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Ismail, Humza and Wagenseil, Jessica, "Hydrogel Viscoelastic Properties Pertaining to Cell Phenotype" (2017). *Mechanical Engineering and Materials Science Independent Study*. 45.
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Hydrogel Viscoelastic Properties Pertaining to Cell Phenotype

Humza Ismail

Introduction

It has long been established that the material stiffness, or the elastic modulus, of a material will modulate the properties and the behavior of cells that interact with material. In tissues, the cells often interact with the three-dimensional extracellular matrix (ECM).[1] The ECM can be characterized as scaffold made of proteins and other polymers that support and communicate with cells forging their tissue specific identities. This model has been dubbed dynamic reciprocity.[2] Studies that analyze the effects of ECM conditions on cell behavior are an important field of study. The application of such research can be used to analyze how specific defects in genotype will affect phenotype. These studies have also been proven valuable in cancer studies.

When analyzing how specific material properties of an ECM and how a specific property might affect cell phenotype, one of the most commonly studied properties is the elastic modulus. Much work has been done to see how the modulus or stiffness of a given substrate will affect how a cell behaves. For example, work done by Engler, Sen et al. analyzed how ECM stiffness directs stem cell lineage.[3] The team discovered that substrates with lower elastic moduli, like the conditions found in the brain (0.1-1 kPa), would exhibit stem cells that were increasingly branched. Stiffer substrates however, would lead to cells that were spindle shaped (8-17 kPa) and polygon shaped (25-40 kPa) as the elastic modulus increased as seen in Figure 1.

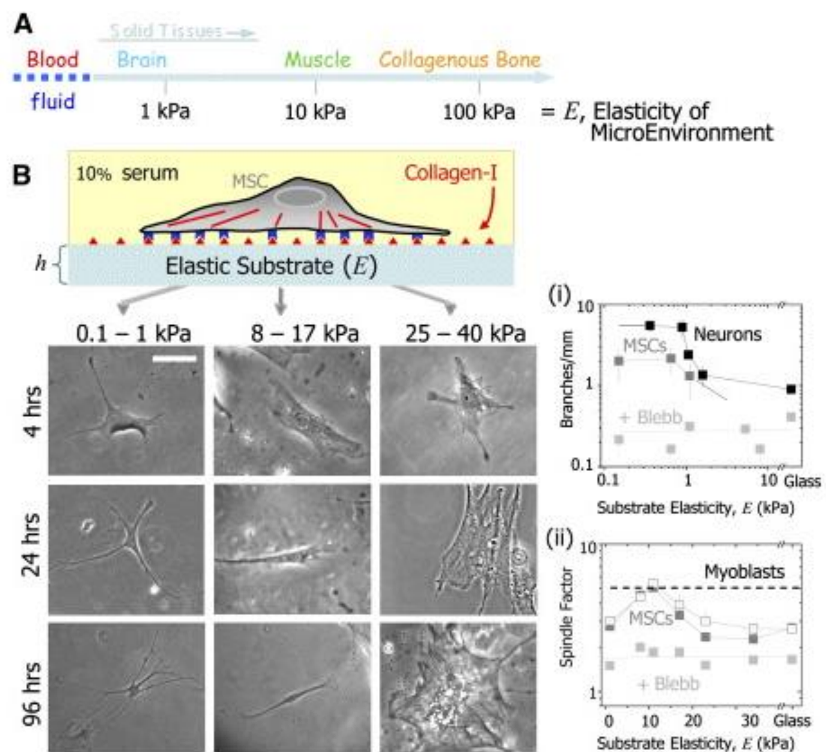


Figure 1- Cell lineage and shape can be dependent of the material stiffness of the substrate that the cells reside on.[3]

Many other laboratories have done similar research into how the elastic modulus of an ECM affects the phenotypic properties of the overlying cells. For example, work by Pathak and Kumar have shown that the elastic modulus of the matrix regulates the migration speed of tumor cells that are growing on the substrate. As ECM stiffness increases, the migration speed of tumor cells also increases as shown in figure 2.[4]

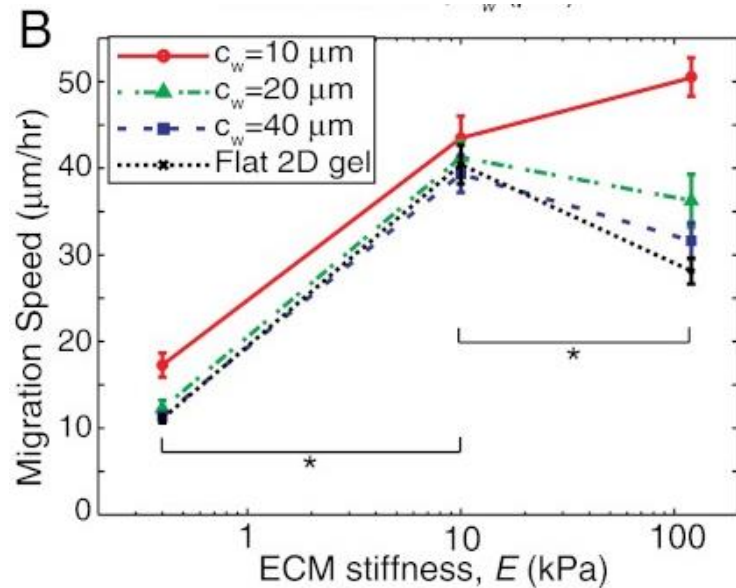


Figure 2- Tumor cell migration speeds on different stiffness ECMs and channel width.[4]

The elastic modulus has a well-established roll in the modulation of cell phenotype through the ECM. However, the same analysis has not been completed extensively for the viscoelastic properties of an ECM.

While many studies have addressed the effects of the elastic modulus of an ECM changes cell behavior, studies that have focused on solely the viscous properties of an ECM and its effect on cell phenotype have not been completed thoroughly

However, some preliminary work has been completed to determine at how viscoelastic properties affect cell behavior. Work done by Cameron, Firth, and Copper-White has shown that dynamic creep (found in more viscous substrates) has an influence over human mesenchymal stem cell behavior. When looking at how cell proliferation was affected by loss modulus, it was found that the higher the loss modulus, or the more viscous the substrate, the higher the rate of proliferation for the cells was observed.[5] Despite the recent progress that has been made in analyzing the effects that viscoelastic properties of a substrate have on cell behavior, more work needs to be done.

To characterize the material properties of an ECM, polyacrylamide hydrogels are the first choice to model different elastic and viscous properties of ECMs. Hydrogels have a high water content making them ideal candidates for being used as synthetic ECMs for 3D cell analysis.[1]

The most common hydrogel used in cell research is the polyacrylamide (PAM) hydrogel. By varying the amount of acrylamide monomer and bis-acrylamide crosslinker, the elastic modulus of a PAM gel can be modulated without much issue. Work done by Yeung et al. has shown that at bis-acrylamide (crosslinker) concentration increases for a specific PAM gel, the elastic modulus increases in a linear fashion for a set acrylamide monomer concentration as seen in figure 3.[5] The study also showed that hydrogels with elastic moduli ranging from 10 Pa to 50,000 Pa can be achieved by varying the acrylamide (monomer) concentration of the gel from 3 to 12 percent. Some attempts have been made recently to look at how viscoelasticity affects cell morphology. Work done by Cameron et al. looked at how varying the viscoelastic properties of a PAM gel while keeping the elastic modulus of the gel constant affected how the affected focal adhesion and cell proliferation. The team was able to create three gels of varying loss modulus and constant storage modulus by varying the amount of acrylamide and bis-acrylamide used to crosslink the gels as seen below in figure 4.[6]

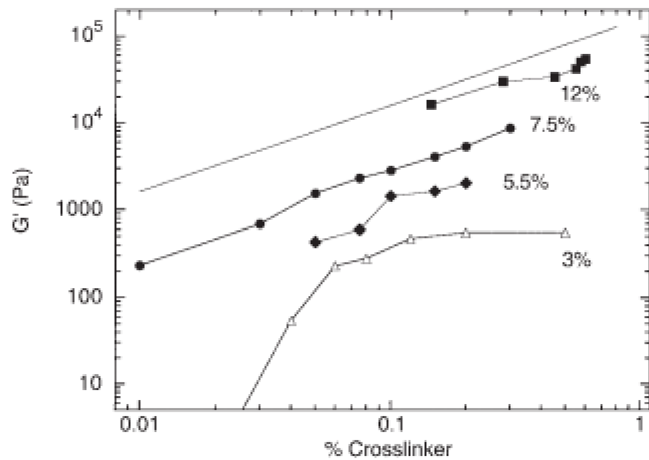
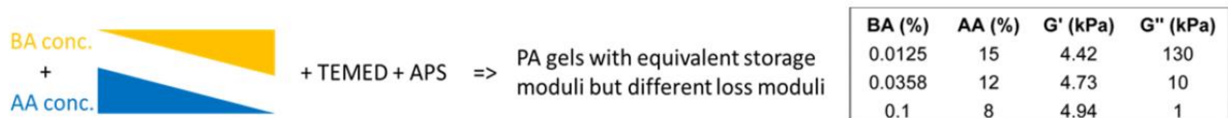


Figure 3- A graph showing the different shear moduli achieved by changing the percent monomer and crosslinker present in a PAM hydrogel.[5]



BA (%)	AA (%)	G' (kPa)	G" (kPa)
0.0125	15	4.42	130
0.0358	12	4.73	10
0.1	8	4.94	1

Figure 4- The varying amounts of acrylamide (AA) and bis-acrylamide (BA) used to make the gels with the varying loss moduli.[6]

While many methods exist to test the mechanical properties of a hydrogel, one of the most effective ways to measure the viscoelastic properties of a hydrogel is through the use of shear rheology. Specifically, oscillation tests that increase the angular frequency of oscillation of a hydrogel while keeping either the stress or strain on the gel constant will lead to values of the storage (G') modulus and the loss (G") modulus. Often times, care must be taken to tune the

specific settings of the oscillation test. The frequency of oscillation and amount of stress or strain applied to the gel need to be set to values that ensure the measurements taken by the rheometer are accurate. If the amount of oscillation or stress/strain applied to the gel is too high, the G' and G" values will be influenced by the inertial effects of the motor that is used to power the rheometer.[1] This technique was used by Cameron et al. to take data for the hydrogels seen in figure 4. Data was collected by oscillating the gels from 0.05 rad/s to 100 rad/s at a constant strain of 1 percent as seen in figure 5.[6] Data collected from rheology can be fit to a number of viscoelastic models such as the Maxwell model.

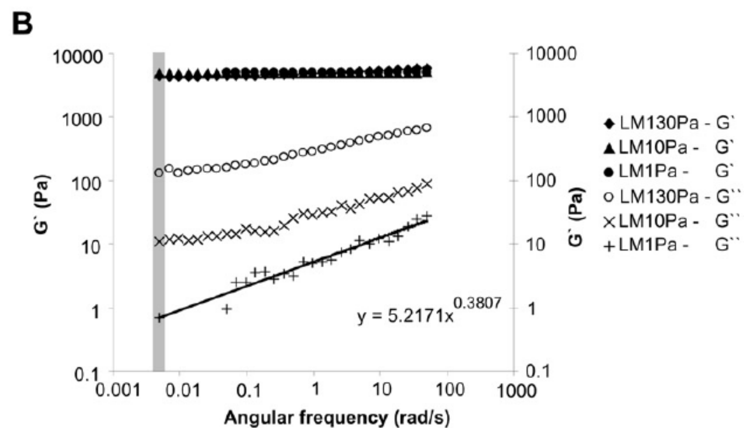


Figure 5- Results of oscillation tests on hydrogels with varying loss moduli. [6]

With the knowledge of how cells behave when exposed to ECMs of different stiffness values, and the fact that there is a severe lack of knowledge regarding how viscoelastic properties of an ECM affect cell phenotype, several research goals were set. Those goals included: fabricating PAM gels with similar G' values and different G" values, growing cells on different PAM gels, and tracking cell proliferation rates on different PAM gels.

Gel Fabrication

To make PAM gels, 4 main ingredients are needed:

1. Acrylamide (monomer)
2. Bis-acrylamide (crosslinker)
3. Ammonium Persulfate (APS)
4. Tetramethylethylenediamine (TEMED)

Stock solution of acrylamide (Sigma-Aldrich 01696) was made at a 40% w/v concentration and bis-acrylamide (Sigma-Aldrich 146072) stock solution was made at a w/v concentration of

2%. The monomer and crosslinker stock solution were stored in a 4° C freezer. APS (Sigma-Aldrich A3678) was made at a 10% w/v composition and was stored in small aliquots (40μL) for use. Freeze-thaw cycles should be avoided whenever possible when using APS. Ideally, a fresh solution of APS should be made when polymerizing hydrogels. All solutions were mixed in ultra-pure water.

Before crosslinking PAM gels, the amount of monomer and crosslinker (acrylamide and bis-acrylamide) used in the gels must be decided. As discussed in the Introduction, varying the amount of acrylamide and bis-acrylamide concentration can give a wide range of G' and G" values for a PAM gel.[5] In an effort to demonstrate that the G" of a PAM gel can be modulated independently of G' in our lab, PAM gels were made as specified in figure 4 in an attempt to replicate the results published by Cameron et al. 15mL aliquots of PAM gels were made with the ratios listed below in Table 1 and stored in the 4° C refrigerator.

40% Acrylamide (mL)	2% BIS (mL)	UPW (mL)	Acrylamide Percentage	BIS Percentage
5.625	.09375	9.28125	15	.0125
4.5	.2685	10.2315	12	.0358
3	.75	11.25	8	.1

Table 1- The volumes and concentrations of monomer and crosslinker to make PAM gels used in the experiment.

Before PAM gels are plated on any surface, special care must be taken to ensure that the PAM gels will adhere to only the desired surface. For cell proliferation, PAM gels were plated on 20 mm diameter glass coverslips. To make sure that the coverslips were hydrophilic so that PAM gels would adhere to the coverslip surface throughout the cell proliferation process, the coverslips were treated with a 0.5% 3-aminopropyl-trimethoxysilane (Sigma-Aldrich 281778) solution, dried in an oven at 115° C and then a 0.5% glutaraldehyde (Sigma-Aldrich G6257) in PBS solution. This process activates the coverslips so that they are hydrophilic and will make sure that PAM gels will adhere to the coverslip throughout the cell proliferation analysis.

Hydrophobic glass slides and imaging chambers are also needed for cell proliferation and rheology studies. To make the glass slides and the 20 mm imaging chambers hydrophobic, both

were submersed in a solution of Sigmacote® (Sigma-Aldrich SL2). This siliconizing agent is designed to make sure that the treated surface is hydrophobic, ensuring that the PAM gels would only adhere to the desired 20 mm coverslips.

To make PAM gels the following steps were taken:

1. 1 mL of gel precursor was put into at 1.5 mL microcentrifuge tube
2. 10 µL of APS was added to the precursor and mixed thoroughly
3. 10 µL of TEMED (Sigma-Aldrich 411019) was added to the solution and mixed thoroughly
4. For cell proliferation, 50 µL of gel solution was plated onto the 20 mm coverslip and sandwiched between the coverslip and the hydrophobic glass slide.
5. For rheology, 500 µL of gel solution was plated onto a 20 mm imaging chamber
6. The gels were allowed to set for at least 30 minutes
7. The gels (for both cell proliferation and rheology) were submerged in PBS and stored at 4° C.

Cell Culture

RFL6 cells were used as the cell of choice for the cell proliferation experiments. They were grown in T-75 culture flasks in a DMEMF12 and 10% FBS growth medium. A fully confluent T-75 culture flask contained 8.5 million RFL6 cells.

To plate RFL6 cells onto the PAM gels, the gels were first sterilized in a UV crosslinker for 45 minutes. While the gels were being sterilized, an activation solution was made. For 12 mL of activation solution, 60µL of 10 mM Sulfo-Sanpah solution with 600 µL of 1M HEPES solution and 11.34 mL of PBS. Note, all work with Sulfo-Sanpah solution must be done in the dark as Sulfo-Sanpah is extremely light sensitive. 200 µL of the activating was put on each gel and were put back in the UV crosslinker for 8 min to activate the Sulfo-Sanpah solution. An 8 min crosslink will give the experimenter the full 20 minute half-life of the activated Sulfo-Sanpah to complete the next steps. While the Sulfo-Sanpah is being activated, a collagen I and PBS solution was made on ice. 1 mL was plated on each gel and were left in the 4° C freezer to crosslink overnight. After the collagen was crosslinked onto the gels, the gels were washed twice

in PBS and RFL6 cells treated with trypsin were diluted into 5 mL of growth media for each gel and plated onto the gels. The gels were then placed in a 37° C incubator with 5% CO_2 content to grow.

Rhology

For rheological testing, an AR-G2 rheometer was used to measure mechanical properties of the gels. A 20 mm smooth geometry was used for all rheological oscillation tests. The gap thickness used was 1 mm. Oscillation tests were done from 0.5 rad/s to 60 rad/s at constant strains at 1%, 5%, 10% and 20%. G' and G" values were plotted versus angular frequency.

Results

The following figures how the results of the oscillation tests to determine the storage (G') and loss (G") moduli of different PAM gels. The PAM gels are identified by their final acrylamide percentage. Error bars were emitted.

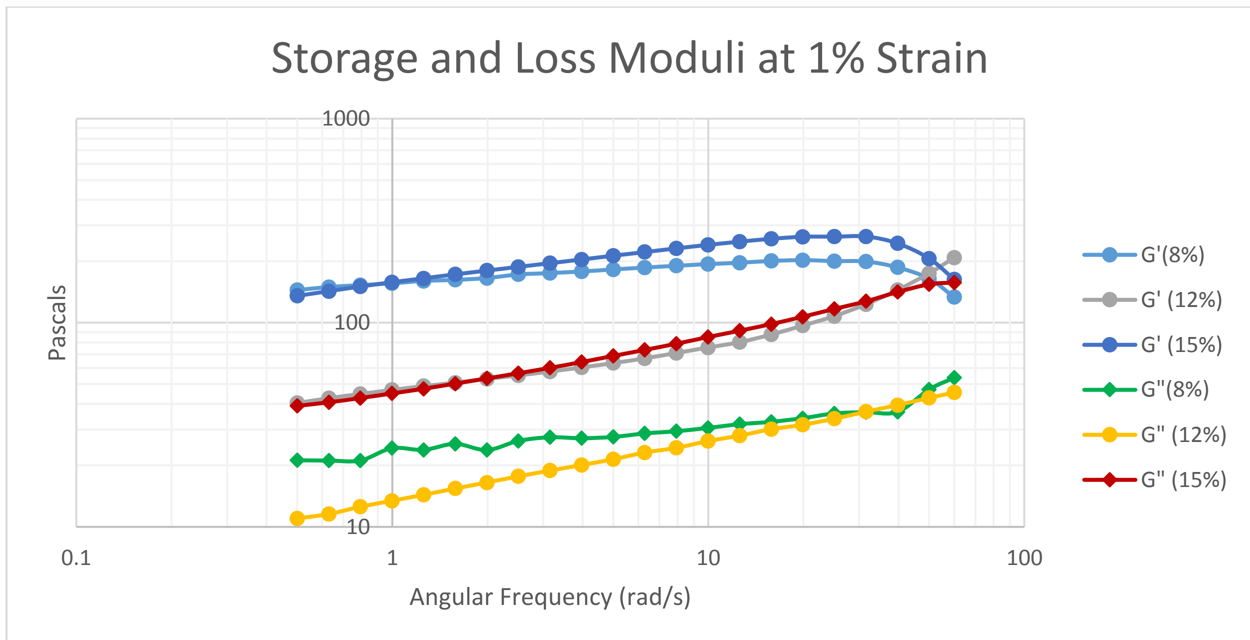


Figure 6- Oscillation test results completed at a constant 1% Strain.

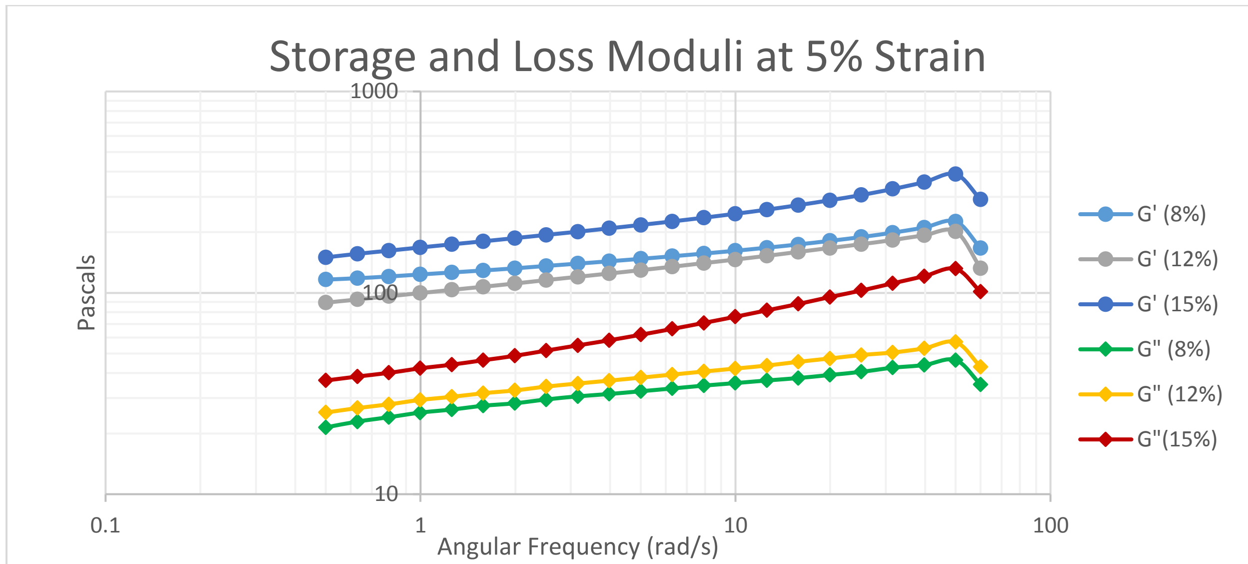


Figure 7- Oscillation test results completed at a constant 5% Strain.

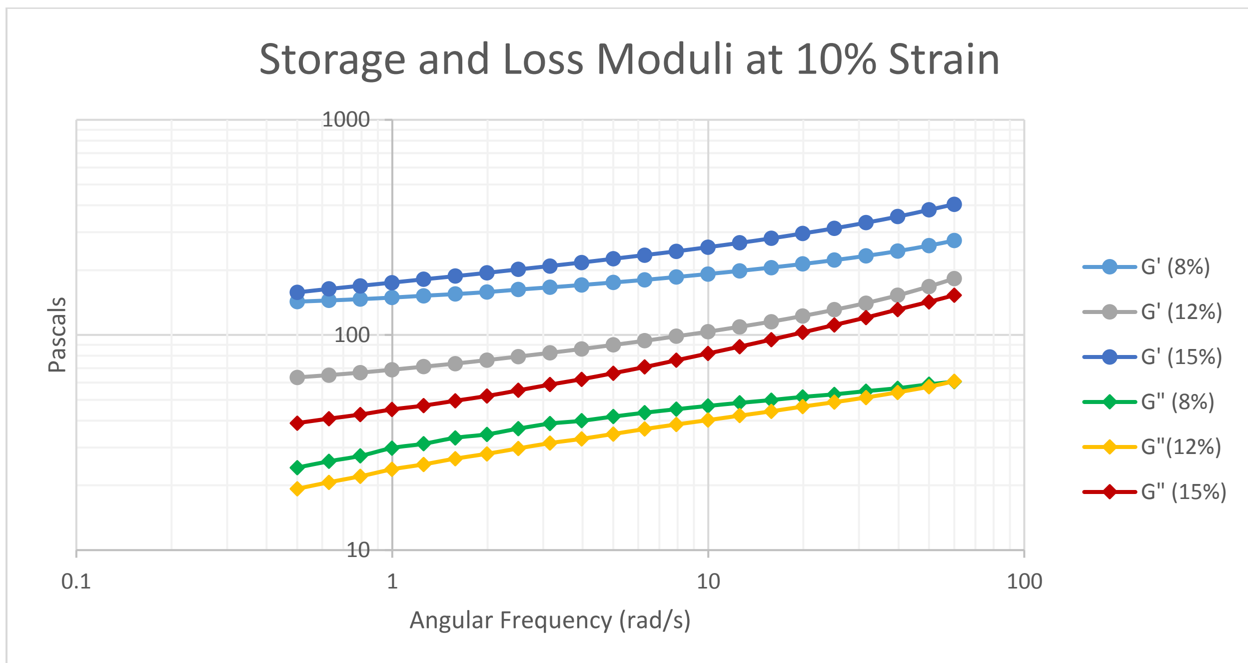


Figure 8- Oscillation test results completed at a constant 10% Strain.

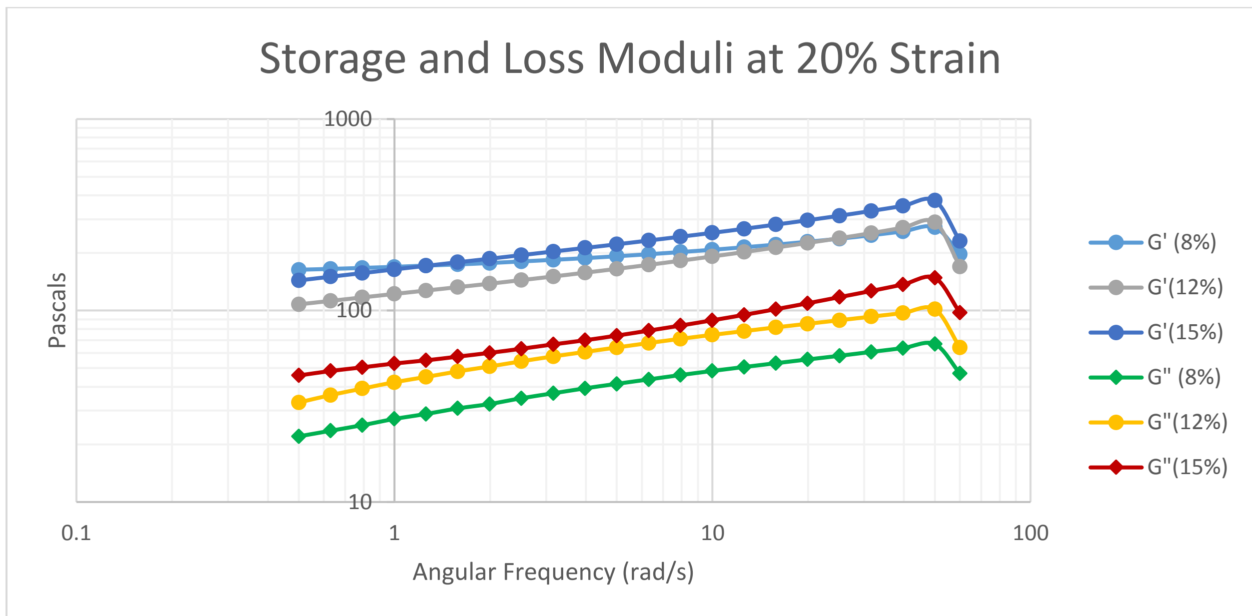


Figure 9- Oscillation test results completed at a constant 20% Strain.

The following figures show the average values of G' and G" at .05 rad/s and 1% strain. These figures help illustrate that under the same testing conditions, data at collected was not consistent. It also shows that the PAM gels tested do not follow the hypothesized trends.

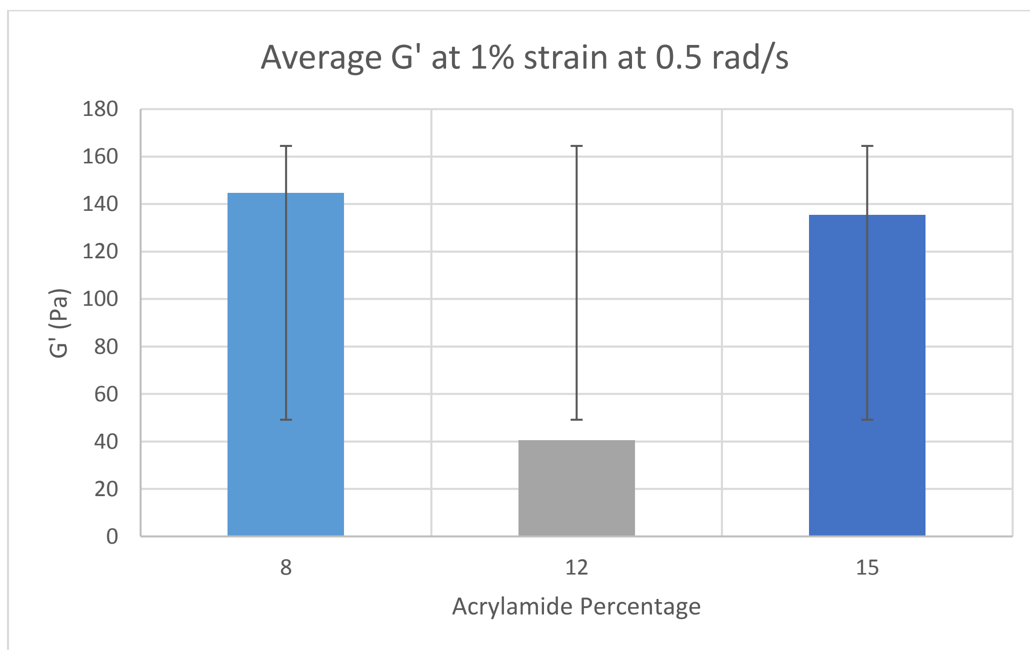


Figure 10- Average G' values for all three PAM gels. Error bars presented to show inconsistency of data.

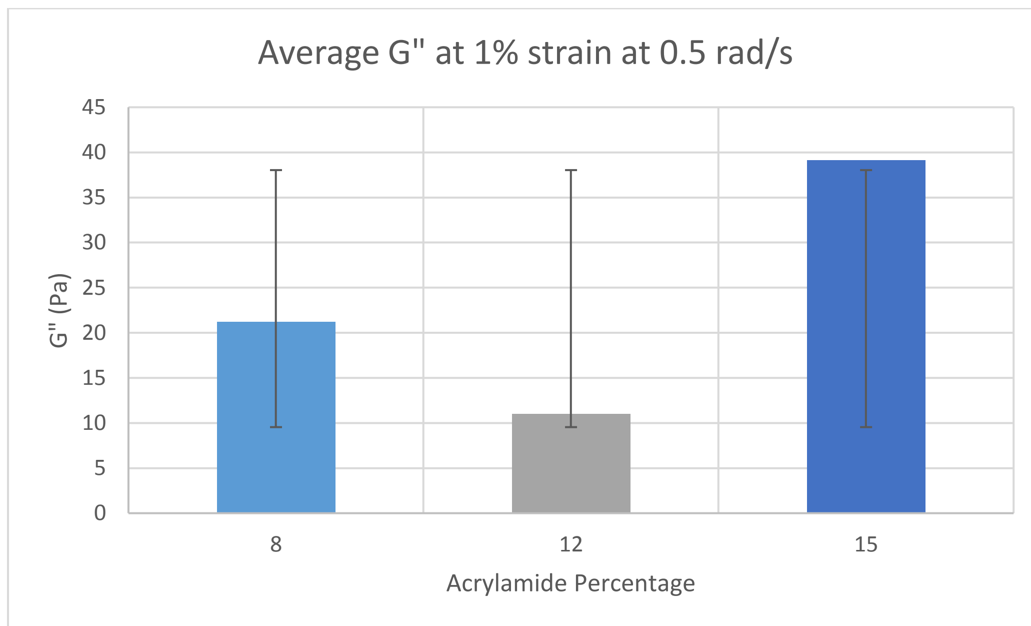


Figure 11- Average G" values for all three PAM gels. Error bars presented to show inconsistency of data.

The following pictures show RFL6 cells growing on glass coverslips. It should be noted that it is hypothesized that the cells pictured are not growing on PAM gels but on the surface of the coverslips. This is due to the incorrect activation of the glass coverslips that the PAM gels were mounted on so the gels did not adhere to the coverslips correctly. The pictures are taken at 10x zoom.

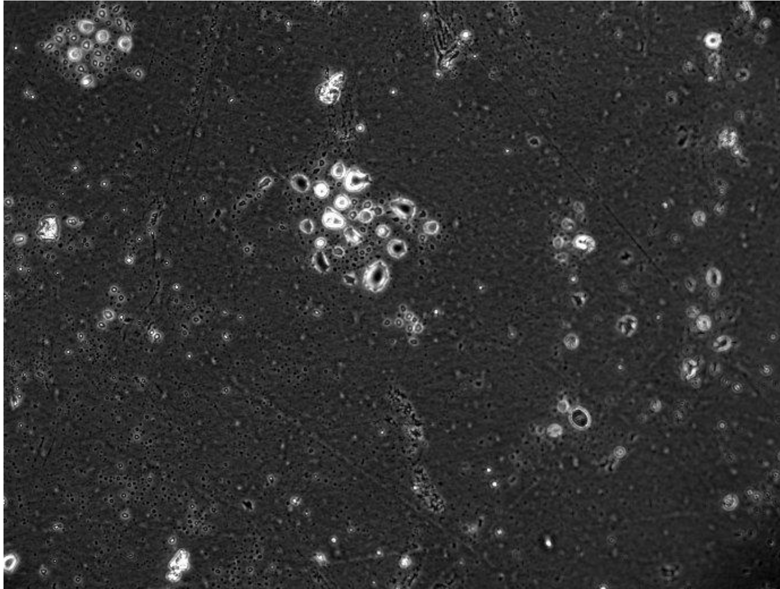


Figure 12- RFL6 cells growing on a glass coverslip. The 12% acrylamide gel that the cells were supposed to grow on floated away from the surface of the coverslip, making it difficult to analyze cell proliferation.

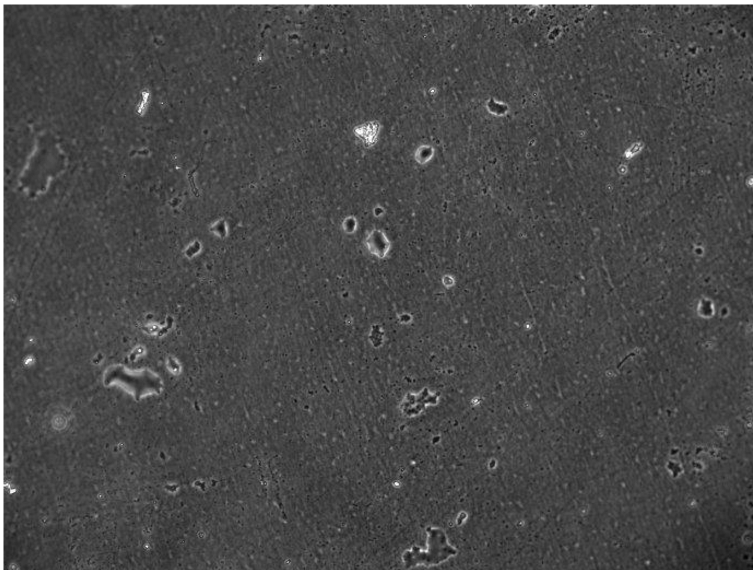


Figure 13- RFL6 cells growing on a glass coverslip. The 12% acrylamide gel that the cells were supposed to grow on floated away from the surface of the coverslip, making it difficult to analyze cell proliferation.

Discussion

It is immediately obvious that the results seen in figure 5 were not replicated through rheological testing. As seen in figure 4, the values of G' found by Cameron et al. were on average around 4.7 kPa for the three PAM gels tested. The highest G' value achieved in this investigation was seen at 10% strain in the 15% acrylamide hydrogel with a value of 405 Pascals. Comparing the maximum G' value found in this experiment to the value found in figure 4 is not very meaningful. The values found by Cameron et al. were measured at an angular frequency of .05 rad/s whereas the maximum value found in this investigation were measured at 60 rad/s. 60 rad/s was used as an upper limit for data collection because after that point, PAM gels were prone to break apart and data collected was dominated by inertial effects. When analyzing data, from a physiological viewpoint, almost never will there be a situation where the ECM that is being studied will be undergoing shear that is occurring at a rate of 60 rad/s. When thinking about how to apply the data collected and the goal of eventually applying it to physiological applications, only data measured at the lower end of angular frequencies have merit, especially when comparing values to other publications.

When looking at values that were measured at 0.5 rad/s, two major points can be seen. First, the data suggests that the three different PAM gels did not have a constant storage modulus. The values were not expected to be exactly the same, but were never supposed to be so far apart as well. Data taken at 20% constant strain seen in figure 9 has G' values that are the closest together throughout the range of angular frequencies that were tested. Again, the main issue seen with the G' values is that they were expected to be in the range of 4.4 kPa but instead hit a maximum of 405 Pa.

The second point that can be seen is that the loss moduli for the three hydrogels are not as different as expected. Loss moduli of 1 Pa, 10 Pa, and 130 Pa were expected at an angular frequency of 0.05 rad/s. As it can be seen, even at 0.5 rad/s, all of the data collected for G" is above 10 Pa. Also, the difference between loss moduli is not as significant as desired. The greatest difference can be seen at a constant strain of 1%, where at 0.5 rad/s the loss moduli range from 11 to 30.15 Pa. This data was represented in figures 10 and 11. Figure 10 shows that even at the lowest oscillation frequency, where inertial effects of the rheometer's motor are

negligible, the G' values for the three PAM gels were not equivalent or even close to equivalent. Figure 11 shows that the desired G" values from figure 4 using the concentrations outlined by Cameron et al. and summarized in table 1 were not achieved. In fact, the data shows that G" values decreased from the 8% acrylamide to 12% acrylamide gels, a phenomenon that was not expected.

The inability to produce loss moduli that are different on even one order of magnitude is the most concerning conclusion from the data collected. With the goal of the investigation to be eventually determine how changes in viscoelastic properties of a hydrogel affect the behavior of cells. If a clear methodology cannot be determined on how to tune the loss modulus while keeping the storage modulus relatively constant, than any cell behavioral studies will be testing changes in elastic properties instead of viscoelastic properties. The logical next step in researching this topic is to firmly derive hydrogel chemistries that have constant storage moduli while varying the loss moduli. Those chemistries must also be repeatable.

The data regarding cell proliferation was not as troublesome in its nature. As mentioned before, the hydrogels floated away from the coverslips that they were supposed to adhere to. This however is something that can be easily fixed by ensuring that the activating protocol described in the Methods section is carefully followed so that the PAM gels stick to the glass coverslips over time. One concerning aspect of the images taken of the RFL6 cell growth is the fact that the RFL6 cell do not look normal or healthy. The cells did not flatten and stick to the surface that they were growing on as they should and is seen in figure 1. However, this is most likely a cell strain specific problem as new cells that have been plated on PAM gels (not pictured) have grown normally and grown to confluence.

The next steps regarding researching how viscoelastic properties affect cell proliferation is to ensure that the PAM gels mounted onto the coverslips are able to remain attached to the gels. Once that is completed, a more in depth analysis of how cells proliferate under different viscoelastic environments may occur. However, before any cell proliferation studies are completed, PAM gels of desired G' and G" values must still be fabricated.

Investigating how viscoelastic properties rather than elastic properties of an artificial ECM affect the behavior of the cells growing on that ECM is a relatively untested field of study. Thus, results may not be as easily obtainable as first imagined.

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