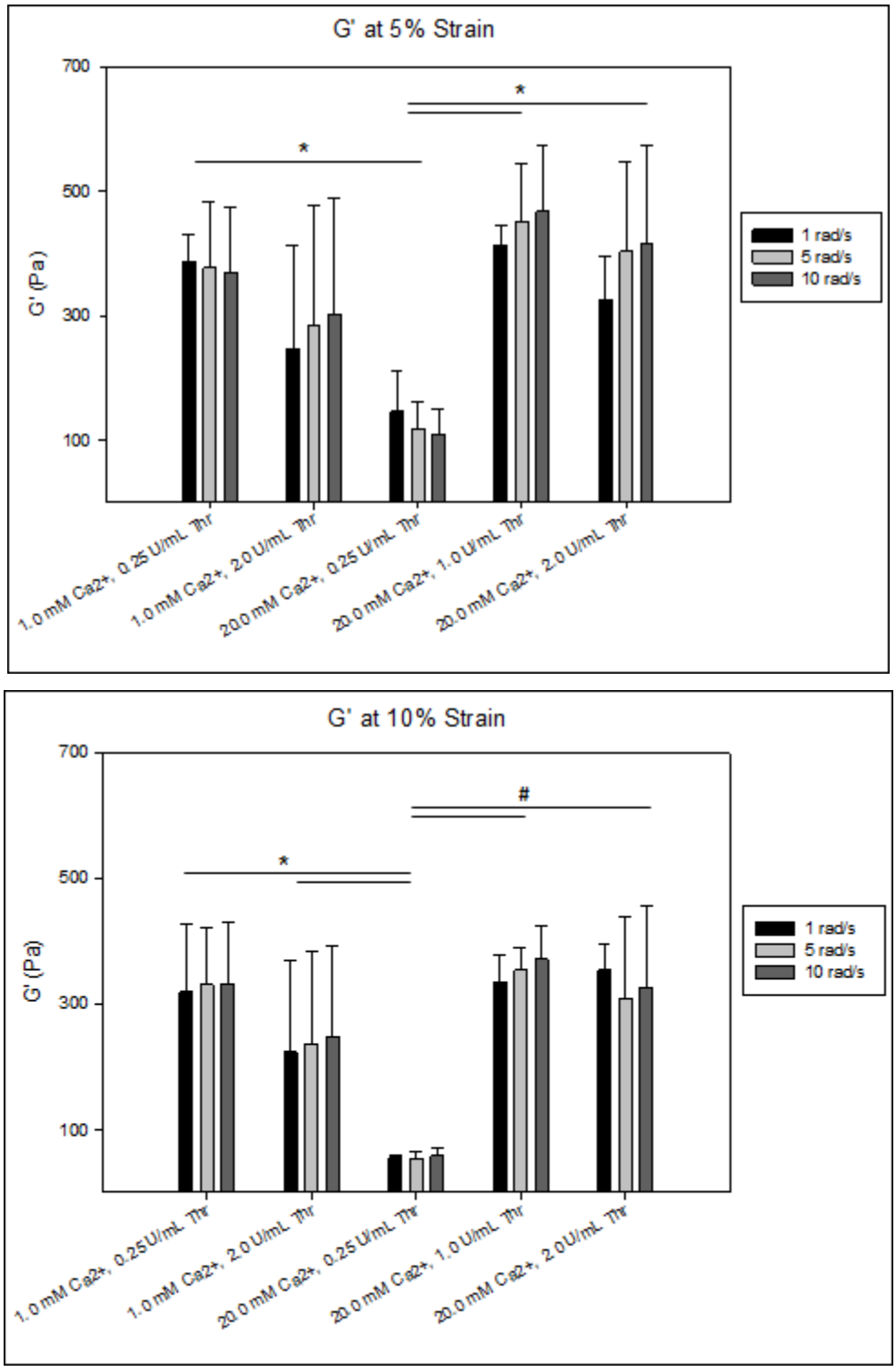


Figure 7: Variations in storage modulus in gels created under different concentrations of calcium and thrombin when subjected to different shear frequencies and percent strains. Measurements were taken using a rheometer at 5.0% or 10.0% strain over angular frequencies of 1 to 10 rad/s. Data is reported as mean ± standard deviation with n=2. (*) denotes a p-value of ≤0.05 between indicated conditions, while (#) denotes a p values of <0.001

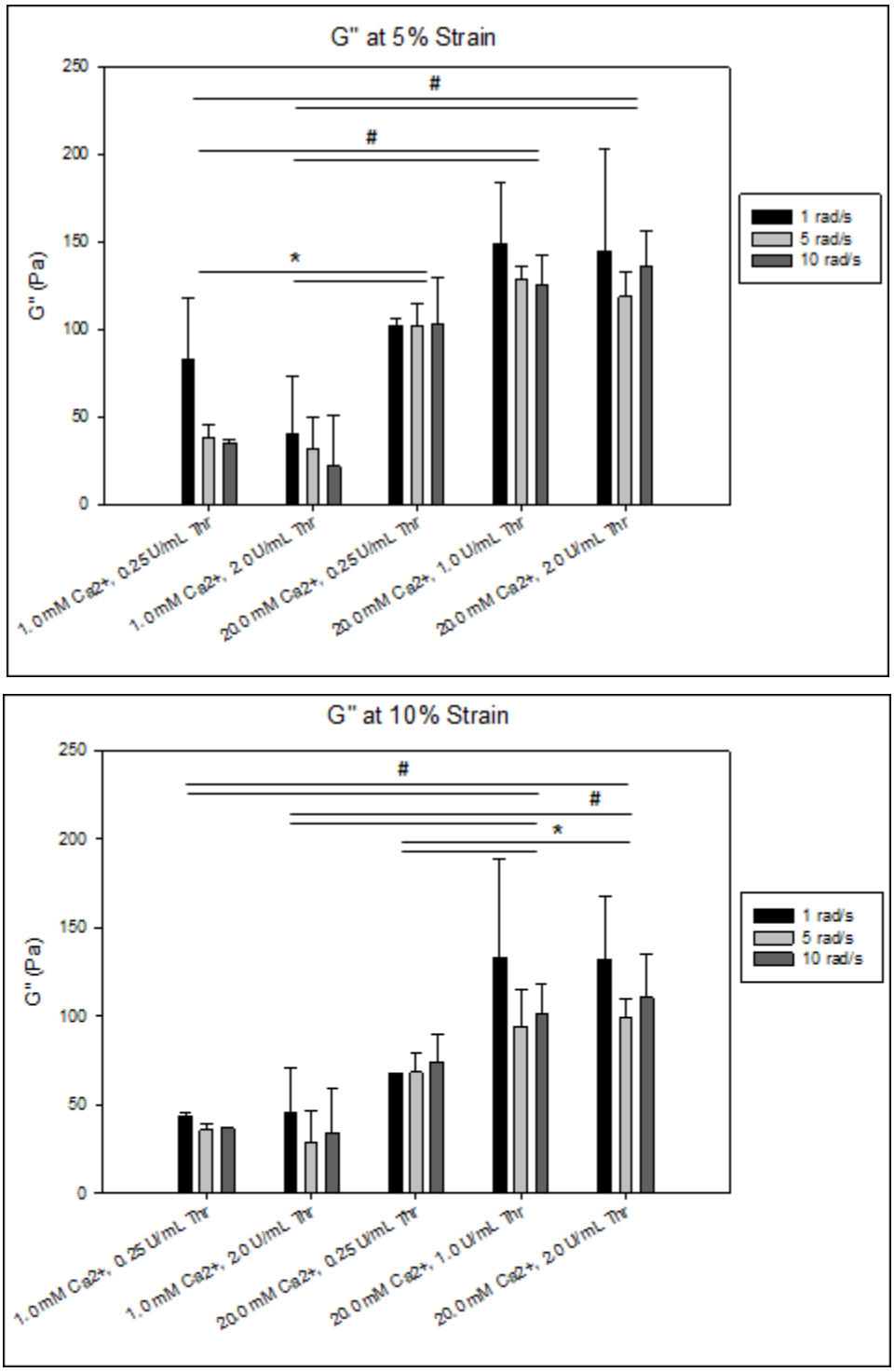


Figure 8: Variations in loss modulus in gels created under different concentrations of calcium and thrombin when subjected to different shear frequencies and percent strains. Measurements were taken using a rheometer at 5.0% or 10.0% strain over angular frequencies of 1 to 10 rad/s. Data is reported as mean ± standard deviation with n=2. (*) denotes a p-value of ≤0.05 between indicated conditions, while (#) denotes a p values of <0.001.

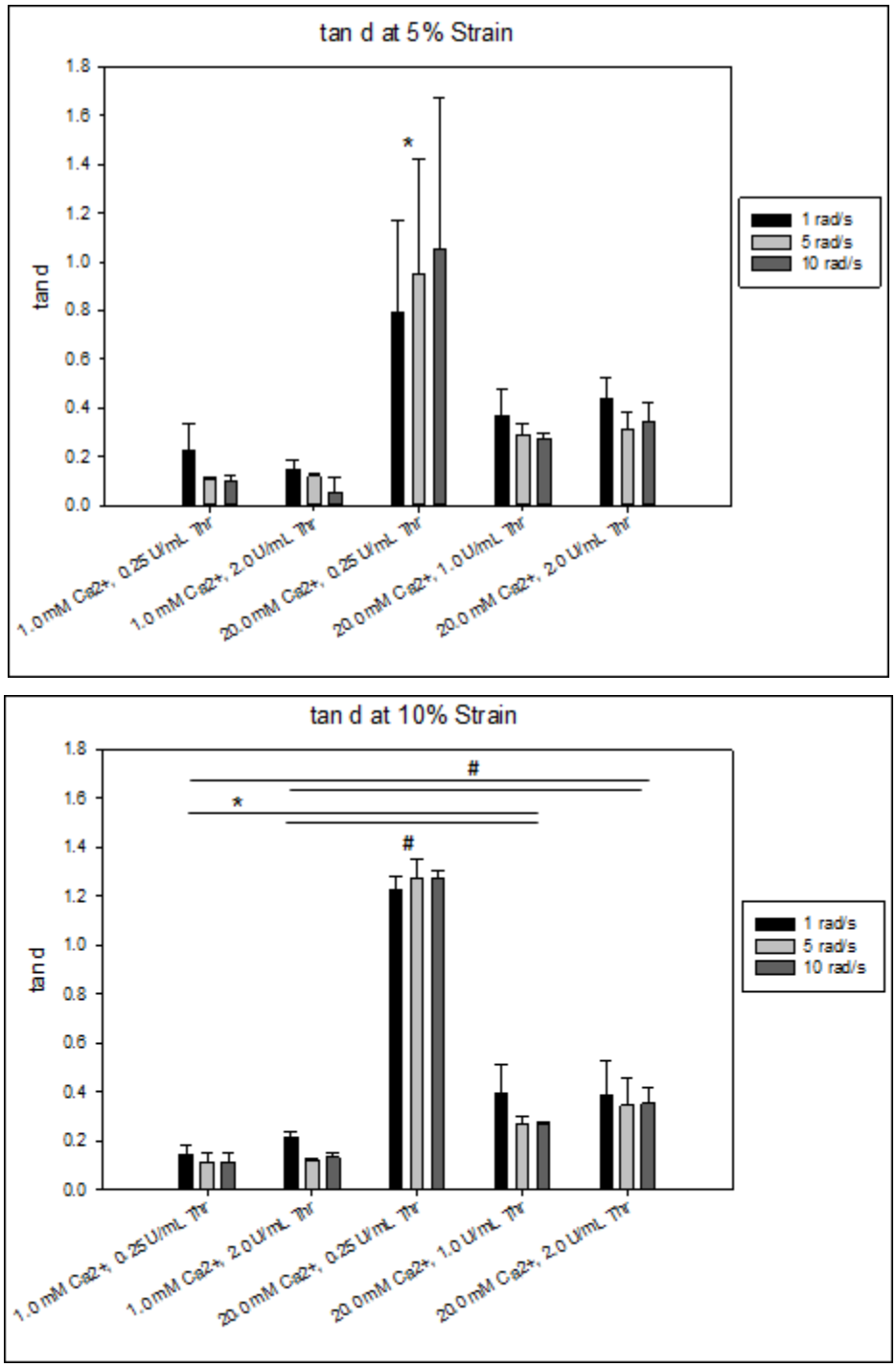


Figure 9: Variations in loss tangent in gels created under different concentrations of calcium and thrombin when subjected to different shear frequencies and percent strains. Measurements were taken using a rheometer at 5.0% or 10.0% strain over angular frequencies of 1 to 10 rad/s. For each set of test parameters, loss tangent was calculated by dividing loss modulus by storage modulus. Data is reported as mean ± standard deviation with n=2. (*) denotes a p-value of ≤0.05 between indicated conditions, while (#) denotes a p values of <0.001.

4.4 Cellular Compatibility

a. Growth of Cellular Constructs

Native cells were isolated from neonatal rat hearts and seeded into fibrin gels made under different formulations on Day 0 to form constructs. By Day 2, cellular spreading was visible in all conditions (Figure 10). In addition, due to relatively low populations of cells within constructs, differences in porosity of gels were noticeable. By Day 4, spontaneous contraction of cardiomyocytes was visible in all conditions. By Day 7, all constructs had become noticeably thinner due to degradation by their constituent cells (Figure 10). Cellular growth was noted across all conditions between Days 2 and 4 and between Days 4 and 7. However, no obvious differences in cell survival or proliferation were noted between conditions.

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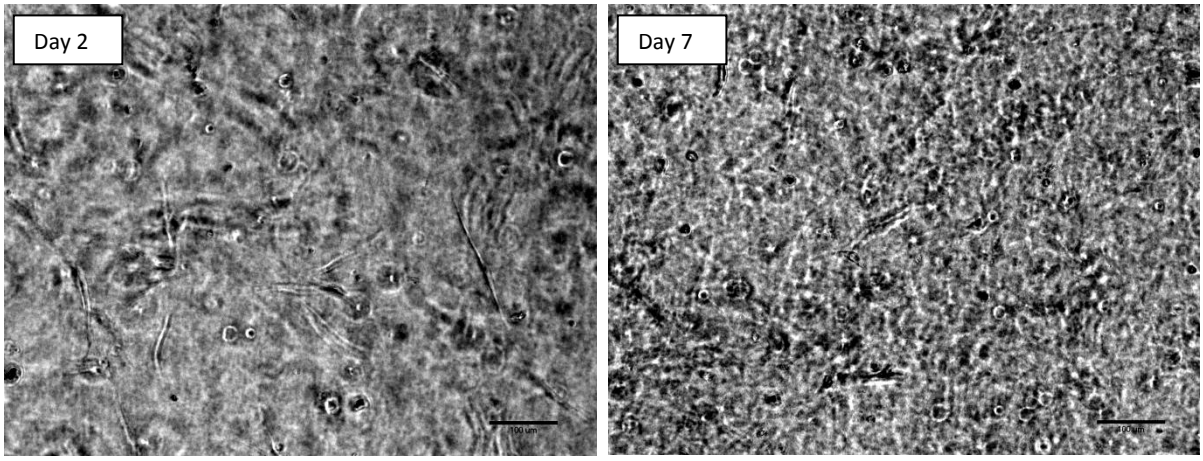
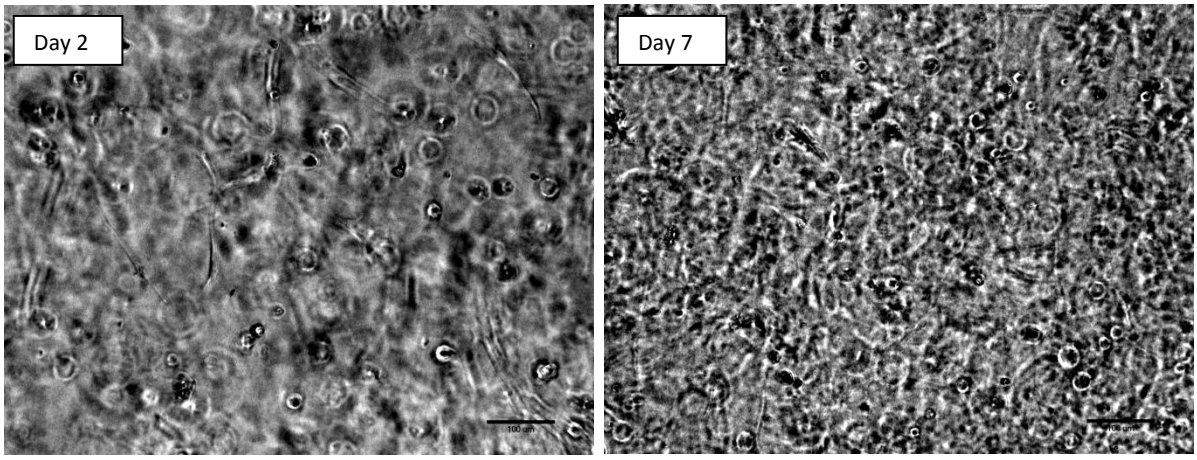
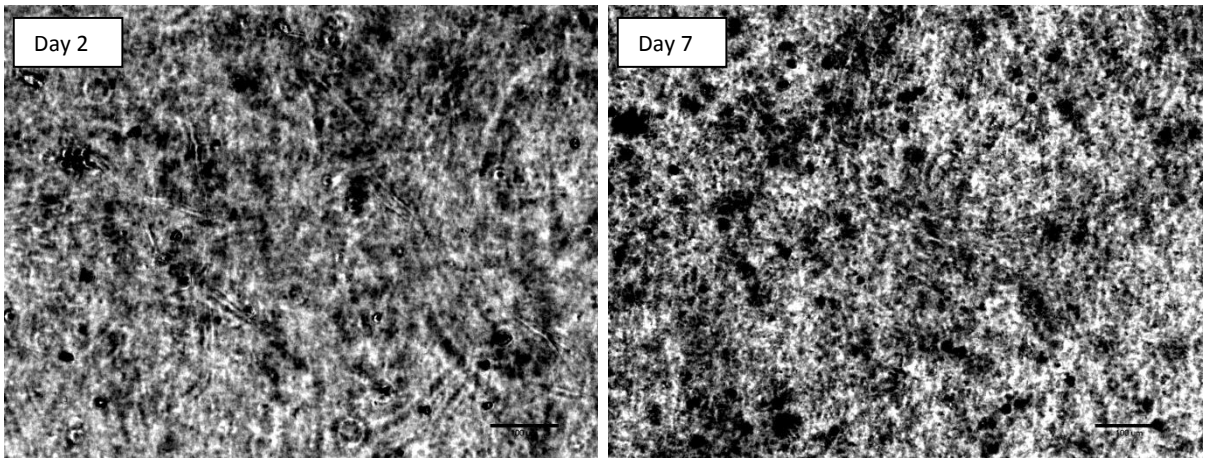


1.0 mM Ca^{2+}, 2.0 U/mL Thr



20.0 mM Ca^{2+}, 1.0 U/mL Thr



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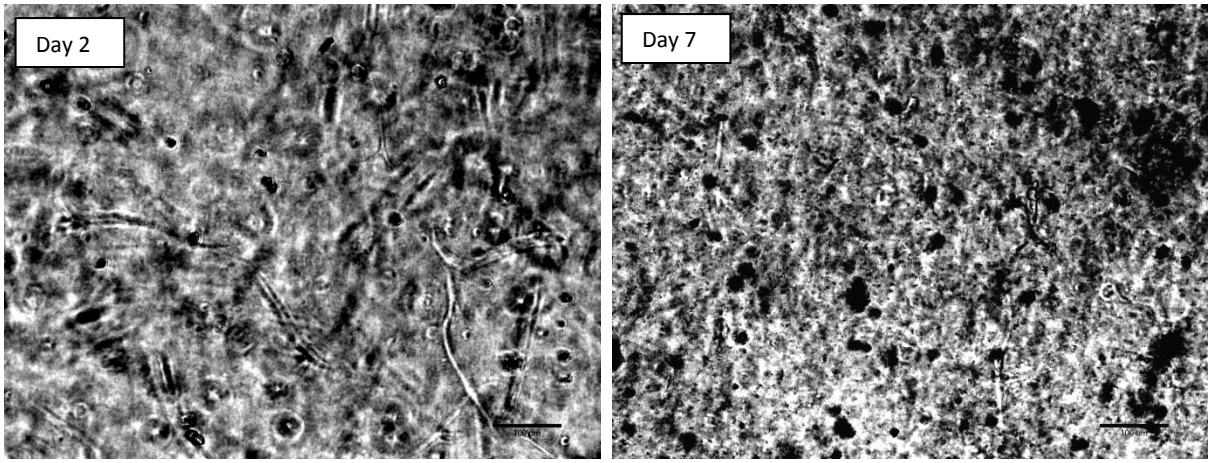

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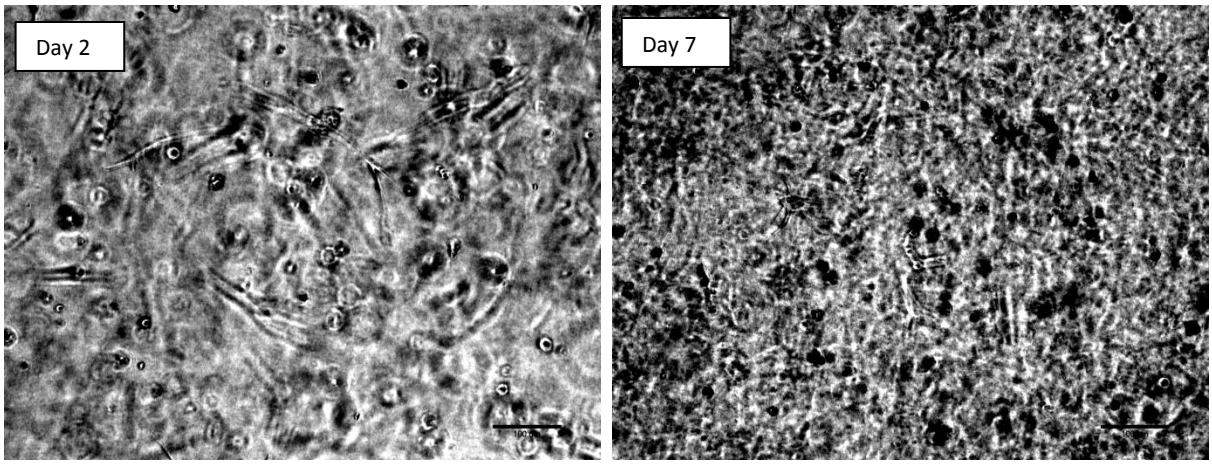


Figure 10: Images of cellular constructs on Days 2 and 7 of growth. Each construct was created on Day 0 by seeding 250,000 cells into 500 μL fibrin gels formed under various thrombin and calcium concentrations. Scale bars are set at 100 μm.

DNA assays were performed on Day 7 to measure quantify differences in cell number between conditions. Over the course of one week, all constructs experienced an approximate two-fold increase in cell number (Figure 11). Fewer cells were found in gels created under high calcium and high thrombin concentrations than in gels made under conditions of low calcium and low thrombin, low calcium and high thrombin, and high calcium and low thrombin ($p \leq 0.030$). In addition, fewer cells were found in gels created under high calcium and

intermediate thrombin concentrations than in gels made under low calcium and high thrombin concentrations (p=0.034). In general, cell numbers were smallest when grown in gels that were created under high concentrations of both reagents.

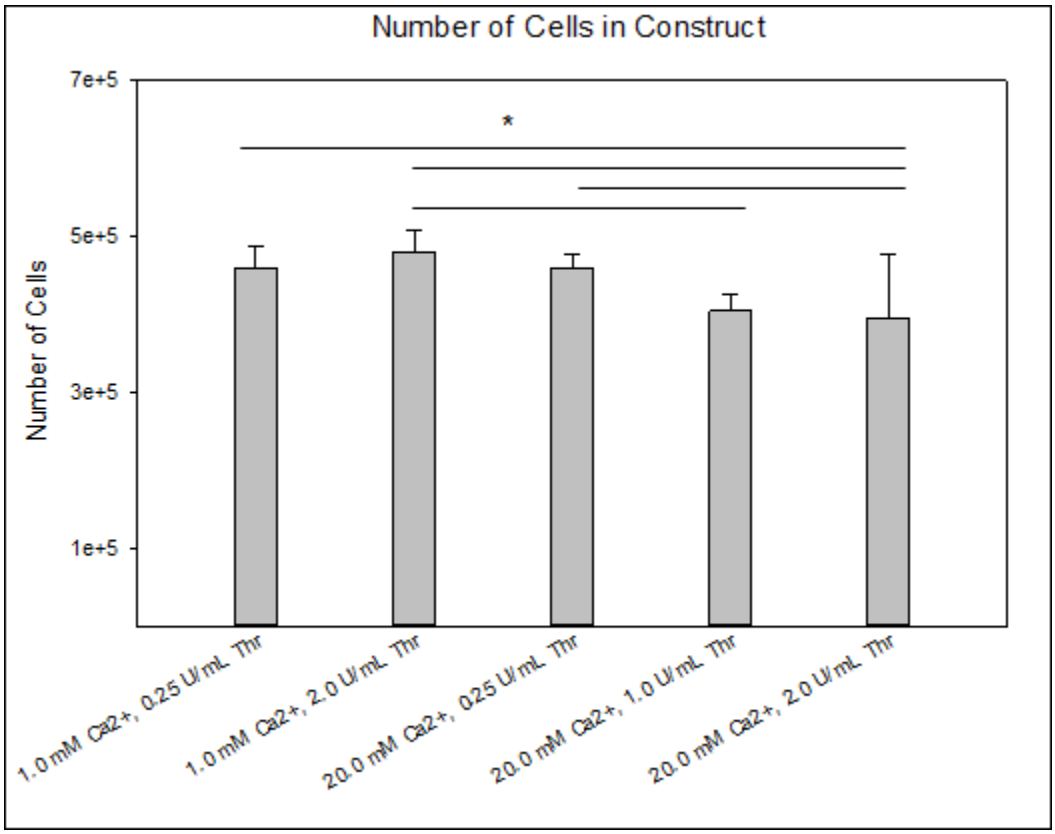


Figure 11: Cell counts on Day 7 of growth within cellular constructs created under by seeding cells into fibrin gels made under different formulations. Data is reported as mean ± standard deviation with n=2. (*) denotes a p-value of ≤0.05 between indicated conditions.

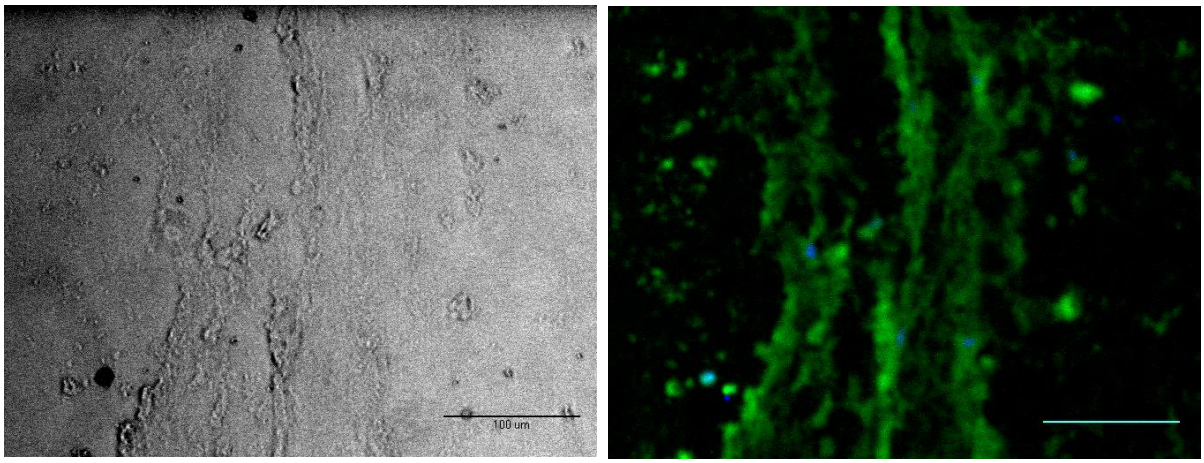
b. Histology

Cellular constructs were fixed on Day 7, sliced, and stained for expression of collagen I (Col I) to determine the success of cells in degrading their fibrin gels and laying down their own ECM. By Day 7, collagen deposition was clearly visible in all gels (Figure 12). In addition, the pattern of Col I stain matched up with the shape of the construct slice for each condition, indicating uniform collagen deposition throughout the construct. Collagen deposition appeared

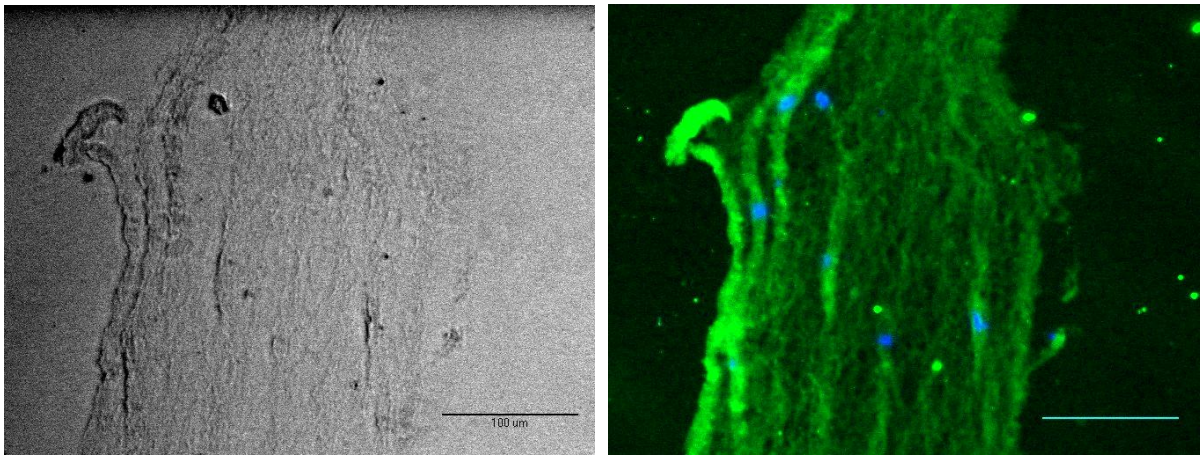
highest in gels made under low calcium and high thrombin concentrations and lowest in gels made under high calcium and low thrombin concentrations.

Other whole constructs were stained on Day 7 for myosin heavy chain (MHC) and connexin 43 (Cxn43) to probe for expression of cardiac markers. A small but noticeable population of cells within each construct stained for MHC, indicating successful survival of cardiomyocytes when grown in gels made under all formulations (Figure 13). There were no obvious differences in relative survival of cardiomyocytes between conditions. On the other hand, no cells within constructs stained strongly for Cxn43 (Figure 13).

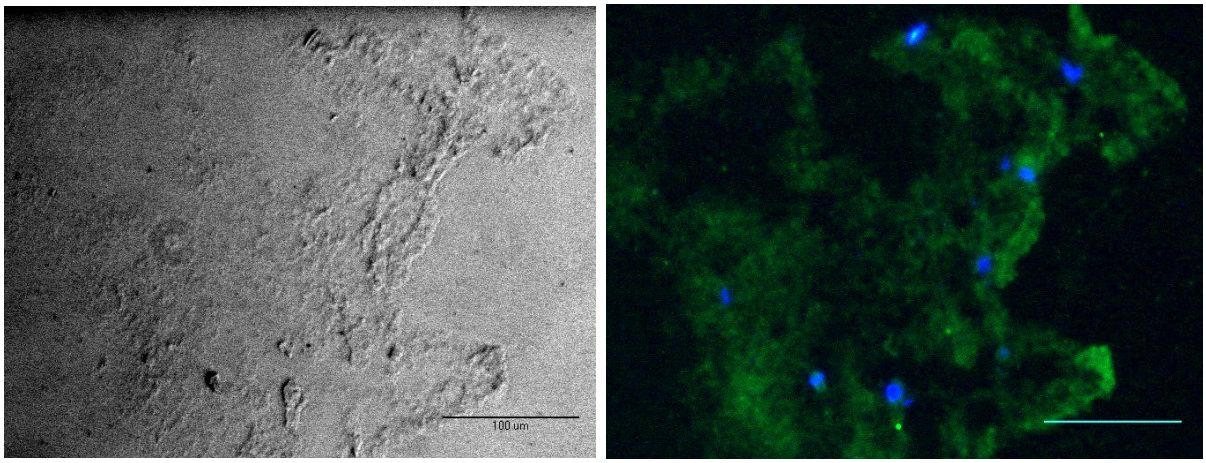
1.0 mM Ca^{2+}, 0.25 U/mL Thr



1.0 mM Ca^{2+}, 2.0 U/mL Thr



20.0 mM Ca^{2+}, 1.0 U/mL Thr



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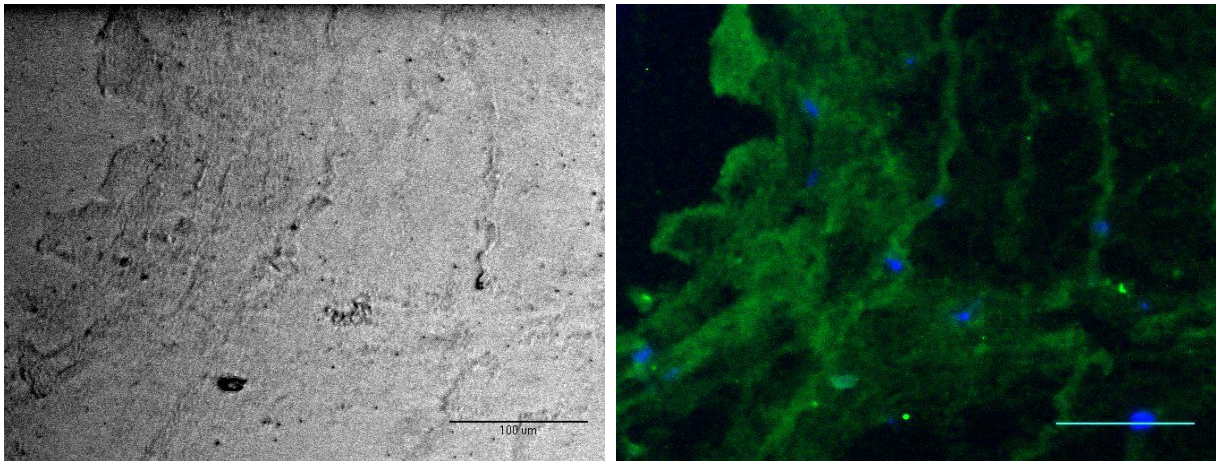


20.0 mM Ca^{2+}, 2.0 U/mL Thr

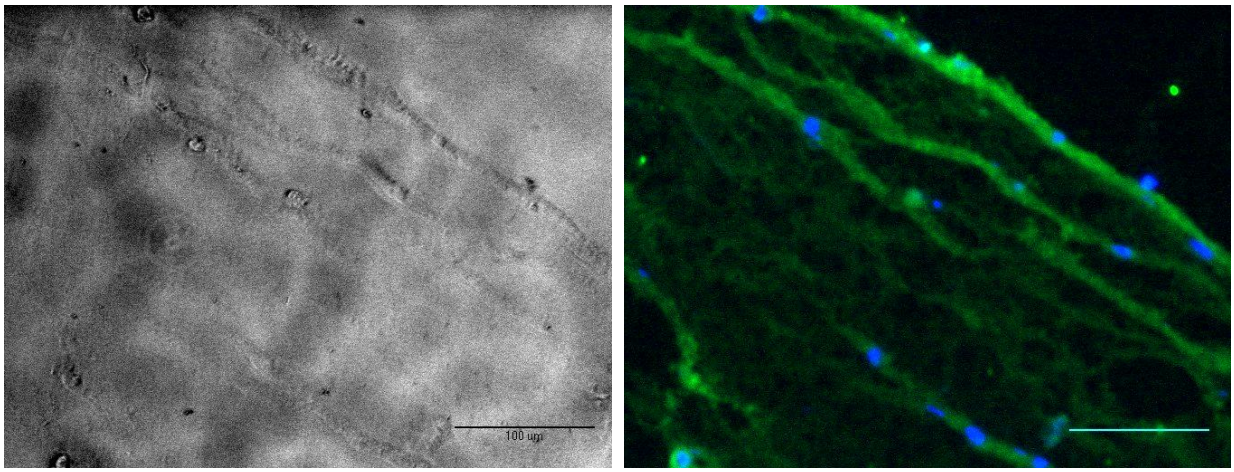
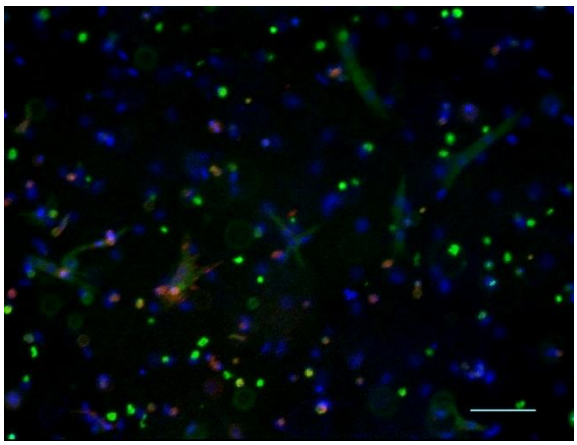
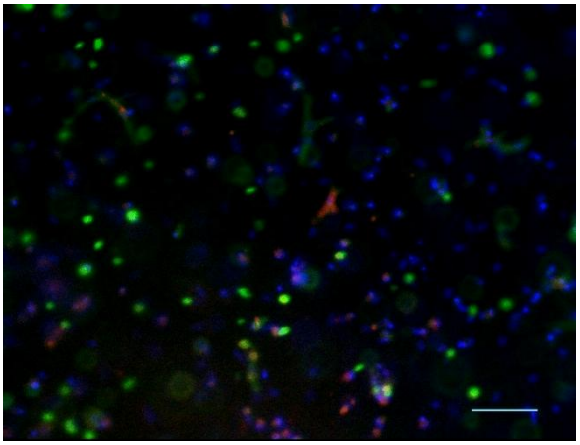


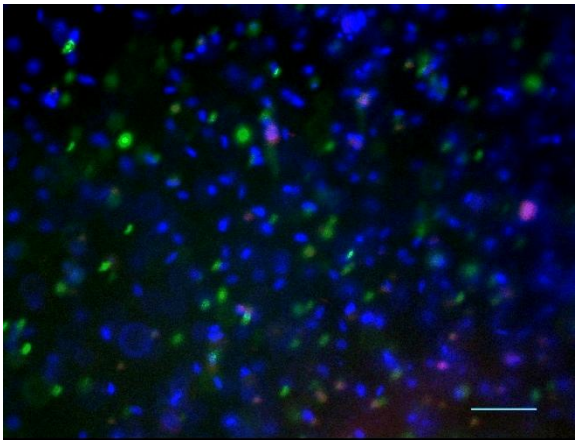
Figure 12: Images of sliced cellular constructs fixed on Day 7 of growth and stained for deposition of col I (green). Fluorescent images are shown alongside transmitted images to allow comparison of the shape of each construct slice. Scale bars are set at 100 μm.



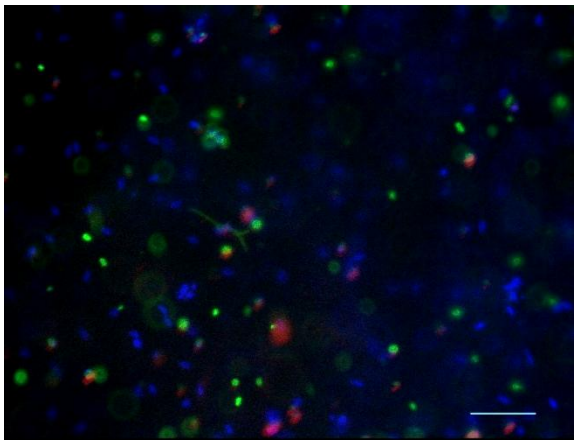
1.0 mM Ca^{2+}, 0.25 U/mL Thr



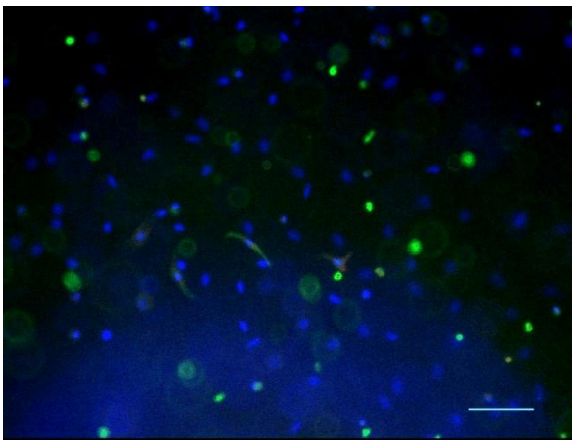
20.0 mM Ca^{2+}, 0.25 U/mL Thr



20.0 mM Ca^{2+}, 1.0 U/mL Thr



1.0 mM Ca^{2+}, 2.0 U/mL Thr



20.0 mM Ca^{2+}, 2.0 U/mL Thr

Figure 13: Images of whole cellular constructs fixed on Day 7 of growth and stained for expression of MHC (green) and Cxn43 (red). Scale bars are set at 100 μm.

5. Discussion

5.1 Analysis of Structure

a. Quantification of Physical Parameters

Light-scattering methods showed a decrease in fiber diameter with increasing thrombin concentration and an increase in fiber diameter with increasing calcium concentration. The observation of diminished fiber diameter at higher thrombin concentrations is consistent with previous results collected by light-scattering methods (Shah et al., 1985; Carr, 1988; Blomback et al., 1990), permeation methods (Blomback et al, 1990), and electron microscopy (Weisel and Nagaswami, 1992; Ryan et al., 1999). These results can be explained by considering the mechanism behind fibrin clot formation. Fiber diameter is determined by a balance between the rate of lateral aggregation of monomers and the rate of fibrinopeptide cleavage (Weisel and Nagaswami, 1992). The amount of thrombin present during clot formation is directly related to the rate of fibrinopeptide cleavage (Weisel and Nagaswami, 1992). Thus, at low thrombin concentrations, the rate of fibrinopeptide cleavage is diminished, allowing greater lateral aggregation of protofibrils and production of thicker fibers. On the other hand, at higher thrombin concentrations, the rate of fibrinopeptide cleavage surpasses than the rate of lateral aggregation, giving rise to a network of thin fibers.

Similarly, the observation of increased fiber diameter at elevated calcium concentrations is consistent with previous results collected by light-scattering methods (Okada and Blomback, 1983; Carr, 1988) and electron microscopy (Ryan et al., 1999). The presence of calcium is believed to increase the rate of fibrin clot formation by enhancing polymerization events that occur after fibrinopeptide cleavage (Brass et al., 1978, Blomback et al., 1978). It has been

suggested that the presence of calcium promotes the lengthwise aggregation through stabilization of the growing protofibril (Hantgan and Hermans, 1979; Fowler et al., 1981).

In addition, light-scattering methods showed an increase in density of each fiber as a function of increasing thrombin concentration and a decrease in density of each fiber as a function of increasing calcium concentration. This trend was the exact opposite from that observed for fiber diameter (Figure 14). This implied that smaller fibers tended to be denser, while larger fibers tended to be less dense, which is logical when considering the larger picture of fibrin gel formation. Because the overall fibrin concentration is consistent between all gels, it would make sense for smaller fibers to be denser, because each fiber would need to fit the same amount of material into a smaller amount of space. On the other hand, it would make sense for larger fibers to be less dense, because the same amount of material would be give a larger amount of space to occupy. However, one factor that this explanation fails to account for is the difference in packing densities of fibers between conditions. Previous experiments have demonstrated that smaller fibers tend to pack more tightly together than larger fibers, giving rise to a greater density of fibers per unit volume (Ryan et al., 1999). In reality, changes in fiber diameter arising from variations in thrombin and calcium concentrations likely result in changes in both in the density of each fiber and in the packing densities of these fibers.

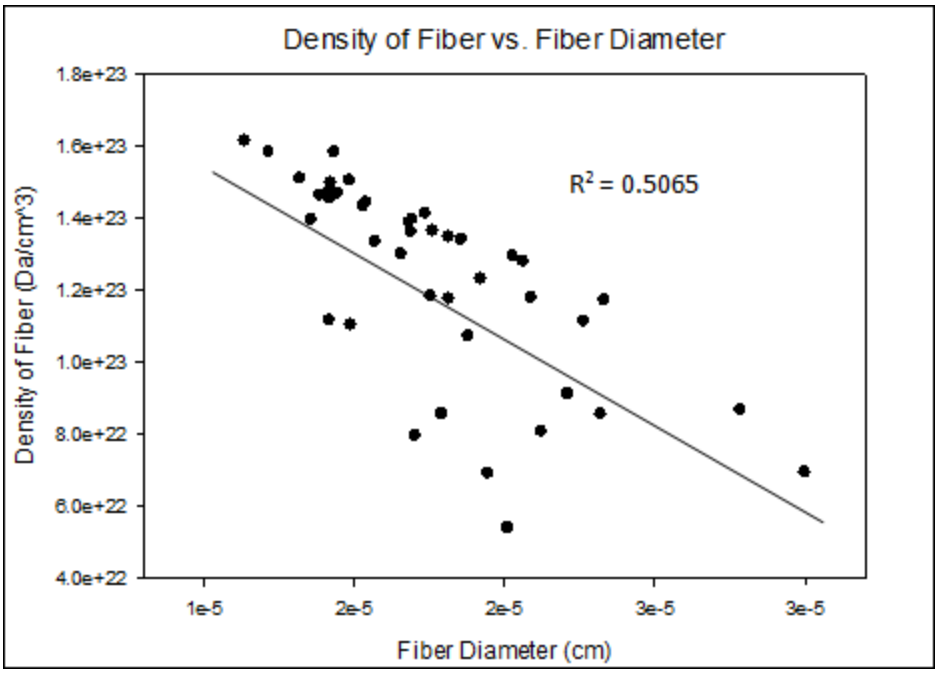


Figure 14: Dependence of density of fiber on fiber diameter. Data for fiber diameter and density of fibers was obtained using light-scattering experiments. An inverse relationship between the parameters is indicated by a negatively-sloped regression line.

b. Visualization of Fibrin Gels

Images of fibrin gels created under different concentrations of thrombin and calcium confirmed the results obtained by light-scattering. These images, which allowed visualization of porosity within fibrin gels, could be used to estimate the spaces filled by fibers within fibrin gels. Previous literature has demonstrated that smaller fibers pack together more densely than larger fibers within fibrin gels (Ryan et al., 1999). Thus, within these images, it was assumed that larger pores indicated the presence of larger fibers that packed together more loosely, while smaller pores indicated the presence of smaller fibers that packed together more closely. Consequently, these images showed that increasing the concentration of thrombin in the gel resulted in a decrease in fiber diameter, while increasing the concentration of calcium in the gel caused an

increase in fiber diameter. These results are consistent with previous images taken by another lab using electron microscopy (Figure 15; Ryan et al., 1999).

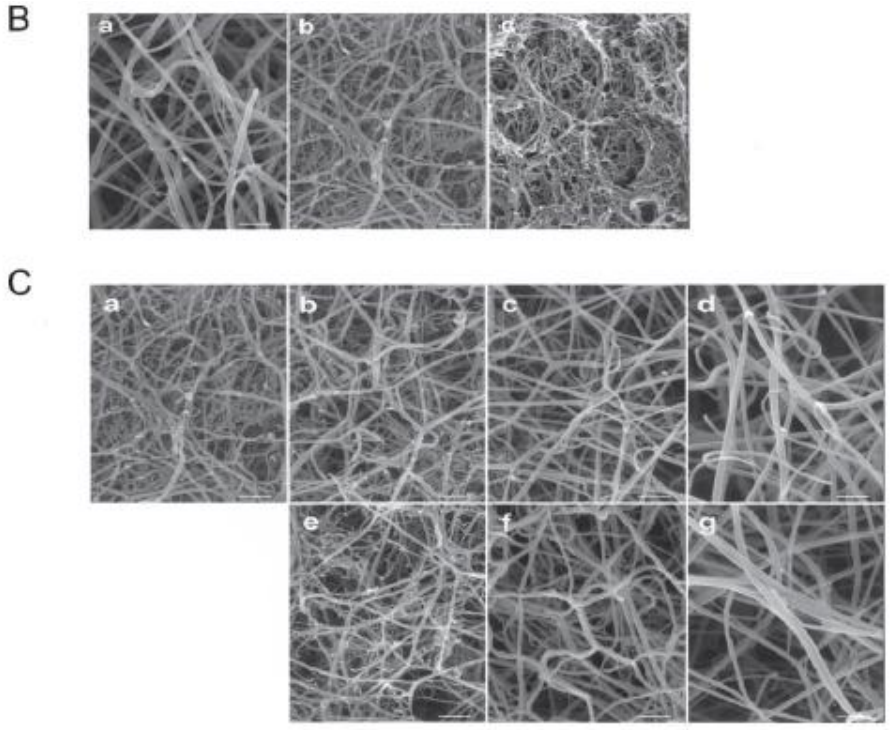


Figure 15: Scanning electron microscope images depicting changes in fiber diameter with (b) increasing thrombin concentration and (c) increasing calcium concentration (Ryan et al., 1999). These trends are consistent with the trends obtained by our experiments.

5.2 Analysis of Mechanical Properties

a. Uniaxial Stretching

The Young's moduli of gels were measured through uniaxial stretching using a tissue stretcher. Fibrin gels were stretched up to a value of 25.0% strain, and Young's modulus was determined at 5.0%, 10.0%, 15.0%, and 20.0% strain. These particular values were chosen because they correspond to percent strains to which engineered heart tissues have been subjected during growth in previous experiments (Syedain et al., 2008; Smith et al., 2010). At all percent strains, Young's modulus was highest in gels created under of calcium and high thrombin

concentrations, followed by gels created under high calcium and low thrombin concentrations. Gels formulated under conditions of low calcium and low thrombin, low calcium and high thrombin, and high calcium and intermediate thrombin contained somewhat Young's moduli. While differences between these conditions were small at lower percent strains, they became more significant as the percent strain applied to the gel approached 20.0%.

Differences between conditions were more prominent when gels were tested at higher strains. This is because the linear region of the stress-strain curve was more likely to be reached at higher strains. Furthermore, since it was difficult to determine the correct preload to apply to gels, it is possible that gels were undergoing compression at lower percent strains, confounding potential differences between measurements. In addition, Young's modulus for each gel was different at different percent strains. This observation is logical when considering the material properties of fibrin gels. Since fibrin gels are hydrogels, they behave as viscoelastic materials, and the slope of their stress-strain curve is likely to vary with strain.

b. Rheology

Viscoelastic properties of gels were measured through rheology. There were no obvious trends in G' as a function of calcium or thrombin concentration. Increasing the concentration of calcium within the gel led to a decrease in G' at low thrombin concentrations and an increase in G' at high thrombin concentrations. Our results obtained for G' as a function of calcium at low thrombin concentrations matched with published results, which depicted a decrease in G' as calcium concentration was increased from 1.5 to 20 mM (Ryan et al., 1999). However, because this published experiment was performed at a thrombin concentration of 1.0 U/mL, which fell almost halfway between our "low" and "high" values for thrombin concentration, no direct comparison could be made between the two experiments. Increasing the concentration of

thrombin led to a decrease in G' at low calcium concentrations and an increase in G' at high calcium concentrations. Our results obtained for G' as a function of thrombin at low calcium concentrations matched with published results, which showed a sharp decline and subsequent plateau upon increases in thrombin concentration from 0.25 to 5.0 U/mL (Ryan et al., 1999). Since the published experiment was performed in the absence of calcium, it makes sense that its results matched the results that we obtained at low calcium concentrations. In general, gels with intermediate fiber diameters had the highest G' values, which implied that they were the most mechanically robust (Figure 16a). On the other hand, gels with fiber diameters at each extreme had lower G' values and were more mechanically weak. These results matched with previously published results, which showed that maximal rigidities where achieved fibrin gels with intermediate fiber diameters and packing densities (Ryan et al., 1999)

Increasing the concentration of calcium within the gel led to an increase in loss modulus (G"). These results matched partially with published results, which depicted an increase in G" as calcium concentration was increased from 0 to 5.0 mM, followed by a decrease in G" upon subsequent increases in calcium concentration to 20.0 mM (Ryan et al., 1999). The value of G" in the published experiment was higher at a calcium concentration of 20.0 mM than at a calcium concentration of 1.0 mM, which was consistent with our results. Increasing the concentration of thrombin within the gel led to an increase in G" at high calcium concentrations. These results were in contrast with published results, which indicated a sharp decline and subsequent plateau in G' upon increases in thrombin concentration from 0.25 to 5.0 U/mL (Ryan et al., 1999). However, the published experiment was conducted in the absence of calcium, while our trend in G" as a function of thrombin concentration was observed at only high calcium concentrations. Our results indicated that increasing the concentration of each reagent involved in fibrin gel

formation led to an increase in its final viscosity. However, no noticeable trends in G" were observed a function of fiber diameter (Figure 16b).

Lastly, information about G' and G" was combined to determine loss tangent (tan δ), which served as a measure of the degree of viscoelasticity of gels. Increasing the concentration of calcium within the gel led to an increase in tan δ. These results were consistent with published results, which indicated an increase in tan δ as calcium concentration was increased from 0 to 20.0 mM (Ryan et al., 1999). Increasing the concentration of thrombin within the gel led to a decrease in tan δ at only high thrombin concentrations. These results are in contrast with previous results, which showed a sharp decrease in tan δ upon an increase in thrombin concentration from 0.1 to 0.5 U/mL, followed by a smaller decrease and plateau upon subsequent increases in thrombin concentration up to 5.0 U/mL (Ryan et al., 1999). But again, the published experiment was conducted in the absence of calcium, while our trend in tan δ as a function of thrombin concentration was observed at high calcium concentrations. In general, tan δ was lowest in gels with small fiber diameters and largest in gels with large fiber diameters (Figure 16c). The increase in tan δ as a function of fiber diameter was not linear.

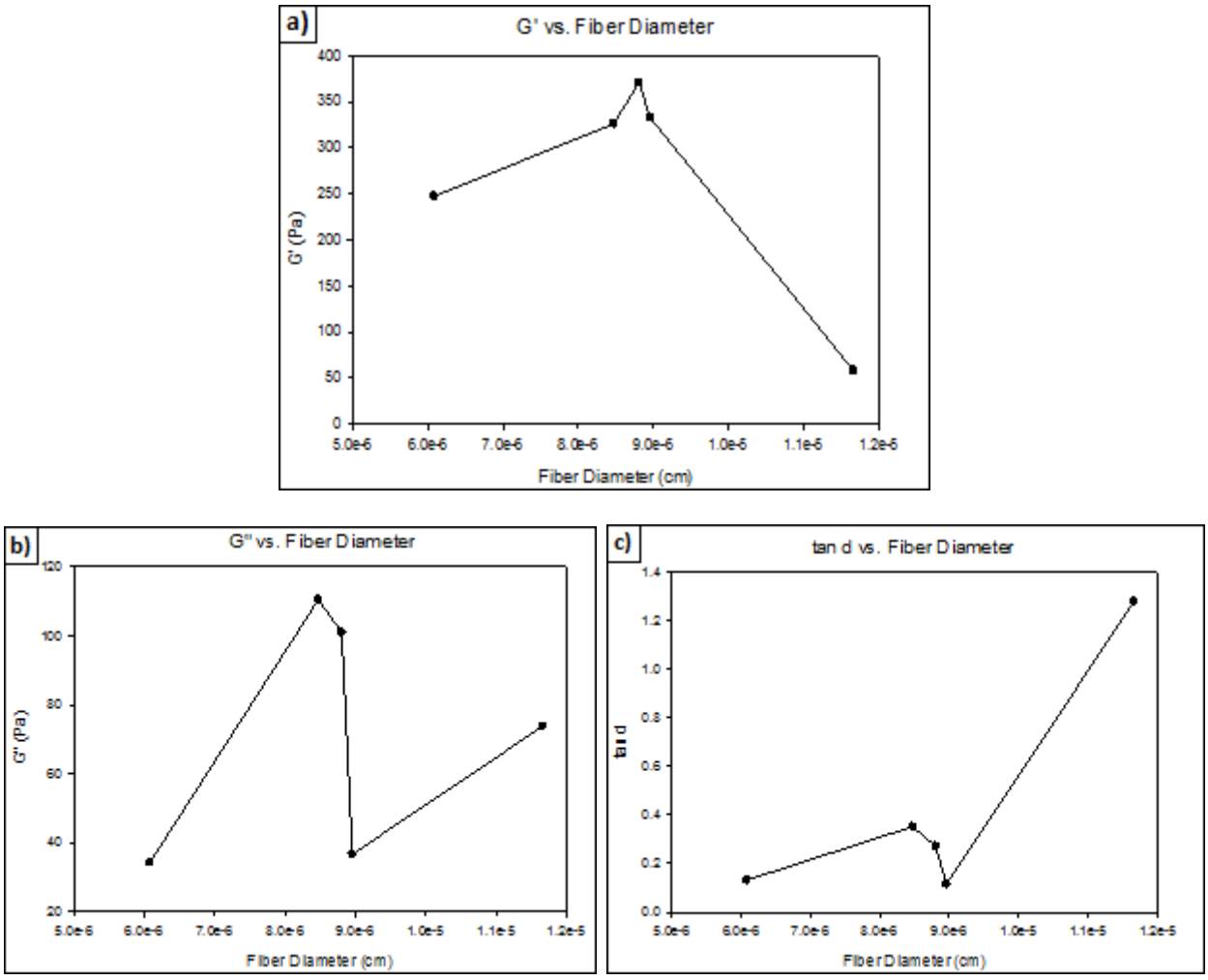


Figure 16: Dependence of (a) storage modulus, (b) loss modulus, and (c) loss tangent on fiber diameter. Data for fiber diameter was obtained using light-scattering experiments, while rheological data was measured using a rheometer at 10.0% strain and 10.0 Hz shear frequency.

c. Comparison of Tests

These results obtained by these different mechanical tests should be comparable, because storage modulus should be approximately equal to Young's modulus at low frequencies of shear. However, in reality, results obtained from unaxial stretching were vastly different from results obtained through rheology. Results obtained using uniaxial stretching indicated large differences in stiffnesses of gels created under different conditions, with values for Young's modulus ranging from around 5 kPa to over 100 kPa. However, results obtained using rheology for gels

created under the same formulations indicated relatively differences between conditions, with values for storage modulus ranging from just 50 to 400 Pa. One possible explanation for these differences is that, while the tests measured the same mechanical parameters in principle, they did so through different mechanisms. While uniaxial stretching involved the application of tension, rheology involved the application of shear.

It is likely that the results obtained through rheology were more representative of the actual mechanical properties of fibrin gels. First of all, because previous studies demonstrated poor mechanical properties in fibrin gels prior to compaction by cells (Jockenhoevel et al., 2001), it would not make sense for acellular fibrin gels to achieve Young's moduli in the range of 50 to 100 kPa within just 30 minutes of formation. In addition, there are many variables associated with unaxial testing that were difficult to control. One confounding factor was that small variations in amount of glue applied to hold the clasps to the gels could have had a large effect on the measured stiffness of the fibrin gel. In addition, it is likely that the integrity of the fibers within gels was disturbed during loading onto the tissue stretcher, which would have changed the force generated upon stretching. Furthermore, because our fibrin gels were so thin, it was difficult to accurately measure their dimensions, which could have led to error in our calculations of Young's modulus. Finally, it was difficult to determine the necessary amount of preload to apply to each fibrin gel to prevent compaction during stretch testing. It is possible that in some cases, the linear area of the stress-strain curve was never achieved.

d. Comparison with Native Cardiac Tissue

Previous experiments have shown that the stiffness of healthy adult cardiac tissue is approximately 18±2 kPa, while the stiffness of infracted adult cardiac tissue increases to a value of approximately 55±15 kPa (Berry et al., 2006). Correlating these values from literature from

the values obtained by rheology, it seems that gels created under all formulations were too weak to mimic cardiac tissue, containing stiffnesses that were around two orders of magnitude too low. Thus, from our results, it appears that fibrin formulations with intermediate fiber diameters are the most promising in terms of mimicking the native cardiac environment, because they are the stiffest. However, because our mechanical testing was performed on acellular gels, we do not account for the changes in mechanical properties of fibrin gels upon seeding with and subsequent degradation by cells. In fact, previous literature has shown that fibrin gels are initially weak upon formation, but that their mechanical strength increases upon compaction by seeded cells (Jockenhoevel et al., 2001). Thus, in the future, it would be important to perform mechanical testing on cellular constructs to allow a more direct correlation with literature to be drawn.

5.3 Analysis of Cellular Compatibility

a. Growth of Cellular Constructs

During the course of growth, there were no visible differences in cellular survival between conditions. All fibrin gel formulations were able to facilitate cellular adhesion, as evidenced by the spreading of cells on Day 2 within all constructs. In addition, all formulations were able to support the growth of cardiomyoctes, as evidenced by the presence of beating of cells starting on Day 4 of growth across all conditions. Lastly, all cellular constructs became significantly thinner over the course of cellular growth, indicating the capability of cells to remodel their surrounding fibrin networks.

DNA assays performed on Day 7 indicated some differences in cell number between conditions. In general, all constructs experienced an approximate two-fold increase cell number over the course of a week. The smallest numbers of cells were found in gels created under high

concentrations of both reagents. However, there were no obvious trends with respect to fiber diameter. Though significant, these differences in cell number were not exceedingly large, and it was difficult to tell whether these differences arose from differences in the physical structures of the fibrin gels or from differences in reagents concentrations to which the cells were subjected to immediately after seeding. For example, the presence of lower cell numbers within gels created under high concentrations of both reagents might have resulted from greater initial cell death due to hypertonic environmental conditions upon seeding.

b. Histology

Histological staining for collagen I (Col I) showed collagen deposition by cells when grown gels created under all formulations. Comparing fluorescent images of construct slices alongside their transmitted counterparts, it was apparent that collagen deposition was pretty consistent throughout the entirety of each construct. Also, for some conditions, the collagen stain was visibly brighter along the long axes of cells, indicating that the fluorescent signal was not due to nonspecific binding throughout the construct. Collagen stain was brightest in constructs created under high thrombin concentrations, which corresponded to gels containing fibers of smaller diameter and greater density. On the other hand, collagen stain was weakest in constructs created under high calcium and low thrombin concentrations, which corresponded to gels containing fibers of larger diameter and lesser density. Thus, it can be concluded that cells are best able to remodel fibrin gels with smaller fiber diameters, and that the effect of fiber diameter outweighed the effect of having a denser fiber in determining collagen deposition. This is likely because gels with smaller fiber diameters provided cells with a larger amount of surface area to which to make focal adhesions, giving them a greater degree of contact with fibrin. This increase in contact likely led to an increase in fibrin degradation, which allowed enhanced collagen

deposition. To test this theory, it would be valuable to measure fibrin degradation in the future to confirm this reciprocal relationship with collagen deposition.

Staining of constructs for myosin heavy chain (MHC) confirmed the successful growth of cardiomyocytes in gels of all formulations. However, since cells within constructs were not aligned, it was difficult to find areas with more than one successfully stained cardiomyocyte within a single plane to image. Thus, in reality, there were most likely a greater number of cardiomyocytes growing within constructs than visualized using MHC staining. To circumvent this problem in the future, confocal imaging could be used to generate a three-dimensional image by taking cross sections of multiple planes within the construct. Staining for connexin 43 (Cxn43) showed minimal communication between cardiomyocytes in all conditions. This result matches with the lack of observation of synchronously contracting patches of cardiomyocytes during growth. One possible explanation for this lack of signaling between cells is the lack of alignment of cells within our constructs. Therefore, in the future, it would be worthwhile to explore the potential upregulation of Cxn43 upon functionalization of constructs through alignment.

6. Conclusion

Our experiments demonstrated that varying the concentrations of calcium and thrombin during the formation of a fibrin gel gives rise to differences to its architecture, which in turn caused in differences in its mechanical properties and cellular compatibility. Increasing the concentration of calcium within the gel led to an increase in fiber diameter and a decrease in the density of each fiber. Conversely, increasing the concentration of thrombin within the gel led to a decrease in fiber diameter and an increase in the density of each fiber. There was an inverse relationship between the diameter and density of each fiber.

Though mechanical properties were determined through uniaxial stretching and through rheology, it was concluded that rheology gave rise to more accurate measurements. The storage modulus of gels with intermediate fiber diameters was largest, implying that these gels were most mechanically robust. The loss tangent of gels increased with increasing fiber diameter, although this relationship was not linear. All gels contained stiffnesses that were two orders of magnitude below the stiffness of cardiac tissue. This discrepancy likely arose from the fact that testing was performed on acellular gels, while the presence of cells within fibrin constructs enhances mechanical strength through compaction.

All tested fibrin formulations were able to support the adhesion of cells and enabled a roughly two-fold increase in cell number over a period of one week. While collagen deposition was exhibited by cells seeded into constructs created under all conditions, it was greatest in when cells were grown in gels with smaller fiber diameters and greater individual fiber densities. Though cardiomyocytes were able to grow and beat in gels of all formulations, they underwent minimal communication with one another. This lack of signaling most likely arose from the fact that cells grew in an isotropic, rather than aligned, manner within constructs.

7. Future Direction

The results of our study have indicated that variations in the structural properties of fibrin gels that arise from their different formulations lead to differences in their mechanical properties and cellular compatibility. However, further experiments should be conducted to verify and expand the scope of these results. Though our study of fiber structure as a function of reagent concentration was comprehensive, it would be valuable to expand our mechanical and cellular studies to include a larger number of conditions. In this study, five conditions were tested, corresponding to two calcium concentrations (1.0 and 20.0 mM) and three thrombin concentrations (0.25, 1.0, and 2.0 U/mL). Although our results gave insight into trends as a function of calcium and thrombin concentration, these results would more conclusive if testing were performed on a greater number of formulations.

With respect to mechanical testing, our study has demonstrated that results obtained by rheology are more reliable than the results obtained by uniaxial stretching, due to the presence of a large number of variables within uniaxial testing that cannot be controlled for. Thus, in the future, mechanical studies will be conducted solely using rheology. In the future, testing will be performed on a wider variety of gel formulations, as previously mentioned. In addition, testing will be performed under a greater number of shear frequencies and strains to give a more comprehensive understanding of the viscoelastic properties of fibrin gels. Lastly, it would be important to perform mechanical testing on cellular constructs in order to determine the changes in mechanical properties in gels that occur due to cellular remodeling.

In the future, we will repeat our cellular studies by growing cells on fibrin gels with a larger number of formulations, as previously mentioned. Our study will be expanded to include two week and four week time points to allow examination of the fate of cellular fibrin constructs

in the long term. In addition, DNA assays will be performed throughout the course of growth, rather than just on the last day of growth, to give a more comprehensive view of cellular proliferation on different fibrin gel formulations over time.

Cellular constructs will be fixed and stained at one, two, and four week time points. Confocal microscopy will be performed to assess cardiomyocyte growth and communication by probing for myosin heavy chain (MHC) and connexin 43 (Cxn43). In addition, collagen deposition will be assessed. Our results have shown preliminary differences in collagen deposition under different fibrin formulations. However, it would be valuable to quantify collagen deposition by performing a hydroxyproline assay (Grassl et al., 2003). In addition, it would be important to measure the amount of fibrin degradation in these cellular constructs, in order to correlate fibrin degradation with collagen deposition. This will be accomplished by creating cellular constructs using fluorescent fibrinogen and measuring fluorescence in culture medium as a function of time (Grassl et al., 2002).

Lastly, functional experiments using different fibrin formulations will be performed. Aligned ring constructs will be created as previously described (Black et al., 2009), and contraction force generated by these constructs will be measured to determine the effects of fibrin gel on cardiomyocyte contractility. In addition, staining for MHC, Cxn43, and Col I will be performed on these aligned constructs, and these results will be compared results obtained using isotropic constructs. Specifically, because our study indicated low expression of Cxn43 for all conditions, it would be valuable to assess whether or not Cxn43 expression was increased when cells were grown in conditions that better mimicked native cardiac conditions.

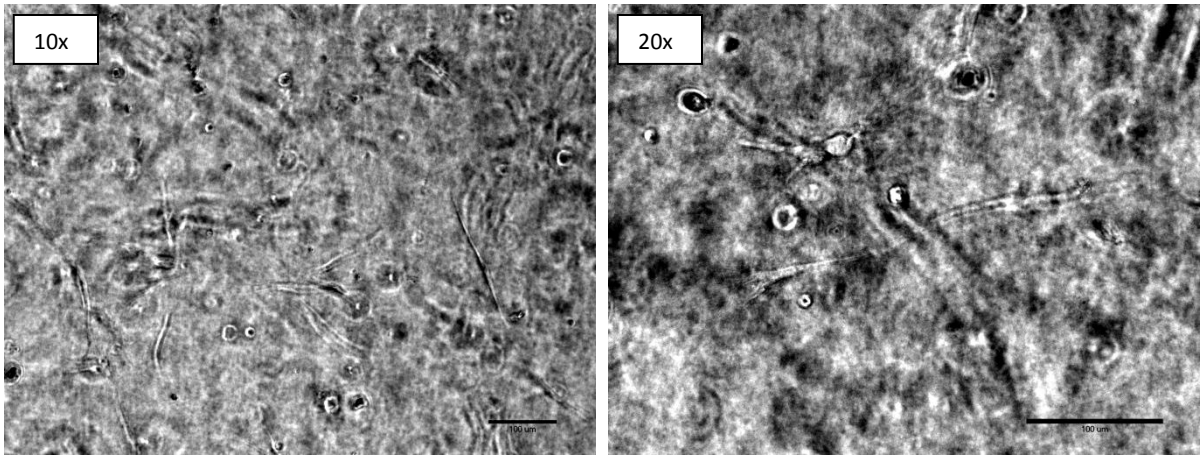
8. Appendix

8.1 Additional Images of Cellular Growth

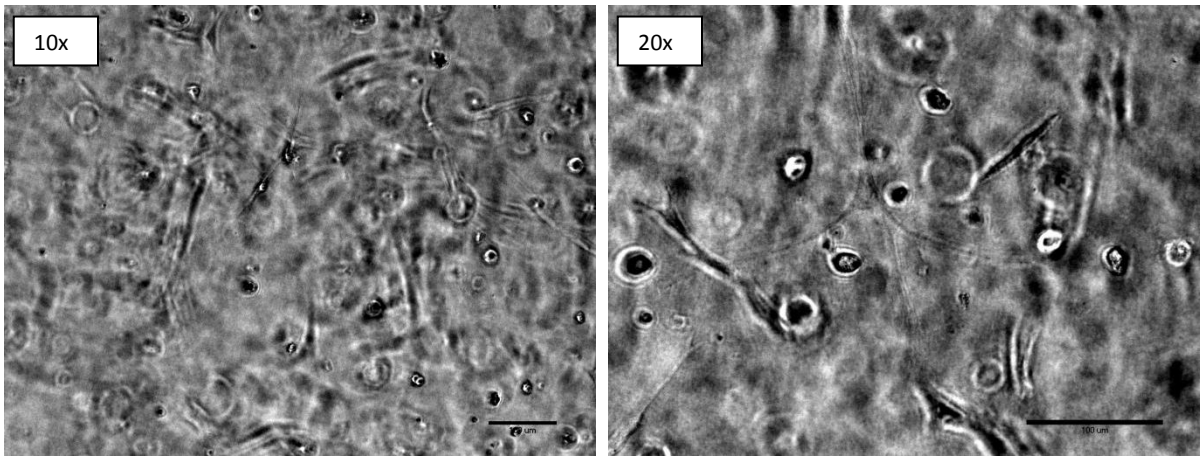
This section of the Appendix includes additional images taken of native cells growing within fibrin gels created under various calcium and thrombin concentrations. Cells were seeded into constructs on Day 0, and representative images for each condition are included for Days 2, 4, and 7 of cellular growth. All scale bars are set at 100 μm.

a. Day 2

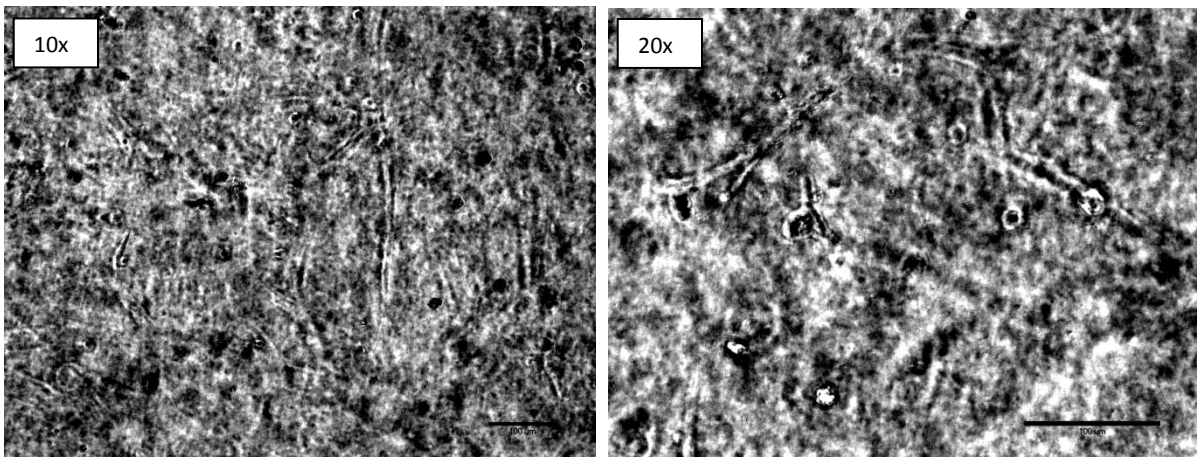
1.0 mM Ca^{2+}, 0.25 U/mL Thr



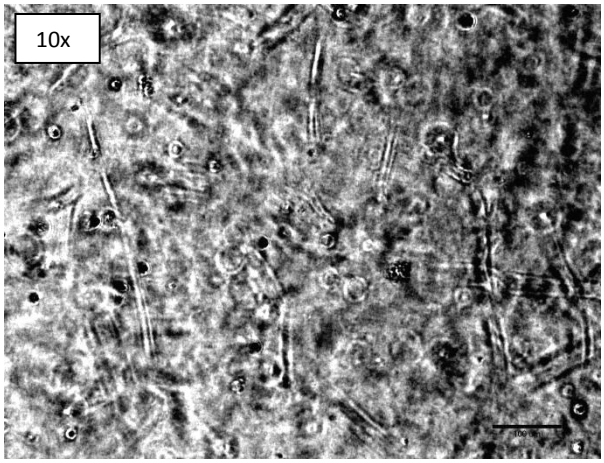
1.0 mM Ca^{2+}, 2.0 U/mL Thr

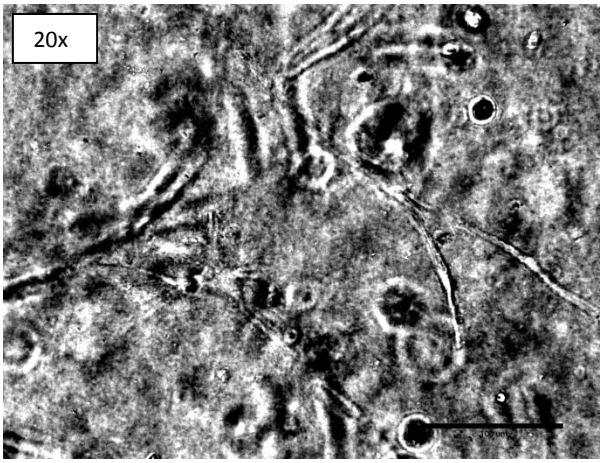


20.0 mM Ca^{2+}, 1.0 U/mL Thr

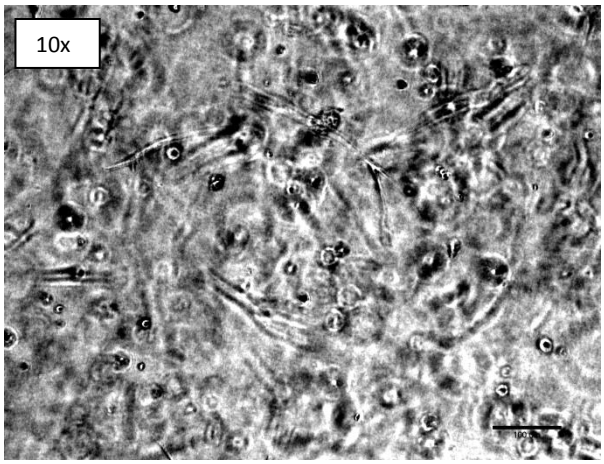


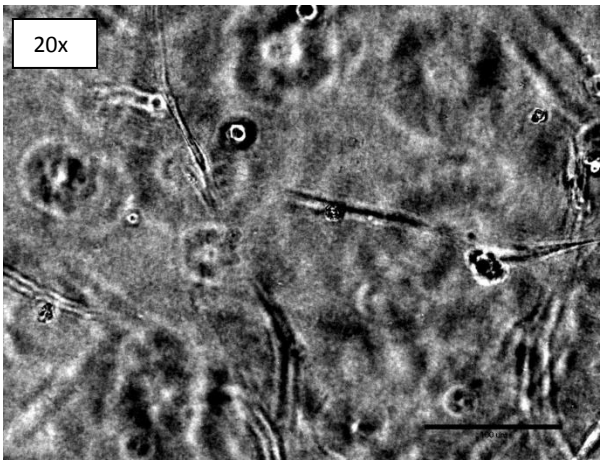
20.0 mM Ca^{2+}, 1.0 U/mL Thr





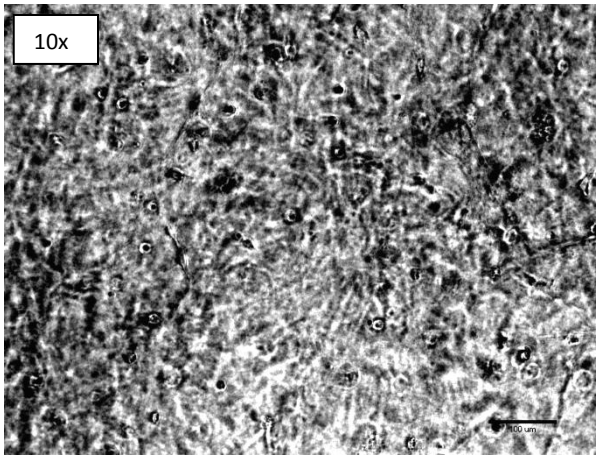
20.0 mM Ca^{2+}, 2.0 U/mL Thr

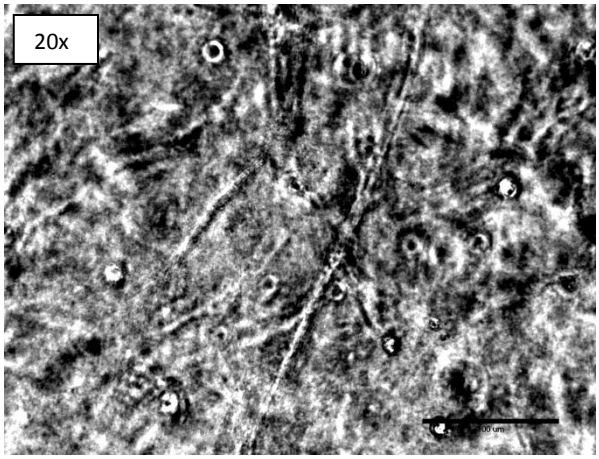




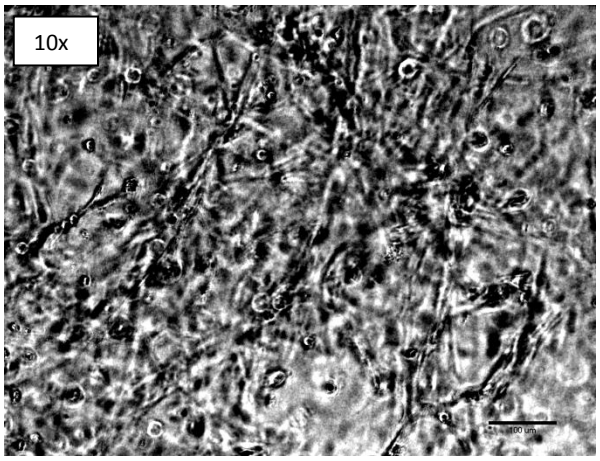
b. Day 4

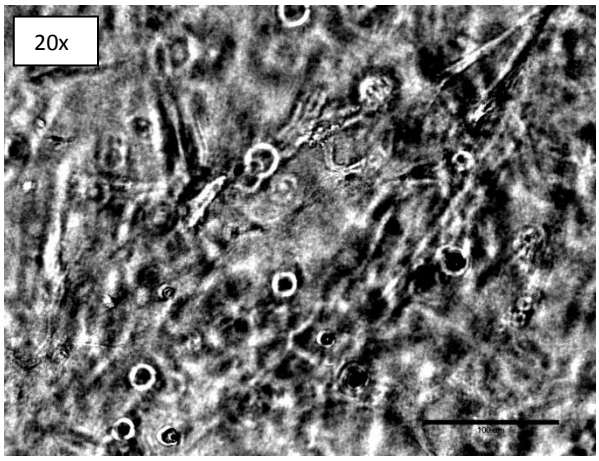
1.0 mM Ca^{2+}, 0.25 U/mL Thr



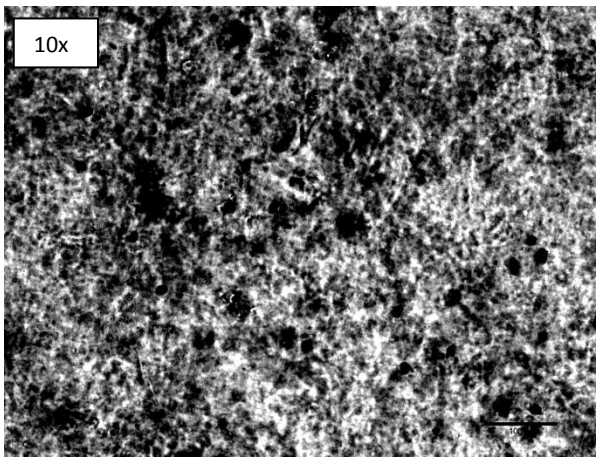


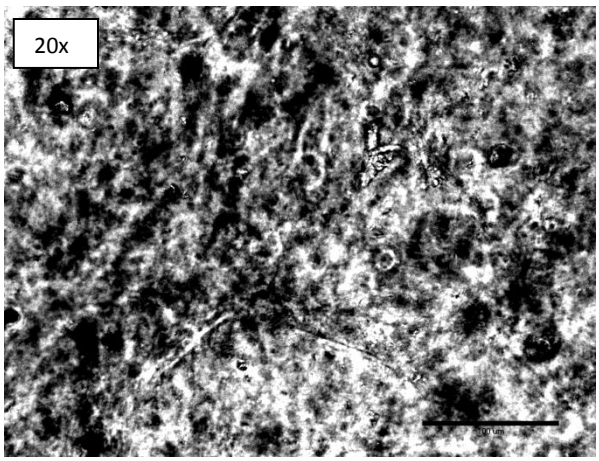
1.0 mM Ca^{2+}, 2.0 U/mL Thr



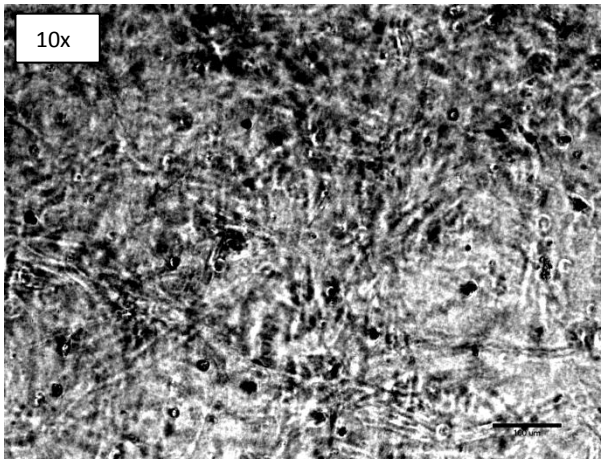


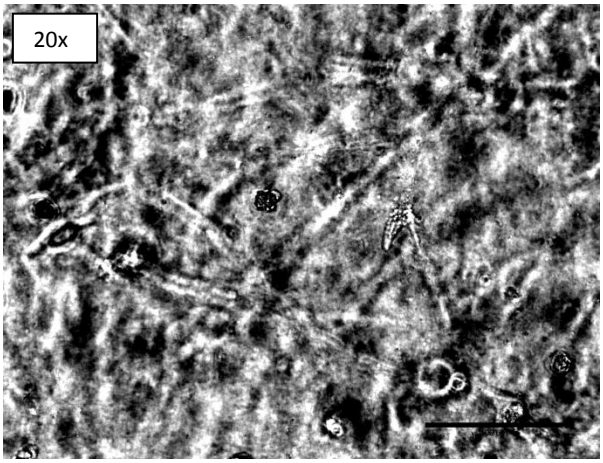
20.0 mM Ca^{2+}, 1.0 U/mL Thr



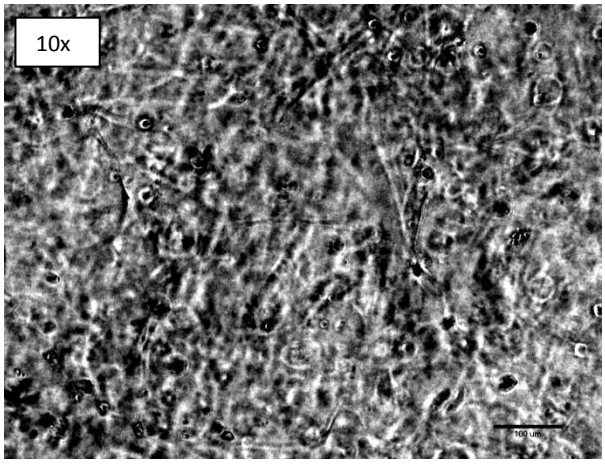


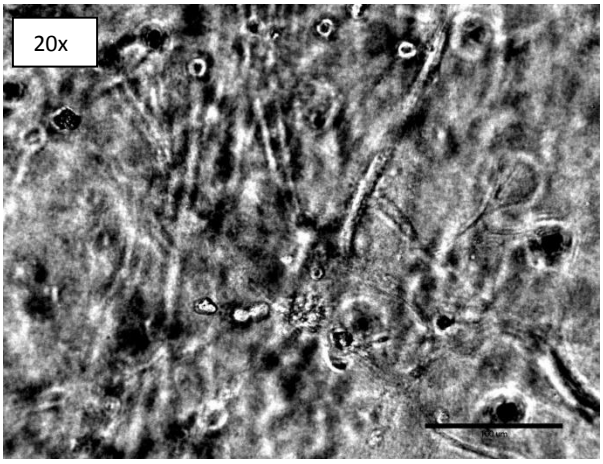
20.0 mM Ca^{2+}, 1.0 U/mL Thr





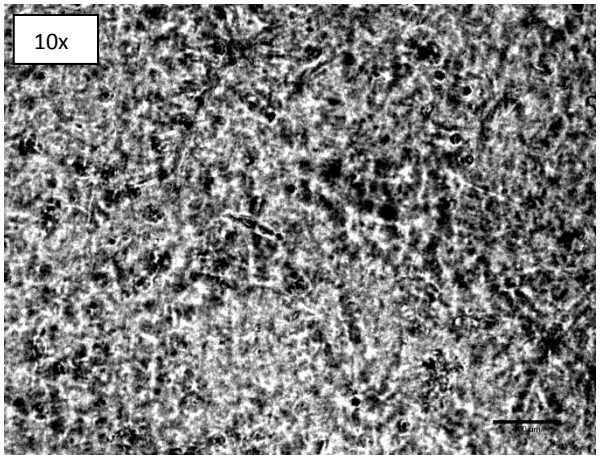
20.0 mM Ca^{2+}, 2.0 U/mL Thr

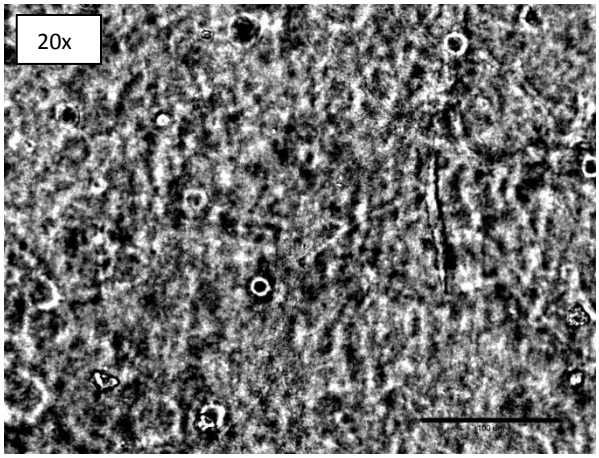




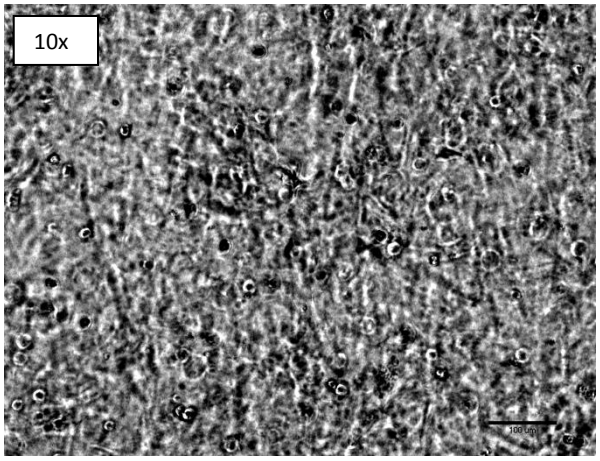
c. Day 7

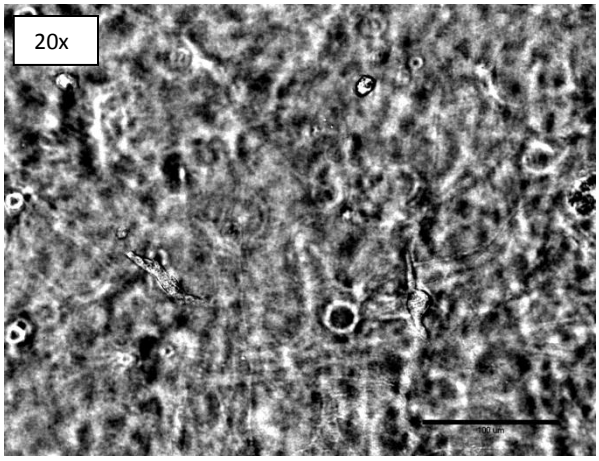
1.0 mM Ca^{2+}, 0.25 U/mL Thr



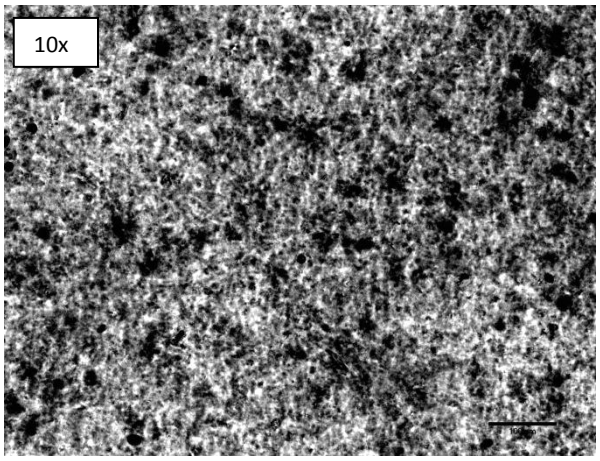


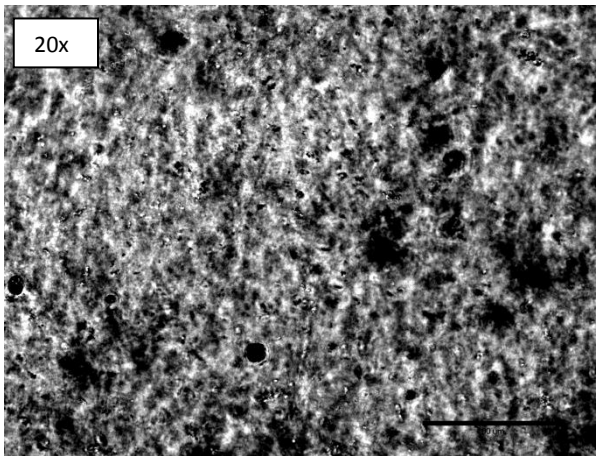
1.0 mM Ca^{2+}, 2.0 U/mL Thr



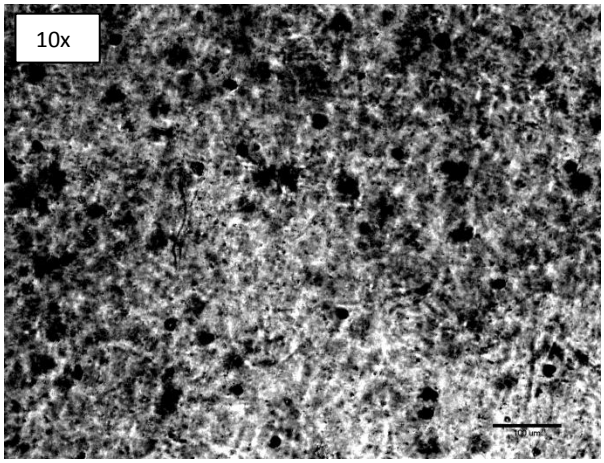


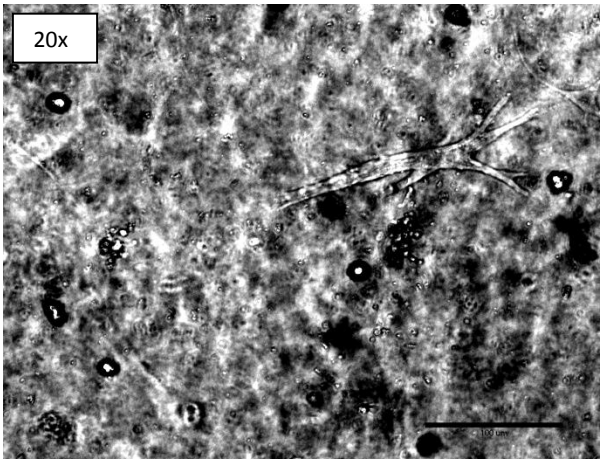
20.0 mM Ca^{2+}, 1.0 U/mL Thr



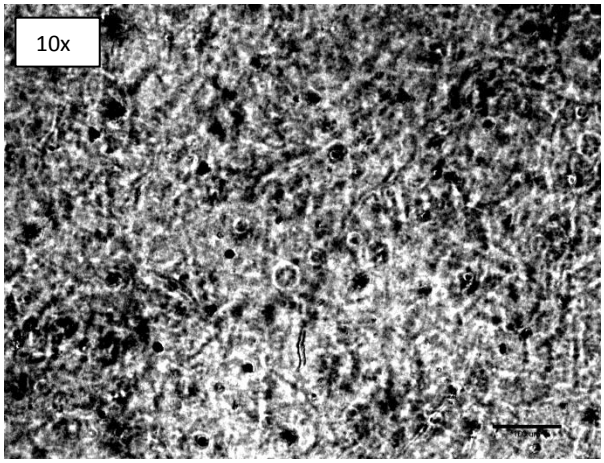


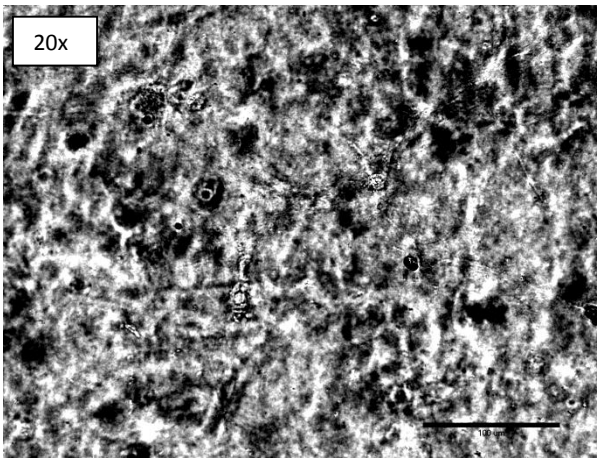
20.0 mM Ca^{2+}, 1.0 U/mL Thr





20.0 mM Ca^{2+}, 2.0 U/mL Thr





8.2 Statistical Analysis

This section of the Appendix contains the ANOVA tests performed using SigmaPlot on results obtained by light-scattering to determine differences in structure between conditions, uniaxial stretching and rheology to determine differences in mechanical properties between conditions, and DNA assay to determine differences in cellular compatibility between conditions. One-way or two-way ANOVAs were used detect differences between experimental groups, depending on the number of variables that were tested for. Important values or statements in each table are bolded. A p value of <0.05 was used to denote statistical significance.

a. Fiber Diameter from Light-Scattering Experiment

Two Way Analysis of Variance Thursday, March 29, 2012, 3:33:16 PM

Data source: Fiber Diameter in Combined Data

Balanced Design

Dependent Variable: Fiber Diameter

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Calcium Conc.	6	6.358E-011	1.060E-011	5.418	<0.001
Thrombin Conc.	5	0.000000000208	4.160E-011	21.274	<0.001
Calcium Conc. x Thrombin Conc.	30	4.819E-011	1.606E-012	0.821	0.711
Residual	42	8.213E-011	1.956E-012		
Total	83	0.000000000402	4.842E-012		

The difference in the mean values among the different levels of Calcium Conc. is greater than would be expected by chance after allowing for effects of differences in Thrombin Conc.. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Thrombin Conc. is greater than would be expected by chance after allowing for effects of differences in Calcium Conc.. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Calcium Conc. does not depend on what level of Thrombin Conc. is present. There is not a statistically significant interaction between Calcium Conc. and Thrombin Conc.. (P = 0.711)

Power of performed test with alpha = 0.0500: for Calcium Conc. : 0.972
Power of performed test with alpha = 0.0500: for Thrombin Conc. : 1.000
Power of performed test with alpha = 0.0500: for Calcium Conc. x Thrombin Conc. : 0.0500

Least square means for Calcium Conc. :

Group	Mean
0.1000	0.00000757
1.000	0.00000803
5.000	0.00000865
10.000	0.00000897
20.000	0.0000101
35.000	0.0000101
50.000	0.00000875

Std Err of LS Mean = 0.000000404

Least square means for Thrombin Conc. :

Group	Mean
0.1000	0.0000113
0.250	0.0000104
0.500	0.00000903
1.000	0.00000826
2.000	0.00000741
3.000	0.00000683

Std Err of LS Mean = 0.000000374

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Calcium Conc.**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
20.000 vs. 0.100	0.00000250	4.376	**0.002**	Yes
35.000 vs. 0.100	0.00000248	4.351	**0.002**	Yes
20.000 vs. 1.000	0.00000203	3.560	**0.018**	Yes
35.000 vs. 1.000	0.00000202	3.536	**0.018**	Yes
20.000 vs. 5.000	0.00000142	2.486	0.253	No
10.000 vs. 0.100	0.00000141	2.462	0.252	No
35.000 vs. 5.000	0.00000141	2.461	0.239	No
20.000 vs. 50.000	0.00000132	2.308	0.308	No
35.000 vs. 50.000	0.00000130	2.284	0.304	No
50.000 vs. 0.100	0.00000118	2.067	0.424	No
20.000 vs. 10.000	0.00000109	1.913	0.509	No
5.000 vs. 0.100	0.00000108	1.890	0.493	No
35.000 vs. 10.000	0.00000108	1.889	0.458	No
10.000 vs. 1.000	0.000000940	1.647	0.596	No
50.000 vs. 1.000	0.000000715	1.252	0.820	No
5.000 vs. 1.000	0.000000613	1.074	0.871	No
1.000 vs. 0.100	0.000000466	0.815	0.934	No
10.000 vs. 5.000	0.000000327	0.573	0.966	No
10.000 vs. 50.000	0.000000225	0.395	0.972	No
50.000 vs. 5.000	0.000000102	0.178	0.980	No
20.000 vs. 35.000	0.0000000140	0.0245	0.981	No

Comparisons for factor: **Thrombin Conc.**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
0.100 vs. 3.000	0.00000444	8.396	**<0.001**	Yes
0.100 vs. 2.000	0.00000386	7.295	**<0.001**	Yes
0.250 vs. 3.000	0.00000360	6.809	**<0.001**	Yes
0.250 vs. 2.000	0.00000302	5.708	**<0.001**	Yes
0.100 vs. 1.000	0.00000300	5.682	**<0.001**	Yes
0.100 vs. 0.500	0.00000224	4.236	**0.001**	Yes
0.500 vs. 3.000	0.00000220	4.160	**0.001**	Yes
0.250 vs. 1.000	0.00000216	4.095	**0.002**	Yes
0.500 vs. 2.000	0.00000162	3.058	**0.027**	Yes
1.000 vs. 3.000	0.00000143	2.714	0.056	No
0.250 vs. 0.500	0.00000140	2.650	0.055	No
1.000 vs. 2.000	0.000000852	1.613	0.385	No
0.100 vs. 0.250	0.000000839	1.587	0.319	No
0.500 vs. 1.000	0.000000764	1.446	**0.287**	**No**
2.000 vs. 3.000	0.000000582	1.101	0.277	No

b. Density of Fiber from Light-Scattering Experiment

Two Way Analysis of Variance Thursday, March 29, 2012, 7:44:47 PM

Data source: Density of Fiber in Density of Fiber Data

Balanced Design

Dependent Variable: Density of Fiber

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Calcium Conc.	6	>1e40	>1e40	3.374	0.008
Thrombin Conc.	5	>1e40	>1e40	34.297	<0.001
Calcium Conc. x Thrombin Conc.	30	>1e40	>1e40	0.693	0.852
Residual	42	>1e40	>1e40		
Total	83	>1e40	>1e40		

The difference in the mean values among the different levels of Calcium Conc. is greater than would be expected by chance after allowing for effects of differences in Thrombin Conc.. There is a statistically significant difference (P = 0.008). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Thrombin Conc. is greater than would be expected by chance after allowing for effects of differences in Calcium Conc.. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Calcium Conc. does not depend on what level of Thrombin Conc. is present. There is not a statistically significant interaction between Calcium Conc. and Thrombin Conc.. (P = 0.852)

Power of performed test with alpha = 0.0500: for Calcium Conc. : 0.752
Power of performed test with alpha = 0.0500: for Thrombin Conc. : 1.000
Power of performed test with alpha = 0.0500: for Calcium Conc. x Thrombin Conc. : 0.0500

Least square means for Calcium Conc. :

Group	Mean
0.1000	4.884E+023
1.000	5.018E+023
5.000	5.141E+023
10.000	5.293E+023
20.000	5.034E+023
35.000	4.894E+023
50.000	4.156E+023

Std Err of LS Mean = 1.983E+022

Least square means for Thrombin Conc. :

Group	Mean
0.1000	3.037E+023
0.250	4.277E+023
0.500	5.198E+023
1.000	5.451E+023
2.000	5.694E+023
3.000	5.848E+023

Std Err of LS Mean = 1.836E+022

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Calcium Conc.**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
10.000 vs. 50.000	1.137E+023	4.055	**0.004**	Yes
5.000 vs. 50.000	9.846E+022	3.511	**0.021**	Yes
20.000 vs. 50.000	8.777E+022	3.130	0.059	No
1.000 vs. 50.000	8.621E+022	3.074	0.065	No
35.000 vs. 50.000	7.379E+022	2.631	0.183	No
0.100 vs. 50.000	7.277E+022	2.595	0.189	No
10.000 vs. 0.100	4.094E+022	1.460	0.915	No
10.000 vs. 35.000	3.993E+022	1.424	0.916	No
10.000 vs. 1.000	2.750E+022	0.981	0.995	No
10.000 vs. 20.000	2.595E+022	0.925	0.995	No
5.000 vs. 0.100	2.568E+022	0.916	0.993	No
5.000 vs. 35.000	2.467E+022	0.880	0.992	No
10.000 vs. 5.000	1.526E+022	0.544	1.000	No
20.000 vs. 0.100	1.500E+022	0.535	0.999	No
20.000 vs. 35.000	1.398E+022	0.498	0.999	No
1.000 vs. 0.100	1.344E+022	0.479	0.998	No
1.000 vs. 35.000	1.242E+022	0.443	0.995	No
5.000 vs. 1.000	1.225E+022	0.437	0.987	No
5.000 vs. 20.000	1.069E+022	0.381	0.974	No
20.000 vs. 1.000	1.556E+021	0.0555	0.998	No
35.000 vs. 0.100	1.017E+021	0.0363	0.971	No

Comparisons for factor: **Thrombin Conc.**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
3.000 vs. 0.100	2.811E+023	10.828	**<0.001**	Yes
2.000 vs. 0.100	2.657E+023	10.234	**<0.001**	Yes
1.000 vs. 0.100	2.415E+023	9.300	**<0.001**	Yes
0.500 vs. 0.100	2.161E+023	8.325	**<0.001**	Yes
3.000 vs. 0.250	1.571E+023	6.051	**<0.001**	Yes
2.000 vs. 0.250	1.417E+023	5.457	**<0.001**	Yes
0.250 vs. 0.100	1.240E+023	4.777	**<0.001**	Yes
1.000 vs. 0.250	1.174E+023	4.523	**<0.001**	Yes
0.500 vs. 0.250	9.211E+022	3.548	**0.007**	Yes
3.000 vs. 0.500	6.498E+022	2.503	0.094	No
2.000 vs. 0.500	4.958E+022	1.909	0.278	No
3.000 vs. 1.000	3.966E+022	1.528	0.438	No
1.000 vs. 0.500	2.531E+022	0.975	**0.706**	**No**
2.000 vs. 1.000	2.426E+022	0.934	0.584	No
3.000 vs. 2.000	1.540E+022	0.593	0.556	No

c. Young's Modulus from Uniaxial Stretching

Two Way Analysis of Variance Monday, April 16, 2012, 8:47:00 PM

Data source: Raw Data 2 in Mechanical Data

Balanced Design

Dependent Variable: Young's Modulus

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Condition	4	133828.854	33457.214	1054.198	<0.001
Percent Strain	3	13811.422	4603.807	145.061	<0.001
Condition x Percent Strain	12	14760.311	1230.026	38.757	<0.001
Residual	40	1269.485	31.737		
Total	59	163670.072	2774.069		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of Condition depends on what level of Percent Strain is present. There is a statistically significant interaction between Condition and Percent Strain. (P = <0.001)

Power of performed test with alpha = 0.0500: for Condition : 1.000
Power of performed test with alpha = 0.0500: for Percent Strain : 1.000
Power of performed test with alpha = 0.0500: for Condition x Percent Strain : 1.000

Least square means for Condition :

Group	Mean
Cond 1	23.239
Cond 2	5.121
Cond 3	63.832
Cond 4	11.360
Cond 5	132.326

Std Err of LS Mean = 1.626

Least square means for Percent Strain :

Group	Mean
5.0%	34.848
10.0%	34.194
15.0%	48.000
20.0%	71.662

Std Err of LS Mean = 1.455

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Condition within 5.0%**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Cond 5 vs. Cond 2	100.438	21.835	**<0.001**	Yes
Cond 5 vs. Cond 4	98.475	21.409	**<0.001**	Yes
Cond 5 vs. Cond 1	88.980	19.344	**<0.001**	Yes
Cond 5 vs. Cond 3	55.452	12.055	**<0.001**	Yes
Cond 3 vs. Cond 2	44.986	9.780	**<0.001**	Yes
Cond 3 vs. Cond 4	43.023	9.353	**<0.001**	Yes
Cond 3 vs. Cond 1	33.528	7.289	**<0.001**	Yes
Cond 1 vs. Cond 2	11.458	2.491	0.050	**No**
Cond 1 vs. Cond 4	9.495	2.064	0.089	**No**
Cond 4 vs. Cond 2	1.963	0.427	0.672	**No**

Comparisons for factor: **Condition within 10.0%**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Cond 5 vs. Cond 2	83.474	18.147	**<0.001**	Yes
Cond 5 vs. Cond 4	80.547	17.511	**<0.001**	Yes
Cond 5 vs. Cond 1	68.503	14.893	**<0.001**	Yes
Cond 3 vs. Cond 2	46.813	10.177	**<0.001**	Yes
Cond 3 vs. Cond 4	43.887	9.541	**<0.001**	Yes
Cond 5 vs. Cond 3	36.661	7.970	**<0.001**	Yes
Cond 3 vs. Cond 1	31.842	6.922	**<0.001**	Yes
Cond 1 vs. Cond 2	14.971	3.255	0.007	**Yes**
Cond 1 vs. Cond 4	12.045	2.619	0.025	**Yes**
Cond 4 vs. Cond 2	2.926	0.636	0.528	**No**

Comparisons for factor: **Condition within 15.0%**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Cond 5 vs. Cond 2	129.334	28.117	**<0.001**	Yes
Cond 5 vs. Cond 4	122.627	26.659	**<0.001**	Yes
Cond 5 vs. Cond 1	105.974	23.039	**<0.001**	Yes
Cond 5 vs. Cond 3	69.538	15.118	**<0.001**	Yes
Cond 3 vs. Cond 2	59.796	13.000	**<0.001**	Yes
Cond 3 vs. Cond 4	53.089	11.542	**<0.001**	Yes
Cond 3 vs. Cond 1	36.436	7.921	**<0.001**	Yes
Cond 1 vs. Cond 2	23.360	5.078	<0.001	**Yes**
Cond 1 vs. Cond 4	16.653	3.620	0.002	**Yes**
Cond 4 vs. Cond 2	6.707	1.458	0.153	**No**

Comparisons for factor: **Condition within 20.0%**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Cond 5 vs. Cond 2	195.578	42.519	**<0.001**	Yes
Cond 5 vs. Cond 4	182.215	39.614	**<0.001**	Yes
Cond 5 vs. Cond 1	172.892	37.587	**<0.001**	Yes
Cond 5 vs. Cond 3	112.324	24.419	**<0.001**	Yes
Cond 3 vs. Cond 2	83.253	18.099	**<0.001**	Yes
Cond 3 vs. Cond 4	69.890	15.194	**<0.001**	Yes
Cond 3 vs. Cond 1	60.567	13.167	**<0.001**	Yes
Cond 1 vs. Cond 2	22.686	4.932	<0.001	**Yes**
Cond 4 vs. Cond 2	13.363	2.905	0.012	**Yes**
Cond 1 vs. Cond 4	9.323	2.027	0.049	**Yes**

d. Storage Modulus (G') at 5% Strain from Rheology

Two Way Analysis of Variance Thursday, April 19, 2012, 10:54:22 PM

Data source: G' Raw Data in Rheology Data 2

Balanced Design

Dependent Variable: G' at 5%

Normality Test (Shapiro-Wilk) Passed (P = 0.157)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Condition	4	375607.989	93901.997	6.988	0.002
Frequency	2	4680.751	2340.376	0.174	0.842
Condition x Frequency	8	12504.364	1563.046	0.116	0.998
Residual	15	201572.668	13438.178		
Total	29	594365.773	20495.371		

The difference in the mean values among the different levels of Condition is greater than would be expected by chance after allowing for effects of differences in Frequency. There is a statistically significant difference (P = 0.002). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Frequency is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Condition. There is not a statistically significant difference (P = 0.842).

The effect of different levels of Condition does not depend on what level of Frequency is present. There is not a statistically significant interaction between Condition and Frequency. (P = 0.998)

Power of performed test with alpha = 0.0500: for Condition : 0.940
Power of performed test with alpha = 0.0500: for Frequency : 0.0500
Power of performed test with alpha = 0.0500: for Condition x Frequency : 0.0500

Least square means for Condition :

Group	Mean
Cond 1	377.241
Cond 2	277.469
Cond 3	124.133
Cond 4	443.892
Cond 5	381.698

Std Err of LS Mean = 47.325

Least square means for Frequency :

Group	Mean
1 rad/s	303.597
5 rad/s	326.392
10 rad/s	332.670

Std Err of LS Mean = 36.658

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Condition**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Cond 4 vs. Cond 3	319.759	4.778	**0.002**	Yes
Cond 5 vs. Cond 3	257.565	3.848	**0.014**	Yes
Cond 1 vs. Cond 3	253.108	3.782	**0.014**	Yes
Cond 4 vs. Cond 2	166.424	2.487	0.163	No
Cond 2 vs. Cond 3	153.336	2.291	0.202	No
Cond 5 vs. Cond 2	104.229	1.557	0.530	No
Cond 1 vs. Cond 2	99.772	1.491	0.494	No
Cond 4 vs. Cond 1	66.651	0.996	0.706	No
Cond 4 vs. Cond 5	62.194	0.929	0.600	No
Cond 5 vs. Cond 1	4.457	0.0666	0.948	No

Comparisons for factor: **Frequency**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
10 rad/s vs. 1 rad/s	29.073	0.561	0.928	No
5 rad/s vs. 1 rad/s	22.795	0.440	0.889	No
10 rad/s vs. 5 rad/s	6.278	0.121	0.905	No

e. Storage Modulus (G') at 10% Strain from Rheology

Two Way Analysis of Variance Thursday, April 19, 2012, 10:54:42 PM

Data source: G' Raw Data in Rheology Data 2

Balanced Design

Dependent Variable: G' at 10%

Normality Test (Shapiro-Wilk) Passed (P = 0.093)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Condition	4	362319.747	90579.937	10.028	<0.001
Frequency	2	667.415	333.708	0.0369	0.964
Condition x Frequency	8	3409.123	426.140	0.0472	1.000
Residual	15	135492.799	9032.853		
Total	29	501889.084	17306.520		

The difference in the mean values among the different levels of Condition is greater than would be expected by chance after allowing for effects of differences in Frequency. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Frequency is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Condition. There is not a statistically significant difference (P = 0.964).

The effect of different levels of Condition does not depend on what level of Frequency is present. There is not a statistically significant interaction between Condition and Frequency. (P = 1.000)

Power of performed test with alpha = 0.0500: for Condition : 0.994
Power of performed test with alpha = 0.0500: for Frequency : 0.0500
Power of performed test with alpha = 0.0500: for Condition x Frequency : 0.0500

Least square means for Condition :

Group	Mean
Cond 1	327.600
Cond 2	235.939
Cond 3	55.752
Cond 4	353.095
Cond 5	329.676

Std Err of LS Mean = 38.800

Least square means for Frequency :

Group	Mean
1 rad/s	257.318
5 rad/s	256.842
10 rad/s	267.077

Std Err of LS Mean = 30.055

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Condition**

Comparison	Diff of Means	t	P	P<0.050
Cond 4 vs. Cond 3	297.343	5.419	**<0.001**	Yes
Cond 5 vs. Cond 3	273.924	4.992	**0.001**	Yes
Cond 1 vs. Cond 3	271.848	4.954	**0.001**	Yes
Cond 2 vs. Cond 3	180.187	3.284	**0.035**	Yes
Cond 4 vs. Cond 2	117.157	2.135	0.263	No
Cond 5 vs. Cond 2	93.738	1.708	0.436	No
Cond 1 vs. Cond 2	91.661	1.670	0.388	No
Cond 4 vs. Cond 1	25.496	0.465	0.957	No
Cond 4 vs. Cond 5	23.419	0.427	0.895	No
Cond 5 vs. Cond 1	2.077	0.0378	0.970	No

Comparisons for factor: **Frequency**

Comparison	Diff of Means	t	P	P<0.050
10 rad/s vs. 5 rad/s	10.235	0.241	0.993	No
10 rad/s vs. 1 rad/s	9.759	0.230	0.968	No
1 rad/s vs. 5 rad/s	0.477	0.0112	0.991	No

f. Loss Modulus (G") at 5% Strain from Rheology

Two Way Analysis of Variance Thursday, April 19, 2012, 10:55:03 PM

Data source: G" Raw Data in Rheology Data 2

Balanced Design

Dependent Variable: G" at 5%

Normality Test (Shapiro-Wilk) Passed (P = 0.987)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Condition	4	53366.081	13341.520	19.892	<0.001
Frequency	2	2597.867	1298.933	1.937	0.179
Condition x Frequency	8	1951.844	243.980	0.364	0.924
Residual	15	10060.690	670.713		
Total	29	67976.481	2344.017		

The difference in the mean values among the different levels of Condition is greater than would be expected by chance after allowing for effects of differences in Frequency. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Frequency is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Condition. There is not a statistically significant difference (P = 0.179).

The effect of different levels of Condition does not depend on what level of Frequency is present. There is not a statistically significant interaction between Condition and Frequency. (P = 0.924)

Power of performed test with alpha = 0.0500: for Condition : 1.000
Power of performed test with alpha = 0.0500: for Frequency : 0.176
Power of performed test with alpha = 0.0500: for Condition x Frequency : 0.0500

Least square means for Condition :

Group	Mean
Cond 1	52.161
Cond 2	31.381
Cond 3	102.294
Cond 4	134.830
Cond 5	133.227

Std Err of LS Mean = 10.573

Least square means for Frequency :

Group	Mean
1 rad/s	103.936
5 rad/s	83.984
10 rad/s	84.415

Std Err of LS Mean = 8.190

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Condition**

Comparison	Diff of Means	t	P	P<0.050
Cond 4 vs. Cond 2	103.449	6.919	**<0.001**	Yes
Cond 5 vs. Cond 2	101.846	6.811	**<0.001**	Yes
Cond 4 vs. Cond 1	82.669	5.529	**<0.001**	Yes
Cond 5 vs. Cond 1	81.065	5.422	**<0.001**	Yes
Cond 3 vs. Cond 2	70.913	4.743	**0.002**	Yes
Cond 3 vs. Cond 1	50.133	3.353	**0.022**	Yes
Cond 4 vs. Cond 3	32.536	2.176	0.172	No
Cond 5 vs. Cond 3	30.933	2.069	0.159	No
Cond 1 vs. Cond 2	20.780	1.390	0.336	No
Cond 4 vs. Cond 5	1.603	0.107	0.916	No

Comparisons for factor: **Frequency**

Comparison	Diff of Means	t	P	P<0.050
1 rad/s vs. 5 rad/s	19.952	1.723	0.284	No
1 rad/s vs. 10 rad/s	19.521	1.685	0.212	No
10 rad/s vs. 5 rad/s	0.431	0.0372	0.971	No

g. Loss Modulus (G") at 10% Strain from Rheology

Two Way Analysis of Variance Thursday, April 19, 2012, 10:55:28 PM

Data source: G" Raw Data in Rheology Data 2

Balanced Design

Dependent Variable: G" at 10%

Normality Test (Shapiro-Wilk) Passed (P = 0.941)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Condition	4	33260.082	8315.020	15.977	<0.001
Frequency	2	1908.951	954.475	1.834	0.194
Condition x Frequency	8	1336.567	167.071	0.321	0.945
Residual	15	7806.515	520.434		
Total	29	44312.115	1528.004		

The difference in the mean values among the different levels of Condition is greater than would be expected by chance after allowing for effects of differences in Frequency. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Frequency is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Condition. There is not a statistically significant difference (P = 0.194).

The effect of different levels of Condition does not depend on what level of Frequency is present. There is not a statistically significant interaction between Condition and Frequency. (P = 0.945)

Power of performed test with alpha = 0.0500: for Condition : 1.000
Power of performed test with alpha = 0.0500: for Frequency : 0.161

Power of performed test with alpha = 0.0500: for Condition x Frequency : 0.0500
Least square means for Condition :

Group	**Mean**
Cond 1	38.611
Cond 2	36.227
Cond 3	69.829
Cond 4	109.419
Cond 5	113.914

Std Err of LS Mean = 9.313

Least square means for Frequency :

Group	**Mean**
1 rad/s	84.349
5 rad/s	65.260
10 rad/s	71.192

Std Err of LS Mean = 7.214

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Condition**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Cond 5 vs. Cond 2	77.687	5.898	**<0.001**	Yes
Cond 5 vs. Cond 1	75.303	5.717	**<0.001**	Yes
Cond 4 vs. Cond 2	73.191	5.557	**<0.001**	Yes
Cond 4 vs. Cond 1	70.808	5.376	**<0.001**	Yes
Cond 5 vs. Cond 3	44.085	3.347	**0.026**	Yes
Cond 4 vs. Cond 3	39.589	3.006	**0.044**	Yes
Cond 3 vs. Cond 2	33.602	2.551	0.086	No
Cond 3 vs. Cond 1	31.218	2.370	0.092	No
Cond 5 vs. Cond 4	4.495	0.341	0.931	No
Cond 1 vs. Cond 2	2.384	0.181	0.859	No

Comparisons for factor: **Frequency**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
1 rad/s vs. 5 rad/s	19.089	1.871	0.224	No
1 rad/s vs. 10 rad/s	13.157	1.290	0.386	No
10 rad/s vs. 5 rad/s	5.932	0.581	0.570	No

h. Loss Tangent (tan δ) at 5% Strain from Rheology

Two Way Analysis of Variance Thursday, April 19, 2012, 10:55:52 PM

Data source: tan d Raw Data in Rheology Data 2

Balanced Design

Dependent Variable: tan d at 5%

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Condition	4	2.639	0.660	12.249	<0.001
Frequency	2	0.00784	0.00392	0.0727	0.930
Condition x Frequency	8	0.117	0.0146	0.272	0.966
Residual	15	0.808	0.0539		
Total	29	3.572	0.123		

The difference in the mean values among the different levels of Condition is greater than would be expected by chance after allowing for effects of differences in Frequency. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Frequency is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Condition. There is not a statistically significant difference (P = 0.930).

The effect of different levels of Condition does not depend on what level of Frequency is present. There is not a statistically significant interaction between Condition and Frequency. (P = 0.966)

Power of performed test with alpha = 0.0500: for Condition : 0.999
Power of performed test with alpha = 0.0500: for Frequency : 0.0500
Power of performed test with alpha = 0.0500: for Condition x Frequency : 0.0500

Least square means for Condition :

Group	Mean
Cond 1	0.141
Cond 2	0.107
Cond 3	0.931
Cond 4	0.310
Cond 5	0.362

Std Err of LS Mean = 0.0948

Least square means for Frequency :

Group	Mean
1 rad/s	0.392
5 rad/s	0.355
10 rad/s	0.363

Std Err of LS Mean = 0.0734

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Condition**

Comparison	Diff of Means	t	P	P<0.050
Cond 3 vs. Cond 2	0.824	6.148	**<0.001**	Yes
Cond 3 vs. Cond 1	0.790	5.894	**<0.001**	Yes
Cond 3 vs. Cond 4	0.621	4.637	**0.003**	Yes
Cond 3 vs. Cond 5	0.569	4.249	**0.005**	Yes
Cond 5 vs. Cond 2	0.255	1.899	0.381	No
Cond 5 vs. Cond 1	0.221	1.646	0.474	No
Cond 4 vs. Cond 2	0.202	1.511	0.482	No
Cond 4 vs. Cond 1	0.168	1.257	0.540	No
Cond 5 vs. Cond 4	0.0521	0.389	0.912	No
Cond 1 vs. Cond 2	0.0339	0.253	0.803	No

Comparisons for factor: **Frequency**

Comparison	Diff of Means	t	P	P<0.050
1 rad/s vs. 5 rad/s	0.0378	0.364	0.978	No
1 rad/s vs. 10 rad/s	0.0290	0.280	0.953	No
10 rad/s vs. 5 rad/s	0.00881	0.0848	0.934	No

i. Loss Tangent (tan δ) at 10% Strain from Rheology

Two Way Analysis of Variance Thursday, April 19, 2012, 10:56:08 PM

Data source: tan d Raw Data in Rheology Data 2

Balanced Design

Dependent Variable: tan d at 10%

Normality Test (Shapiro-Wilk) Passed (P = 0.987)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Condition	4	5.231	1.308	278.649	<0.001
Frequency	2	0.0132	0.00658	1.401	0.277
Condition x Frequency	8	0.0226	0.00283	0.603	0.762
Residual	15	0.0704	0.00469		
Total	29	5.337	0.184		

The difference in the mean values among the different levels of Condition is greater than would be expected by chance after allowing for effects of differences in Frequency. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Frequency is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Condition. There is not a statistically significant difference (P = 0.277).

The effect of different levels of Condition does not depend on what level of Frequency is present. There is not a statistically significant interaction between Condition and Frequency. (P = 0.762)

Power of performed test with alpha = 0.0500: for Condition : 1.000
Power of performed test with alpha = 0.0500: for Frequency : 0.100

Power of performed test with alpha = 0.0500: for Condition x Frequency : 0.0500
Least square means for Condition :

Group	Mean
Cond 1	0.124
Cond 2	0.156
Cond 3	1.257
Cond 4	0.309
Cond 5	0.359

Std Err of LS Mean = 0.0280

Least square means for Frequency :

Group	Mean
1 rad/s	0.470
5 rad/s	0.423
10 rad/s	0.429

Std Err of LS Mean = 0.0217

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Condition**

Comparison	Diff of Means	t	P	P<0.050
Cond 3 vs. Cond 1	1.133	28.648	**<0.001**	Yes
Cond 3 vs. Cond 2	1.101	27.841	**<0.001**	Yes
Cond 3 vs. Cond 4	0.948	23.970	**<0.001**	Yes
Cond 3 vs. Cond 5	0.898	22.694	**<0.001**	Yes
Cond 5 vs. Cond 1	0.236	5.955	**<0.001**	Yes
Cond 5 vs. Cond 2	0.204	5.147	**<0.001**	Yes
Cond 4 vs. Cond 1	0.185	4.678	**0.001**	Yes
Cond 4 vs. Cond 2	0.153	3.871	**0.005**	Yes
Cond 5 vs. Cond 4	0.0505	1.277	0.393	No
Cond 2 vs. Cond 1	0.0319	0.807	0.432	No

Comparisons for factor: **Frequency**

Comparison	Diff of Means	t	P	P<0.050
1 rad/s vs. 5 rad/s	0.0472	1.540	0.373	No
1 rad/s vs. 10 rad/s	0.0410	1.338	0.362	No
10 rad/s vs. 5 rad/s	0.00622	0.203	0.842	No

j. Cellular Quantification on Day 7 from DNA Assay

One Way Analysis of Variance Sunday, April 29, 2012, 8:04:46 PM

Data source: Cell Numbers in DNA Assay Combined

Normality Test (Shapiro-Wilk) Passed (P = 0.130)

Equal Variance Test: Passed (P = 0.170)

Group Name	N	Missing	Mean	Std Dev	SEM
Cond #1	9	0	457253.229	29333.959	9777.986
Cond #2	9	0	479142.095	45005.800	15001.933
Cond #3	9	0	459151.876	20404.210	6801.403
Cond #4	9	0	403045.153	36729.127	12243.042
Cond #5	9	0	395082.116	71611.316	23870.439

Source of Variation	DF	SS	MS	F	P
Between Groups	4	50146617749.151	12536654437.288	6.410	<0.001
Residual	40	78236353596.207	1955908839.905		
Total	44	128382971345.358			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.963

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Cond #2 vs. Cond #5	84059.979	4.032	**0.002**	Yes
Cond #2 vs. Cond #4	76096.941	3.650	**0.007**	Yes
Cond #3 vs. Cond #5	64069.760	3.073	**0.030**	Yes
Cond #1 vs. Cond #5	62171.114	2.982	**0.034**	Yes
Cond #3 vs. Cond #4	56106.723	2.691	0.060	No
Cond #1 vs. Cond #4	54208.076	2.600	0.063	No
Cond #2 vs. Cond #1	21888.865	1.050	0.760	No
Cond #2 vs. Cond #3	19990.219	0.959	0.717	No
Cond #4 vs. Cond #5	7963.038	0.382	0.913	No
Cond #3 vs. Cond #1	1898.647	0.0911	0.928	No

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