Fabrication of Hydrogels with Controllable Heterogeneity in Stiffness

A Major Qualifying Project Report:
Submitted to the Faculty
Of the
Worcester Polytechnic Institute
In partial fulfillment of the requirements for the
Degree of Bachelor of Science
By

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Date: May 2014

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1. Hydrogel
2. Stiffness
3. Heterogeneity
Abstract

Tissues in the body are heterogeneous in stiffness, yet there has been little investigation regarding how cells work cooperatively in heterogeneous environments. The mechanical properties of the extracellular matrix can affect cell morphology, proliferation, differentiation, and migration. Polyacrylamide hydrogels with regions of varying stiffnesses were fabricated to better represent in vivo tumor-like conditions to study cell behavior and cell-cell interactions. A method to fabricate hydrogels with uniform thickness and controllable heterogeneity in stiffness was developed. Atomic force microscopy was used to characterize stiffness of the fabricated hydrogels. NIH 3T3 cells were plated on the hydrogels and experimental tests were conducted to quantify cell viability and morphology.
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Authorship

All authors contributed equally to the writing and editing of this report.
Acknowledgements

The team would like to thank Worcester Polytechnic Institute, Department of Biomedical Engineering, Lisa Wall (Lab Manager, Biomedical Engineering), Gawain Thomas (PhD Student, Physics) for his time and talent with Atomic Force Microscopy, and our advisors, Professor Qi Wen (Physics) and Professor Sakthikumar Ambady (Biomedical Engineering) for their guidance and assistance throughout the course of this project.
Chapter 1: Introduction

Tissues in the body are heterogeneous in stiffness, yet there has been little investigation regarding how cells work cooperatively in heterogeneous environments. Stiffness of the extracellular matrix (ECM) in body tissue affects cell adhesion, migration, differentiation, and morphology. Because ECM stiffness is not uniform across tissues, cells may not all behave in the same way.

Current *in vitro* systems used to study cell behavior are not representative of biological tissue. If tissue culture substrates better mimicked specific cellular environments, further research and experimentation could be conducted that more accurately represents cell behavior *in vivo*. Hydrogels have recently gained popularity as substrates in cell and tissue engineering research. These networks of highly hydrophilic polymer chains have mechanical properties similar to body tissue, and are therefore becoming more widely used as a substrate. Hydrogels have tunable mechanical properties and show promise to develop into research tools that better represent *in vivo* conditions.

The goal of this project was to develop a system to fabricate hydrogels that more accurately represent in vivo conditions. Specifically, a cancerous, tumor-like environment. By developing a hydrogel that mimics the stiffness of tumors in the body, further research can be conducted regarding what specific changes in adhesion, morphology, migration, differentiation, and proliferation cells undergo and how far from the stiffness area these changes can be seen.

Background research was conducted to narrow down and ultimately select viable polymers for hydrogel fabrication. Laboratory experiments were performed to select the most effective crosslinking method for altering regions of stiffness within the hydrogel. Tests were performed to verify stability and quantify stiffness by measuring the elastic modulus of the
hydrogel. Cells were plated on the hydrogel and cell adhesion, proliferation, and morphology were observed.

The following report will include a literature review, in Chapter 2, providing background information about the ECM and the effects of tumors on cell behavior. Information on fabrication processes and methods to measure hydrogel properties will also be discussed. Chapter 3 will discuss the client statement, objectives, constraints, and overall project approach. Design alternatives and verification of our product design will be explained in Chapters 4 and 5. The results of the design verification will be discussed in Chapter 6 and Chapter 7 will detail our final product design and validation. Finally, conclusions and recommendations for future work are discussed in Chapter 8.
Chapter 2: Literature Review

Extra Cellular Matrix

A significant volume of tissue in the body is not composed of cells, but a complex system of macromolecules known as the extracellular matrix (ECM) (Alberts et al, 2002). The ECM is composed of locally secreted polysaccharides and fibrous proteins organized into a network outside the cells. Cells in their natural biological environment are anchorage dependent and adhere to this matrix, which functions to support cellular structure and intercellular communication (Sternlicht et al, 2001). Properties of the ECM influence aspects of cell behavior such as cell adhesion, morphology, migration, proliferation, and differentiation. Cell behavior in response to mechanical properties of the ECM plays a role in processes ranging from embryogenesis (Ingber et al, 2006) to the pathogenesis of disease states.

ECM stiffness in particular is a factor in cellular function. Cells have the ability to grow and develop on a range of stiffness. The ECM stiffness however, impacts the ways in which cells behave. Changes in stiffness have been associated with fibrosis, cancer, and cardiovascular disease (Cretu et al, 2010). A normal tissue cell anchors and pulls on its surroundings; this processes depends on myosin-based contractility and cell-ECM adhesions. Cells apply forces to the substrate and respond to the resistance it senses, through cytoskeleton organization. This process occurs whether resistance is from tissue matrix, synthetic substrate, or adjacent cells. The cell responds to this resistance by adjusting its adhesions, cytoskeleton, and overall state (Dischter et al, 2005).

To gain a better understanding of how tissue cells like fibroblasts, myocytes, and neurons sense ECM stiffness, quantitative studies where cells adhere to gels elasticity similar to tissue have been carried out (Komatsu et al, 2010). Although there is cell-to-cell variability, and
cellular force–response relationships are not linear, generally cells on stiffer substrates have stiffer, more organized cytoskeletons and more stable focal adhesions (Wells, 2008). The feedback of local matrix stiffness on cell state likely has important implications for development, differentiation, disease, and regeneration.

**Stiffness In Vitro and In Vivo**

Young’s modulus, also known as elastic modulus (E), is defined as resistance to deformation. This value can be measured by applying force to a defined area in a material (stress) and calculating the resulting change in length (strain). The slope of the stress versus strain plot is the stiffness value, also known as the elastic modulus (Hibbler, 2012). Atomic Force Microscopy (AFM) is a common microindentation technique used to measure E values on a small scale, such as individual cells (Wells, 2008). AFM technique will be discussed in more detail in following sections.

The elastic modulus of living tissues varies over orders of magnitude. The E of cells is largely determined by actin-myosin–based cytoskeletons. The brain for example, has an E of several hundred pascals, the E of muscle is around 12 kPa, and tendon and cartilage is in the range of megapascals. When compared to the stiffness of tissue culture plastic or glass with an E in the gigapascal range, it is apparent that these *in vitro* surfaces are significantly stiffer than any living tissue (Wells, 2008). Cells in culture are in an environment with mechanical conditions not physiologically similar to living tissue. Substantial differences in E raises concern that behaviors attributed to cells in culture including cytoskeletal organization, proliferation, and differentiation may not be representative of cell behavior *in vivo*. 
Effects of Tumors on Cellular Environment

Tumors are masses of abnormal cells that result from rapid and uncontrolled cell division. Two types of tumors exist, malignant (cancerous) and benign (noncancerous). Both of which can pose threats to surrounding tissues (Baba et al, 2007). Malignant tumors invade surrounding tissues and can further shed cells, enter the bloodstream, and invade tissues in various parts of the body. Tumors compete for nutrients and space with healthy tissues and can be fatal if left untreated. Benign tumors can also be detrimental to surrounding cells as they compress adjacent cells and inhibit cell function (Lerner et al, 2008).

Tumors have been found to be stiffer than the tissue which surrounds it. Healthy breast tissue, for example has an elastic modulus of approximately 0.2kPa, while breast tumor stiffness is approximately 4kPa. Liver tissue ranges in stiffness (0.4-0.6Pa) however, diseased liver tissue can be as stiff as 15 kPa (Janmey et al, 2011). In studies of cancerous tissue it has been observed that when cells form tumors, forces are applied to surrounding cells. These forces change the mechanical properties of the cellular environment and can potentially change the fate of cells (Tee et al, 2011). The effects of stiffness and other mechanical properties on cell behavior has not been studied as extensively as chemical signals, however these properties continue to prove significant.

Hydrogels

Hydrogels are hydrophilic polymer chains, crosslinked to form an interconnected network resulting in an insoluble gel. Due their crosslinked structure, hydrogels can absorb several hundred up to one thousand times their dry weight in water giving them solid-like properties. This means hydrogels have a defined shape and mechanical properties, such as an elastic modulus. However, hydrogels also exhibit liquid-like properties such as the ability of
solute molecules to diffuse through the matrix. The properties of a hydrogel can be manipulated in a variety of ways. The modulus can be altered by increasing concentrations of a particular polymer or crosslinking agent, or by changing the density of crosslinking. A hydrogel can become irreversible by covalently crosslinking or reversible by being physically crosslinked. Degradability of the gel can be altered based on the selection of polymers or introduction of degradable crosslinks (Lee et al, 2004).

Hydrogels are currently used in a variety of biomedical applications including soft contact lenses, drug delivery systems, wound healing, cartilage repair, and most recently as substrates for tissue engineering (Hoffman, 2012). The ability of a hydrogel to swell and retain water, its porous matrix, tunable mechanical properties, and biocompatibility, provides reason to further investigate gels for use as scaffolds and substrates on which to grow cells. Hydrogel properties are similar to soft biological tissue ECM making this material an ideal candidates for studying cells in vitro with in vivo properties accounted for (Jia, 2011). There are many possible combinations of materials that can be used (natural vs. synthetic, copolymer vs. homopolyer, crosslinking methods) in the fabrication of hydrogels for cell culture. The following section provides background for potential materials that have been chosen for the design of this hydrogel.

Polyacrylamide

Polyacrylamide has become a common material in hydrogel fabrication; this polymer also has many advantages in cell culture applications. By changing relative concentrations of the monomer acrylamide and crosslinker bis-acrylamide, the stiffness of the gel can be varied, allowing for a wide range of controllable mechanical properties (Tse et al, 2010). Polyacrylamide can form thin, translucent gels, ideal for observing cell interactions in culture.
In order to fabricate a polyacrylamide hydrogel, crosslinking is necessary. A unique trait of polyacrylamide hydrogels is the thermal solution-gel transition. At lower temperatures polyacrylamide can be a liquid; it solidifies, forming a gel, at body temperature. Polyacrylamide crosslinked hydrogels provide mechanical support throughout the gel, preventing excessive amounts of shrinkage from occurring (Muniz et al., 2001). Another advantage of polyacrylamide gels is that changing the mechanical properties will not alter hydrogel surface chemistry. This is important in cell culture studies that are trying to demonstrate cell dependence on ligand density, when cells are in movement. In cell culture it is critical that cells can adhere to the hydrogel. Nonspecific binding of cell surface receptors is generally negligible with polyacrylamide, meaning only adhesive molecules that are chosen may be attached to the surface of the gel. A protein coating such as collagen must be added to the surface of a polyacrylamide hydrogel to allow for cell adhesion (Tse et al., 2010).

Polyacrylamide hydrogels are fabricated by combining different concentrations of acrylamide and bis-acrylamide, then polymerization is initiated through the use of chemical or photoinitiator compounds. These compounds aid in polymerization by binding the crosslinker, bis-acrylamide to the monomer acrylamide (Tse et al., 2010). Disadvantages of polyacrylamide include the inability to readily absorb proteins and a need to covalently bind proteins in order to form a hydrogel.

Poly(ethylene glycol) diacrylate (PEG)

Poly(ethylene glycol) (PEG) has been used extensively in hydrogel applications including tissue engineering, controlled drug release, and cell culture. Its high hydrophilicity, biocompatibility, and easily tunable mechanical properties makes it a very attractive candidate in hydrogel fabrication. Mechanical and swelling properties can be easily altered by changing the
PEG chain length or varying the degree of crosslinking with UV exposure (Beamish et al., 2010). PEG diacrylate utilizes a single component as both a crosslinker and the crosslinked chain. This creates a dense network of crosslinks without chemical reactants being absorbed and later released by the gel, potentially harming cells plated on it. Photopolymerization can be employed to form PEG hydrogels. Many studies have been conducted altering the ratio of PEG to water to adjust the swelling (Beamish et al., 2010). PEG does not absorb proteins and will not allow for cell attachment unless modified. Collagen, gelatin or another natural polymer must be added to the surface to allow cell adhesion.

Stiffening Methods

In addition to selecting a polymer with favorable mechanical properties, there are various techniques that can be employed not only to finely tune the properties of the bulk material, but also achieve controlled regions of stiffness required for this project. This section will focus on the use of ultraviolet (UV) light for crosslinking of polymer chains to increase stiffness. Alternative means of creating controlled regions of stiffness will also be explored.

UV Crosslinking

In a laboratory setting, ultra violet crosslinking is achieved using a UV lamp at a specific wavelength and a photoinitiator to activate a photo-reactive crosslinking reagent. By varying the wavelength of ultraviolet light and the amount of exposure time, one can finely tune crosslinking within a material (Thermo Scientific, 2013).

Irgacure Photoinitiators

Irgacure photoinitiator is a component of the UV curing process. When added to solution, 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone, also known as Irgacure 2959, decomposes into free radicals and initiates the polymerization process due to the
absorption of light. This photoinitiated polymerization process involves UV light of a wavelength of 365nm which catalyzes free radical polymerization of acrylamide and bis-acrylamide by cleaving the Irgacure 2959 molecules into free radicals. Chemical crosslinking, using the substances Tetramethylethylenediamine (TEMED) and Ammonium Persulfate (APS), is inconsistent in its ability to control spatial parameters and mechanical properties therefore, UV light and Irgacure are used as a more consistent and controllable means of crosslinking. Drawbacks to Irgacure 2959 include its low solubility in water and relatively narrow excitation spectrum (Tse et al, 2010).

Controlling the Regions of Stiffness

As previously stated, UV crosslinking on its own can change the mechanical properties of a hydrogel. In order to create differing regions of stiffness within the same hydrogel, a method of blocking UV light from particular regions of the gel is necessary. Sunyer et al. developed a method to create a gradient of stiffness across a hydrogel using ultraviolet light. An opaque film was positioned over the material, then pulled at a constant speed while the entire set up was exposed to UV light (Sunyer et al, 2012). This allowed the far end of the material to be exposed for a longer period of time, increasing the amount of crosslinking and yielding a stiffer region, than the near end which was only exposed to the UV light for a short time. Although a gradient of stiffness is not the goal of this project, the technique of the opaque mask and exposing different regions of the same material to different amounts of UV light, is one possible method for acquiring controlled regions of stiffness.

Transglutaminases

Enzymes often used in cross-linking are transglutaminases. These enzymes depend on calcium and catalyze a reaction between various polymers and solutions. There are numerous
forms of transglutaminases, many of which catalyze the formation of hydrogels. Polymer hydrogels formed by tissue transglutaminase crosslinking are continuously changing and adapting with new findings. Research has been conducted on the role transglutaminases have in crosslinking hydrogels. Current studies suggest that transglutaminase cross-linked hydrogels may be used as surgical tissue adhesives (Hu et al, 2005).

A study completed by the Department of Chemical and Biomolecular Engineering at the University of Maryland created a hydrogel from gelatin that was thermally stable by using a transglutaminase, microbial transglutaminase (mTG), for crosslinking. This protein is not harmful and is often used in food manufacturing as it has been approved by the U.S. Food and Drug Administration (Yung et al, 2007). Gels formed using mTG as a crosslinking enzyme are resistant to thermal degradation. The mTG-cross-linked-hydrogels are also biocompatible, a necessary component in cell culture. Through this method of crosslinking, a denser hydrogel can be created, which will reduce susceptibility to degradation. Utilizing transglutaminases to form cross-linked polymer hydrogels is an effective means of producing gels with varying mechanical properties.

Measuring Techniques

An important aspect of this project is the characterization and quantification of the varying mechanical properties within the fabricated hydrogel.

Atomic Force Microscopy

A commonly used technique to measure the elastic modulus (stiffness) of a material is Atomic Force Microscopy (AFM). The basic framework of an atomic force microscope consists of a cantilever with a nanometer scale pyramidal shaped tip, which is moved over the surface of a material. A laser is shined on the top surface of the cantilever and the reflection of the laser
beam is measured with a photodetector. As the cantilever is moved up and down, in response to
the surface topography, the angle of the laser reflection changes and is analyzed to create an
“image” of the surface (Angewandte Physik, 2012). A schematic of the AFM setup is shown in
Fig. 2-1.

Figure 2-1: Atomic Force Microscopy Setup

There are several types of AFM available, but for measuring mechanical properties either
force mode or PeakForce quantitative nanomechanical mapping is used. In force mode, the tip of
the cantilever is moved over the surface of the material as in AFM imaging, however it is also
moved vertically over each point. The force with which the tip presses on each point can be
controlled and the distance of deflection it causes in the sample can be measured. From these
measurements, elastic modulus can be calculated (Shi et al, 2012). A more recent technology
known as PeakForce quantitative nanomechanical mapping is similar in its overall mechanism
but oscillates the cantilever tip vertically at a controllable frequency. This technique allows for
more data points per area and improves the precision and accuracy of measurements (Shi et al,
A major drawback with these methods is that the material being measured can be altered. The cantilever tip can scratch the material surface or cause imperfections if too much force is generated. In most cases however, the amount of force applied can be controlled and relatively little force needs to be applied to obtain these measurements.

**Magnetic Resonance Elastography**

Another method for characterizing the elastic modulus (stiffness) is Magnetic Resonance Elastography (MRE). MRE creates a two-dimensional, color-coded map of the material based on stiffness measurements. This method is currently being considered as a diagnostic tool for tumor detection, especially in regions of the body, like the brain, where manual palpation is not possible (Manduca et al., 2000). MRE technique consists of exposing a material to controlled magnetic waves, which induces a shear stress on the material. The original wave and the returned wave are measured and the phase shift is used to calculate the strain caused in the material (Manduca et al., 2000). From the measured stress and strain values, elastic modulus can be calculated. These values are then used to create images that show the distribution of stiffness within the material as shown in Fig. 2-2 (Manduca et al., 2000). A disadvantage to this method is that the imaging capabilities are on the scale of millimeters. This project requires greater accuracy.
Fig. 14.  MRE elastogram of the breast of a patient with a 4 cm diameter, biopsy-proven breast cancer. The image was obtained with shear waves at 100 Hz, applied to the skin of the medial and lateral aspects of the breast (Laumierc et al., 1999). The field of view is approximately 10 cm and the section thickness is 5 mm. The MRE elastogram indicates that the shear stiffness of the tumor in the posterior-medial aspect of the breast (arrowhead) is substantially higher than that of normal fibroglandular and adipose tissues in the breast.

Figure 2-2: Magnetic Resonance Elastography
Chapter 3 : Project Strategy

Initial Client Statement

The team’s initial task was to develop a method to fabricate a hydrogel with controlled heterogeneity in stiffness. A transparent gel should be created so that cells can be imaged using a regular light microscope. The gel should be uniform in thickness, about 100-500 microns, but preferably in the 100-200 micron range. The gels should be easily mounted on a microscope cover glass slip. It should be possible to control the size, shape and stiffness of the heterogeneous regions of the gel. A measurement technique should be standardized in order to consistently characterize heterogeneity. A method should be developed to visually distinguish the heterogeneous regions across the gel, using a microscope. Cell studies should be conducted on the gels.

The design team discussed the initial client statement together. After completing basic background research, the team met with the client in order to gain further understanding of the propositioned task. This meeting provided more background on the project details and helped form specific project objectives and constraints. This then helped the group to refine the initial client statement.

Objectives & Constraints

After gaining a detailed understanding of the project, a list of objectives was developed based on the client need. These objectives outlined the characteristics and standards of the design that were necessary to meet both the user requirements and expectancies of the client. The objectives for the design are for it to be measureable, biocompatible, reproducible, stable, able to be manufactured, and have controllable heterogeneity. These objectives were compiled in an
objective tree, with categories and sub-objectives adding further detail as shown in Fig. 3-1.

The first objective identified was for the design to be measurable. This includes both the gel surface and the cells plated on the gel. This stipulates that once cells are cultured onto the gel, the area, migration and stiffness of those cells need to be measured. An important aspect of this objective is accuracy. If the group is able to satisfy all other objectives, but design properties cannot be accurately measured, then the data collected is unserviceable for both the project group and any future researchers that would potentially use this design. Another objective is for the device to be biocompatible. This is of importance, because if cells are plated on the hydrogel but unable to grow or survive in culture, then it will not be possible to gather data. Another objective is for the hydrogel to have controllable heterogeneity. This will enable the group to investigate how cells plated on the hydrogel will respond to heterogeneous materials, an essential aspect of the project. It is necessary for the size and stiffness of the hydrogel to be controllable, because this allows the group to collect a larger span of data regarding how the cells interact. A sub-objective of controllable is identifiable. This will come into play when the team is using a microscope to visually determine the heterogeneity across the gel. Reproducible was identified as an objective; if the team is only able to create a single hydrogel that exhibits all of the...
necessary characteristics, it will not be applicable to researchers. Creating a standard protocol for fabricating the hydrogel will ensure that the team is able to reproduce the design. Another objective is for the design to be stable, more specifically the hydrogel should be non-degradable. This is critical for cell culture in which researchers may study cells for extended periods of time. If the hydrogel degrades prior to gathering necessary data, the design would be inadequate to researchers. The final objective is for the designed gel to be manufacturable. The hydrogel should be made using readily available materials and simple to create. Improving simplicity will increase the manufacturability of the hydrogel and increase the appeal of this design for researchers and potential buyers.

The team created a pairwise comparison chart to rank objectives in order of importance as shown in Fig. 3-2. All objectives were ranked against one another, using a numbering system of 0, 0.5, or 1. Higher numbers indicate that the objective is of greater significance. Biocompatible was ranked as the most important objective, followed by controllable heterogeneity. Measurable and reproducible were tied next, then stable, and finally able to be manufactured.
Once the team finalized the design objectives, a list of constraints was compiled. One
constraint is the timeframe for project completion; the team was given one academic year to
complete all necessary requirements for the project. There is a budget of $156.00 per student for
purchasing any necessary materials in order to complete the design. The size and range of
stiffness is also constraints the group will need to consider while working on the design. The
stiffness of the gels should represent soft body tissues and fall in the range of 0.1-100kPa. If the
group works without these constraints in place, the final design will not be acceptable for the
client and the developed hydrogel would be inadequate.

Though one academic year is a significant constraint for the design project, creating strict
deadlines and limiting time spent on brainstorming design ideas will help the team remain within
the time limit of the project. The team believes testing different hydrogel fabrication techniques

<table>
<thead>
<tr>
<th>Objective</th>
<th>Measureable</th>
<th>Controllable Heterogeneity</th>
<th>Reproducible</th>
<th>Stable</th>
<th>Able to be Manufactured</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measureable</td>
<td>X</td>
<td>0</td>
<td>½</td>
<td>1</td>
<td>1</td>
<td>2 ½</td>
</tr>
<tr>
<td>Controllable Heterogeneity</td>
<td>1</td>
<td>X</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Biocompatible</td>
<td>1</td>
<td>1</td>
<td>X</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Reproducible</td>
<td>½</td>
<td>0</td>
<td>0</td>
<td>X</td>
<td>1</td>
<td>2 ½</td>
</tr>
<tr>
<td>Stable</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>X</td>
<td>1</td>
</tr>
<tr>
<td>Able to be Manufactured</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>X</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 3-2: Pairwise Comparison Chart
in the laboratory will be most beneficial, once enough background information has been
gathered.

Keeping within the budget is less of a concern for the team. However, if testing proves
that expensive materials are necessary to create the final design, this will pose more of a
challenge. The team will re-evaluate designs that are more costly in an attempt to develop a
successful design with less expensive materials, and a more feasible design price. Keeping in
mind the manufacturability of the gel, a constraint on the cost to fabricate each gel was imposed.
The team must be able to produce gels for less than $1 for each gel.

The size of the hydrogel is should be 100-200 microns in thickness. The team will also
have to control stiffness values within the hydrogel. This will pose a challenge for the team, as a
design must be developed so that stiffer regions on the gel can be created while maintaining
softer regions of stiffness. This stiffness variation is necessary to mimic tumor like conditions
within the human body. Stiffness values must correlate to organ and tumor tissue stiffness
similar to human body tissue (0.1-100kPa).

The final constraint for our design was that Atomic Force Microscopy must be used to
measure gel stiffness. An atomic force microscope was made available to the team to verify gel
stiffness.

Revised Client Statement

This project aims to design and fabricate a hydrogel with controllable heterogeneity in
stiffness. The hydrogel should be transparent, uniform in thickness (<500 microns), able to be
mounted on a microscope cover glass, and imaged using a light microscope. Size, shape, and
stiffness of heterogeneous regions should be controllable and visually apparent. Cell behavior on
the developed gel specifically morphology will be examined.
Project Approach

Technical

The team chose polyacrylamide (PA) to fabricate the hydrogel. This polymer was selected because of its advantages. There is a vast amount of information available on PA because it has been used in cell culture and is considered a standard material for hydrogels in research. This polymer has tunable mechanical properties and can be compatible with cell culture when prepared appropriately. PA is also a readily available material for the team.

In order to ensure accurate hydrogel fabrication, with consistent size and shape, the team designed a fabrication chamber to produce uniform gels. This was an important consideration, as the hydrogels were designed for manufacturability. Once the chamber is exposed to UV light, polymerization begins, which causes cross-linking. A method to selectively inhibit ultraviolet light from reaching specific areas of the hydrogel was designed. This method needed to be impermeable to UV light, and protects the underlying hydrogel from ultraviolet radiation, except in the regions that we intended to become more crosslinked and therefore stiffer.

To study cell behavior and determine the biocompatibility of the fabricated hydrogels the team worked with NIH 3T3 cells. NIH 3T3 cells are a robust mouse fibroblast cell line; these cells will be used in testing initially to prove our developed gels are compatible to study cell behavior. All general laboratory precautions and aseptic cell culture technique were carried out in order to ensure the safety of the team and the validity of the results gathered throughout the experiments.

Financial

With any design project there are financial constraints that must be considered. Not only does this project have budgetary considerations but the cost per hydrogel must also be considered
because it is being designed for manufacturability. With this in mind the cost and time of production must be reduced but a high level of quality must be maintained. Since the design has the potential to be sold as a research tool the cost per gel must be minimized to less than $1 per gel to make it a desirable product.
Chapter 4: Alternative Designs

The project contains two, almost independent, aspects of design: the what and the how. The what component refers to the protocol for making a hydrogel; the how component refers to the system designed to make the hydrogel controllable and reproducible. For ease of explanation, these two parts to the design will be evaluated separately in this section.

Hydrogel Protocol

Needs Analysis

The initial client statement did not specify any particular type of hydrogel must be used, only that its stiffness could be controlled and that the hydrogel be biocompatible. Early on in the design process, the team narrowed its search to polyacrylamide hydrogels, as this material was widely used in the literature for similar applications, and it was available at no cost to the team. In order to develop an effective hydrogel that meets the needs of the user and its intended purpose, a thickness of less than 500 microns must be obtained. Ideally, the gel will be 100-200 microns when complete. The gels must have defined regions of stiffness; these regions should fall within the range of 0.1-100kPa to mimic body tissue.

Functions and Specifications

The function of the fabricated hydrogel will be to promote cell adhesion and growth. The specification that follows is that more than 90% of plated cells adhere and survive on the gel.

Preliminary Testing

Before selecting polyacrylamide as the final gel fabrication material, the team tested a number of hydrogel fabrication protocols using chemical and UV crosslinking. Hydrogels were successfully fabricated using these protocols. After identifying challenges within the different
protocols, a finalized hydrogel fabrication protocol utilizing UV crosslinking was developed by the team. Based on successful fabrication, the team moved forward using polyacrylamide gel.

Fabrication System

To produce accurate and reproducible gels with controllable heterogeneity, the team determined that standardized fabrication system was necessary. To meet these objectives, the team brainstormed, designed, and conceptualized several original systems, most of which include a chamber within which the gel will be fabricated.

Needs Analysis

In addition to making the gels accurately, in a repeatable fashion, the team also identified the need for gel manufacturability. In order to do this a system needed to be created that would allow for the fabrication of more than one gel at a time. Because ultraviolet light was chosen to crosslink the hydrogel, the fabrication chamber needs to be UV resistant. The chamber cannot deform due to UV exposure as this may alter gel size and shape. A means to block UV light from specific regions of the gel is necessary to control heterogeneity. The chamber design should cost less than $100 to print, due to financial constraints; less than $20 however, is preferable. The chamber also needs to hold coverslips securely and so gel consistency is maintained.

Functions and Specifications

The developed chamber must control gel swelling such that gel thickness does not exceed 500 microns. There is a need for the chamber to hold glass coverslips to satisfy the constraint that gels must be mounted on glass coverslips. The chamber needs to ensure minimal coverslip movement to make certain the gels will not be disturbed when placing the gels under UV light or removing the gels from the chamber. There is a need for the chamber to withstand UV light of 365nm, 85W (power), as this was the UV box provided.
Conceptual Designs

Based on the client need and team defined objectives, functions and constraints, several conceptual designs were developed as design alternatives for a hydrogel fabrication chamber. The designs that remained conceptual designs are discussed in this section. One component that remained consistent throughout all designs was the means to control heterogeneity. The team designed a UV resistant mask. The mask design allows for defined regions to be exposed to UV light while blocking the remainder of the gel. These masks provide a versatile way to define and control regions of stiffness.

One of the earliest designs consisted of using the sandwich method to hold the gel, placing a mask directly on top of the sandwiched cover glass slips, and holding all of these components in place with metal clips in a chamber, as shown in Fig. 4-1. The sandwich method involves placing gel solution on one coverslip and putting the second coverslip directly on top of the first, creating a “sandwich” with the gel solution in between both coverslips. This design did not possess the necessary accuracy with respect to consistent mask placement, nor the desired control over hydrogel thickness.

![Figure 4-1: Chamber with Clips](image)

The next design was to make a Polydimethylsiloxane (PDMS) mold for hydrogel fabrication as shown in Fig. 4-2A. This concept involved creating the inverse of the desired
PDMS mold as a master using 3D printing technology. The 3D printed part would be used to create the desired profile in the cast PDMS mold. The bottom chamber of the PDMS mold would be filled with hydrogel solution and a treated coverslip would be placed directly above it. There would be a raised edge higher up the mold to hold a mask. One fault of this design is that the UV light source would have to come from above the chamber; the team had access to a UV box with a light source coming from below only. The primary reason an alternative design was chosen is due to anticipated difficulty of removing the hydrogel from the mold. A final shortcoming of this design was a way to hold the mask securely in place; as shown in Fig. 4-2A, the mask would simply sit on the top raised edge, and could easily be moved out of place. This issue was addressed in the next iteration of this design as shown in Fig. 4-2B. This design would also be cast from PDMS using a 3D printed inverse master. In this design a mask would be held securely in place.

![PDMS Chamber Design](image)

Figure 4-2: (A) PDMS Chamber Design, (B) Improved PDMS Chamber Design

**Alternative Designs**

**Original 3D Printed Design (1 chamber with thin wall)**

The team was able to create hydrogels of the desired size and thickness (fitting on a 25x25 coverslip and no greater than 500 microns in thickness) using the design shown in Fig. 4-
3. This chamber was printed in VeroClear on the Objet260 Connex Rapid Prototype Machine. This design contained a thin wall that separated the coverslips, but allowed room for a gel to form. Another aspect of this design was a larger slot for a mask to slide in over the coverslips. This improved the versatility of the design, allowing for different masks to be placed over the gels; the possibility of varying placements of the stiff regions was realized with this design aspect. A major drawback with this design was that after approximately ten hours of UV exposure, the thin wall separating the coverslips began to deform, making it difficult to place the coverslips within these slots. This design did not provide for adequate manufacturability. The chamber only allowed for the fabrication of one gel at a time; this was time consuming and tedious. In order to improve design manufacturability and to allow for sufficient data collection it was necessary to design a chamber that will allow for increased gel production.

Second 3D Printed Design (10 chambers with thin wall)

In order to improve the manufacturability of this design, the team developed a similar chamber to the one shown in Fig. 4-3. This new chamber, shown in Fig. 4-4, contained a thin
wall separating the coverslips, but had ten separate sections for hydrogel fabrication. Again this
design had a larger slot in order to maintain adequate spacing between the mask and the
coverslips. This design increased manufacturability by allowing for an increased number of gels
to be fabricated in the same amount of time. While these changes helped to improve the
manufacturability of the chamber, it exhibited mechanical flaws. Upon repeated exposure to UV
light, there was considerable residue buildup within the slots. This made it difficult to place the
coverslips within the slots. The resin also attached to the gels, causing them to be imperfect and
inconsistent.

![Final 3D Printed Design (10 chambers without thin wall)](image)

**Figure 4-4: Chamber for 10 with thin wall**

*Final 3D Printed Design (10 chambers without thin wall)*

The final chamber design removed the thin wall separating the two coverslips, shown in
Fig. 4-5. This allowed for a sandwich-method to be employed while creating the hydrogels,
which is a less challenging means of placing the polyacrylamide between the two coverslips. The
final chamber had ten sections for hydrogel fabrication, each with a larger slot above in order to
insert masks. Without the thin wall, the team was able to have the chamber printed on the
Dimension SST 1200es Rapid Prototype Machine, which allowed the chamber to be printed in ABS plastic, a less expensive material. The chamber was able to withstand repeated exposure to UV light without deforming. Residue was not observed in this chamber because of the material used and the method of printing. This enabled the team to create consistent hydrogels, while maintaining the versatility, accuracy, and manufacturability of this design.

Figure 4-5: Chamber for 10 with thin wall

Feasibility Studies

Given the time and financial constraints of the project, a 3-Dimensional printer was used to fabricate a chamber made of ABS plastic. Based on the given resources and constraints for the project the team determined this was the most feasible option. However, with additional time and resources, a metal printed chamber would be a more viable option. A fabrication chamber made of metal would provide increased UV resistance, durability, and accuracy. This design can be produced on a machine with higher precision than the Dimension machine used to fabricate the current design. Numerous metal printing resources were considered however (Table 4-1), the team did not possess the time or money to pursue this option.
Table 4-1: Metal Printing Prices

<table>
<thead>
<tr>
<th>Company</th>
<th>Process</th>
<th>Quote</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FedTech</td>
<td>Laser Cutting</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Stainless Supply</td>
<td>Laser Cutting</td>
<td>/</td>
<td>could not cut slots inside - straight cuts only</td>
</tr>
<tr>
<td>GPI Prototype</td>
<td>DMLS</td>
<td>Several hundred</td>
<td>minimum wall thickness 0.381mm</td>
</tr>
<tr>
<td>Solid Concepts</td>
<td>DMLS</td>
<td>$2,212.00</td>
<td>feature &lt;0.012&quot; thick will be considered on a best effort basis</td>
</tr>
<tr>
<td></td>
<td>Plastic 3D Printing</td>
<td>/</td>
<td>minimum feature 0.030&quot;</td>
</tr>
<tr>
<td>Incodema</td>
<td>DMLS</td>
<td>$1,756</td>
<td>made in 2 pieces, welded together</td>
</tr>
<tr>
<td>Fineline Prototyping</td>
<td>Micro Fine Metal Direct</td>
<td>$1,900</td>
<td>10 slots</td>
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<td></td>
<td></td>
<td>$1,200</td>
<td>4 slots</td>
</tr>
<tr>
<td>Shapeways</td>
<td>Stainless Steel</td>
<td>$471.65</td>
<td>not certain they are capable of producing thin feature</td>
</tr>
<tr>
<td></td>
<td>Ceramic</td>
<td>$26.37</td>
<td></td>
</tr>
</tbody>
</table>

The iterative process used by the team to develop a fabrication chamber allowed for improvements to be made to the chamber that could not be foreseen in the conceptual design stage. The team utilized rapid prototyping technology to identify issues with the preliminary designs and applied changes to future design alternatives.

**Decisions**

There were a number of decisions that had to be made regarding chamber design. Decisions made would affect the final outcome of the polyacrylamide gels.

The first decision was choosing between a PDMS cast mold and a 3D printed mold. The PDMS mold would be less durable than a 3D printed part and would require multiple castings. The 3D printed design was also much simpler to produce than a PDMS mold. Ultimately the team selected 3D printing as a means to fabricate the chamber. Two 3D printers were available to the team on campus, the Dimension SST 1200es and the Objet260 Connex. The Dimension prints in durable, ABS plastic. This option is less expensive than the Objet machine, but also less
accurate. The Objet machine provides increased accuracy, allowed for the thin wall feature to be printed. This machine prints VeroClear, which is a transparent material with decreased UV resistance. The team initially chose to print the chamber using the Object machine due to its increased accuracy. However, when the design changed to exclude the thin wall feature, the Dimension machine was used to fabricate the chamber in ABS plastic, reducing costs as well as providing and opaque material for UV resistance.

The decision to eliminate the thin wall feature was made based on the observed deformation of the wall in the VeroClear 10 gel chamber. The team determined that the thin wall was not vital to design, the swelling of the gel could be controlled without it. The spacing was kept the same as in the original design, but without the thin wall. This allowed coverslips to be inserted into the chamber via sandwich method. This larger space still prevented the coverslips from moving too far apart when the gel forms between, swells and expands. The new chamber cost less than one third of the VeroClear 10 gel chamber price.

The final component of the design that required important decisions were the masks. The mask could be created with any design pattern, the only limit being the tolerances of the 3D printer the mask is being printed on. The team created many potential mask designs, but ultimately, chose to fabricate masks with one 3 mm circle in the exact center of the gel. A size of 3 mm was chosen due to machine constraints. Masks could be printed for about $1 each using the Dimension 3D printer. Another consideration that led to the selection of this mask design was input from the Atomic Force Microscope technician. A hole was placed at the center of the mask to create symmetry in the final gel. This symmetry made the stiff region easier to locate. To improve UV resistance the team decided to paint the masks using black acrylic paint. This helped
to insure that no UV light was reaching the blocked regions intending to remain softer than the exposed regions of the gel.
Chapter 5: Design Verification

This section provides verification for each of our ranked objectives: biocompatible, controllable heterogeneity, measureable, reproducible, stable, and manufacturable. Observation of the degradation of gels over time determined the stability, live/dead assays confirmed biocompatibility, Atomic Force Microscopy data demonstrates the controllable heterogeneity, and that gels were measureable. Fabrication of hydrogels using the developed protocol throughout the course of the project confirmed the reproducibility and manufacturability of the system. Also included are methods to incorporate fluorescein o-acrylate into the gel to visually identify stiff regions of the gels. Finally, testing was completed using NIH 3T3 to study cell behavior on heterogeneous gels.

Hydrogel Fabrication Protocol

The following protocols were created to satisfy the objective of reproducible hydrogel production.

Preparing Coverslips

Two coverslips were used to make one gel. The gel attached to one hydrophilic coverslip while the other was removed after gel polymerization.

1. Coverslips were oxygen plasma treated for removal of impurities on the coverslip surface.

2. All coverslips were completely submerged in 1% APTMS for 3 minutes to create a hydrophobic surface that the gel will not adhere.

3. Coverslips were removed from APTMS, rinsed with deionized (DI) water and dried.
4. One half of the coverslips were soaked in 0.5% Glutaraldehyde for a minimum of two hours in the refrigerator. This made the coverslips hydrophilic so that the gel would attach.

5. Coverslips were removed, rinsed with DI water, and air dried.

Preparing Gel Solution
1. To obtain a gel of a desired stiffness (listed in the far right column of the table below) the appropriate volumes of 40% stock acrylamide, 2% stock bis-acrylamide, and DI water were mixed.

<table>
<thead>
<tr>
<th>Acrylamide %</th>
<th>Bis-acrylamide%</th>
<th>Acrylamide from 40% stock solution (ml)</th>
<th>Bis-acrylamide from 2% stock solution (ml)</th>
<th>Water (ml)</th>
<th>E ± St. Dev. (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.03</td>
<td>0.75</td>
<td>0.15</td>
<td>9.1</td>
<td>0.20 ± 0.03</td>
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<td>3</td>
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<td>0.75</td>
<td>0.3</td>
<td>8.95</td>
<td>0.48 ± 0.16</td>
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<td>1.10 ± 0.34</td>
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<tr>
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<td>0.75</td>
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<td>1.78 ± 0.19</td>
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<td>1.16 ± 0.54</td>
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<td>2.01 ± 0.75</td>
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<tr>
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<td>0.75</td>
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<td>2.55 ± 0.17</td>
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<td>3.13 ± 0.42</td>
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<td>1</td>
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<td>7.5</td>
<td>3.24 ± 0.58</td>
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<td>0.15</td>
<td>8.6</td>
<td>1.00 ± 0.31</td>
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<td>8.25</td>
<td>3.15 ± 0.85</td>
</tr>
<tr>
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<td>0.75</td>
<td>8</td>
<td>4.47 ± 1.19</td>
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<td>19.66 ± 1.19</td>
</tr>
<tr>
<td>8</td>
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<td>2</td>
<td>2.4</td>
<td>5.6</td>
<td>40.40 ± 2.39</td>
</tr>
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<td>0.3</td>
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</tr>
<tr>
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<td>2.5</td>
<td>0.5</td>
<td>7</td>
<td>10.61</td>
</tr>
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<td>0.75</td>
<td>6.75</td>
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</tr>
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<tr>
<td>10</td>
<td>0.3</td>
<td>2.5</td>
<td>1.5</td>
<td>6</td>
<td>34.88</td>
</tr>
</tbody>
</table>

*This table shows the relative concentrations of acrylamide and bis-acrylamide and their expected modulus of elasticity after polymerization in water. Acrylamide and bis-acrylamide can be kept premixed in solution for weeks to months at 4°C. At least 3 hydrogels per sample and 50 indentations per hydrogel were assessed for these measurements.*
2. 0.05mg/ml of Irgacure 2959 was added to the gel solution. The tube was covered in foil to protect the Irgacure from the light until it was ready to be used. The solution was put into a water bath (37°C) for 10 minutes to dissolve the Irgacure into the solution. After it was removed from the water bath, the solution was vortexed until all of the Irgacure was completely dissolved.

3. The gel solution was vacuumed for 20 minutes to remove all dissolved gases. This enhances polymerization.

Gel Fabrication

1. 300µl of the gel solution was added to the glutaraldehyde treated coverslip.

2. An APTMS treated coverslip was placed over the gel solution to create a coverslip-solution-coverslip “sandwich”.

3. The coverslips were added to the chamber with the APTMS treated coverslip closest to the mask.

4. The chamber was placed in the Bio Rad Gel Doc 1000 UV box and exposed to 365nm wavelength UV light at 85 W power. The box was turned off after a predetermined exposure time. The mask was added to the chamber to inhibit UV light from reaching certain regions of the gel. This allowed the exposed regions to be further polymerized.

5. After polymerization was complete, the gels were rinsed three times with DI water.

6. A 1 mg/ml solution of (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) HEPES buffer and Sulfo-SANPAH prepared for each gel. 250µl of solution was used for each gel. 2µl Dimethyl Sulfoxide (DMSO) was added to the 250µl of HEPES-Sulfo-SANPAH solution to aid in the dissolution Sulfo-SANPAH.

7. 250µl lightly was dropped onto the gel, covering the entire surface.
8. Gels were exposed to UV light for 6 minutes.

9. In a cell culture hood, the gels were rinsed 5 times with HEPES buffer. After the final rinse all liquid was aspirated.

10. 0.1mg/ml collagen solution was prepared and 250µl of the solution was lightly dropped onto the surface of the gel, covering the entire surface.

11. The gel was either left at room temperature for 2 hours or left in the refrigerator overnight before cells could be plated.

Hydrogel Degradation

To test the stability of the hydrogels a basic degradation test was completed. Four gels were fabricated using a 19.0 kPa gel solution and polymerized for 20 minutes. The gels were stored in DI water at room temperature on the laboratory shelf. Gels were observed weekly and no signs of degradation were seen after 28 days, as shown in Fig. 5-1. No gel debris was observed at any time point. Cells were plated on these gels and confluent layer formed. After a 28 day storage period it was confirmed that the hydrogels supported cell viability.

Figure 5-1: Polyacrylamide Hydrogel
Biocompatibility Testing

Hydrogels must be biocompatible to support cell studies on the developed gels. The gels had to be tested to investigate if the cells are viable once plated on the gels. Cells were plated at a density of 100,000 cells per gel. Cells were given three days to attach, spread, and proliferate on the gel. Cells were trypsinized and removed from the gels per proper cell culture protocol. 40µl of the cell suspension was removed and added to 40µl of Trypan blue. The solution was mixed by repeated pipetting to create a homogeneous mixture. The solution was then placed into a hemocytometer. Cells were counted and viability was determined based on dead (stained blue) or alive (no color) cell counts. Calculated results determined an average cell viability of 93% (n=3).

Hydrogel Characterization: Atomic Force Microscopy (AFM) Data

Gels were measured using AFM to determine stiffness profiles of the fabricated gels. Homogeneous gels were produced to determine the relationship between gel stiffness and exposure time. Heterogeneous gels were fabricated using masks (as previously described) and were measured to determine a stiffness profile. Effects of the exposure time on stiffness within the same gel was determined.

Homogeneous Gels

Several (n=5) gels were prepared using the gel fabrication protocol described above with acrylamide and bis-acrylamide concentrations anticipated to achieve a stiffness of 19.66 ± 1.19 kPa. Each gel was exposed for either 1, 2, 10, 15, 17, or 20 minutes. Gels were measured at 5 random points per gel. Each time point on the graph represents the average of 25 measurements (Fig. 5-2). It appears that a linear relationship between UV exposure time and stiffness exists with a linear coefficient of 0.9451.
Heterogeneous Gels

Heterogeneous gels were fabricated using the same protocol as previously described in conjunction with the UV resistant mask. The entire gel was exposed to UV light for one minute, then the mask was inserted to the system, and exposed for an additional nine minutes. The stiff region had a total UV exposure time of ten minutes and the soft region, one minute. The gel was measured using AFM starting at the center of the gel, taking a measurement every 200 microns, in a straight line to a distance of about 3.5 mm from the center, shown in Fig. 5-3. The region exposed for ten minutes ranged in stiffness from 10 – 12 kPa. There was a transitional distance of about 700 microns where stiffness decreased as a function of distance. After the transitional region leveled off, the region exposed for one minute was measured to be less than 2 kPa.
An additional stiffness profile is shown in Fig. 5-4. This gel was prepared and measured in the same manner as the gel described above, with the soft and stiff regions exposed for one and ten minutes.
Proof of Concept: Cell Testing

Preliminary testing was completed using NIH/3T3 cells cultured on the developed hydrogels to test cell morphology on heterogeneous gels. Cells were plated onto heterogeneous gels at a density of 100,000 and allowed to attach for 24 hours. Cells were fixed and stained using DAPI and Phalloidin fluorescent stains. Cells were then imaged using a fluorescent light microscope. Cells on the stiff region of the gel are shown in Fig. 5-5 and on the soft region in Fig. 5-6. Cells were plated on heterogeneous gels at a density of 300,000, fixed, and imaged using a light microscope. Images were taken at various regions of the gel as shown in Fig. 5-7.

Figure 5-5: NIH 3T3-Hard region (12kPa)-20X DAPI/Phalloidin
The following protocols were attempted to incorporate fluorescein o-acrylate dye into gels through UV exposure. The ability to visually identify regions of stiffness would provide useful and further cell behavior research.

The first protocol was as follows:

1. A gel solution was prepared as previously described.
2. 0.001g/ml of fluorescein o-acrylate was added to the gel solution.
3. The tube was covered in foil as to not expose the dye to light before it was activated in the gel. The tube was placed in a water bath (37 degrees Celsius) for 10 minutes to accelerate the dissolution of the dye. The solution was to completely dissolve the dye.

4. 250µl of the dye solution was added to the glutaraldehyde treated coverslip.

5. An APTMS treated coverslip was placed over the gel solution to create a coverslip-solution-coverslip “sandwich”.

6. The coverslips and mask were added to the chamber with the APTMS treated coverslip closest to the mask.

7. The chamber was placed in the Bio Rad Gel Doc 1000 UV box and exposed to 365nm wavelength UV light at 85 W power. The box was turned off after 15 minutes exposure time. The mask was removed from the chamber to allow the remainder of the gel to be exposed to the UV light for 5 minutes.

8. The gel was rinsed 3 times and stored in DI water.

9. The gels were viewed using a fluorescent microscope.

When viewed under the microscope, there was no noticeable difference in the intensity of the fluorescence of the stiff region (exposed to UV light for a total of 20 minutes) when compared to the soft region (exposed to UV light for 5 minutes).

A second method was attempted to selectively attach dye to the stiff region of the gel. The protocol was as follows:

1. Gels were polymerized for a brief period of time (exposure time depends upon the desired stiffness of the soft region of the gel). For the following test gels were exposed to UV light for 1, 2, 3, and 5 minutes.
2. A fluorescein o-acrylate dye solution was made by weighing 0.001g of dye and adding it to 10 ml of HEPES buffer. The tube was covered in foil as to not expose the dye to light before it was activated in the gel. The tube was placed in a water bath (37 degrees Celsius) for 10 minutes to accelerate the dissolution of the dye. The solution was vortexed to dissolve the dye.

3. The 10 ml HEPES-dye solution was added to a petri dish and the gel “sandwich” was submerged in this solution and allowed to soak overnight.

4. The gels were removed from the solution and coverslips were dried. Gels were placed into the chamber with a mask, as previously described, to further crosslink the exposed area of the gel and crosslink the dye to the exposed area as well.

5. The gels were exposed such that the stiff region of the gel would be exposed for a total of 10 minutes per gel. For example, the gel exposed for 1 minute initially was exposed to the UV light for an additional 9 minutes with the mask, while the gel initially exposed for 5 minutes was exposed for an additional 5 minutes with the mask.

6. Gels were rinsed with HEPES buffer three times and then stored in fresh HEPES buffer for observation under the fluorescent microscope.

When viewed under the fluorescent microscope the entire gel fluoresced.
Chapter 6: Discussion

The hydrogel fabrication approach is innovative in that gels with small controllable regions of stiffness have yet to be developed and used in cell behavior studies. Protocols exist to fabricate homogeneous hydrogels with different stiffness values; stiffness gradients within gels have also been produced. The versatility of the team’s hydrogel makes it unique; different shapes and sizes of stiffness regions are controllable with masks used to fabricate the gel. Regions are well defined and accurate. This approach provides consistency and accuracy better than competitors due to the fabrication techniques.

UV light exposure had a direct correlation to stiffness. Homogeneous gels with varying exposure times were prepared based on the developed protocol. The longer the exposure time, the stiffer the gel became, as shown in Fig. 5-2. It appears that a linear relationship between UV exposure time and stiffness exists with a linear coefficient of 0.9451. Gels can be produced to match research needs, such as a stiffness value for particular body tissue that may be of interest, by varying exposure time.

The consistency of the fabrication method is shown in the similarities between the stiffness profiles of Fig. 5-3 and Fig. 5-4. Both gels have a relatively linear stiffness value in the center of the gel. As measurements continue across the gel a region of variability exists. This region is approximately 700 microns, followed by the gel leveling out to a consistent value of less than 2 kPa. The 700 micron transition region is likely due to the scattering of ultraviolet light within the provided UV light box.

At the start of the project, a list of objectives and constraints was developed. The fabricated hydrogels had to be measurable; this objective was met as stiffness measurements using AFM were calculated for the gels. Heterogeneity was deemed controllable, as different UV light
exposure times yielded varying stiffness values in regions of the gel. Distinct gels exposed to UV light for the same amount of time were measured to have similar stiffness, with little variation.

When viewed under the fluorescent microscope it was hypothesized that the stiff region in the center of the gel would fluoresce, however this was not the case. The entire gel fluoresced, which did not allow for the visual identification of the stiff region in the center of the gel. Fabricated hydrogels were biocompatible as cells successfully adhered and proliferated on the gels. Further cell studies conducted on gels demonstrated potential applications for the design as a research tool to study cell behavior. NIH/3T3 cells were plated at a density of 300,000 cells on to heterogeneous gels with a stiff region of 12 kPa and a soft region of 2 kPa. When observed at 5X objective, as shown in Fig. 5-7, the cell density was much higher on and around the stiff region of the gel when compared to the soft region. NIH/3T3 cells are accustomed to stiff substrates and therefore, it was expected that the cells would favor the stiff region of the gel. Figure 5-5 is a 20X image of NIH/3T3 cells stained with DAPI and Phalloidin fluorescent stains on the stiff 12 kPa region of a heterogeneous gel. The cells observed were larger and more spread when compared to the cells plated on the soft region (2 kPa), shown in Fig. 5-6. It should also be noted that there is a greater number of cells present on the stiff region compared to the soft region of the gel, despite the fact cells were plated at a uniform density. This result is likely due to the fact that cells are expected to proliferate on stiffer substrates, similar to cell behavior in tumors. These preliminary cell studies demonstrate that cell behavior changes as a result of substrate stiffness, and that this design can be a useful tool for research to study cell behavior as a result of substrate stiffness.

A consistent protocol was designed to ensure gels were reproducible; this was confirmed by the team’s ability to manufacture gels throughout the project. The developed protocol results
in consistency and low variability in the quality of the gels. Gels also proved to be stable, as they could successfully support cell adhesion and proliferation after a 28 day storage period. The chamber’s design allowing the fabrication of ten gels at a time met the manufacturability objective.

The project design also satisfied identified constraints. The design was chosen, developed, and tested over one academic year (the given timeframe). The project remained within budget. The designed fabrication chamber was approximately $25.00 to produce and masks were fabricated for $1.00 each. Each gel produced cost less than $1.00 as shown in Table 6-1.

Table 6-1: Cost Breakdown

<table>
<thead>
<tr>
<th>Material</th>
<th>Bulk Cost</th>
<th>Cost per Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (Bio-Rad)</td>
<td>$49.00</td>
<td>$.20</td>
</tr>
<tr>
<td>Bisacrylamide (Bio-Rad)</td>
<td>$50.00</td>
<td>$.13</td>
</tr>
<tr>
<td>Coverslips (VWR Int.)</td>
<td>$92.00</td>
<td>$1.84</td>
</tr>
<tr>
<td>APTMS (Sigma-Aldrich)</td>
<td>$54.60</td>
<td>$.82</td>
</tr>
<tr>
<td>Glutaraldehyde (Crescent)</td>
<td>$56.70</td>
<td>$.57</td>
</tr>
<tr>
<td>Igracure 2959 (BASF)</td>
<td>$41.20</td>
<td>$.01</td>
</tr>
<tr>
<td>Collagen (Krackeler Scientific)</td>
<td>$176.00</td>
<td>$.19</td>
</tr>
<tr>
<td>Sulfo-SANPAH (ProteoChem)</td>
<td>$157.00</td>
<td>$3.93</td>
</tr>
<tr>
<td>HEPES Buffer (Amresco)</td>
<td>$122.78</td>
<td>$.59</td>
</tr>
<tr>
<td>DMSO (Amresco)</td>
<td>$34.06</td>
<td>$.01</td>
</tr>
</tbody>
</table>
Hydrogel stiffness was measured between 1 and 20 kPa. These stiffness ranges are similar to those found within the body, both healthy and tumor tissue. Hydrogel thickness did not exceed 250 microns, which remained within the design constraints.

Human error is one limitation of the project. To fabricate the hydrogel, components were weighed and measured by the team in lab, with provided equipment. Inaccuracy and inconsistencies in laboratory equipment is an additional source of error. In cell testing, human error in cell counts and calculation inaccuracies may exist. Gel uniformity is assumed in the gel because masks and coverslips of the same size were used in fabrication. The gels were prepared and placed in chamber compartments of the same size and shape. All gels were prepared using a sandwich method with the same amount of liquid so even distribution is expected on the glass.

Economics

The final design has a direct economic impact on scientific research, specifically those studying cellular behaviors and interactions. There is a need in the scientific community for more affordable ways to grow cells in culture that better mimic in vivo conditions. The design will provide researchers with hydrogels that can meet the specific needs of their individual research. Compared to similar technology on the market, these hydrogels will be more individualized and can be sold at a fraction of the cost. Current multi-well culture plates with stiffness less than plastic are sold for $50-$100. The design can produce heterogeneous gels for less than $1.00. This new fabrication method for hydrogels will make cell behavior studies more affordable to research laboratories, allowing for more experimentation at a lower cost.

Environmental & Societal Impact

This design would have little environmental and societal impact. Some of the materials used in the hydrogel fabrication process are toxic. However, if handled appropriately using
proper safety precautions and laboratory procedures, this risk is minimal. Fabrication of the gels requires small amounts of the chemicals used, keeping the environmental impact to a minimum. The final product is biocompatible and a minimal risk for the user. The societal impact is indirect, as the product would only be used by scientific researchers. Ideally this hydrogel design will allow for more realistic in vitro experimentation leading to an increased understanding of cell behavior. This could provide potential improvements to current tumor treatment methods and other disease treatments that would impact society more directly.

**Political Ramifications & Ethical Concern**

There are currently no foreseen political ramifications of the design. This product would primarily impact the scientific community with furthering their research in a more cost efficient way. Ethical concerns are also minimal, as the goal of the design is to assist in the development of improved disease treatments through cell behavior studies researchers can conduct with the hydrogels.

**Health and Safety Issues**

As discussed in the environmental and society impacts section, there is minimal concern for public safety. The scientist fabricating these gels has minor exposure to toxic chemicals, but proper safety protocols have been described. The expected outcome of the research tool is an improvement in public health due to better detection and treatment of cancer.

**Manufacturability**

This design was developed with manufacturability in mind. The fabrication chamber can produce ten hydrogels at one time, and could be redesigned to produce more depending on the size of UV light space. The protocol is fairly simple and reproducible as well. This approach will provide consistency and accuracy better than competitors due to the fabrication techniques. The
design helps produce consistent gel thickness, shape, and size, all important features for manufacturability. Most hydrogel fabrication methods utilize chemical polymerization; this leads to more variability in the gel. The team’s developed method uses UV light, producing more controlled and accurate stiffness values. When addressing sustainability, as previously mentioned, there is potential danger in using some of the materials used to fabricate the hydrogel. These chemicals are used in small quantities however and the final product is biocompatible. The hydrogels are a onetime use disposable product however, the designed fabrication chamber and masks are reusable; gels can be produced with these repeatedly.
Chapter 7 : Final Design and Validation

The final design constructed by the team successfully met objectives defined during the design process. By revising the initial design in an iterative fashion, the team was able to create a final design that allowed for the fabrication of hydrogels, which were measureable, controllable in heterogeneity, biocompatible, reproducible, manufacturable, and stable.

The final chamber had slots for ten gels to be fabricated at one time. This increased the manufacturability of the device, whereas initial designs allowed for only one gel to be fabricated at one time. Another means by which the team improved the manufacturability of the design was by selecting readily available materials. Acrylamide and bis-acrylamide, along with other materials used, were available in the lab, and are easily accessible through online vendors. When ordering new masks or chambers overall wait time was no longer than four days. If a consumer wanted a variation in the mask it would be possible to obtain this within a week, which further confirms the manufacturability of the device.

The ten slots in the chamber enable the user to employ a "sandwich" method while creating the hydrogels. This simplified the overall process, creating an easier way to place the gel solution between two coverslips. While ease to the user was not a major objective the team sought to meet, increasing the simplicity of the process can increase the number of users that benefit from the design. A simpler method allows for less experienced researchers to use this chamber, which increases the consumer market for the design.

Another aspect of the final design is the thicker slot in order to insert masks into the chamber. This slot is located above the coverslips. Designed masks contain holes of varying sizes and location. By selecting specific masks geometries the user can control the size and location of the stiff regions on the hydrogels. Circles were the only shape this team chose to
create, however various shapes could be constructed depending on client need. Exposing the gels
to different UV times, while the mask is in place, creates hydrogels containing areas of stiffer
and softer regions. This creates gels with a controllable heterogeneity, another objective the team
met.

Atomic Force Microscopy was used to measure the stiffness of the hydrogels. Gels of the
same UV exposure time were sent in weekly to be measured. Hydrogels formed by varying UV
exposure times were also measured, in order to determine a correlation between stiffness and UV
exposure time. Data gathered from Atomic Force Microscopy showed that the gels exposed to
UV light at the same amount of time were of like stiffness. This data met another objective set by
the team, to create measurable hydrogels.

While fabricating the hydrogels the team followed an exact protocol, without variation, in
order to determine that the process was reproducible. The data collected using AFM determined
that the hydrogels fabricated were reproducible numerous times, while using the procedure
created by the team. The team was able to hydrogel reproducibility throughout the entire design
process, by maintaining a consistent and detailed protocol.

After creating gels using the chamber several were set aside to soak in DI water, in order
to assess the rate at which the hydrogels would degrade. After a period of 28 days there was no
noticeable degradation of the gels. This addressed one of the design objectives. If the gels
degrade, it shortens the time available to distribute the gels as well as the time customers have to
use the gels. Creating stable gels that did not quickly degrade helped to increase the
manufacturability of the design.
Chapter 8: Conclusions and Recommendations

The team successfully established a method of constructing a hydrogel, which had both uniform thickness and controllable heterogeneity in stiffness. Creating a chamber for the hydrogels to be formed within, improved the overall manufacturability of the design while consistently creating gels of varying yet controllable stiffness. Fabricating hydrogels with controllable heterogeneity in stiffness will provide scientific researchers with a precise and consistent tool, which will aid in research within the biomedical field, regarding cell-cell interactions, cell behavior, and tumor studies. The fabricated hydrogels will provide researchers with the necessary means to complete experiments that can lead to a further understanding of cell behavior, and can aid in the discovery of new tumor treatment methods and experiments, as well as other disease treatments. This design will allow for the expansion of research and development within the field of cancer research, as described in Fig. 8-1.

![Figure 8-1: Overarching Goals](image)

While this device is novel in its potential for future research, there are a few recommendations the team has to further develop the design. Potential goals for this design would be to improve the hydrogel to more accurately represent body tissue in vivo. In particular, advancing the hydrogel from 2D to a 3D system would be beneficial. The more representative and accurate hydrogel would further research in and applications for cell behavior and tumor research; this could lead to additional discovery and improved therapies for disease.
Given additional time and finances, 3D metal printing of the fabrication chamber and masks is recommended. This would provide a more durable, UV resistant product. This will help confirm that neither the masks nor the chamber are altered upon exposure to UV light. This also helps to ensure that the hydrogels will not be contaminated due to residue from the chamber. Machinery used in 3D metal printing would also offer more precise and accurate measurements for the chamber and masks produced. With more advanced printing technology, masks could be printed that allow for smaller stiffness areas on the gel or a more intricate shape to be formed within these masks.

The team also recommends further investigation regarding improving the visualization of stiff regions within the hydrogel. Specifically, a means of integrating a fluorescent dye in order to visually distinguish regions of stiffness, either under a microscope or to the naked eye is suggested. Methods for incorporating fluorescein o-acrylate into the stiff regions of the hydrogels were tested using various protocols, but remained unsuccessful.

The fabrication of hydrogels with controllable heterogeneity in stiffness provides researchers with a tool that will aid in the fields of study of cell biomechanics and tumor research.
References


Mask 3mm center
Scale 4:1

Worcester Polytechnic Institute

Mask: 4 holes further apart

Size: A

Scale: 2:1

Weight:

Sheet 1 of 1
Appendix B: Alternative Mask Designs

All diameters = 500 microns (0.50 mm)

Distances between outer holes to center, adjacent
Top left = 250 microns (0.25 mm), 560 microns (0.56 mm)
Top right = 500 microns (0.50 mm), 910 microns (0.91 mm)
Bottom right = 750 microns (0.75 mm), 1270 microns (1.27 mm)
Bottom left = 1000 microns (1 mm), 1620 microns (1.62 mm)

Shortest distance between separate designs (between bottom left and right): 3.82 mm
Shortest distance to edge of mask: 7.36 mm
All diameters = 500 microns

Distances between outer holes to center, adjacent
Top left = 250 microns (0.25 mm), 380 microns (0.38 mm)
Top right = 500 microns (0.50 mm), 680 microns (0.68 mm)
Bottom right = 750 microns (0.75 mm), 970 microns (0.97 mm)
Bottom left = 1000 microns (1 mm), 1260 microns (1.26 mm)

Shortest distance between separate designs (between bottom left and right): 3.96 mm
Shortest distance to edge of mask: 7.44 mm (bottom left to left edge)
All diameters = 500 microns

Distances between outer holes to center, adjacent*
Top left = 250 microns (0.25 mm)
Top right = 500 microns (0.50 mm)
Bottom right = 750 microns (0.75 mm)
Bottom left = 1000 microns (1 mm)
*Distance between all holes in pattern the same

Shortest distance between separate designs (between bottom left and right): 4.19 mm
Shortest distance to edge of mask: 7.36 mm (bottom left to bottom edge)
Mask lines 500 microns thick varying distances 100-1000 microns

Slots = 500 microns wide (0.50 mm) constant

Distance between slots varies from 100 microns (0.100 mm) at bottom by increments of 100 microns up to 1000 microns (1 mm) at the top
Mask lines varying 100-1000 microns thick 500 micron distances

Thickness of slots vary from 100 microns (0.100 mm) at bottom by increments of 100 microns up to 1000 microns (1 mm) at the top

Distance between slots = 500 microns (0.50 mm) constant