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3D Hydrogel System with Continuous Stiffness Gradient

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Table of Contents

AUTHORSHIP 5

ACKNOWLEDGEMENTS 6

ABSTRACT 7

LIST OF FIGURES 8

LIST OF TABLES 9

CHAPTER 1: INTRODUCTION 10

CHAPTER 2: BACKGROUND 12

2.1 Tissue Engineering and Applications 12
2.2 Scaffolds 12
2.2.1 Properties 13
2.2.2 Importance of Environment 14
2.2.3 Hydrogels 14
2.2.4 Hydrogel Precursors 14
2.2.5 Properties 15
2.2.6 Cross-linking 16
2.3 Cell Response to Stiffness *In vivo* 20
2.3.1 Stiffness of Tissues *In vivo* 20
2.3.2 Effects of Stiffness on Cell Processes 20
2.4 Stiffness Gradient 21
2.4.1 Hydrogel Creation 22
2.4.2 Gradient Creation 22
2.4.3 Stiffness Measurement Techniques 24

CHAPTER 3: PROJECT STRATEGY 27

3.1 Initial Client Statement 27
3.2 Design Objectives 27
3.3 Design Constraints 30
3.4 Revised Client Statement 31
3.5 Project Approach 31
3.5.1 Project Considerations 32

CHAPTER 4: DESIGN ALTERNATIVES 38

4.1 Needs Analysis 38
4.2 Design Functions and Specifications 39
4.2.1 Functions 40
4.2.2 Specifications 40

4.3 DESIGN ALTERNATIVES **41**
4.3.1 POLYMER OPTIONS 41
4.3.2 GRADIENT GENERATION METHODS 42
4.4 CONCEPTUAL DESIGN **46**
4.4.1 DESIGN EVALUATION MATRIX 46
4.4.2 GROUP BRAINSTORMING 47
4.4.3 6 HATS EVALUATION METHOD 48
4.5 DECISIONS **49**
4.6 FEASIBILITY STUDIES & EXPERIMENTS **49**
4.6.1 CALCULATIONS 49
4.6.2 GELATION TIME STUDIES 51
4.6.3 AFM BASELINE STIFFNESS MEASUREMENTS 53
4.6.4 BIOCOMPATIBILITY OF POLYMER ASSESSMENT 53
4.7 MODELING **55**
4.8 PRELIMINARY DATA **55**
4.8.1 GELATION TIME DATA 55
4.8.2 STIFFNESS OF PRELIMINARY SAMPLES 57
4.9 OPTIMIZATION **58**
4.9.1 ADDITIONAL AFM MEASUREMENTS 58
4.9.2 HYDROGEL FORMULA MODIFICATIONS 58
4.9.3 ADDITIONAL GELATION TIME STUDIES 59

CHAPTER 5: DESIGN VERIFICATION **61**

5.1 BASELINE AFM MEASUREMENTS **61**
5.2 VALIDATION OF GRADIENT CREATION COMPONENTS **62**
5.2.1 VALIDATION OF GRADIENT 62
5.2.2 VALIDATION OF CELL FLOW 62
5.3 GRADIENT CREATION AND VISUALIZATION **63**
5.3.1 CREATION OF GRADIENT WITH CONJUGATES 63
5.3.2 ENCAPSULATION OF CELLS WITHIN GRADIENT 65
5.3 CELL CULTURE **66**
5.3.1 CELL CULTURE 66

CHAPTER 6: DISCUSSION **70**

6.1 GELATIN-HPA HYDROGEL STIFFNESS **70**
6.2 GRADIENT VISUALIZATION **71**
6.3 CELL BIOCOMPATIBILITY **71**
6.4 CELL BEHAVIOR **72**
6.5 DESIGN CONSIDERATIONS **74**
6.5.1 ECONOMICS 74
6.5.2 ENVIRONMENTAL IMPACT 74
6.5.3 SOCIETAL INFLUENCE 74
6.5.4 POLITICAL RAMIFICATIONS 74

6.5.5 ETHICAL CONCERN 74
6.5.6 HEALTH AND SAFETY ISSUES 75
6.5.7 MANUFACTURABILITY 75
6.5.8 SUSTAINABILITY 75
6.6 FINANCIAL CONSIDERATIONS 75

CHAPTER 7: FINAL DESIGN AND VALIDATION 78

7.1 FINAL DESIGN 78
7.2 DESIGN METHODS 78

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS 82

8.1 CONCLUSIONS 82
8.2 RECOMMENDATIONS FOR FUTURE WORK 83

REFERENCES 87

GLOSSARY 89

APPENDICES 90

APPENDIX A: GANTT CHART 90
APPENDIX B: 6 HATS METHOD EXERCISE 91
APPENDIX C: SOP: PREPARING GELATIN-HPA CONJUGATE 93
APPENDIX D: SOP: PREPARING GELATIN-HPA HYDROGELS 97
APPENDIX E: MICROFLUIDIC GRADIENT GENERATORS PREPARATION 99
APPENDIX F: CONDUCTING AFM ON GTN-HPA HYDROGELS 101
APPENDIX G: PLASMA TREATING 103
APPENDIX H: FLOWING GRADIENT-HYDROGELS 105
APPENDIX I: GELATION TIME DATA 107
APPENDIX J: ATOMIC FORCE MICROSCOPY DATA 108

Authorship

This project report, titled 3D Hydrogel System with Continuous Stiffness Gradient, was created with equal, cohesive efforts by Christopher Gorgone, Claire Hambright, Lindsay Hock, and Melanie Wiater. Each student deserves to earn equal credit for the authorship of this report.

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Abstract

Traditional 2D cell culture protocols poorly represent *in vivo* conditions. Methods to allow culturing of patient derived cells in scaffolds that closely mimic the 3D environment would be of great clinical value. Parameters to consider are tissue stiffness, chemical and stiffness gradients, and the ability to co-culture various cell types. This project aimed to create a 3D hydrogel system in a range of stiffness comparable to body tissues while allowing cell growth and differentiation in a 3D environment. The design utilized a fast gelling hydrogel and a cross-linker that allowed controlled stiffness. This was combined with a microfluidic gradient generator to produce a 3D hydrogel with a continuous stiffness gradient. This approach can be a valuable tool for tissue engineering research.

List of Figures

Figure 2.1: Source Sink Diffusion 22
Figure 2.2: Gradient Generator 23
Figure 2.3: Dynamic Mixing 23
Figure 2.4: Convective Spreading 24
Figure 2.5: Measurement through Atomic Force Microscopy 25
Figure 3.1: Objective Tree 28
Figure 3.2: Work Breakdown Structure 36
Figure 4.1: Dynamic Mixing Sketch 42
Figure 4.2: Gradient Generator Mold Sketch 43
Figure 4.3: Dynamic Flow Middle Sketch 44
Figure 4.4: Dynamic Flow Side Sketch 44
Figure 4.5: Radial Cross-linker Sketch 45
Figure 4.6: Vertical Diffusion Sketch 45
Figure 4.7: 1.96 mM H_2O_2 Gelation Results 56
Figure 4.8: 1.47 mM H_2O_2 Gelation Results 56
Figure 4.9: 0.98mM H_2O_2 Gelation Results 57
Figure 4.10: Test gels for 1%, 1.5%, and 2% gelatin-HPA. 60
Figure 4.11: Gelation time data 60
Figure 5.1: Average AFM Measurements for Gtn-HPA Hydrogels. 61
Figure 5.2: Gradients generated with water and food dye 62
Figure 5.3: Cells Flowing through Gradient generator 63
Figure 5.4: Gelatin-HPA percentage and H_2O_2 concentration in each syringe 64
Figure 5.5: Gelatin-HPA with H_2O_2 Gradient Gel ranging from softer to stiffer 64
Figure 5.6: Cells encapsulated within the 3D Hydrogel with a stiffness gradient 65
Figure 5.7: Mouse Fibroblast cells (3T3) in 3D Gel 67
Figure 5.8: Primary Human Fibroblast cells (2097 line) in 3D Gel 68
Figure 5.9: Mouse embryonic fibroblast cell line (STO) in 3D gel 69
Figure 6.1: Gel Conjugate Preparation Costs 76
Figure 6.2: Cost Analysis for the Creation of Gradient Gels 76
Figure 6.3: Cost Analysis for the Gradient Generator Creation 77
Figure 7.1: Final Design Methods 78
Figure 7.2: Syringe Pump Setup 81
Figure 7.3: Gradient Illustration 81

List of Tables

Table 3.1: Pairwise Comparison Chart……29
Table 3.2: Design Evaluation Matrix for Hydrogel Precursor……33
Table 3.3: Design Evaluation Matrix for Cross-linking Reagent……34
Table 3.4: Preliminary Design Evaluation Matrix for Gradient Generation Method……35
Table 4.1: Design Specifications……41
Table 4.2: Revised Design Evaluation Matrix for Gradient Generation Method……46
Table 4.3: 1.5% Gelatin-HPA Hydrogels……51
Table 4.4: 1.5% Gelatin-HPA Hydrogels (half volume)……54
Table 4.5: Preliminary AFM Measurements……57
Table 4.6: Additional AFM Measurements……58
Table 4.7: Revised Gelatin-HPA Hydrogel Formula……58
Table 4.8: Revised H_2O_2 Stock Solutions……59
Table 7.1: Gelatin-HPA Hydrogel Formulas……79
Table 7.2: H_2O_2 Stock Solutions……79

Chapter 1: Introduction

Cell development and behavior is influenced by various chemical and mechanical stimuli. Growth factors, surface characteristics, loading conditions, biochemical factors, and stiffness are some of the many factors that affect cell behavior. Studies suggest that among these stiffness is one of the more selective factors (Mason *et al.*, 2012; Breuls *et al.*, 2008). Stiffness plays an important role in deciding cell morphology, differentiation, proliferation, and striation. A current challenge in cell culture is that most cell culture studies are performed under conditions that are unrepresentative of native tissue stiffness.

The body exhibits a select range of stiffness: Brain tissue is one of the softest tissues, at approximately 0.1 to 1 kPa, while bone is one of the stiffest, at approximately 25 to 40 kPa (Tse and Engler, 2011). Cell culture is generally conducted using extremely stiff 2D polystyrene well plates that have two main limitations. First, these plates have a stiffness of 1 GPa, a value several magnitudes stiffer than what is experienced *in vivo* (Kolahi *et al.*, 2012). Second, these plates are 2D, which does not allow for the 3D cell growth, morphologies, and migration that occurs within the *in vivo* environment. To address these limitations, hydrogels are being developed to provide more realistic tissue-like environments.

Hydrogels provide a highly hydrophilic environment similar to native tissue conditions and can be easily modified to possess specific mechanical properties. Hydrogel scaffolds are currently being engineered and used in research to study cell behavior at different stiffness levels. A novel approach to survey the effects of stiffness on cellular behavior in a 3D environment is the use of hydrogels containing continuous stiffness gradients.

Various microfluidic techniques have been used to successfully create stiffness gradients in 3D hydrogels (Sant *et al.*, 2010). However, these techniques are intended for cell seeding on the surface of the hydrogel for 2D culture. A major challenge in developing cell laden 3D hydrogels is the paucity of a combination of biomaterials and cross-linkers that allow fast gelation times (<10 minutes) to allow uniform cell distribution in 3D hydrogels. Moreover, most of the currently available methods for gradient generation depend on cross-linking reactions using exposure to UV light.

In order to observe cell behavior most representative of cell behavior *in vivo*, an *in vitro* model needs to be developed that permits cells to experience natural levels of stiffness in a three-dimensional environment. The goal of this project was to overcome current cell culture limitations by designing a three-dimensional, stiffness-gradient containing, cell-encapsulating hydrogel. This design combined the use of the Gelatin-Hydroxyphenylpropionic acid (Gelatin-HPA) conjugate described by Wang *et al.* (2012) that gels rapidly, and a microfluidic gradient generator that produces a continuous gradient. The team formulated hydrogel precursors that enabled a stiffness gradient within the range of 300-1500 Pa. Once the design was completed, the gradient-hydrogels were tested and validated to ensure continuity and consistency of mechanical properties, as well as biocompatibility. Formation of a continuous gradient ranging within 300 to 1500 Pa will more accurately represent natural *in vivo* stiffness, allowing for a variety of cell types to be evaluated along very specific increments of stiffness.

This gradient-hydrogel can be used as an *in vitro* model for researchers to study how cells migrate, proliferate, and differentiate in response to different stiffness levels. The creation of an innovative three-dimensional cell encapsulating hydrogel with a stiffness gradient has the potential to revolutionize cell culture due to its many potential applications. This scaffold could allow for the co-culturing of cells to create more successful cell therapies, the creation of organoids for drug and device testing, and could provide a better simulation of an *in vivo* environment. With these many applications in mind, this gradient-hydrogel for cell encapsulation could serve as the basis for the future of biomedical research.

Chapter 2: Background

The goal of the project was to create a 3D hydrogel with a continuous stiffness gradient to allow cell behavior to be studied more accurately and advancements to be made in the field of tissue engineering. To assist in the understanding of this project, this chapter reviews topics such as the tissue engineering field, scaffolds, hydrogels, *in vivo* systems, and stiffness gradients.

2.1 Tissue Engineering and Applications

Tissue engineering is the creation of artificial human tissue in order to repair, replace or enhance the function of organs and tissues in the human body. Natural and synthetic materials are used to design scaffolds that aim to simulate the *in vivo* environment. These scaffolds can then be seeded with cells of interest and cultured into working tissues.

Through tissue engineering, researchers aim to mimic native human tissue as closely as possible for a variety of applications. One such application is the engineering of cartilage tissue. Cartilage is naturally made up of type two collagen and glycosaminoglycans, and has a similar macromolecular structure to many natural and synthetic hydrogels. Hydrogel scaffolds have been developed with embedded chondrocytes and growth factors to stimulate the development of cartilage tissue, and many have been tested *in vitro* and *in vivo* (Drury and Mooney, 2003).

Another application of tissue engineering scaffolds is the development of skin grafts for wound healing. In this application, a single layer or bi-layer ECM is produced and seeded with skin cells preferably patient-derived. Tissue engineered skin has successfully been developed to provide a barrier to the outside world, but more research is required to develop the complex functions such as temperature control through sweat glands and immune response through Langerhans cells (Metcalfe and Ferguson, 2007). Tissue engineering is an advancing field in biomedical research and regenerative medicine; however current methods are far from the ultimate goal of producing fully functioning organs.

2.2 Scaffolds

Scaffolds are important in tissue engineering because they act as a supportive matrix structure for the cells to grow in. They can be made from a variety of different materials, allowing for a range of mechanical properties to be achieved.

2.2.1 Properties

There are many properties that can be determined depending on the type of scaffold material used. For example, the stiffness of the scaffold will affect how the cells proliferate and grow (Chan and Leong, 2008). Also, the cell adhesion of the scaffold can be modified depending on whether the cell line being used is highly anchorage-dependent or not. In addition, specific cells require more nutrients and factors than others, so it is important to change the vascularization and diffusivity of each scaffold in accordance with this need. Overall, scaffolds must be biocompatible so that they can promote the growth, proliferation, maturation, and migration of the cells (Chan and Leong, 2008).

To tailor these properties, scaffolds can be made out of a variety of different materials. These materials fall under two categories, natural scaffold materials and synthetic scaffold materials.

Natural Scaffold Materials

Naturally occurring materials are one option for the creation of scaffolds. Natural materials include collagen, chitosan, fibrin, alginate, and many more. Natural materials are commonly used because of their high biocompatibility levels. In one specific example, a scaffold made of collagen and chitosan was created as a scaffold for tissue engineered skin. Since these natural materials are found in the body, the scaffold is biocompatible to both the cells in the engineered construct as well as in the body itself (Ma, 2004).

Synthetic Scaffold Materials

Synthetic materials are another option for the fabrication of scaffolds. Synthetic materials include HEMA, PEG, PGA, NIPAM, and a variety of others. Since these materials are fabricated in a lab, their properties can be easily manipulated. For example, P(NIPAM-HEMA) is a thermosensitive polymer (Gan *et al*., 2009). This means that the material is a solution when the temperature is below the Lower Critical Solution Temperature (LCST) and is a gel when it is above the LCST. One study used P(NIPAM-HEMA) as a cellular scaffold and drug delivery device by injecting it into the body. As it was injected, it became a gel because of its properties, allowing it to properly serve as a scaffold and drug delivery system (Gan *et al*., 2009). Many other synthetic materials can be altered or combined in similar ways to create materials with different properties.

2.2.2 Importance of Environment

It is important for a scaffold to possess certain properties in order to mimic an *in vivo* environment for testing. In the body, most cells grow in their own extracellular matrix (ECM). To replicate native ECM, a scaffold must serve as an artificial structure *in vitro* that permits cells to behave like they would in the body. For tissue applications, it would be best to use a material that has similar properties to the natural ECM of the targeted tissue so that the cells will be in a realistic environment (Chan and Leong, 2008). There are varying degrees of stiffness throughout the body, ranging from brain tissue to bone. Some cells need to have a stiffer surface to adhere to while others may not need as much support and anchorage as they grow (Chan and Leong, 2008). All of these properties must be taken into consideration when creating a scaffold for tissue engineering applications.

2.2.3 Hydrogels

A hydrogel consists of hydrophilic polymer chains that are cross-linked together using either physical or chemical means. Either natural or synthetic polymers can be used for the creation of hydrogels. Hydrogels swell greatly in the presence of water, and may have a water content of more than 30% by weight (Drury and Mooney, 2003). Hydrogels are a promising material for tissue engineering scaffolds because they effectively simulate the properties of native human tissue.

2.2.4 Hydrogel Precursors

Collagen is a natural protein that is native to the human body. Within the human body it comes in five types, I-V, but there are at least 14 other types of collagen found in nature (Drury and Mooney, 2003). Hydrogels made out of collagen are relatively biocompatible, although some forms of collagen, such as those that are animal-derived, have been shown to be weakly immunogenic (Gorgieva and Kokol, 2011). Collagen hydrogels are also biodegradable, being naturally degraded by collagenase and serine proteases in the human body (Drury and Mooney, 2003). The mechanical properties of collagen are difficult to fine-tune during the production process, and there may be variation between different batches of collagen due to sourcing (Tronci, 2010).

Gelatin is a naturally derived protein that is produced by the denaturing collagen. Gelatin hydrogels are very biocompatible, showing none of the immunogenic responses of collagen due to very careful purification and sterilization during manufacturing. This manufacturing process also eliminates batch variation that is seen in collagen (Gorgieva and Kokol, 2011). Hydrogels made from gelatin have also been shown to biodegrade naturally through hydrolysis over the course of several weeks. In addition, gelatin hydrogels are also proangiogenic, supporting the growth of new vascular tissue from existing vascular tissue (Tronci, 2010).

Poly (ethylene glycol), or PEG, is a synthetic polymer used in the creation of hydrogels. Because it is synthetic, it has mechanical properties superior to natural hydrogels, and these properties can be fine-tuned during the production process. It can also be altered to be biodegradable. However, there is a possibility of immune response due to PEG (Kasko, 2013).

Acrylamide is another synthetic polymer used to make hydrogels. It has superior mechanical properties to natural hydrogels, because they can be specified during production. Many acrylamide hydrogels are cross-linked using UV light, making them unsuitable for cell encapsulation and 3D culture (Tse and Engler, 2011).

2.2.5 Properties

The physical properties of hydrogels including tensile strength, elasticity and compressibility, can be greatly affected by the characteristics of both the polymer and the cross-linker. The mechanical properties can also change over time due to swelling and degradation, both of which tend to weaken the gel (Drury and Mooney, 2003). Stiffness in particular has been shown to vary greatly with both the concentrations of polymer precursor (Tse and Engler, 2011) and of cross-linking agent (Wang *et al*., 2012).

The degradation rate of a hydrogel can also be designed during production. The degradation rate of gelatin-HPA-Tyr hydrogels has been linked to their stiffness, with an increasing stiffness corresponding to a slower degradation rate (Wang *et al*., 2012). The degradation rate of some synthetic gels, such as PEG, can also be controlled through the addition of degradable ester linkages (Kasko, 2013).

Hydrogels swell greatly in the presence of water do to their hydrophilic properties. This swelling can be quantified using a degree of swelling, which can be calculated using the following equation:

$$DS = \frac{(W_s - W_d)}{W_d}$$

In this equation, W_s is the weight of the hydrogel after complete swelling and W_d is the weight of the hydrogel when it is completely dry (Andreaopoulos *et al*., 1998).

The mesh size is the space between the polymer chains of a hydrogel. It can be calculated using the following equation:

$$\xi = \alpha_s (C_n n l^2)^{1/2}$$

In this equation, ξ is the mesh size, α_s is the linear deformation in distance between cross-links from a swollen to a non-swollen state, C_n is the characteristic ratio of the polymer, l is the bond length of the polymer backbone and n is the number of bonds between two cross-links (Andreaopoulos *et al*., 1998).

Both the polymer and the cross-linking method affect the biocompatibility of a hydrogel. In order for a hydrogel to be biocompatible a polymer must be chosen that is neither cytotoxic to the cells, nor creates cytotoxic degradation products. If a chemical cross-linker is used, it must not be toxic to the cells or degrade into toxic products. If a physical cross-linking method is used it must not be harmful to the cells, such as those that involve high shear stresses or UV light.

Finally, a hydrogel can be either 2D or 3D. In 2D hydrogels, cells are cultured only on the surface of the gel. In a 3D hydrogel, cells are cultured throughout the entire gel. Proper diffusion of nutrients and waste products is an important concern when working with 3D hydrogels.

2.2.6 Cross-linking

As aforementioned, a hydrogel is a cross-linked polymer network. There are a variety of cross-linking mechanisms that can be used to form the networked structure of hydrogels. The method used to create cross-links must be selected with consideration of how it will affect the properties, such as gelation time, mechanical modulus, and biocompatibility, of the hydrogel. Generally, there are three main components involved in cross-link formation: monomers, initiators, and cross-linkers (Ottenbrite *et al*., 2010). Highlighted in this section are several cross-linking

techniques and a description of how they each can be used to manipulate specific hydrogel characteristics.

Cross-linking Methods

Cross-linking methods can be categorized into physical and chemical processes. Physically formed hydrogels are held together by networks of physically connected junctions (Ottenbrite *et al*., 2010). These linkages can be made from molecular entanglements, crystalline regions, phase-separated microdomains, and secondary forces such as ionic bonds, Hydrogen bonds, and hydrophobic forces. Physical gels tend to be unstable and degrade at a fast, unpredictable rate. Chemically bonded hydrogels are more commonly used in biomedical applications because covalently bonded linkages allow the gel to swell while maintaining structural integrity. Several methods commonly used to form cell-encapsulating hydrogels for biomedical applications are described below.

Radical Chain Polymerization

This method of cross-linking is commonly used to form hydrogels with vinyl-bearing macromers (Ottenbrite *et al*., 2010; Nicodemus and Bryant, 2008). This process is typically used with the synthetic polymers poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG), or with the natural polymers hyaluronic acid (HA), chitosan, and dextran. Polymers can be modified to contain two or more vinyl groups, making them capable to undergo this cross-linking method. During radical chain polymerization, radicals are created when a cross-linking initiator is exposed to a stimulus, such as change in temperature or exposure to light. These radicals propagate through carbon-carbon double bonds to create covalently linked kinetic chains, forming a networked structure. Common ways to initiate the cross-linking reaction are to introduce redox or thermal initiators or to use UV light to induce photopolymerization. Redox-initiating systems use a two-component initiator, for example a peroxide oxidizing agent and an amine reducing agent, that initiate polymerization when mixed. Photoinitiation with UV light specifically is advantageous because it provides rapid cross-linking, but exposing encapsulated cells to UV light can have an adverse effect on cell viability. Another drawback is that the heat produced during cross-linking can cause cellular necrosis. In general, some initiators and generated radicals can be toxic to cells. Consideration of the chemistry of the reaction, concentration of the initiator, and length of exposure to UV light can help to reduce these

cytotoxic effects. Some methods of cross-linking that use visible light photoinitiators, such as eosin-Y, triethanolamine, and camphorquinone, have been used to avoid the adverse effects of UV light exposure.

Cross-linking of Functional Groups

This method of cross-linking occurs through reactions between the functional groups of water-soluble monomers. Some categories of these reactions include: Schiff-base formation, and Michael-type additions (Ottenbrite *et al*., 2010; Nicodemus and Bryant, 2008).

Schiff-base formation of a hydrogel involves linking an aldehyde and an amino group (Ottenbrite *et al*., 2010). Glutaraldehyde is commonly used as a cross-linker in hydrogels, but has been found to be highly toxic to cells. Alternatively, reactive aldehyde groups can be created through the oxidation of a polysaccharide such as dextran, hyaluronic acid, or alginate. Hydrogels formed using Schiff-bases can be subject to degrade through the hydrolysis of imine bonds at low pH, but the addition of borax, a basic component, can produce stable hydrogels with fast gelation times.

Michael-type addition reactions are most common in making injectable hydrogels (Ottenbrite *et al*., 2010). The reactions occur when two polymers that contain nucleophilic groups, such as an amine or thiol, and electrophilic groups, such as a vinyl, acrylate, or maleimide, are mixed. Numerous polymers can be conjugated with these groups, but most commonly hyaluronic acid, dextran, PVA, and PEG are used. Hydrogels synthesized from Michael-type addition have a moderate gelation time that ranges from 0.5 to 60 minutes. Properties of these hydrogels can be modified by adjusting the reactivity of the functional groups and cross-linking density. Another characteristic of hydrogels formed through this reaction is that they usually provide favorable environments for cell proliferation as long as there are no excess thiol groups, which can cause cell death.

Enzymatic Reactions

Hydrogels can be cross-linked from polymers and polypeptides through enzymatic reactions (Ottenbrite *et al*., 2010). Commonly used to initiate these reactions is transglutaminase, a calcium-dependent enzyme that forms amide linkages between carboxamide and primary amines on polymers or polypeptides. Another initiator, horseradish peroxidase, causes linking of phenols

or aniline derivatives when exposed to hydrogen peroxide. This method has been used to formulate hydrogels from phenol conjugated poly(aspartic acid), as well as with hyaluronic acid, dextran, cellulose, alginate, and gelatin (Wang *et al*., 2012). Hydrogel properties can be modified by a variation in the horseradish peroxidase/hydrogen peroxide ratio. The gelation time for this method of cross-linking is under one minute, and can be adjusted by increasing or decreasing the enzyme concentration.

Thermo-Gelation

Thermo-gelation occurs when a change in temperature stimulates a hydrophobic interaction to occur (Ottenbrite *et al*., 2010). Biodegradable thermo-sensitive hydrogels can be made from block or graft copolymers that contain hydrophilic PEO and hydrophobic PLA, however they are slightly cytotoxic. Naturally occurring polymers, such as gelatin and agarose, can also form thermo-sensitive biodegradable hydrogels.

Combining Physical and Chemical Cross-linking

Physical and chemical cross-linking processes can be combined to create hydrogels with improved mechanical properties (Ottenbrite *et al*., 2010). Physical cross-linking reactions create reversible hydrogels with low mechanical properties. Chemical cross-linking reactions create irreversible hydrogels with high mechanical properties, but in many cases the reagents can adversely affect the biocompatibility of the gel. By applying two cross-linking methods, one physical and one chemical, the hydrogel becomes double cross-linked and can have improved mechanical properties, biocompatibility, and gelation times.

Cross-linking Considerations

The properties of a hydrogel, such as gelation time, mechanical modulus, swelling, degradation, and biocompatibility, are largely determined by the method of cross-linking that is used during formulation (Ottenbrite *et al*., 2010; Nicodemus and Bryant, 2008). Generally, an increase in cross-linking density increases mechanical properties of a hydrogel while decreasing swelling and mesh sizes. When forming a 3D hydrogel for cell encapsulation, the effect of the cross-linking method on cell viability must be considered. This is because cells are being mixed with gel precursor prior to being cross-linked, and are therefore exposed to the reactions occurring during the cross-linking process. Some cross-linking mechanisms, like glutaraldehyde initiators

and exposure to UV light, can have a high level of toxicity to cells, and therefore cannot be used for cell encapsulation methods.

2.3 Cell Response to Stiffness *In vivo*

Cells are anchorage dependent, meaning they must attach to a surface in order to proliferate. Cells also exhibit a characteristic called durotaxis, meaning they show a preference for a specific level of stiffness. Depending on the type of cell, this preference can range from a very soft surface, to a very rigid one. The various degrees of stiffness *in vivo* and their effect on cells is explored in this section.

2.3.1 Stiffness of Tissues *In vivo*

Within the body there is a wide range of stiffness that occurs. Stiffness can be defined as the ability of a material to resist deformation (Mason *et al*., 2012). Some tissues have a very low stiffness, such as brain tissue (260-490 Pa), a moderate stiffness, such as muscle (10kPa) and some tissues have a very high level of stiffness, such as pre-mineralized bone (30kPa) (Breuls *et al*., 2008; Sunyer *et al*., 2012). Tissue stiffness can be subject to change throughout certain physiological processes such as embryonic development, wound healing, and tumorigenesis (Mason *et al*., 2012).

Natural stiffness gradients can be caused by a pathological condition, such as myocardial infarction, which can cause a gradient of 8.7± 1.5 kPa/mm, or due to normal variation in tissue, such as mycocardium, which can have a gradient of 0.6±0.9 kPa/mm (Tse, 2011). At interfaces in the body, such as when rigid bone is connected to soft ligament, natural stiffness gradients occur. This transition consists of a shift from ligament to fibro-cartilage to bone. Natural gradients occur in teeth, where the composition and mineral density varies, and in bone structures, where bone composition and porosity ranges from compact (5-30% porosity) and spongy (30-90% porosity). Another cause of localized tissue stiffness variation is the formation of malignant tumors, which are stiffer than surrounding healthy tissue (Sunyer *et al*., 2012).

2.3.2 Effects of Stiffness on Cell Processes

Cells are very responsive to stiffness, as it has an effect on multiple cell processes such as proliferation, migration, morphology, and differentiation. As cells interact with their ECM

environment, they probe and sense the stiffness of their surroundings (Mason *et al*., 2012). There have been several cell behavior studies conducted to observe these effects.

Studies involving chondrocytes have shown that when cultured in a stiff hydrogel with a modulus of approximately 500 kPa, chondrocytes are retained in a proliferative state (Drury and Mooney, 2003). However, when these same cells are cultured in a less stiff hydrogel they were found to differentiate. A different study that plated heart tissue explants on stiff and soft matrices found that at high stiffness cells migrated out of the explant to cover the culture plate, while at low stiffness they did not (Mason *et al*., 2012). Some cells, such as rat kidney epithelial and fibroblasts, migrate at faster rates on softer matrices, while vascular smooth muscles migrate faster on intermediately stiff matrices (Breuls *et al*., 2008). Other studies show that matrix stiffness influences cell morphology, as it regulates polarization and alignment of stress fibers within cells. Stiffer substrates tend to cause spreading of cells, while soft substrates cause a more-rounded morphology.

Cellular differentiation is influenced by genetic, chemical, and mechanical factors (Mason *et al*., 2012). During embryonic development, the cytoskeletal contraction of cells can increase the level of stiffness in surrounding tissue by as much as 50-fold over the course of eight hours. Many studies have been conducted to observe the effects of stiffness on stem cell differentiation, and have determined that stiffness plays an integral role in regulating cell differentiation. When cultured on substrates that have a stiffness corresponding to native tissue, stem cells undergo lineage-specific differentiation (Tse and Engler, 2011). For example, mesenchymal stem cells can be differentiated into neurons when cultured on soft matrices, or into osteoblasts when plated on a stiff matrix of similar chemical composition (Engler *et al*., 2006).

2.4 Stiffness Gradient

Many research groups have been able to model hydrogels with a constant stiffness throughout a hydrogel. The ability to create hydrogels with varying stiffness is crucial to properly model an *in vivo* environment. As stated in the previous section, cells display durotaxis, which cannot be properly modeled on a constant stiffness. To create a hydrogel with varying stiffness there are two major components to consider. The first is the molecular level. As discussed in previous sections the cross-linking agent chosen to form a hydrogel will affect its mechanical properties.

Secondly, the physical method for gradient generation must be determined. Microfluidics, controlled mixing, and injections of reagents are currently used processes.

2.4.1 Hydrogel Creation

There are two main components of a hydrogel: the gel precursor and the cross-linker. Either of these materials can be altered to vary the resulting mechanical properties. A gradient can be formed by varying the molecular weights of the material used. A higher molecular weight will result in a higher stiffness upon hydrogel formation. A gradient can also be formed by altering the cross-linking agent's intensity. For chemical cross-linking methods, such as heat and photocross-linking, extending the exposure to the cross-linker will result in a greater density of cross-linking and therefore a stiffer hydrogel. An increase in intensity can also have this effect (Nemir *et al*., 2010). There are however limits to these cross-linking methods. Certain hydrogel materials may become denatured or damaged if exposed to dramatically high temperatures or intense light. In addition, when preparing 3D hydrogels, only biocompatible materials and methods can be used, greatly narrowing the available options.

2.4.2 Gradient Creation

Different methods have been developed to physically form a stiffness gradient. The combination of materials must be a precise process to ensure that gradient is smooth and uniform. Some methods are compatible will either the change in molecular weight or the change in cross-linker intensity as primary gradient generation techniques, while others are applicable to just one. Several current methods for forming gradients are summarized below.

Source Sink Gradient Generation

The Source Sink method differs from most that will be discussed as it does not involve syringes or pumps to control flow. This method depends on the natural diffusivity of materials. In order to work with this method, the materials used to make the hydrogel must have significantly different diffusivity coefficients. A mold with two inlets on opposite ends are connected by a channel (Figure 2.1). Each inlet is filled with a different material. The materials then fill into the channel and, based on their respective properties, a sink and a source

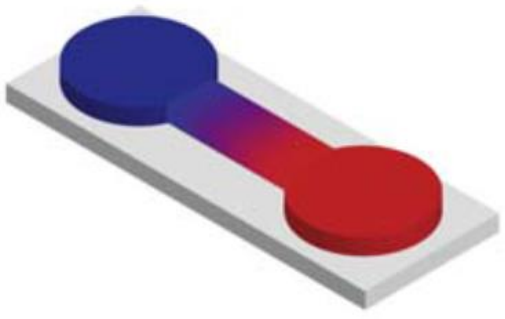


Figure 2.1: Source Sink Diffusion (Sant *et al*., 2010)

will develop. The natural mixing that happens as a result of this development creates as gradient. This process however should not be used if time is a concern for gradient creation as this natural gradient process can take extensive amounts of time (Abhyankar *et al*., 2006).

Gradient Generator

A gradient generator can also be used to produce a stiffness gradient. This generator is composed of microchannel networks that attach to a mechanism to force the material through the channels to form a gradient. There are many different patterns that can be formed using this method depending on the structure of the generator's network. Gradient generators are capable of mixing different materials, such as chemical cross-linking agents of varying intensity or varying molecular weight. This results in a different concentration or weight to be expelled from each channel. For this system to be effective, the flow of the materials into the generator must be steady and constant. A main advantage of this process is that it can occur very quickly. In some cases injection can occur in under a minute depending on the desired size of the hydrogel. One consideration is that this tool creates a gradient perpendicular to the direction of the flow. A visual representation of this variation can be seen in Figure 2.2 (Sant *et al*., 2010).

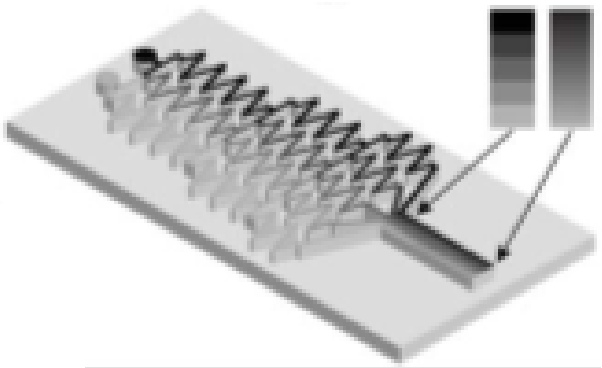


Figure 2.2: Gradient Generator (*Sant et al*., 2010)

Dynamic Mixing

Another generation method that involves the injection of materials into a mold is dynamic mixing. This is a simple method that uses two syringes and a mixer. The syringes can be filled with various solutions and then injected at different rates. The solutions are then mixed when they reach the mixing attachment. The gradient that results can be varied depending on the input

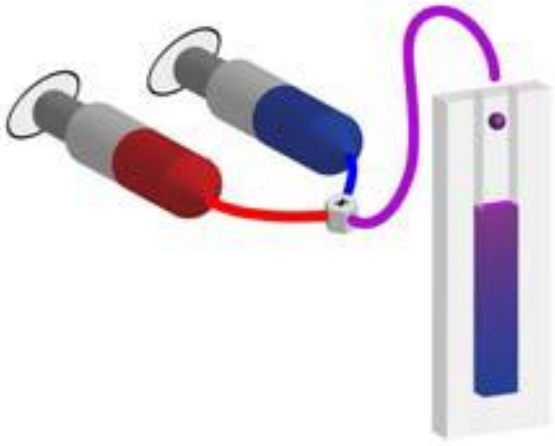


Figure 2.3: Dynamic Mixing (*Sant et al*., 2010)

from the two syringes. Through constant variation in the injection rates a gradient can be formed. This method is can create a gradient through a fairly short injection time.

Convective Spreading

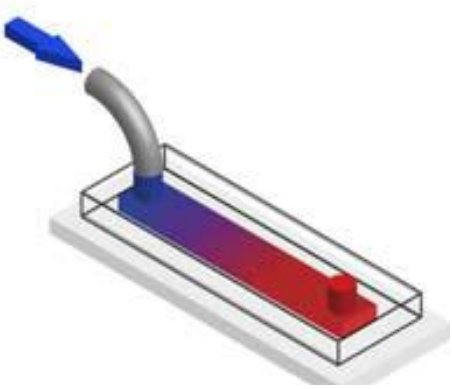


Figure 2.4: Convective Spreading *(Sant et al.*, 2010)

Convective Spreading involves the injection of two material solutions into a mold. The mold has two inlets on either side in which the solutions can be injected. The lower concentration or weight solution is injected into the first inlet while a filled second syringe is connected into the remaining inlet. After the mold has been filled with the first solution, the initial syringe is drawn back. This results in the solution of the second syringe being drawn into the mold. The first syringe should then again be expelled to inject the contained solution back into the mold. Repeating this process several times will eventually result in gradient formation. The more time this process occurs the longer the resulting gradient will be.

Ultraviolet Exposure

Certain material used to make hydrogels, such as collagen, can be cross-linked through exposure to Ultraviolet (UV) light. Creating a gradient with this method is a simple concept. The cross-linking density is directly related to the amount of time of exposure. Processes to create a controlled gradient have been developed by controlling the exposure to UV light. One technique is the use of a greyscale mask. This mask has a greyscale gradient, which is placed on top of the hydrogel so that a gradient forms as the intensity of UV light varies through the mask. This is an example of a variation in intensity that results in different stiffness. The gradient can also be created through longer or shorter exposure periods. For this method, the entire hydrogel is initially exposed to UV light. Then a mask is slid at a controlled rate over the hydrogel to block the light (Sant *et al*., 2010).

2.4.3 Stiffness Measurement Techniques

There are several ways to measure stiffness gradients. Two main methods are using Atomic Force Microscopy and Rheology.

Atomic Force Microscopy

In Atomic Force Microscopy, a cantilever spring that has a tip with a radius of 10 to 50 nanometers is pulled across the surface of the material (Helm, 2012). As the tip is pulled across, a laser reflects off of the cantilever spring and into a photodetector. Depending on where the laser's reflection hits the photodetector, the microscope can record the displacement of the tip, therefore allowing for the calculation of force and substrate stiffness (Helm, 2012).

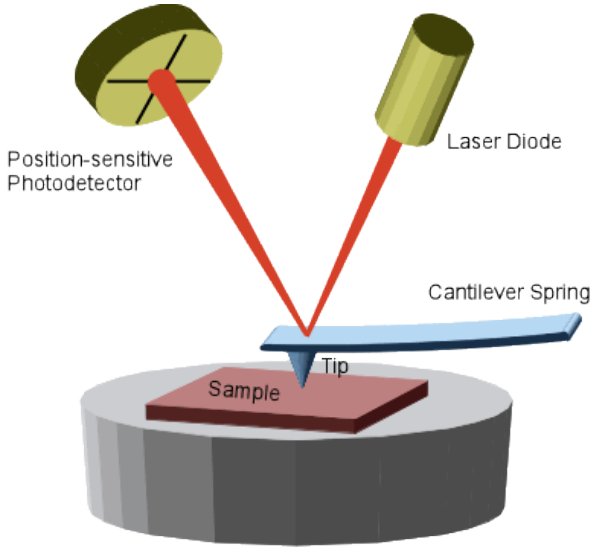


Figure 2.5: Measurement through Atomic Force Microscopy (Helm, 2012)

There are various modes of use for Atomic Force Microscopes. In contact mode, the tip is pulled across the surface as it is making direct contact with the material. This mode is generally used for stiffer materials since the tip does not leave the surface of the material. Another method commonly used is noncontact mode. In noncontact mode, the tip on the cantilever spring oscillates directly above the surface of the sample material without ever touching it. By sensing the van der Waals forces, the tip is able to oscillate right above the surface, allowing for measurements to be recorded that calculate the forces and stiffness (Nanoscience Intruments, 2013). A final commonly used mode is intermittent contact mode. In this mode, the tip of the Atomic Force Microscope again oscillates directly above the surface of the material. As its name suggests, the tip touches down on the surface occasionally to collect more data on the stiffness and strength of the sample material (Nanoscience Instruments, 2013). Both noncontact and intermittent contact modes are commonly used when measuring the characteristics of hydrogels and other soft materials because the tip does not damage the surface of the materials.

Acoustic Rheology

In Acoustic Rheology, a piezoelectric crystal launches contraction and extensional waves through the material that is being tested. These waves cause extensional stresses to oscillate through the material. Once these waves travel through the material, the amplitude, frequency, sound waves, and other factors can be recorded. In this method, the rheometer does not ever make contact with the material, ensuring that no damage occurs (McDonagh, 2010).

Chapter 3: Project Strategy

3.1 Initial Client Statement

The team was given the following initial Client Statement in order to define project objectives, constraints, and functions.

> "Substrate stiffness and external mechanical stimuli can affect cell behavior such as cell locomotion, morphology, adhesion, and cytoskeletal protein expression. Traditionally, studies on the effect of substrate stiffness on cellular response have been done on materials of discrete stiffness separately. Inside the body, tissue stiffness is not uniform and therefore, adjacent cells may experience different stiffness leading to different responses. This phenomenon needs to be explored *in vitro* in order to better understand cell behavior *in vivo*.

The needs of this project are

1. Develop a method to manufacture 3D gel (scaffold) with a continuous stiffness gradient.
2. The gel should be transparent so that cells can be imaged using a regular microscope.
3. Scaffold should be of uniform thickness of about 100-500 microns, preferably in the 100-200 micron range.
4. Choose from natural ECMs (e.g. collagen), commercially available hydrogels or synthetic polymers to prepare the gel (preferably biodegradable).
5. Ability to embed (mix) cells in the scaffold before cross-linking.
6. Choose a suitable cross linker that does not affect cell viability.
7. A method to generate a gradient of the cross-linker across the scaffold to complete cross-linking within 2 to 20 minutes, preferably less than 10 minutes.
8. Standardize a measurement technique to consistently measure and determine the stiffness gradient across the gel(s).
9. Develop a method to visually determine the gradient (under a microscope) across the gel.
10. Determine cell behavior in the gradient gel."

3.2 Design Objectives

From the initial Client Statement, the team created a compilation of items that would be important to meet all goals. From this compilation, a final list of objectives was created in accordance with the needs of the client. These objectives are shown in an objectives tree (Figure 3.1) and are explained in further detail below.

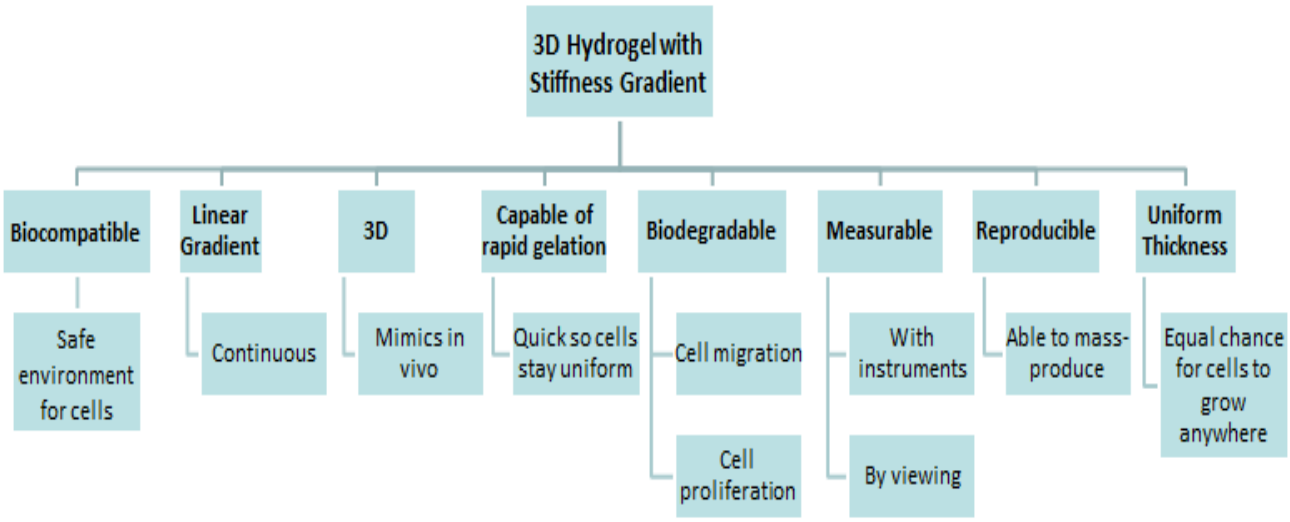


Figure 3.1: Objective Tree

Biocompatible: The hydrogel must be biocompatible in order for cells to survive inside of it. The hydrogel should be a safe environment for the cells to mature, proliferate, and move about. If the environment is toxic to the cells, they will not survive within it and the team will not be able to study cell behavior.

Biodegradable: The hydrogel should be biodegradable so that the cells can move throughout it. Also, in order for the cells to proliferate and grow in place of the hydrogel scaffold, it will be necessary for degradation to occur. If the hydrogel is not biodegradable, the cells will not be able to easily move throughout the gel and they won't be able to proliferate to form their own scaffold.

Capable of rapid gelation: The scaffold will need to be capable of rapid gelation so that cells can be uniformly distributed throughout the hydrogel without sedimenting.

Continuous Gradient: The hydrogel should have a continuous stiffness gradient in order to study cell behavior. By incorporating a wide range of stiffness, the hydrogel will more accurately represent the varying degrees of stiffness found throughout the body.

Measurable: The hydrogel will need to be measurable in order to determine the stiffness at any given location. If the hydrogel is not measurable, the team will not be able to determine the cells behavior in different stiffness regions.

Reproducible: The hydrogel scaffold should be reproducible. For the team to be able to test a variety of cell types and situations, it will be necessary to create multiple hydrogel scaffolds that all have the same exact properties. If the hydrogels do not have the same properties, then it will not be possible to accurately determine cell behavior.

Three-dimensional (3D): The hydrogel scaffold should be three-dimensional so that cell behavior can be studied. Since cells *in vivo* are able to grow in all planes rather than just a flat surface, it will be important to make the hydrogel 3D. By having a 3D hydrogel, cells will be able to grow in an environment that better simulates an *in vivo* setting.

Uniform Thickness: The hydrogel should have a uniform thickness so that cells have an equal opportunity to grow anywhere in the structure. If the hydrogel is not uniform in thickness, it will make it more difficult to determine whether the cells are responding the gradient or to the amount of space available.

From these objectives, the team created a joint pairwise comparison chart. In a pairwise comparison chart, each objective is listed and ranked against the other objectives. The objective that is determined to be more important receives a 1 while the other objective receives a 0. If the objectives are determined to be equally important, each receives a 0.5. After the entire chart is filled in, the objective totals are determined.

Table 3.1: Pairwise Comparison Chart

	Continuous Gradient	3D	Biodegradable	Biocompatible	Reproducible	Uniform thickness	Measurable	Capable of rapid gelation	**Score**
Continuous Gradient		0.5	1	0	1	1	1	1	**5.5**
3D	0.5		1	0	1	1	1	1	**5.5**
Biodegradable	0	0		0	1	1	1	0	**3**
Biocompatible	1	1	1		1	1	1	1	**7**
Reproducible	0	0	0	0		1	0	0	**1**
Uniform thickness	0	0	0	0	0		0	0	**0**
Measurable	0	0	0	0	1	1		0	**2**
Capable of rapid gelation	0	0	1	0	1	1	1		**4**

The results obtained from the team's pairwise comparison chart were presented to the client. After discussion, the team and clients agreed to rank the objectives in the following order:

1. Biocompatible
2. Continuous Gradient and 3D (tie)
4. Capable of rapid gelation
5. Biodegradable
6. Measurable
7. Reproducible
8. Uniform Thickness

3.3 Design Constraints

After reviewing the needs identified in the initial client statement and developing design objectives, the team identified the following constraints for the project design:

- The hydrogel must be between 100-500 microns thick
- Cross-linking must complete between 5-20 minutes
- The stiffness gradient must range within 1-100 kPa
- The hydrogel must be at least 90% transparent

The hydrogel design must be able to sustain cell culture and illustrate the cellular response to a stiffness gradient *in vitro*. This means that sufficient nutrient and cell waste diffusion must be able to occur to promote cell proliferation. The distance between a cell and capillary *in vivo* rarely exceeds 100 microns (Sherwood, 2010), so the thickness of the hydrogel must replicate this diffusion distance as accurately as possible. One of the most important features of the hydrogel design is its stiffness gradient. The hydrogel design is intended for research where a variety of cell types are cultured, and so it must encompass the range of stiffness that can be experienced in biological tissues. In order for cell behavior to be studied during culture, the hydrogel needs to be transparent enough for cells to be viewed under a microscope. These constraints aim to promote a design that can be repeatedly fabricated with consistent properties every time.

3.4 Revised Client Statement

After meeting with the team's project advisors to clarify the initial client statement and to discuss the project objectives and constraints, the team revised the initial client statement as follows:

> "As research in the Tissue Engineering field is evolving, there is a need for a scaffold that can simulate an *in vivo* environment as accurately as possible. To achieve this, the scaffold should be a hydrogel that is three-dimensional, biocompatible, and biodegradable. It should have a continuous stiffness gradient ranging from 1 kPa to 100 kPa, a gelation time of under 20 minutes, and the ability to embed cells within the gel prior to cross-linking without affecting cell viability."

3.5 Project Approach

In order to successfully create a 3D cell encapsulating hydrogel with a continuous stiffness gradient, the team developed a strategy for approaching the project. First is to develop a hydrogel formula that will serve as the basis for every scaffold. Once the gel is created, it will be tested to verify that the stiffness gradient within it is continuous. Finally, cell behavior within the hydrogel will be studied.

To create the hydrogel, the team must consider possible gel precursors, as well as a method of cross-linking and gradient creation. Since cells will be embedded into the hydrogel, the team must consider cell viability when choosing the cross-linking and gradient formation method.

To actually create the stiffness gradient the team will conduct research on existing gradient generation methods, evaluate which method would be optimal for the purpose of this project, and then make appropriate modifications to enable cell encapsulation.

Once the hydrogel is created, the team will need to measure the mechanical properties of the hydrogel. Mainly, the levels of stiffness must be evaluated throughout the hydrogel to verify that a continuous stiffness gradient has been formed. To do this, Atomic Force Microscopy (AFM) will be used. The team will continue to test hydrogels and refine the design until it is determined that a hydrogel with a continuous stiffness gradient has been created.

Upon finalizing the hydrogel formula and determining its stiffness gradient to be continuous, the team will begin a cell culture study over the course of three to four weeks. Cells will be prepared using immunocytochemistry prior to embedment to allow for fluorescence microscopy to be conducted, distinguishing live from dead cells. Imaging will provide qualitative results to show

the effect of stiffness on cell behavior over the period of time it was cultured, as well as validate the capacity of the hydrogel to promote cell growth.

3.5.1 Project Considerations

For project planning purposes, the project approach is broken down into specific technical, management, and financial considerations in the following section. The design challenges, design alternatives, timeline, work breakdown, and projected costs will be specifically evaluated.

Technical Considerations

There are several challenges the design must overcome in order to be successful. The most important of these is the encapsulation of cells uniformly throughout the hydrogel without affecting their viability. To accomplish this the design must employ a method of cross-linking and gradient generation that is neither toxic to the cells nor induces a large amount of shear stress on them. Cross-linking of the hydrogel must also occur in less than twenty minutes, so that the cells do not fall out of suspension before gelation is complete.

Once the hydrogel is created, the cells must be imaged in order to determine their viability, mobility, and differentiation. A variety of stains and assays must be employed in order to distinguish between live and dead cells and between different cell types. The cells must also be imaged on a regular basis over a period of four weeks in order to observe their movement through the gel and to ensure long-term viability. It is also important that cells can be imaged in the center of the hydrogel, not just at the surface. It may also be necessary to perform histological sections to view the center of the culture, even though this would ultimately destroy the hydrogel.

Another design challenge is the small size of the hydrogel. The hydrogel must be between 100 and 500 microns thick. In order to work at this scale, the design must incorporate microfluidic methods that will allow for precise control of fluids at a small scale.

Design Evaluation Matrices

In order to quantitatively compare the design alternatives and finalize the primary design concept, a series of design evaluation matrices were generated. The design was broken into three components: hydrogel precursor, cross-linking method, and gradient generation method. Several

options for each of these design components were measured against the design constraints and objectives. Each objective was assigned a weighted percentage based on its relative importance determined in the pair-wise comparison chart.

The first design component to be analyzed was the hydrogel precursor. Collagen, gelatin, PEG and acrylamide were compared using the weighted objectives. Table 3.2 shows the design evaluation matrix for the hydrogel precursor.

Table 3.2: Design Evaluation Matrix for Hydrogel Precursor

Design Constraints		**Collagen**		**Gelatin**		**PEG**		**Acrylamide**	
Between 100-500 Microns Thick		Y		Y		Y		Y	
Cross-linking between 5-20 minutes		Y		Y		Y		Y	
Stiffness gradient from 1-100 kPa		Y		Y		Y		Y	
At least 90% transparent		Y		Y		Y		Y	
Design Objectives	**Weight (%)**	Score	W. Score	Score	W. Score	Score	W. Score	Score	W. Score
Biocompatible	25	0.7	17.5	0.9	22.5	0.6	15	0.4	10
Continuous Gradient	20	1	20	1	20	1	20	1	20
3D	20	1	20	1	20	1	20	1	20
Capable of Rapid Gelation	15	0.9	13.5	1	15	0.8	12	0.8	12
Biodegradable	10	1	10	1	10	0.8	8	0.4	4
Measurable	5	1	5	1	5	1	5	1	5
Reproducible	3	0.6	1.8	0.8	2.4	0.9	2.7	0.9	2.7
Uniform thickness	2	1	2	1	2	1	2	1	2
TOTAL			89.8		**96.9**		84.7		75.7

Using the design evaluation matrix, it was decided that gelatin is the most suitable hydrogel precursor for the needs of the project with a score of 96.9. Gelatin was chosen oven the other options due to its biocompatibility, biodegradability and its capability of being gelled very rapidly.

Once the hydrogel precursor was chosen, a comparison was done for possible cross-linking reagents. Genipin, carbodiimide, and horseradish peroxidase (HRP) coupled with hydrogen peroxide (H_2O_2) were compared against the weighted objectives. Table 3.3, below, shows the design evaluation matrix for the cross-linking reagents.

Table 3.3: Design Evaluation Matrix for Cross-linking Reagent							
Design Constraints		**Genipin**		**Carbodiimide**		**HRP and H2O2**	
Between 100-500 Microns Thick		Y		Y		Y	
Cross-linking between 5-20 minutes		Y		Y		Y	
Stiffness gradient from 1-100 kPa		Y		Y		Y	
At least 90% transparent		Y		Y		Y	
Design Objectives	**Weight (%)**	Score	W. Score	Score	W. Score	Score	W. Score
Biocompatible	25	0.9	22.5	0.8	20	0.9	22.5
Continuous Gradient	20	0.8	16	0.8	16	0.9	18
3D	20	1	20	1	20	1	20
Capable of Rapid Gelation	15	0.5	7.5	0.8	12	1	15
Biodegradable	10	1	10	1	10	1	10
Measurable	5	1	5	1	5	1	5
Reproducible	3	0.8	2.4	0.8	2.4	0.8	2.4
Uniform thickness	2	1	2	1	2	1	2
TOTAL			85.4		87.4		**94.9**

It was determined that HRP combined with hydrogen peroxide (H_2O_2) is the most viable option for the design. This cross-linking method was chosen due to its biocompatibility, rapid gelation rate, and capability of forming a stiffness gradient that can be controlled by H_2O_2 concentration.

Finally, a preliminary design evaluation matrix was created for the method of gradient generation. Four methods were compared against the constraints and objectives: dynamic mixing, convective spreading, diffusion source sink and a gradient generator. Table 3.4 shows the design evaluation matrix for the gradient generation method

Table 3.4: Preliminary Design Evaluation Matrix for Gradient Generation Method									
Design Constraints		**Dynamic Mixing**		**Convective Spreading**		**Gradient Generator**		**Diffusion Source Sink**	
Between 100-500 Microns Thick		Y		Y		Y		Y	
Cross-linking between 5-20 minutes		Y		Y		Y		**N**	
Stiffness gradient from 1-100 kPa		Y		Y		Y		Y	
At least 90% transparent		Y		Y		Y		Y	
Design Objectives	**Weight (%)**	Score	W. Score	Score	W. Score	Score	W. Score	Score	W. Score
Biocompatible	25	0.9	22.5	0.9	22.5	0.8	20	N/A	N/A
Continuous Gradient	20	0.9	18	0.7	14	1	20	N/A	N/A
3D	20	1	20	1	20	1	20	N/A	N/A
Capable of Rapid Gelation	15	1	15	0.8	12	0.9	13.5	N/A	N/A
Biodegradable	10	1	10	1	10	1	10	N/A	N/A
Measurable	5	1	5	1	5	1	5	N/A	N/A
Reproducible	3	0.8	2.4	0.5	1.5	1	3	N/A	N/A
Uniform thickness	2	1	2	1	2	1	2	N/A	N/A
TOTAL	100		**94.9**		87		93.5	N/A	N/A

The diffusion source sink method was ruled out due to the long time needed to complete the process. Of the remaining three, dynamic mixing was chosen as the most viable option for gradient generation due to its biocompatibility, capability of producing a continuous stiffness gradient and rapid gelation time. The gradient generator is a second option due to its capability of producing a continuous stiffness gradient that is reproducible.

Management

Taking into account all design alternatives, the team created a work breakdown structure. The break down shown in Figure 3.2 incorporates the main components of all possible designs. The first factor that must be determined is which hydrogel formula to use, and, more importantly

which cross-linking method will be compatible. Second, the gradient generation method must be determined. Evaluation of the mechanical properties of the hydrogels must also be performed. Finally the hydrogel will have to be studied for biocompatibility. This will be conducted by evaluating the proliferation of cells encapsulated within the entire hydrogel.

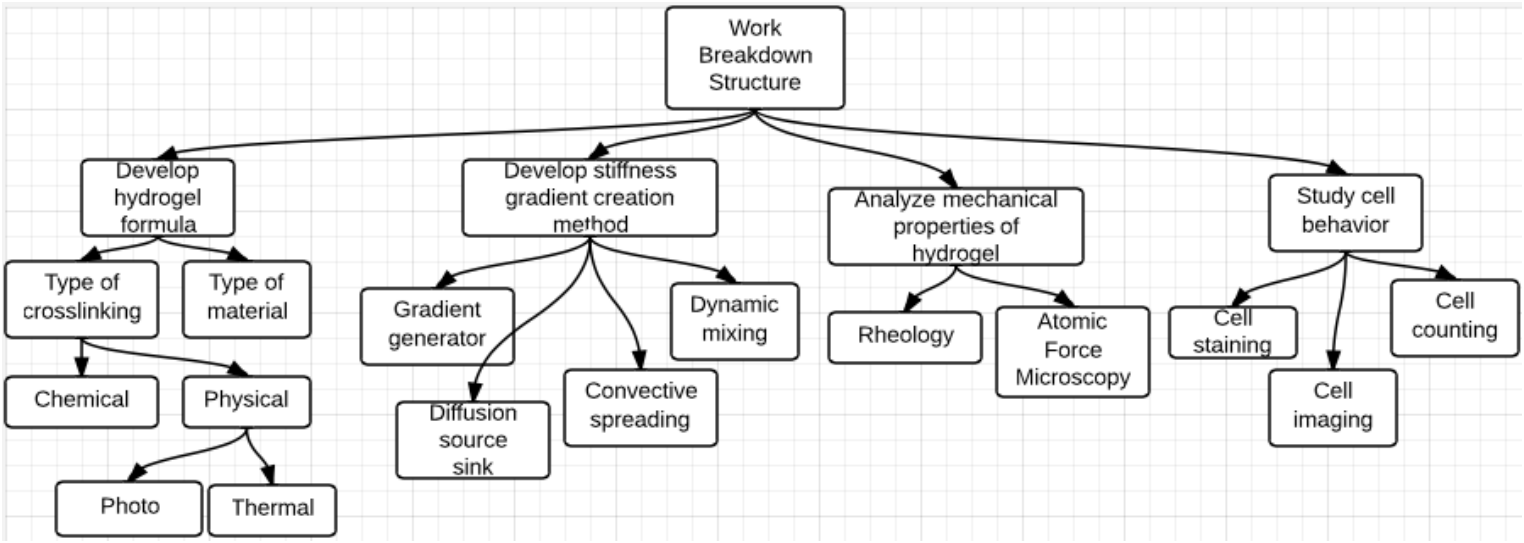


Figure 3.2: Work Breakdown Structure

A Gantt chart (Appendix A) was created to establish the team's deadlines for project milestones. The general goals to be complete for the end of each term are as follows.

A Term: Complete writing chapters 1-3 of report
Familiarize team with lab space and creation of hydrogels
Develop or select hydrogel formula and synthesis method
Finalize primary design and alternatives

B Term: Create hydrogel gradient machine
Create hydrogels using team-developed methodology
Analyze produced hydrogels
Make design alterations as necessary
Record all team progress

C Term: Continue recording progress and write results
Culture cells in hydrogels
Perform assays and cell counts
Track cell response to stiffness

D Term: Finish all experiments
Finalize results
Write discussion
Prepare final presentation

Chapter 4: Design Alternatives

Presented in this chapter are the preliminary developments of the design. The needs, functions, and specifications are summarized, the design alternatives are presented and evaluated, the final design is chosen, and feasibility studies, calculations, and preliminary experiments and results are explored.

4.1 Needs Analysis

After discussions as a team and with the project advisors, and after receiving feedback during presentations on the preliminary development of the team's project, the needs of the project were evaluated and solidified. The top objectives were identified and used to distinguish the functional needs from the needs that are desired but not crucial to the design.

Originally the needs identified by the clients in the Client Statement included:

- Develop a method to manufacture 3D gel (scaffold) with a continuous stiffness gradient.
- The gel should be transparent so that cells can be imaged using a regular microscope.
- Scaffold should be of uniform thickness of about 100-500 microns, preferably in the 100-200 micron range
- Choose from natural ECMs (e.g. collagen), commercially available hydrogels or synthetic polymers to prepare the gel (preferably biodegradable).
- Ability to embed (mix) cells in the scaffold before cross-linking.
- Choose a suitable cross linker that does not affect cell viability.
- A method to generate a gradient of the cross-linker across the scaffold to complete cross-linking within 2 to 20 minutes, preferably less than 10 minutes.
- Standardize a measurement technique to consistently measure and determine the stiffness gradient across the gel(s).
- Develop a method to visually determine the gradient (under a microscope) across the gel.
- Determine cell behavior in the gradient gel.

The top three ranked objectives determined from the teams pairwise comparison chart are that the design must be biocompatible, contain a continuous gradient, and be three-dimensional. Based off of these objectives, the team decided that the necessary characteristics of the design include: biocompatibility with cells, methods to develop a 3D continuous stiffness gradient, and the ability to encapsulate cells through rapid gelation. Additionally, the team will need to create

standard methods for measuring and determining the stiffness gradient across the gel in order to validate the success of the design.

The team also evaluated the other needs based on the results of the pairwise comparison chart. The following characteristics have been classified as wants: transparency of the gel, the ability of the scaffold to biodegrade, uniform scaffold thickness, visualization of the gradient under a microscope, and determination of cell behavior in the final design.

The rationale for classifying these characteristics is as follows. The gel must be transparent enough to image the cells under a microscope.. The client specified the biodegradability of the scaffold as a preference, but not a necessity. Ideally, the team would prefer to create a biodegradable scaffold that would allow cells to replace the hydrogel with their own matrix, however this is not a functional need of the project. Uniform scaffold thickness was the lowest ranked objective, as it is a design parameter that is not crucial to the design functionality and can be refined in the future. The team determined that validation of the gradient visually under a microscope is not a necessity, as the gradient can be validated in other ways by measuring the stiffness across the gel. However the team would ideally prefer to visualize the gradient. Lastly, the clients and the team would both like to study cell behavior within the final gradient gel, however this does not directly impact the functionality of the design and is therefore classified as a want.

The team developed a list of physical limitations that impact the scaffold design. The scaffold thickness must fall within the 100-200 micron range in order for cells to thrive. At a thickness greater than 200 microns, a necrotic core tends to develop. The design must have a gelation less than 20 minutes, otherwise the cells will sink to the bottom of the gel and the system will no longer be 3D. The cross-linking method and polymers used in this design must be biocompatible so that cells can be encapsulated and cultured within the hydrogel.

4.2 Design Functions and Specifications

To more fully develop the design criteria, this section describes the specific functions the design must be capable of. The necessary specifications are also introduced.

4.2.1 Functions

For this scaffold to be an effective cell culture tool there are several necessary functions. Cell culture is a very dynamic process that requires the proper environment for cell survival. In addition, this scaffold will have several more functions than tradition cell culture methods.

Cell Response

The major purpose of this scaffold is to allow for the study of the cell behavior when encapsulated in a range of stiffness. This differs greatly from other traditional cell culture methods and will provide an environment more similar to that found in vivo. This will allow for the study of cell morphology and proliferation as the cells react to the gradient and migrate throughout the gel towards their preferred environment.

Support Cells and Proliferation

The scaffold must be able to support the proliferation of cells within. This means that all stages of its synthesis and the materials that it is composed of must be non-toxic and biocompatible. For the cells to continue to grow and proliferate, nutrients and proper culture environment must be maintained. This means that the gel must be a permeable material. The nutrients must be able to diffuse into the hydrogel to reach the cells and metabolic waste must be able to diffuse out of the system.

Degradation

It is important that the hydrogel is a degradable material. This is because this scaffold is intended to allow for the study of cell morphology as well as monitoring cell proliferation. As the cells continue to proliferate it is possible for them to develop their own structure, independent of the scaffold. For this to happen, however, the scaffold must degrade over time. This degradation must be at a controlled and consistent rate that is slow enough so that the cells are not left without a scaffold for support.

4.2.2 Specifications

Certain specifications have been developed in order to achieve the optimal design. The stiffness gradient will range within 1 to 100 kPa. This allows all stiffness levels of the *in vivo* environment to be represented, allowing any type of cell to be cultured in this hydrogel design. The thickness of the hydrogel will be calculated to be 300 microns. This will prevent cell necrosis from

occurring by allowing appropriate diffusion of nutrients and waste through the hydrogel. A summary of the specifications is below in Table 4.1.

Table 4.1: Design Specifications	
Stiffness gradient range	1-100 kPa
Hydrogel thickness	300 microns

4.3 Design Alternatives

The possible design alternatives are presented in this section of the report. First, the options for the polymer used to form the hydrogel are discussed. Then, the various methods for stiffness gradient formation are presented.

4.3.1 Polymer Options

The material that the hydrogel will be made of must fulfill all the mechanical and biological requirements for the study and survival. There are several biomaterials that are suitable for this application.

Gelatin-HPA Conjugate

This scaffold material consists of gelatin and Hydroxyphenylproprionic Acid (HPA), which is then cross-linked with Hydrogen Peroxide (H_2O_2). This material has been shown to be highly biocompatible and allow for tuning of the stiffness of the final material based on the concentration of the cross-linker. This material is also desirable due to its fast gelation time, which allows for cell encapsulation.

Cross-linked Collagen

Collagen is another biomaterial that is often used for the formation of hydrogels. To achieve appropriate stiffness of the materials a cross-linking method would have to be used. Genipin is a highly biocompatible compound that has been used for the cross-linking of collagen and would be a valid cross-linking option. Another biocompatible cross-linking method of collagen is thermosetting. Both of these options would allow for the fine tuning of the stiffness of the resulting gel.

Gelatin-Genipin

The combination of gelatin cross-linked with genipin would also be able to fill the needs for the hydrogel material. This combination would be produced similarly to the collagen genipin combination. This option would be advantageous as only gelatin and genipin are needed to form this gel, meaning the hydrogel would simply be made of two compounds. This would allow for simpler gel formulation and fewer sources of error.

4.3.2 Gradient Generation Methods

The method used to make the actual gradient must create a gradient that meets the necessary specifications. Several different gradient generation methods have been explored.

Dynamic Mixing: Syringe pumps at controlled rates

This method would require the use of multiple syringe pumps and tubing sets as well as a mold for the gradient to be made in. Each syringe pump would hold a material of a specific molecular weight or percentage. As these materials are injected, the user would be able to change the rates so that specific ratios of the materials were being mixed together. By changing the rates for different pumps, it would be possible to create a higher stiffness on one side and a lower stiffness on the other side. As these materials were mixed, they would be injected into a mold that would allow the gel conjugate and cross-linker to gel.

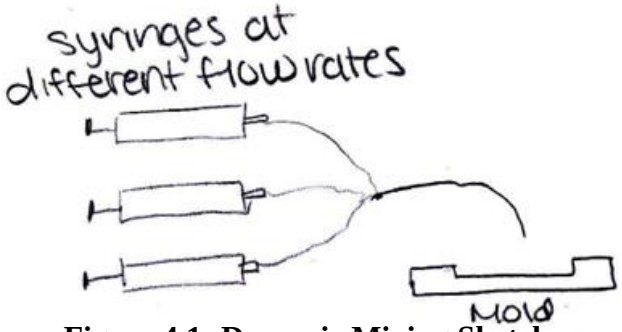


Figure 4.1: Dynamic Mixing Sketch

Gradient generator microfluidic mold

This method would use a microfluidic gradient generator and a multi-port syringe pump. As seen in the diagram below, there would be three ports to inject different molecular weight or percentage conjugate/cross-linker into the microfluidic chamber. On one side, the gel with the lowest stiffness would be pumped through, in the middle, the gel with the medium stiffness, and

on the far side, the gel with the highest stiffness. As these gels traveled through the channels, a gradient would be created due to the microfluidics of the generator.

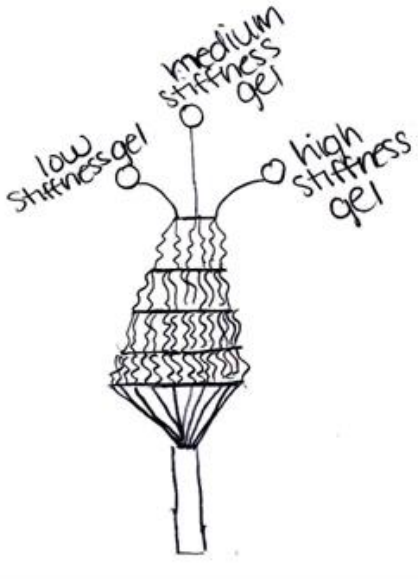


Figure 4.2: Gradient Generator Mold Sketch

Dynamic Flow: Gel creation with cross-linker flow through center

With this method, a mold would be made as shown in the Figure 4.3. In each side chamber of the mold, a gel of a constant concentration would be created. Once this had fully gelled, tubing would be connected to the center channel, and cross-linker of a specific molarity would be flowed through. While it was flowing through this center channel, the cross-linker would diffuse into the hydrogel, creating a continuous gradient. The stiffest gel would be found at the inside of the channel and the softer gel would be found near the outside wall.

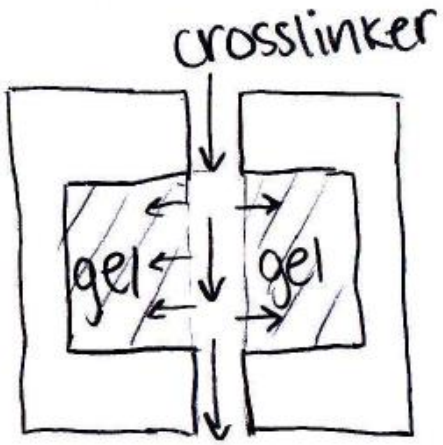


Figure 4.3: Dynamic Flow Middle Sketch

Dynamic Flow: Gel creation with cross-linker flow down the side

With this method, similar to the center-flowing method, a mold would be created. In the center chamber of the mold, gel of a constant concentration would be created and allowed to fully gel. Then, tubing would be connected to each side channel, and cross-linker would be flowed through each side. While it was flowing through, it would diffuse into the hydrogel, creating a continuous gradient. The stiffest gel would be found at the edge of the gel (near the cross-linker channels) and the softest gel would be found at the center of the gel.

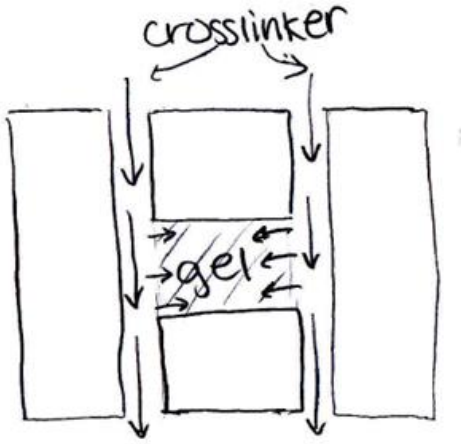


Figure 4.4: Dynamic Flow Side Sketch

Radial cross-linker diffusion

In this method, the hydrogel would be poured into a circular dish and allowed to completely gel. After it had completely gelled, a small circular piece of gel would be removed from the center of the mold. In place of this, cross-linker would be injected into this center hole. Over time, the cross-linker would diffuse into the gel, creating a stiffness gradient. The stiffest gel would be found in the center, closest to the cross-linker hole. The softest gel would be found near the outsides of the dish, since the smallest amount of cross-linker would diffuse out there.

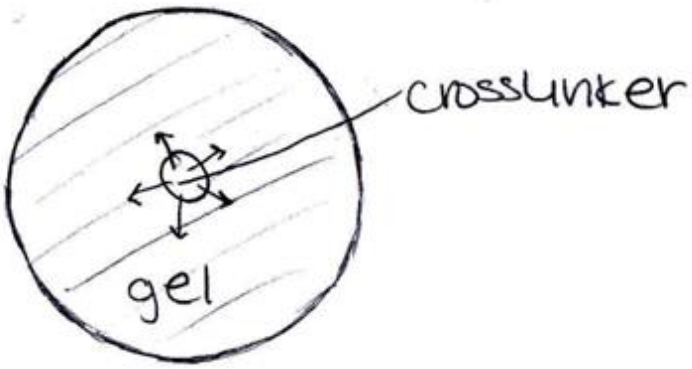


Figure 4.5: Radial Cross-linker Sketch

Vertical diffusion

This method would require a mold to be created in order to hold the gel and cross-linker. First, a gel of constant percentage would be created in the vertical diffusion mold. After the gel had formed, the cross-linker would be poured on top of the mold. The cross-linker would diffuse through the gel due to gravity, creating a stiffness gradient. The stiffest gel would be found at the top of the mold while the softest would be found at the bottom.

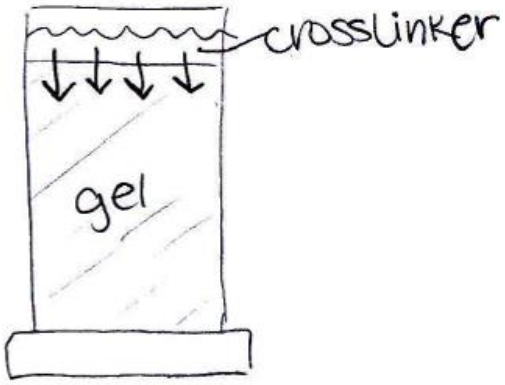


Figure 4.6: Vertical Diffusion Sketch

4.4 Conceptual Design

In this section of the chapter, the design alternatives presented in the previous section are evaluated in order to develop a final design. Methods of evaluation include a design evaluation matrix, group brainstorming, and the 7 Hats exercise.

4.4.1 Design Evaluation Matrix

After investigating all of the design options further, the team chose to reevaluate the options for the gradient generation method using a design evaluation matrix. The results can be seen below in Table 4.2.

Table 4.2: Revised Design Evaluation Matrix for Gradient Generation Method

Design Constraints		**Dynamic Mixing**		**Convective Spreading**		**Gradient Generator**		**Diffusion Source Sink**	
Between 100-500 Microns Thick		Y		Y		Y		Y	
Cross-linking between 5-20 minutes		Y		Y		Y		**N**	
Stiffness gradient from 1-100 kPa		Y		Y		Y		Y	
At least 90% transparent		Y		Y		Y		Y	
Design Objectives	**Weight (%)**	Score	W. Score	Score	W. Score	Score	W. Score	Score	W. Score
Biocompatible	25	0.9	22.5	0.9	22.5	0.9	22.5	N/A	N/A
Continuous Gradient	20	0.8	16	0.7	14	1	20	N/A	N/A
3D	20	1	20	1	20	1	20	N/A	N/A
Capable of Rapid Gelation	15	1	15	0.8	12	0.9	13.5	N/A	N/A
Biodegradable	10	1	10	1	10	1	10	N/A	N/A
Measurable	5	1	5	1	5	1	5	N/A	N/A
Reproducible	3	0.8	2.4	0.5	1.5	1	3	N/A	N/A
Uniform thickness	2	1	2	1	2	1	2	N/A	N/A
TOTAL	100		94.9		87		**96**	N/A	N/A

4.4.2 Group Brainstorming

The following section highlights pros and cons for each alternative for gradient generation methods.

Dynamic Mixing: Syringe pumps at controlled rates

Pros	**Cons**
• Could specifically design according to desired gel percentage/stiffness • Good controllability of gradient generation	• It's possible that the gel/cross-linkers wouldn't mix enough to create consistency • Wouldn't know how to connect tubing to mold to get proper microfluidics

Gradient generator microfluidic mold

Pros	**Cons**
• It will reliably create a gradient • Don't have to worry about mixing	• Unsure of whether or not the cells will be affected • May have dimensional constraints

Dynamic Flow: Gel creation with cross-linker flow through center

Pros	**Cons**
• It's an original idea • Potential to make 2 hydrogel gradients at a time • Would create an ideal gradient if it worked properly • Wouldn't have to worry about the cells moving	• Concerned that the cross-linker wouldn't diffuse through the gel properly • Don't fully understand the microfluidics of the process

Dynamic Flow: Gel creation with cross-linker flow down the side

Pros	**Cons**
• It's an original idea • Would create an ideal gradient if it worked properly • Wouldn't have to worry about the cells moving	• Concerned that the cross-linker wouldn't diffuse through the gel properly • Don't fully understand the microfluidics of the process

Radial cross-linker diffusion

Pros	**Cons**
• If cross-linker diffuses properly, it'll create a nice continuous gradient • Very quick and easy method	• Would need to ensure the gel is viscous enough to not spread everywhere when center hole is created • Cross-linker may not fully diffuse properly

Vertical diffusion

Pros	**Cons**
• Very quick and easy method • Would be an easy mold to create for the application • Gravity would ideally create a good gradient	• Gravity may pull cells down so that they are no longer evenly spaced throughout • If the gel cross-links on top, it's unsure whether the cross-linker could still diffuse through the rest of the gel

4.4.3 6 Hats Evaluation Method

After brainstorming the advantages and disadvantages of each design alternative, the team elected to use the "6 Hats Method" to further evaluate the use of a gradient generator mold as the main design. The "6 Hats Method" was established by Edward de Bono and is intended for teams to brainstorm and evaluate an idea through the use of distinct filters. The six different colored thinking hats are white, red, black, yellow, green, and blue. Each color is representative of information, emotions, discernment, optimism, creativity, and organization respectively. A more detailed description of each color is described in the next paragraph.

When using the white hat, the purpose of the exercise is to identify all available information and facts concerning the design method. The red hat is used to express intuitive and emotional feelings towards a design without providing any justifications as to why one feels a certain way. The black hat is used to describe any logical concerns or criticisms with the proposed design method. The purpose of the yellow hat is to develop logical reasons as to why the design method will be beneficial or advantageous. The green hat is intended to promote creativity by generating and building upon new ideas. Lastly, the blue hat is for mandating how the brainstorming session should begin and end. The blue hat is organizational thinking, meaning that before the team

begins the process of how to proceed should be defined, and that after brainstorming the team must decide how to process and evaluate the results of each hat.

The ideas generated for each hat can be found in Appendix B. After the exercise was completed, the team reviewed the resulting thoughts and ideas to come to a final decision on which design method to proceed with, which will be discussed in the following section of this chapter.

4.5 Decisions

After evaluating the design alternatives, the team was able to decide upon a final design. The team was easily able to choose a polymer for the design, as the gelatin-HPA formula guaranteed rapid gelation of the hydrogel—a critical aspect of the project's design. The gradient generation method took more evaluation and brainstorming to decide upon. After reevaluating each design in a design selection matrix, the gradient generator was prominently the most fitting gradient generation method for the design projects needs. Upon brainstorming pros and cons for each design alternative, the team came to a consensus that the gradient generation method had the least concerning cons. Once the team further investigated each aspect of the gradient generator design using the 7 Hats Method, it was decided that manipulating a microfluidic generator mold to create a stiffness gradient would be the most feasible and promising option for the design.

4.6 Feasibility Studies & Experiments

In preparation for using the gelatin-HPA conjugate and the gradient generator mold as the main design features, certain studies and experiments are needed to determine the success of the final design. Before beginning any experiments, calculations were conducted to determine stock solutions, concentrations, and other design parameters needed to create the hydrogels. Then the gelation time, stiffness, and biocompatibility of these hydrogels were evaluated.

4.6.1 Calculations

In order to create hydrogels out of the gelatin-HPA conjugate, certain calculations were necessary to determine how much of each material and which concentrations would be needed. These calculations include finding stock solution concentrations, a 3% gelatin-HPA stock, determining the gel thickness in a 24-well plate, and plating a known number of cells.

Stock Solutions:

49mM H_2O_2:

$$9.8\ M\ H_2O_2\left(\frac{0.25\ mL}{50\ mL}\right) = 0.049\ M\ H_2O_2 = 49mM$$

36.75mM H_2O_2:

$$49mM\ H_2O_2\left(\frac{VH_2O_2}{20mL}\right) = 36.75mM$$

$$VH_2O_2 = 15mL$$

$$VDPBS(-) = 5mL$$

24.5mM H_2O_2:

$$49mM\ H_2O_2\left(\frac{VH_2O_2}{20mL}\right) = 24.5mM$$

$$VH_2O_2 = 10mL$$

$$VDPBS(-) = 10mL$$

10mM H_2O_2:

$$9.8\ \square_2O_2\left(\frac{VH_2O_2}{100mL}\right) = 10mM$$

$$VH_2O_2 = .102mL = 102\mu L$$

$$VDPBS(-) = 99.898mL$$

15mM H_2O_2:

$$9.8\ H_2O_2\left(\frac{VH_2O_2}{100mL}\right) = 15mM$$

$$VH_2O_2 = .153mL = 153\mu L$$

$$VDPBS(-) = 99.847mL$$

20mM H_2O_2:

$$9.8\ H_2O_2\left(\frac{VH_2O_2}{100mL}\right) = 20mM$$

$$VH_2O_2 = .204mL = 204\mu L$$

$$VDPBS(-) = 99.796mL$$

25mM H_2O_2:

$$9.8\ H_2O_2\left(\frac{VH_2O_2}{100mL}\right) = 25mM$$

$$VH_2O_2 = .255mL = 255\mu L$$

$$VDPBS(-) = 99.745mL$$

30mM H_2O_2:

$$9.8\, H_2O_2 \left(\frac{VH_2O_2}{100mL}\right) = 30mM$$

$$VH_2O_2 = .306mL = 306\mu L$$

$$VDPBS(-) = 99.694mL$$

3% Gelatin-HPA Stock:

$$.03 = \left(\frac{conjugate\ g}{VDistiled\ Water}\right)$$

Gel Thickness:

Area of 24 well plate = 1.93 cm^2

$$\frac{300\mu L \frac{1mL}{1000\mu L}}{1.93cm^2} = 155\ microns$$

Plating of 70,000 Encapsulated Cells:

$$\left(Known\ amount\ of\ cells \frac{Cells}{mL}\right)(V) = 70{,}000\ Cells$$

4.6.2 Gelation Time Studies

In order to determine how quickly the gelatin-HPA hydrogels would be able to fully gel, a gelation time study was conducted. Two microcentrifuge tubes of hydrogel solution were prepared in the hood according to the following protocol:

Table 4.3: 1.5% Gelatin-HPA Hydrogels

	0.98 mM Final [H_2O_2] with cells	**1.47 mM Final [H_2O_2] with cells**	**1.96 mM Final [H_2O_2] with cells**	**No H_2O_2 with cells**	**1.47 mM Final [H_2O_2] without cells**
Gelatin-HPA [3% wt]	300 µL	300 µL	300 µL	300 µL	300 µL
Horseradish Peroxidase	6 µL	6 µL	6 µL	6 µL	6 µL

[10 U/mL] (Final conc. 10U/mL)					
10X DMEM	54 µL	54 µL	54 µL	54 µL	60 µL
DPBS(-)	156 µL	156 µL	156 µL	180 µL	210 µL
Cell Suspension	60 µL	60 µL	60 µL	60 µL	0 µL
H_2O_2	24 µL of 24.5 mM H_2O_2	24 µL of 36.75 mM H_2O_2	24 µL of 49 mM H_2O_2	None	24 µL of 36.75 mM H_2O_2
Total Volume	600 µL	600 µL	600 µL	600 µL	600 µL

1. Dissolve gelatin-HPA powder in distilled H_2O at 65°C to create a 3% weight stock solution.
2. Pipette 300 µL of the 3% gelatin-HPA stock solution into a 1.5 mL microfuge tube.
3. To #2, add 6 µL of horseradish peroxidase at a concentration of 10 Units/mL.
4. Add 54 µL of 10x DMEM to #3.
5. Add 156 µL of DPBS(-) to #4.
6. To #5, add 60 µL of cells suspended in 1X DMEM. If not seeding the gel with cells, substitute 6 µL of 10X DMEM without cells and 54 µL of DPBS(-).
7. To #6, add 24 µL of H_2O_2 (24.5 mM, 36.75 mM or 49 mM depending on desired final concentration). For control without H_2O_2, replace with 24 µL of DPBS(-)
8. Immediately following step #7 pipette the solution up and down ten times to mix, being careful not to create bubbles.

Immediately following the preparation of the hydrogel solution, the gelation time study was conducted for the 1.47 mM H_2O_2 hydrogel according to the following protocol:

1. Pipette 150 µL of the prepared hydrogel onto each of 8 22 mm x 22 mm cover slips. Start a timer once all the hydrogels are pipetted onto the cover slips
2. Starting at 6 minutes, flip one cover slip 90 degrees to a vertical position. Observe whether the hydrogel moves on the slide.
3. Repeat step #2 with the remaining slides in 30-second intervals until all the slides have been flipped. The time at which the hydrogel does not move on the cover slip in a vertical position indicates the gelation time.

The gelation time study was conducted for the 0.98 mM H_2O_2 hydrogel as well. For this study, three microfuge tubes each containing 900 µL of the hydrogel solution were prepared following the same protocol and ratios as above. 150 µL of hydrogel solution was pipetted onto each of 18

cover slips. The gelation time study was conducted according to the protocol above, starting at 6 minutes and ending at 15 minutes with a cover slip flipped every 30 seconds.

4.6.3 AFM Baseline Stiffness Measurements

The stiffness of gelatin-HPA hydrogels was measured using atomic force microscopy (AFM). For the first round of measurements, gelatin-HPA hydrogels were made at a final concentration of 2% gelatin-HPA. The three concentrations of H_2O_2 that were tested were 0.9 mM, 1.35 mM and 1.8 mM. APTMS treated cover slips were placed in separate petri dishes. 300 µL of the prepared hydrogel was pipetted onto a bottom cover slip in the petri dish. A second cover slip was then placed on top of the hydrogel, and the hydrogel was allowed to set for 5 minutes. The petri dishes with the hydrogels were covered in parafilm. The prepared hydrogels were then brought to Gateway for AFM measurements. Three measurements were taken at five different points on each gel.

A second round of AFM measurements was conducted for gelatin-HPA hydrogels at a final concentration of 1.5%. These hydrogels were made with three different concentrations of H_2O_2: 1mM, 1.5mM and 2.0mM. Three measurements were taken at three different points on each gel.

4.6.4 Biocompatibility of Polymer Assessment

In order to determine the biocompatibility of the gelatin-HPA conjugate, cells were cultured under several conditions and then imaged every other day. Hydrogels were prepared in duplicate at 1.5% gelatin-HPA conjugate for both NIH 3T3 Mouse Fibroblasts and for Human Primary Fibroblasts. The conditions used were as follows:

H_2O_2 Concentration: 0mM, 1mM, 1.5 mM, 2mM

- Cells encapsulated: 150,000 cells
- Cells seeded on the surface: 100,000 cells

Controls: Cells cultured on the polystyrene well surface

- 50,000 cells
- 100,000 cells

The hydrogels and controls were prepared in a 24 well plate using the following protocol:

Table 4.4: 1.5% Gelatin-HPA Hydrogels						
	1 mM Final [H_2O_2] with cells	**1.5 mM Final [H_2O_2] with cells**	**2 mM Final [H_2O_2] with cells**	**0mM H_2O_2 with cells**	**50,000 Cells**	**100,000 Cells**
Gelatin-HPA [3% wt]	150 µL	150 µL	150 µL	150 µL	None	None
Horseradish Peroxidase [10 U/mL] (Final conc. 10U/mL)	3 µL	3 µL	3 µL	3 µL	None	None
10X DMEM	27 µL	27 µL	27 µL	27 µL	None	None
DPBS (-)	78 µL	78 µL	78 µL	90 µL	None	None
Cell Suspension	30 µL	30 µL	30 µL	30 µL	30 µL	30 µL
H_2O_2	12 µL of 24.5 mM H_2O_2	12 µL of 36.75 mM H_2O_2	12 µL of 49 mM H_2O_2	None	None	None
Total Volume	300 µL	300 µL	300 µL	300 µL	60 µL	60 µL

1. Dissolve gelatin-HPA powder in distilled H_2O at 65°C to create a 3% weight stock solution.
2. Pipette 150 µL of the 3% gelatin-HPA stock solution into each well of a 24 well plate, leaving 2 columns empty for the controls.
3. To #2, add 3 µL of horseradish peroxidase at a concentration of 10 Units/mL, leaving the control wells empty.
4. Add 27 µL of 10x DMEM to #3, leaving the control wells empty.
5. Add 78 µL of DPBS(-) to #4 for the 1 mM, 1.5 mM, and 2 mM concentrations. Add 90 µL to the 0 mM concentrations, and leave the control wells empty.
6. To #5, add 30 µL of cells suspended in 1X DMEM.
7. To #6, add 12 µL of H_2O_2 (24.5 mM, 36.75 mM, 49 mM, or none depending on desired final concentration).
8. Immediately following step #7 pipette the solution up and down ten times to mix, being careful not to create bubbles.
9. Leave the hydrogels in the hood to cross-link for 30 minutes.

10. After 30 minutes, gently pipette 500 µL of cell culture media into each well.
11. Store the culture plates in the 37C 5% CO_2 incubator.

After 24 hours of incubation, both the plates of NIH 3T3 Mouse Fibroblast cells and the Human Primary Fibroblast cells were imaged using a phase contrast microscope at 20X magnification. Additional images were also taken of each condition at 36 and 52 hours.

4.7 Modeling

A model system was used to illustrate the process of gradient formation through the generator. First DPBS was used as the primary solution that was flowed through the channels to confirm that the fluid flowed correctly through the mold. Concentrated dyes were then added to the water in the syringes connected to the outermost channels of the generator. The dyes visually confirmed the presence of a gradient within the gradient-formation well.

4.8 Preliminary Data

Data obtained from feasibility studies and experiments are included in this section of the report. Quantitative and qualitative results are included for the gelation time studies, the AFM baseline stiffness measurements, and the biocompatibility study.

4.8.1 Gelation Time Data

1.5% Gelatin-HPA 1.96 mM H_2O_2

The baseline experiment for the gelation time of 1.96 mM H_2O_2 cross-linked hydrogels determined that the hydrogel completely gelled within 10 minutes. In the gelation study, the first coverslip was flipped vertically 2:00 minutes after the timer was started. A total of 5 coverslips were flipped at 30-second intervals. The 1.96 mM H_2O_2 cross-linked hydrogels fully gelled at 4 minutes. The resulting vertical coverslips can be seen in the image below.

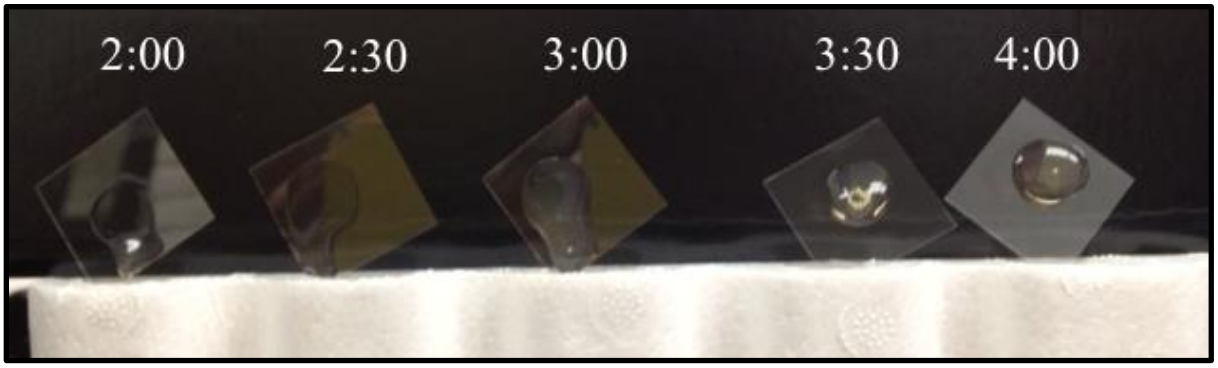


Figure 4.7: 1.96 mM H_2O_2 Gelation Results

1.5% Gelatin-HPA 1.47mM H_2O_2

The baseline experiment for the gelation time of 1.47 mM H_2O_2 cross-linked hydrogels determined that the hydrogel completely gelled within 10 minutes. In the gelation study, the first coverslip was flipped vertically 6:00 minutes after the timer was started. A total of 12 coverslips were flipped at 30-second intervals. The 1.47 mM H_2O_2 cross-linked hydrogels fully gelled at 8:30 minutes. The resulting vertical coverslips can be seen in the image below.

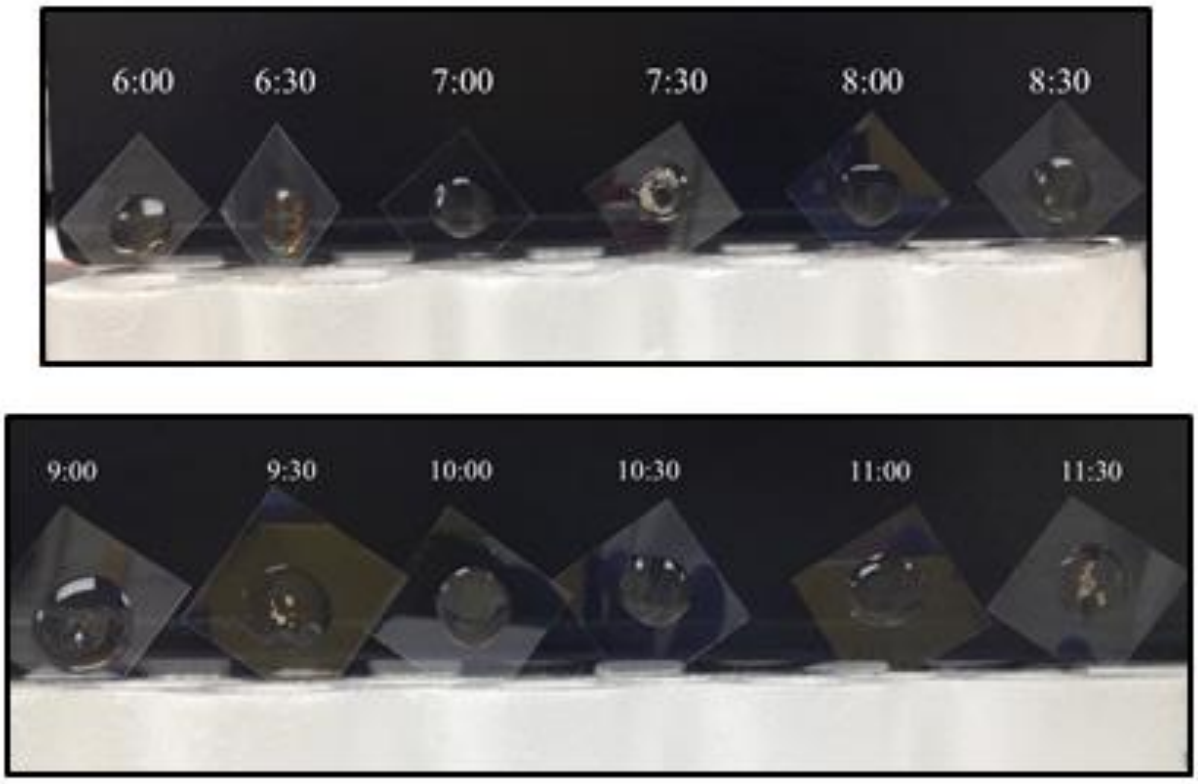


Figure 4.8: 1.47 mM H_2O_2 Gelation Results

1.5% Gelatin-HPA 0.98mM H_2O_2

The baseline experiment for the gelation time of 0.98 mM H_2O_2 cross-linked hydrogels determined that the hydrogel did not completely gel within 10 minutes. In the gelation study, the first coverslip was flipped vertically 6:00 minutes after the timer was started. A total of 16

coverslips were prepared to be flipped at 30-second intervals. The first two coverslips were completely liquid, and therefore the first coverslip was not flipped until 7:00 minutes into the experiment. The 0.98 mM H_2O_2 cross-linked hydrogels fully gelled at 14 minutes. The resulting vertical coverslips can be seen in the images below.

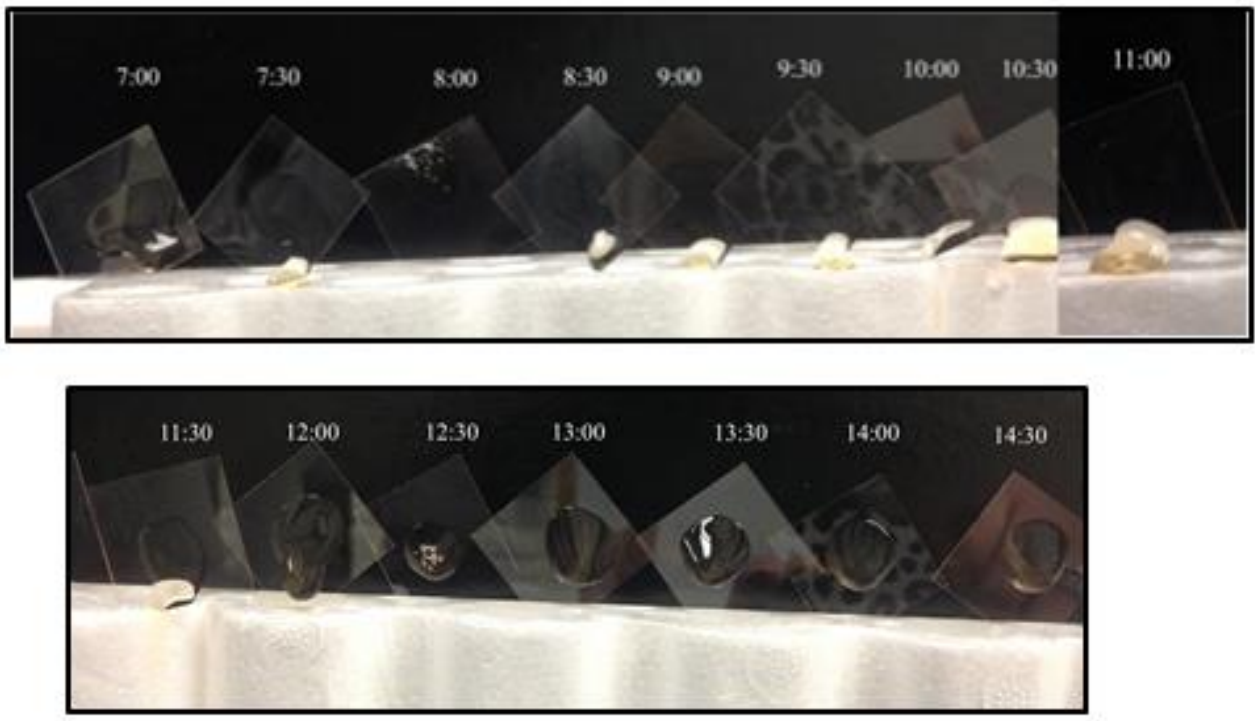


Figure 4.9: 0.98mM H_2O_2 Gelation Results

4.8.2 Stiffness of Preliminary Samples

The average stiffness of the samples measured using AFM are organized in the table below.

Table 4.5: Preliminary AFM Measurements

Gtn-HPA	H_2O_2 (mM)	Average Stiffness	Standard Deviation
2%	1.8 mM	354.72 Pa	± 199.7 Pa
	1.35 mM	424.18 Pa	± 424.1 Pa
	0.9 mM	260,886.09 Pa	± 237,402.1 Pa
1.5%	2 mM	620.50 Pa	± 308.5 Pa
	1.5 mM	285.50 Pa	± 382.9 Pa
	1 mM	1,382,931.86 Pa	± 2,548,988.2 Pa

4.9 Optimization

After developing the design further, some adjustments were needed to achieve the desired functions and specifications.

4.9.1 Additional AFM Measurements

Preliminary AFM measurements revealed that the samples tested did not fulfill the entire stiffness range of 1 to 100 kPa. To create stiffer gels, the team decided to test hydrogels using a higher percentage of gelatin-HPA and a higher concentration of the H_2O_2 cross-linker. The team conducted additional AFM experiments on the gels outlined in Table 4.6.

Table 4.6: Additional AFM Measurements

Gtn-HPA	H_2O_2 (mM)	Average Stiffness	Standard Deviation
2%	1.5 mM	1,753 Pa	± 2,540,472 Pa
	2.0 mM	1,056 Pa	± 560 Pa
	2.5 mM	1,364 Pa	± 484 Pa
	3.0 mM	1,108 Pa	± 352 Pa
	3.5 mM	1,333 Pa	± 531 Pa
1.5%	1.5 mM	277 Pa	± 140 Pa
	2 mM	357 Pa	± 89 Pa
	2.5 mM	318 Pa	± 87 Pa
	3.0 mM	482 Pa	± 452 Pa
	3.5 mM	988 Pa	± 856 Pa

4.9.2 Hydrogel Formula Modifications

Upon additional review the hydrogel formula, the amounts of reagents were recalculated to provide greater accuracy of stock concentrations. The recalculated formula is listed in Table 4.7.

Table 4.7: Revised Gelatin-HPA Hydrogel Formula

	1% Gtn-HPA	1.5% Gtn-HPA	2% Gtn-HPA
Gtn-HPA [3% wt]	40 µL	60 µL	80 µL
HRP [10 U/mL]	5 µL	5 µL	5 µL

DPBS (+)	45 µL	25 µL	5 µL
Cells in 10X DMEM	24 µL	24 µL	24 µL
H_2O_2	6 µL	6 µL	6 µL
Total Volume	120 µL	120 µL	120 µL

New stock solutions were diluted from a 1 M H_2O_2 solution in order to create the final H_2O_2 concentrations listed in Table 4.8.

Table 4.8: Revised H_2O_2 Stock Solutions

Final H_2O_2 Concentration	Stock Concentration
1 mM	20 mM
1.5 mM	30 mM
2 mM	40 mM
2.5 mM	50 mM
3 mM	60 mM
3.5 mM	70 mM

4.9.3 Additional Gelation Time Studies

After making alterations to the hydrogel formulas, additional gelation time studies were conducted for 1%, 1.5%, and 2% gtn-HPA hydrogels at 1 to 3.5 mM H_2O_2 concentrations. These studies were completed using an alternative method from the initial gelation studies. The hydrogels were made using the formulas from Table 4.7, but by following the procedure outlined in Appendix C. The total volume created of each combination was 120 uL, and two 50 uL drops were placed in petri dishes. A timer was started when the hydrogen peroxide was added to the HPA solution. Then the drops were inspected both visually, as the drops would become cloudy during gelation, and also physically, as upon completion of gelation it is no longer possible to draw the gel into a pipette tip. The test gels can be seen in Figure 4.10. The 2.0% gels appeared to have the most opaque appearance, possibly as a result of containing the highest extent of cross-linking. In addition to this they had the quickest gelation times for each respective hydrogen peroxide concentration used.

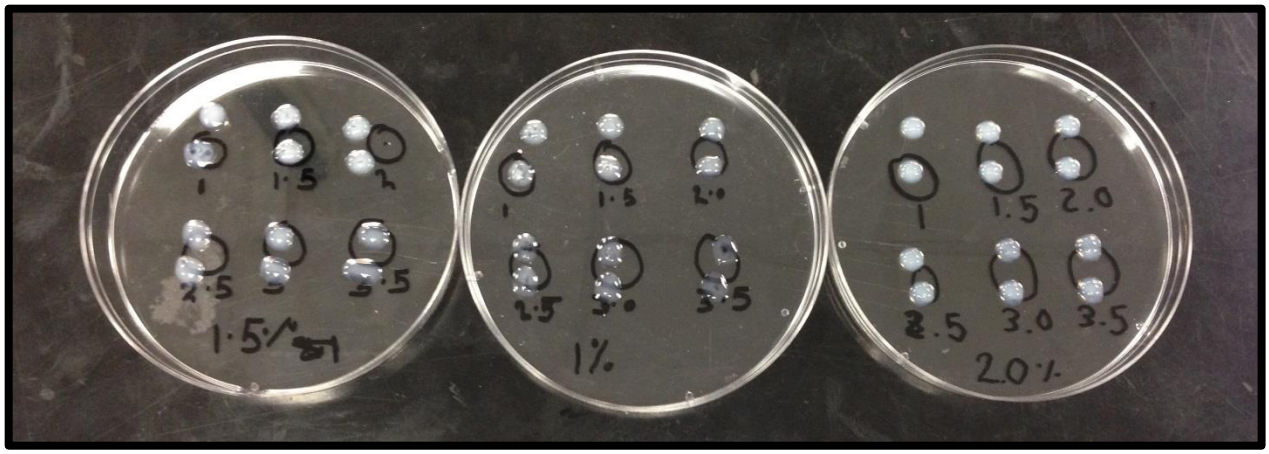


Figure 4.10: Test gels for 1%, 1.5%, and 2% gelatin-HPA.

A direct relation can be seen between an increase in the concentration of both gelatin and hydrogen peroxide and an in increase in the rate of gelation. The data found can be seen displayed in Figure 4.11; in addition this information is available in the table found in Appendix I.

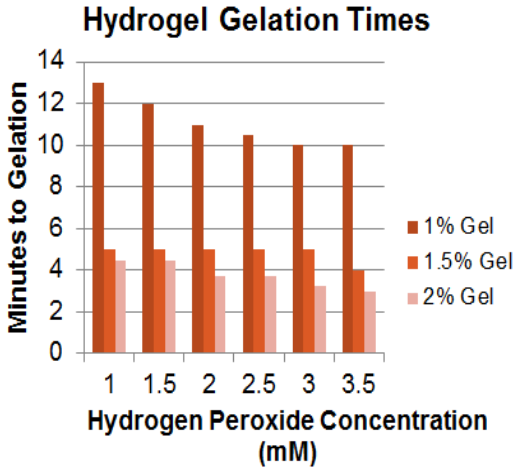


Figure 4.11: Gelation time data

Chapter 5: Design Verification

The raw results of each validation experiment are presented in this chapter. Data was collected from AFM measurements of baseline hydrogels, microscopy of a gradient containing fluorescent dye, and observations from cell biocompatibility and behavior studies.

5.1 Baseline AFM measurements

In Chapter 4, preliminary results were reported for baseline single-stiffness Gelatin-HPA hydrogels. However, these results did not achieve the optimal level of stiffness for the 1 to 100kPa gradient. Additional baseline AFM measurements were taken for 1.5% and 2% Gelatin-HPA hydrogels with cross-linker H_2O_2 concentrations 1.5, 2, 2.5, 3, and 3.5 mM. Three samples were prepared for each testing condition, and three AFM measurements were conducted in three different points of each sample. All AFM data is available in Appendix J. All of the data found in Appendix J was averaged to obtain the following plot.

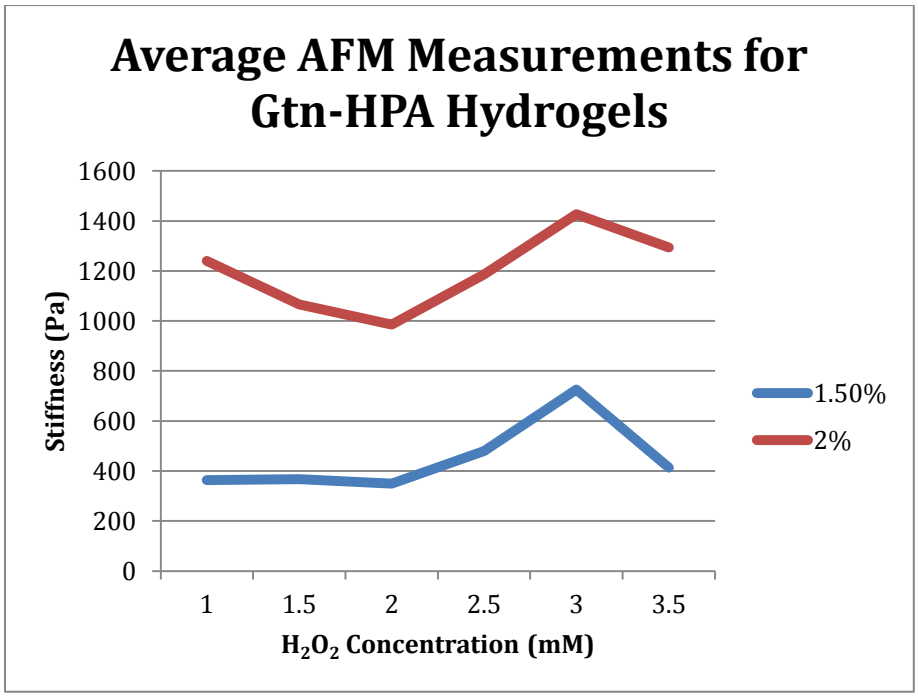


Figure 5.1: Average AFM Measurements for Gtn-HPA Hydrogels.

5.2 Validation of Gradient Creation Components

This section presents the validation of gradient creation within the gradient generator as well as the validation of cell flow throughout the gradient generator system.

5.2.1 Validation of Gradient

To ensure that a gradient could be created with the microfluidic gradient generator, water was mixed with basic food dye and was run through the system. As seen in the figure below, the gradient generator device did indeed create a gradient with the water and food dye.

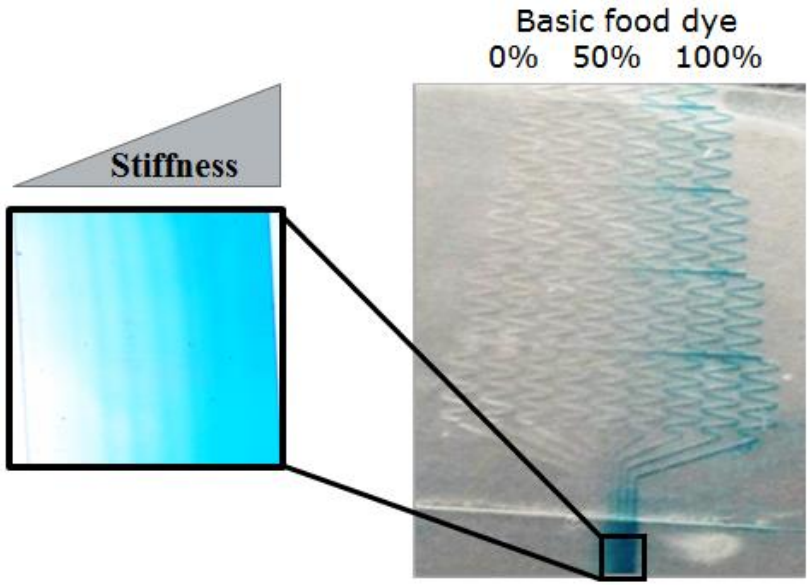


Figure 5.2: Gradients generated with water and food dye

In the figure above, the right image shows the water and food dye as it flows through the gradient maker and mixes together until reaching the final well. The left image shows the water and food dye gradient that was created from this flow system. The stiffness bar shows that the increasing food dye represents the increasing stiffness in a gel.

5.2.2 Validation of Cell Flow

To test if cells were able to flow with ease through the gradient generator, cells were mixed with complete media, loaded into syringes, and were pumped through the system using a syringe pump. The image shown in Figure 5.3 is captured from a video taken of the cells flowing through the system.

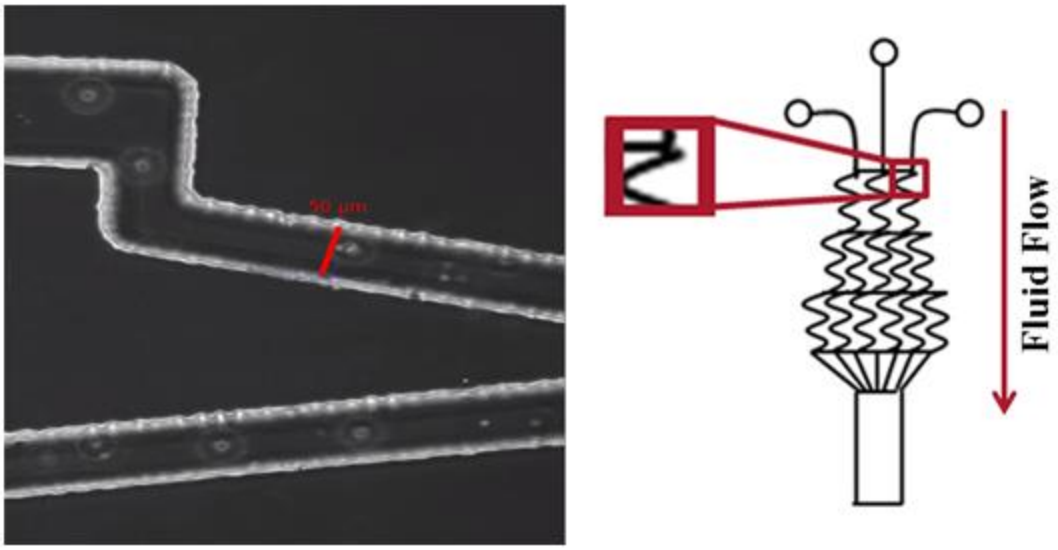


Figure 5.3: Cells Flowing through Gradient generator. The right shows the location of the flow testing within the system and the left shows an image of the cells moving through the system.

While viewing the video, it was determined that there was no problem with cells flowing through the system. As seen in the image above, the cells seemed to flow quite easily through the system and did not appear to block any of the system. This test proved that cell flow through the gradient generator would be simple and would not interfere with any gradient creation.

5.3 Gradient Creation and Visualization

Once gradient creation and cell flow within the generator were validated, the next step was to create the actual 3D hydrogel with a stiffness gradient and encapsulate cells within it.

5.3.1 Creation of Gradient with Conjugates

Following the Standard Operating Procedure in Appendix H, three gel concentrations were flowed through the microfluidic generator to create a gradient. The following table shows which gel conjugate percentage and concentration was in each of the syringes.

	Gtn-HPA	H_2O_2
Syringe 1	1.5%	1.5mM
Syringe 2	2%	1.5mM
Syringe 3	2%	3.0mM

Figure 5.4: Gelatin-HPA percentage and H_2O_2 concentration in each syringe

The syringe pump ran at a flow rate of 15 μL/min for approximately 3 minutes to make the gradient-hydrogel. After this time was complete, the following image was taken of the resulting gel. It is important to note that syringe 1 had no food dye, syringe 2 had the 50% food dye mixed in, and syringe 3 had the 100% food dye mixed in.

Figure 5.5: Gelatin-HPA with H_2O_2 Gradient Gel ranging from softer to stiffer

As seen in the figure, the ranging gradient of blue food dye within the gel is representative of the stiffness level of the gel. The figure shows that the creation of a three dimensional hydrogel system with a continuous stiffness gradient is possible with the use of a microfluidic gradient generator.

5.3.2 Encapsulation of Cells within Gradient

Once it was determined to be possible to create a 3D hydrogel with a stiffness gradient, cells were then encapsulated inside of the 3D environment. Cells were mixed in the gel concentrations listed above in Figure 5.4 and the system was run according to the same protocol. Again, after 3 minutes, the system was stopped and the gel was allowed enough gelation time. After this time was up, the gradient-hydrogel was imaged. The following figure shows cells encapsulated within the gradient gel.

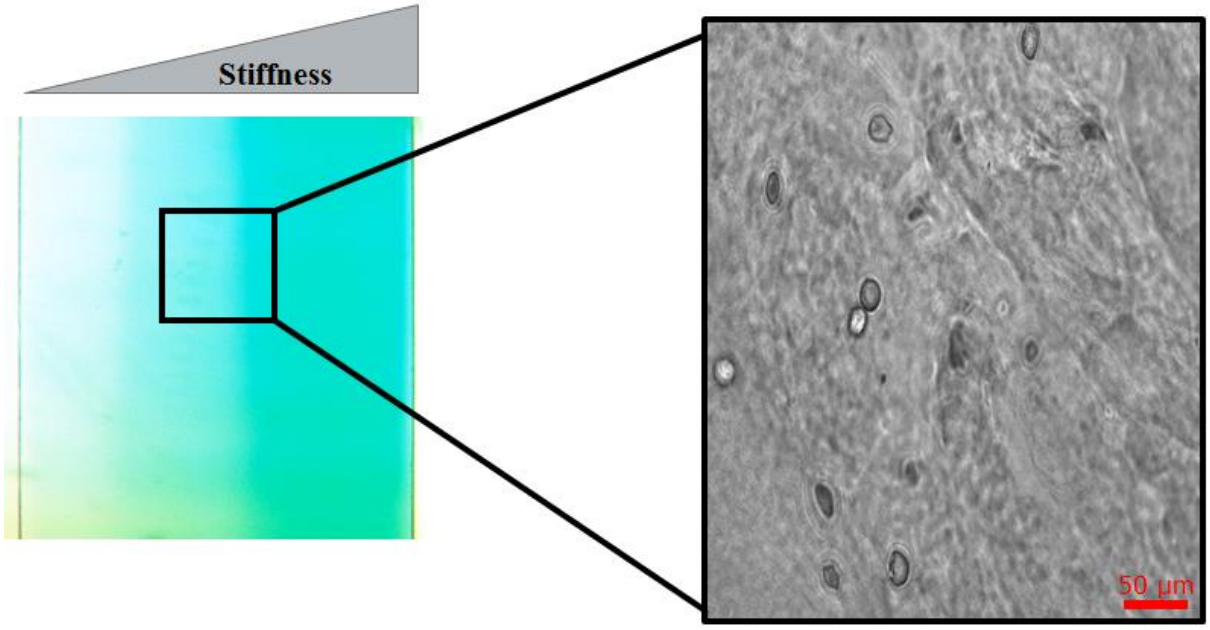


Figure 5.6: Cells encapsulated within the 3D Hydrogel with a stiffness gradient

The figure above shows that some cells are in focus in the image while others are not completely in focus. This tells viewers that the cells are in different planes of the gel, meaning that they are encapsulated in a three dimensional environment within the gel.

With the creation of a gradient and the encapsulation of cells, the final goal of the project was completed; to create a three dimensional hydrogel system with a continuous stiffness gradient for the encapsulation of cells.

5.3 Cell Culture

In Chapter 4 the results of a preliminary cell biocompatibility and behavior study were reported. After modifying the hydrogel formulas, the team decided to conduct a second study using the following formulas:

- 1.5% and 2% Gelatin-HPA
    - 1, 1.5, 2, 2.5, 3, 3.5 mM H_2O_2

For this study NIH 3T3 Mouse Fibroblasts, Primary Human 2097 Fibroblasts, STO Mouse Embryonic Fibroblasts, and Mouse Embryonic Stem Cells were each cultured in 96-well plates. Each plate was prepared with the following conditions:

- Cells encapsulated in hydrogel (100,000 cells)
- Cells seeded on the surface of hydrogel (2,000 cells)
- Cells seeded directly onto the polystyrene well (2,000 cells)

5.3.1 Cell Culture

After imagine the cells at Day 0, the cells were imaged every three days. The first figure shows images taken of the 3T3 Mouse Fibroblasts.

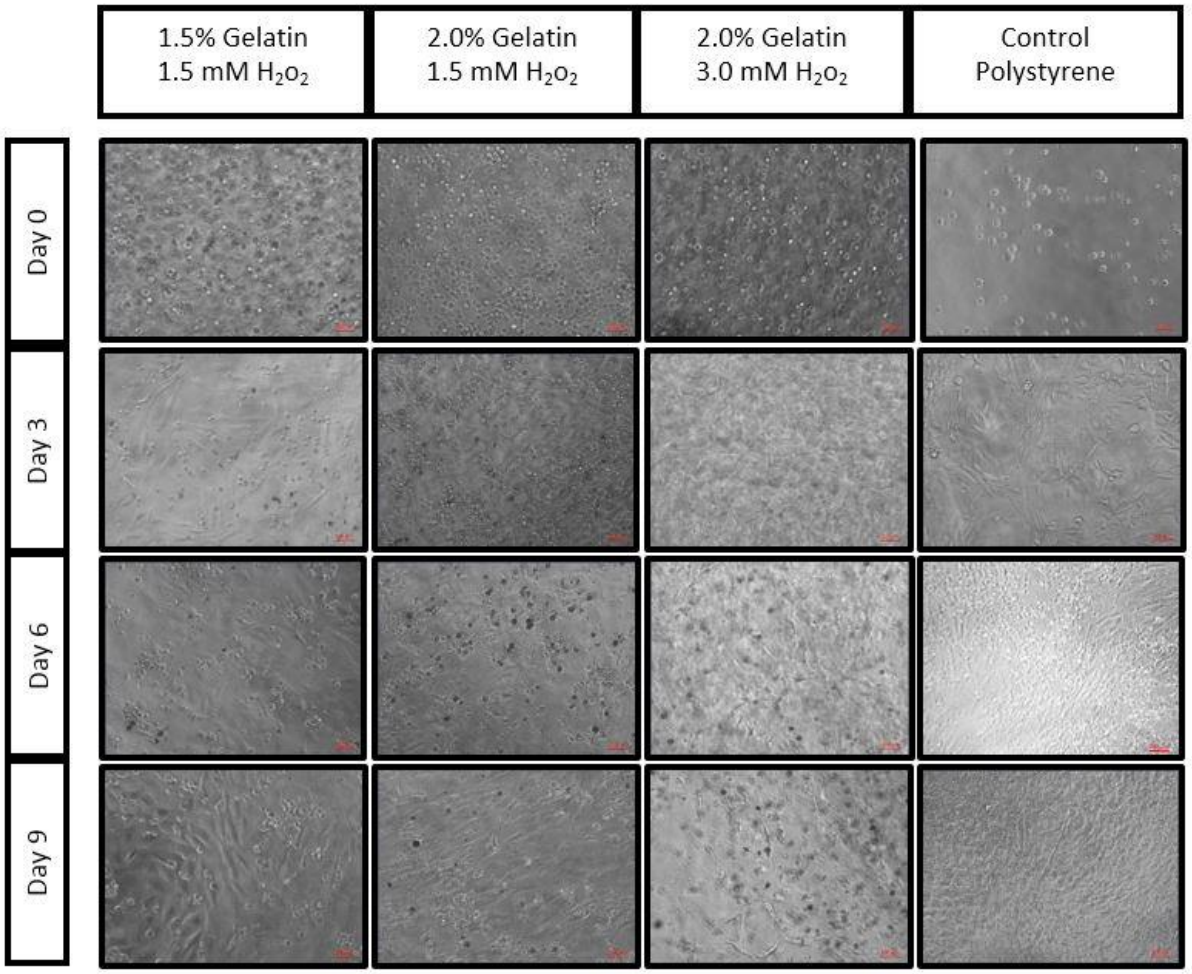


Figure 5.7: Mouse Fibroblast cells (3T3) in 3D Gel

NIH 3T3 mouse fibroblasts encapsulated in the hydrogels (1.5% gtn-HPA 1.5 mM H_2O_2, 2% gtn-HPA 1.5 mM H_2O_2, and 2% 3.0 mM H_2O_2) and plated on polystyrene as a control both showed small balled morphologies at day 0 imaging, indicative of recent plating. By day 3, some spreading was visible among all conditions. By day 6, the control polystyrene wells showed high confluency in comparison to the encapsulated cells. Some dark cells can be seen in the 2% gtn-HPA 1.5 mM H_2O_2 and 2% gtn-HPA 3.0 mM H_2O_2 conditions, indicating cell death. By day 9, a large amount of cells can be seen spread throughout the hydrogels.

Next, the following figure shows the 2097 Primary Human Fibroblast cells over the course of the experiment.

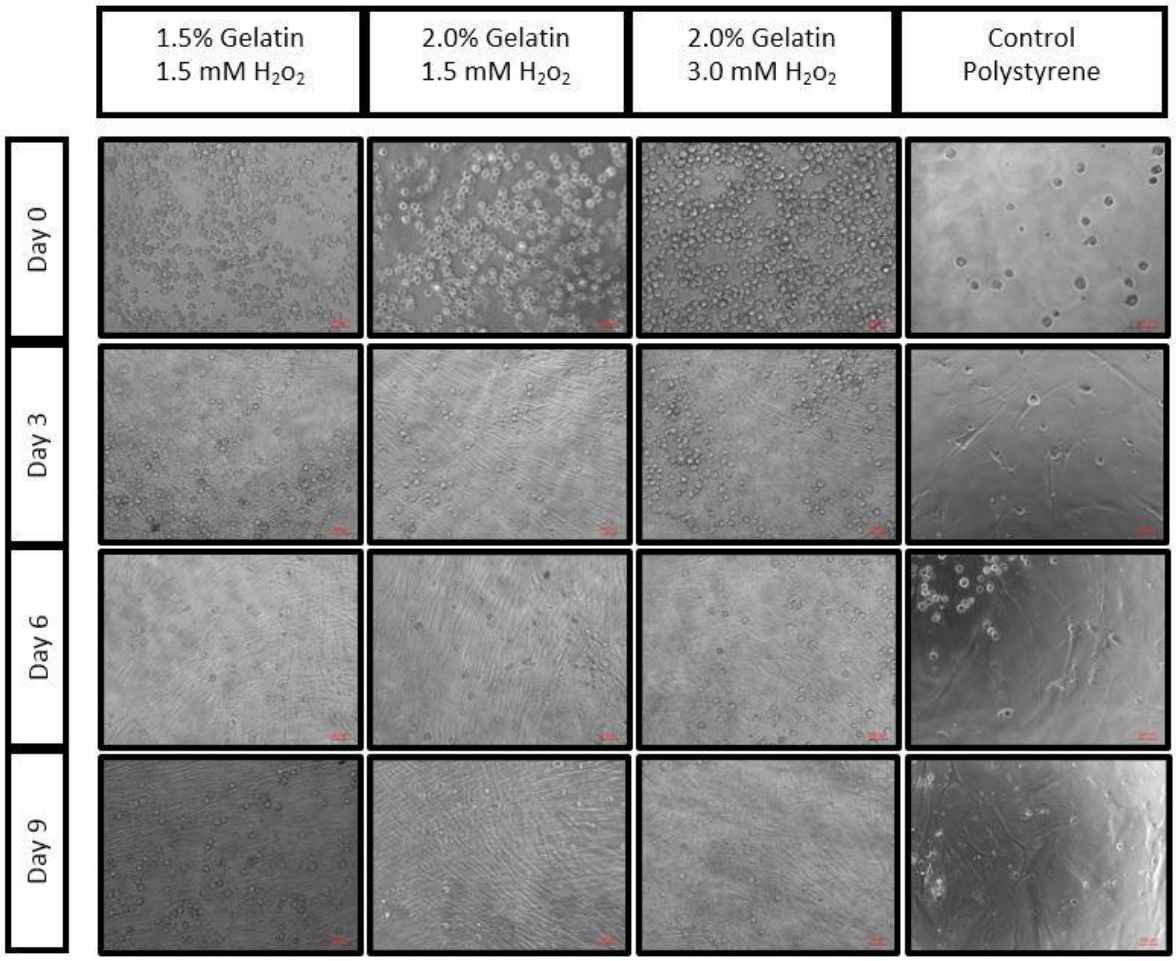


Figure 5.8: Primary Human Fibroblast cells (2097 line) in 3D Gel

2097 primary human fibroblasts encapsulated in the hydrogels (1.5% gtn-HPA 1.5 mM H_2O_2, 2% gtn-HPA 1.5 mM H_2O_2, and 2% 3.0 mM H_2O_2) and plated on polystyrene as a control both showed small balled morphologies at day 0 imaging as well, indicative of recent plating. At day 3, some of the encapsulated cells remain balled while some can be seen spreading through the gel and extending narrow protrusions. The control cells can be seen spreading and developing thicker protrusions. By day 6, some dark cells can be seen across all cell conditions indicating minimal cell death. By day 9, the encapsulated spread cell can be seen overlapping each other, forming a grid-like pattern. Significant amounts of spreading can be seen in the control cells at day 9.

Finally, the results for the STO embryonic mouse fibroblast cell line are shown in the figure below.

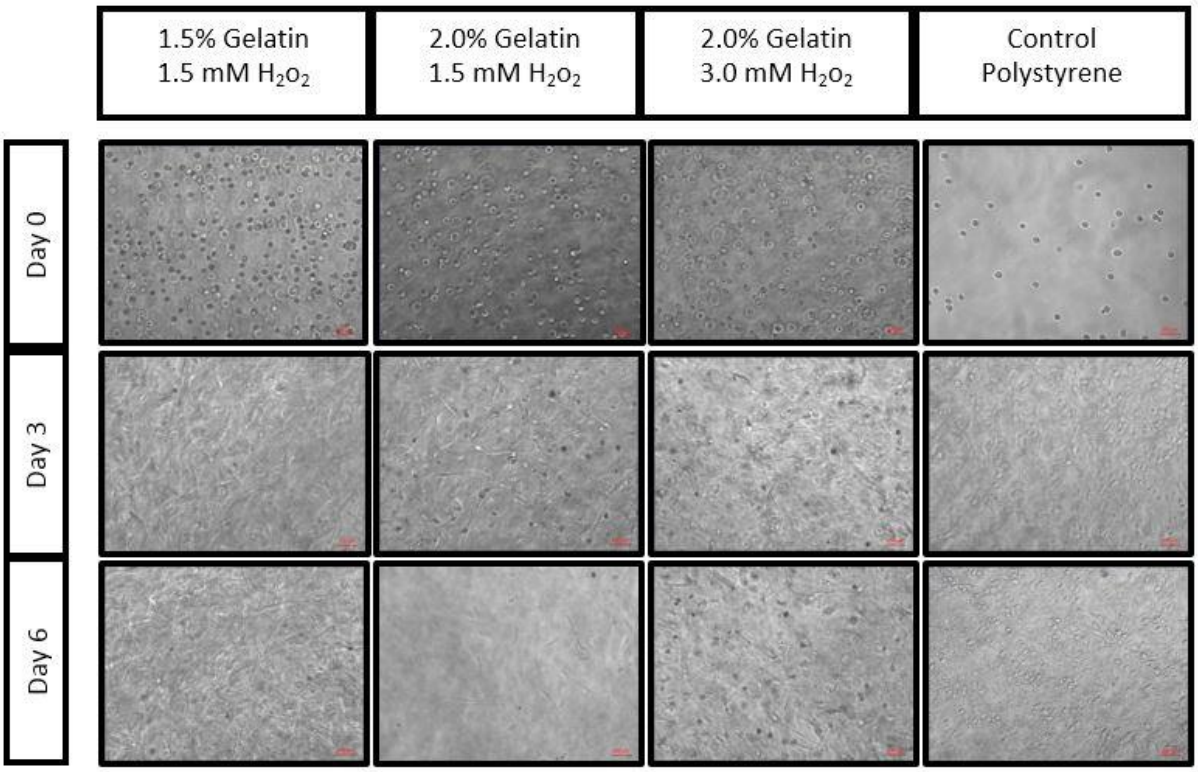


Figure 5.9: Mouse embryonic fibroblast cell line (STO) in 3D gel

STO embryonic mouse fibroblasts encapsulated in the hydrogels (1.5% gtn-HPA 1.5 mM H_2O_2, 2% gtn-HPA 1.5 mM H_2O_2, and 2% 3.0 mM H_2O_2) and plated on polystyrene as a control both also showed small balled morphologies at day 0 imaging, indicative of recent plating. By day 3, the encapsulated cells can be seen very clearly in 3D beginning to spread throughout the hydrogel. By day 6, the cells show similar morphologies to the previous imaging period. The polystyrene control shows a high confluency of cells, indicating the proliferation of cells.

Chapter 6: Discussion

In this chapter, the results for AFM measurements, gradient visualization, cell biocompatibility, and cell behavior are analyzed and discussed.

6.1 Gelatin-HPA Hydrogel Stiffness

Data from the AFM measurements showed that an increase in cross-linker concentration from 2mM to 3mM H_2O_2 resulted in an increase in gelatin-HPA hydrogel stiffness. After the 3mM H_2O_2 concentration, an increase in H_2O_2 concentration resulted in decreased stiffness. At this high concentration of H_2O_2 it is likely that the gelatin-HPA becomes fully saturated with cross-links produced by the H_2O_2. The gelatin-HPA hydrogel is formed through an enzyme-mediated oxidative coupling reaction. The H_2O_2 plays a role in determining the hydrogel stiffness while the HRP determines the gelation rate. The data suggest that at 3 mM H_2O_2 the optimal amount of available phenol groups are coupled into cross-links, forming the stiffest possible hydrogel. At H_2O_2 concentrations greater than 3 mM, additional H_2O_2 creates an excess of cross-linking chains, which diminishes the mechanical properties of the hydrogel.

The stiffness levels that were achieved ranged from 300 Pa (1.5% gtn-HPA, 1mM H_2O_2) to 1500 Pa (2% gtn-HPA, 3.5mM H_2O_2). Reported values from previous 2% gelatin-HPA hydrogel studies conducted by Wang et al. (2012) showed a stiffness range from 20 to 1000 Pa for H_2O_2 concentrations ranging from 0.5 mM to 1.7 mM. These reported values are very similar to the values achieved through the AFM analysis conducted for the team's design.

While the AFM measurements provided data that identified the range of achievable stiffness, there are several limitations that should be considered when evaluating the data. The methods for hydrogel creation introduce a level of variability in the AFM results. The gelatin-HPA hydrogel is formed through a chemical cross-linking reaction. In order for the gel to be completely uniform, it must be thoroughly mixed before pipetting onto coverslips for AFM. Any user error in mixing can result in a hydrogel with variable stiffness. Some of the hydrogel samples that were measured showed high standard deviations between each sampling point. For the purpose of data analysis, any extreme outliers (standard deviation > 2,000) were removed from averages.

6.2 Gradient Visualization

With the assistance of basic food dye, it was possible to view that a continuous gradient was created using the microfluidic gradient generator. The range of food dye color represents the range of stiffness across the hydrogel.

Through various trials, the team was able to flow the Gelatin-HPA with hydrogen peroxide through the system in order to create a gel with a stiffness gradient. More impressively it was found to be possible to flow cells through the gradient generator with the gel precursors, ultimately creating the final goal of a 3D, cell encapsulating hydrogel with a continuous stiffness gradient. Through the successful incorporation of cells, the team showed that it is indeed possible to suspend the cells in a 3D culture system using the design methods.

Throughout the studies, a few limitations of the system were determined. First, the camera on the microscope used for imagining did not have a high enough resolution. This made it difficult to capture cell flow and gradient creation. Next, it was determined that the gradient was easiest to create before the addition of the cross-linker. Although this is beneficial for visualization purposes, the cross-linker is necessary to reach full gelation and properly suspend cells in 3D. It would be ideal if this part of the system could be refined so that gelation can fully occur without clogging gradient channels. Also, when the cross-linker was added prior to flowing the system, the time period for flow was limited to about 3 minutes due to the rapid gelation time of the conjugates. The team needed to mix the conjugates and set up the system very time efficiently in order to create a high quality gradient-hydrogel, which was challenging. Finally, bubbles disturbed the gradient on multiple occasions. While preparing the gradient generator mold and setting up the system, there was always a risk of air bubbles getting inside the system. Once air is trapped within the mold it is almost impossible to remove. It would be beneficial to create a method for prevention or removal of trapped air so that the gradient is not altered by air bubbles in the future.

6.3 Cell Biocompatibility

The gelatin-HPA hydrogel was anticipated to show biocompatible results, as gelatin is naturally experienced *in vivo*. However, a concern that was the H_2O_2 could adversely affect cell viability above a certain concentration. The 2-week cell study showed that cells encapsulated within the

hydrogels thrived. Cells that were seeded on the hydrogel surface thrived all H_2O_2 concentrations except the 3 and 3.5 mM. Dead cells were seen in the 3 and 3.5 mM concentrations starting at day three, and by day twelve all cells in the 3 and 3.5 mM concentrations were dead.

Studies conducted by Wang *et al* (2012) showed that there was a high tolerance towards H_2O_2 for aNSCs encapsulated within gelatin-HPA hydrogels. Experiments showed that the viability of aNSCs did not change when exposed to H_2O_2 concentrations up to 200 µM. At 500 µM the aNSCs showed apparent cell death. The team used H_2O_2 concentrations ranging from 1 to 3.5 mM (1000 to 3500 µM). In comparison, the results of the biocompatibility study suggest that the cells were able to withstand a much higher concentration of H_2O_2 than in previous studies.

AFM measurements revealed that at 3 mM H_2O_2 the hydrogel becomes fully saturated with cross-links, meaning that at increased concentrations there is remaining unused H_2O_2. The most likely reason for the cell death occurring for the cells seeded on the surface of the hydrogels is because the excess H_2O_2 is leeching out from the gel into the media. In future studies, the hydrogels should be rinsed with DPBS several times before seeding the cells in order to remove the excess H_2O_2.

6.4 Cell Behavior

The cell behavior studies showed significant differences in the morphologies of the cells seeded within the hydrogels in comparison to those that were controls on the polystyrene plates. Often in the hydrogels an assortment of morphologies were seen. Within a single hydrogel there was a variance in morphologies as some cells maintained small balled morphologies while others showed significant spreading.

Observations indicated a high rate of cell death in 1.5% and 2.0% hydrogels with a 3.5mM H_2O_2 concentration. However, cells in the hydrogels cross-linked with lower H_2O_2 concentrations thrived. Despite the issue with toxicity in the hydrogels with 3.5mM H_2O_2 concentrations, the cells encapsulated and seeded atop the gels with lower concentration appeared to have a high rate of survival. This conclusion was drawn by visual inspection of the plate. The cells were plated at a low density, allowing for the easy observation of individual cell morphology to assess whether or not the cells were alive and attached to the substrate. Inspection of the culture plates showed that during the cell study period, the media continually turned yellow, suggesting cell survival

and proliferation. In the plates containing human fibroblasts and mouse fibroblasts, there were significant amounts of living cells at the end of the two-week study.

Cell behavior studies conducted on aNSCs showed decreased proliferation in all gelatin-HPA hydrogels, with the highest cross-linking densities showing the most extreme difference. The team observed that the encapsulated cells were proliferating, however attempts at conducting a BrdU assay to quantify proliferation were unsuccessful. In some culture plates, the stains were unable to diffuse through the hydrogel, while in others the stained images were difficult to focus on through the 3D hydrogel.

Wang *et al.* (2012) found that aNSCs encapsulated in gelatin-HPA hydrogels differentiated into neurons and astrocytes when exposed to certain stimuli. It was found that for the aNSCs the density of the cross-linking did not significantly affect differentiation. The team cultured eMSCs on the surface of the gelatin-HPA hydrogels, but differentiation was not observed.

There are several limitations that should be considered when analyzing the cell studies. A major challenge was the inability to count the cells once they were suspended in 3D. It is not possible to extract the cells from the cross-linked hydrogel, and there are no distinguished methods for counting cells within a 3D hydrogel. Until a method can be established to remedy this issue, it will be extremely challenging to obtain accurate counts of the cells within 3D hydrogels. Another challenge with the culturing of the hydrogels is the replenishment and removal of media from the wells. Aspiration using a Pasteur Pipette does not allow for the necessary precision and control and is therefore not an option, however even the use of a micropipette can be concerning. As the softer hydrogels spend more time suspended in media, they swell and become more likely to be accidently aspirated into a pipette when removing depleted media. For this reason, it is important to keep careful records of what amount of media was added to each well to avoid unintentional removal of the hydrogels. Finally, many staining procedures that are currently used are not adapted to use for cells suspended with a medium. When staining cells, it is necessary to account for the time necessary for the stain to be able to diffuse through the hydrogel to the cells.

6.5 Design Considerations

6.5.1 Economics

After additional refinement, the design could become a patented cell culture system available to biomedical research companies. This would introduce competition within current cell culture techniques used for biomedical research and would promote the development of additional 3D culture systems that better represent *in vivo* stiffness levels.

6.5.2 Environmental Impact

The creation of the design will have little impact on the natural environment. The hydrogels form through a cross-linking reaction that does not release harmful toxins into the environment. All design preparation and experiments must be conducted in a laboratory, so there is no direct impact on the outside environment.

6.5.3 Societal Influence

By allowing for the creation of *in vitro* models more reflective of conditions within the body, the design could potentially lead to the discoveries of new treatments and cures for various medical conditions. This can ultimately lead to a healthier population.

6.5.4 Political Ramifications

The design has the potential to revolutionize cell culture methods by making them more representative of *in vivo* conditions, meaning that any stakeholders investing in use of the culture system described in this report could increase the value and integrity of their company. The design pertains to cell culture with various cell types, and can therefore be applied to clinical circumstances such as the co-culturing of cells, the development of organoids for *in vitro* drug testing, and the culturing of patient derived cells. Having wide variety of clinical applications, the 3D hydrogel system containing a realistic stiffness gradient can be used for research by companies on a global scale.

6.5.5 Ethical Concern

A major application of the design would be in the study of stem cells to see their behavior at a range of stiffness within the body. This application could produce some controversy. However, the benefits that could result from using stem cells to develop new methods of treatments may outweigh this ethical conflict.

6.5.6 Health and Safety Issues

There are little safety concerns involving the design. However, the design involves the use of biological materials and therefore must be handled in a biosafety cabinet. Any chemicals must be disposed of in a biohazardous waste bin as appropriate. If the user disposes of hazardous chemicals improperly, for example down the drain, then there could be a potential negative impact on the environment as the chemicals would then be introduced into the local water supply.

6.5.7 Manufacturability

The design could eventually be manufactured, which would produce a marketable product. Currently, the design would need to be manufactured as a kit. The kit would include the gelatin-HPA conjugate, the gradient generator mold, and any small features such as syringes and tubing that are necessary for the design. Instructions provided with the kit would allow for others to create their own 3D stiffness gradient-hydrogel systems.

6.5.8 Sustainability

The hydrogel system is composed largely from the natural polymer gelatin. Gelatin occurs in living organisms, meaning that, unlike synthetic polymers, it can be easily derived without exhausting resources for synthesis.

6.6 Financial Considerations

The financial considerations were analyzed to determine the cost associated with making a single gradient gel from the microfluidic gradient generator. First, the costs to make one gel conjugate batch from a full preparation were determined. Following the scaled-down version of the protocol in Appendix C, the following cost chart (Figure 6.1) was created for a gel conjugate preparation.

Conjugate Preparation Costs				
Material	**Unit**	**Unit Price**	**Quantity Needed**	**TOTAL**
N, N-dimethylformamide	1000 mL	$101.00	30 mL	$3.02
Hydrophenylpropionic (HPA)	25 g	$71.80	0.332 g	$0.95
N-Hydroxysuccinimide	25 g	$36.50	0.32 g	$0.47
Dimethylaminopropyl carbodiimide hydrochloride	1 g	$20.00	0.382 g	$7.64
Gelatin from Porcine Skin	100 g	$20	0.9375 g	$0.25
Sodium Chloride	1000 L	$38	23.37 g	$0.89
Ethanol 190 Proof	4L	$296	1 L	$74
Spectra/Por 6 dialysis tubing, 1K MWCO, 45mm flat width*	1 roll of 33 ft	$272	10 in	$6.87
Standard dialysis tubing closures*	10 pack	79.2	4	$31.68
			TOTAL	$125.78

Materials from Sigma-Aldrich; * Materials from Spectrum Labs
**Produces 1.5806 g of conjugate

Figure 6.1: Gel Conjugate Preparation Costs

From this analysis, it was determined that it takes $125.78 to produce 1.5806 grams of gelatin-HPA conjugate. A single gradient-hydrogel uses 960 µL of gelatin-HPA conjugate. The cost to make a single 960 µL gel was derived through further calculations. Figure 6.2 shows this analysis, based off of the 1.5% gels made using the protocol in Appendix D.

1.5% Gel Cost (960 uL)				
Material	Unit	Unit Price	Quantity Needed	TOTAL
Gtn-HPA	1.5806 g	$125.78	480 uL	$1.15
HRP	10 mg	$71.00	40 uL	$0.03
10x DMEM	500 g	$28.00	192 uL	$0.0001
H2O2	5 mL	$36.80	48 uL	$0.00
DPBS	500 mL	$18.35	200 uL	$0.007
			TOTAL	$1.19

*Materials from Sigma-Aldrich and Life Technologies

Figure 6.2: Cost Analysis for the Creation of Gradient Gels

From this analysis, it was determined that 960 µL of a 1.5% gel could be created for $1.19.

Next, the gradient generator creation and preparation costs were analyzed. These costs can be found in the Figure 6.3.

Gradient Generator Cost				
Material	Unit	Unit Price	Quantity Needed	TOTAL
PDMS	500 g	$49	30 g	$2.94
Coverslides	72 slides	$4.25	1 slide	$0.06
*Materials from Dow Corning and Education Innovations			TOTAL	$3.00

Figure 6.3: Cost Analysis for the Gradient Generator Creation

From this analysis, it was determined that a gradient generator costs $3.00 to make.

The total cost of the gradient gel system was calculated based on these analyses. Since each gradient gel requires the use of three separate 960 μL gels and one gradient generator, the total cost is as follows:

TOTAL COST OF SYSTEM:
(3 gels x $1.19) + (1 gradient generator x $3.00) = **$6.57**

As seen above, the total cost of the gradient generator system is $6.57.

Chapter 7: Final Design and Validation

This chapter describes how each of the design aims were met, and how the project was conducted. The final, validated design is described in detail.

7.1 Final Design

The final design consists of a gelatin-HPA conjugate hydrogel, a microfluidic gradient generator, and a syringe pump system (Figure 7.1). The gelatin-HPA hydrogel as described by Wang *et al*. (2012) was chosen for the hydrogel system because it is biocompatible, biodegradable, transparent enough to image cells, easily tunable mechanically, and can be cross-linked without compromising cell viability. The microfluidic gradient generator was chosen as the method for gradient formation because it creates a continuous gradient, allows for cells to pass through the channels, and is capable of mixing various concentrations.

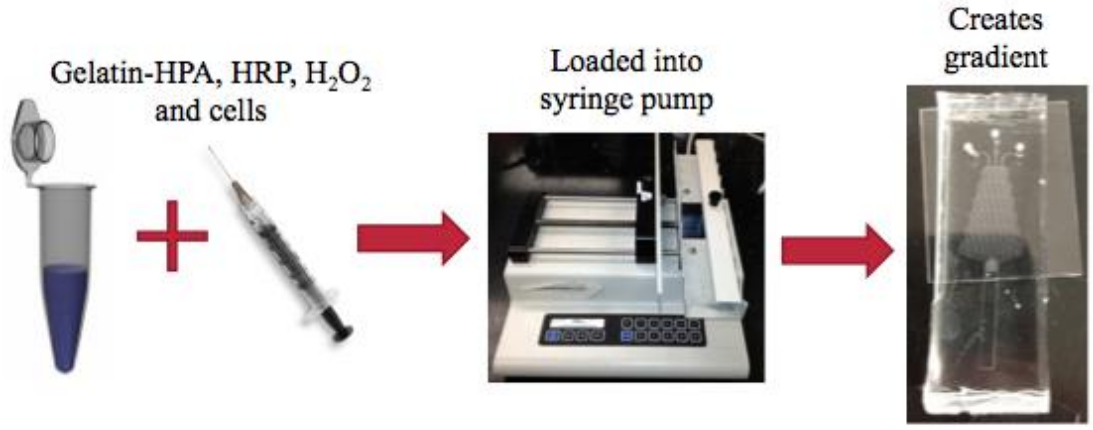


Figure 7.1: Final Design Methods

7.2 Design Methods

The following sections describe the methods that were followed in order to achieve the final design.

7.2.1 Hydrogel Preparation

The gelatin-HPA conjugate was synthesized following Wang *et al.*'s procedure, described in Appendix C. All reagents were mixed and maintained at a pH of 4.7, dialyzed against salt water, ethanol, and water, and lyophilized. In order to create gelatin-HPA hydrogels of certain

concentrations, a stock mixture of 3% gelatin-HPA was created. Table 7.1 summarizes the calculated formulas for 1%, 1.5%, and 2% gelatin-HPA dilutions.

Table 7.1: Gelatin-HPA Hydrogel Formulas

Reagents	1%	1.5%	2%
1. Gelatin-HPA (3% Stock)	40 µL	60 µL	80 µL
2. HRP (10 U/mL) (Final conc. 0.1 U/mL)	5 µL	5 µL	5 µL
3. DPBS (+)	45 µL	25 µL	5 µL
4. 10X DMEM Cell Suspension	24 µL	24 µL	24 µL
5. H_2O_2	6 µL	6 µL	6 µL

When mixing the hydrogel precursors, each reagent was warmed to 30 °C. Hydrogel precursors were mixed in microcentrifuge tubes. The order that each reagent was added when mixing the hydrogel precursors was important. The reagents were added following the sequence outlined in Table 7.1. To achieve varied stiffness, different H_2O_2 stock solutions were used (Table 7.2). After adding the H_2O_2, the gel precursors were mixed 4 times by pipetting up and down with a 100 µL pipette. A detailed procedure for preparing the hydrogels is located in Appendix D.

Table 7.2: H_2O_2 Stock Solutions

Final H_2O_2 Concentration	Stock Concentration
1 mM	20 mM
1.5 mM	30 mM
2 mM	40 mM
2.5 mM	50 mM
3 mM	60 mM
3.5 mM	70 mM

7.2.2 Gradient Generator Preparation

Microfluidic gradient generators were prepared following the protocol outlined in Appendix E. Molds were created from silicone wafers using PDMS. In order to provide adequate surface tension in the microfluidic channels, a glass coverslip was plasma bonded over the mixing portion of the mold. When plasma bonding the glass to the PDMS, it was crucial that both parts

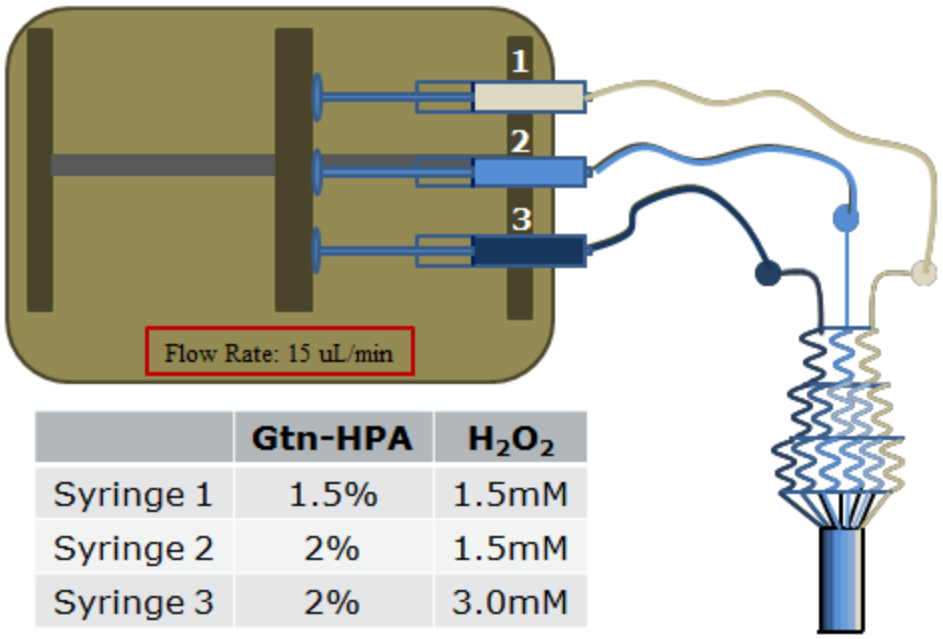
were completely clean and dust free. In order to create a full, permanent plasma bond it is important not to touch any of the treated area when handling the plasma treated glass. To finish preparing the gradient generator for flowing, a second glass coverslip was cleaned and pressed firmly onto the area of the generator where the gradient forms. To seal the system, a piece of Scotch tape was pressed firmly onto the area where the two glass coverslips meet.

7.2.3 Stiffness Validation

Stiffness of the hydrogels was measured using the AFM protocol outlined in Appendix F. Gelatin-HPA hydrogels were formed on treated coverslips the day of AFM testing. The gels were allowed to cross-link for 30 minutes before the top coverslip was removed. DPBS was added and the hydrogels were hydrated for 15 minutes before being measured. For each sample, three measurements were taken at three random points across the gel's surface. The data was then analyzed using a MATLAB script provided by WPI Graduate Student, Gawain Thomas.

7.2.4 Flow Methods

The flow methods used to create gradient-hydrogels are outlined in detail in Appendix H. When flowing the gradient-hydrogel, three different hydrogel combinations were mixed into microcentrifuge tubes. In order to visualize the gradient, concentrated dyes were used in place of the DPBS (+) for validation experiments. The concentrations used were: 1.5% gtn-HPA with 3.0 mM H_2O_2, 2% gtn-HPA with 1 mM H_2O_2, and 2% gtn-HPA with 3.0 mM H_2O_2. Once the H_2O_2 was added to the tubes, syringes were loaded with each individual combination. After removing any air bubbles, locking the syringes in place, and attaching the tubing, flow was started at a rate of 15 μL/min (Figure 7.2). After about 3 minutes of flowing, the hydrogels fully cross-linked and a gradient was visible (Figure 7.3).



	Gtn-HPA	H_2O_2
Syringe 1	1.5%	1.5mM
Syringe 2	2%	1.5mM
Syringe 3	2%	3.0mM

Figure 7.2: Syringe Pump Setup

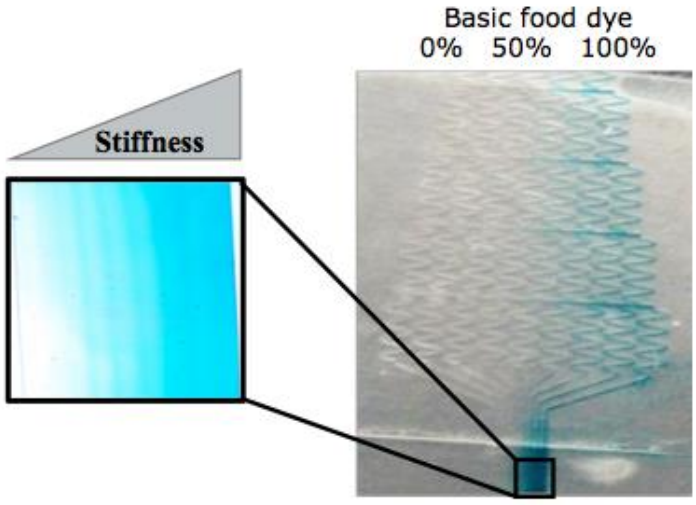


Figure 7.3: Gradient Illustration

Chapter 8: Conclusions and Recommendations

The 3D hydrogel system with a continuous stiffness gradient developed for this project has been fully developed conceptually. While many validations have proved that the concept will yield a functional 3D hydrogel system, the stiffness of a gradient-hydrogel has not yet been measured. To conclude, final points and recommendations for future work are discussed in this chapter.

8.1 Conclusions

The team has developed methods that allow for cells to be encapsulated in a 3D hydrogel with a continuous stiffness gradient. The design incorporates a biocompatible cross-linking method for a hydrogel that gels rapidly within 3 to 10 minutes, allowing for 3D suspension of cells. The gelatin-HPA hydrogel was easily modified to achieve stiffness levels that surpass those of previous studies, allowing for 300 to 1500 Pa to be represented in the gradient. The gradient was validated to be continuous by using concentrated dyes and microscopy, and the biocompatibility and the dimensionality of the hydrogel were validated through a cell culture study.

Traditional cell culture methods do not accurately reflect conditions natively experienced by cells, and can therefore not be used as clinical models. This 3D hydrogel system has the potential to revolutionize cell culture techniques. By encapsulating cells in a biocompatible, 3D environment, cells can interact with one another, develop morphologies, and proliferate at a rate more representative of how they would behave naturally within tissue. The 3D gelatin-HPA hydrogel with a stiffness gradient from 300 to 1500 Pa allows for cell cultures to be conducted in a substrate with a more realistic stiffness level and dimensionality reflective of *in vivo* conditions.

While the gradient 3D hydrogel system provides a highly representative *in vitro* model for cell culture, there are several challenges associated with common use of this design. In particular, it is difficult to focus on cells when imaging a 3D culture because encapsulated cells are suspended throughout various layers of the hydrogel. For this project, videos were taken to easily view cells in all levels of focus. However in order for 3D systems to become a mainstream cell culture technique, imaging methods must be more thoroughly established.

This 3D hydrogel system approach can be a used as an *in vitro* model system to study changes in a multitude of cell behavior as they respond to changes in substrate stiffness in a 3D environment. This design allows for the production of hydrogels engineered to assist in the development of tissue-engineered scaffolds that mimic biomechanical properties specific to tissues for a wide range of clinical applications. Stiffness gradients can occur naturally within tissues, or from pathological causes such as tumors or cardiac infarction. This stiffness gradient 3D hydrogel system can potentially be applied as scaffolds for the development of organoids for *in vitro* clinical testing.

After performing multiple validation experiments, the team was able to show that the gelatin-HPA hydrogel can be used to encapsulate cells in 3D, biocompatible environment, that it can create a range of *in vivo* stiffness levels through increased H_2O_2 concentration, and that it can be flowed through the microfluidic generator with cells to create a hydrogel with a continuous gradient in stiffness. However, due to limitations with time and resources the design can still use future refinement in order to enhance efficiency. The recommendations for future work are discussed in the next section.

8.2 Recommendations for Future Work

In the future, modifications should be made to improve the efficiency and accuracy of the gradient-hydrogel formation. Several of the major design challenges involved the consistency of gels when flowing the gradient generator multiple times, the incorporation of H_2O_2 without obstructive gelation occurring in the generator, the disruption of flow caused by air bubbles in the generator channels, and the clogging of generators after single use.

Development of a more controlled flow system

The current set-up for creating gradient-hydrogels through the gradient generator flow system has many factors that can introduce variability in the hydrogels that are formed. When setting up the system, the gel precursors must be prepared, thoroughly mixed in microcentrifuge tubes, and maintained at a temperature of 30 °C. The gradient generator and the tubing must then be prepared with water to remove any air bubbles prior to flowing the gel precursors. Once all equipment is prepped and in place, the H_2O_2 must be added at the same moment to each of the microcentrifuge tubes. This requires multiple personnel to work together. After the H_2O_2 has

been added, the syringe pumps must be loaded, the air bubbles removed, and then the syringes must be locked in place to start flowing. In about 3 minutes, the gel precursors will have gelled, meaning that the aforementioned steps must completed efficiently in a timely manner. Even following the SOP for gradient-hydrogel formation, there are many steps that could introduce variability into the final gradient-gel. In the future, the development of a system with minimal steps, or more mechanically controlled steps, could help to better control the flow process and produce more consistent gradient-hydrogels.

Downstream incorporation of H_2O_2 to improve gelation time

The gelatin-HPA hydrogel formulas that were used for this project all had gelation times ranging from 3 to 10 minutes. These fast gelation times enable cells to be encapsulated in a 3D environment, however they cause the gradient generator to clog after about 3 minutes of flowing hydrogel precursors. A future modification that would enhance the efficiency of design would be to find a better method of incorporating the H_2O_2 downstream of the inlets. A new microfluidic mold could be designed where additional channels connect closer to the outlet where the gradient-hydrogel will be formed. This would allow more time to set up the whole flow system and could help to avoid clogging of the channels.

Development of a heated platform to slow gelation time

Another proposed method for preventing gelation of the hydrogel within the microfluidic channels is the development of a heated platform upon which the flow system can rest. Gelatin is thermoresponsive because it gels more rapidly at low temperatures. If the entire flow system were heated, then it is possible that the amount of time the hydrogel solution can be flowed without clogging the channels will increase. This would allow for easier handling of the hydrogel solutions during the flow process.

Method for removing air bubbles from microfluidic channels

One of the major limitations in creating a continuous gradient is the distortion of flow caused by air trapped within the gradient generator. The team tried removing air from the channels by increasing the flow rate to blast air out, flowing liquid through each inlet of the generator manually by syringe to push out bubbles, and by placing the generator in a liquid-filled dish under a vacuum. The most effective method of removal was to gently flow liquid through each inlet to prep the generator. However, bubbles were still noticeable in the system. After

conducting research on methods currently used to remove air from microfluidic devices, the team recommends the incorporation of an air filter at the inlets of the generator. This would allow for any air to be filtered out of the generator as the precursors flowed through the inlet, and would aid in generating more consistent continuous gradients.

Method for cleaning used generator molds

The gradient generator is plasma bonded permanently to the glass coverslip covering the microfluidic channels. Ideally, each gradient generator mold should be able to be used repeatedly. However, by flowing the hydrogel precursors mixed with the H_2O_2 the microfluidic channels become clogged and are difficult to completely clean and sterilize. Finding the appropriate solvent to break the cross-links within the gelatin-HPA hydrogel will allow for molds to be reused. For this project, gradient generators were made out of PDMS and plasma treated each time the team needed to flow gradient-hydrogels. Finding a method to reuse the gradient generators will make the process more efficient by saving time and resources.

Refined 3D cell encapsulation methods

The procedure in Appendix D outlines the methods with which the 3D hydrogels were made. For the hydrogels formulated using the higher conjugate percentages (1.5% and 2%) and the higher H_2O_2 concentrations (2mM, 2.5 mM, 3mM, and 3.5mM), cells were consistently suspended throughout the 3D hydrogel. However, for the lower conjugate percentages (1% and 1.5%) and the lower H_2O_2 concentrations (1mM and 1.5mM) cells often sank to the bottom of the well before full gelation occurred. In order to accommodate for the longer gelation times of the lower concentration hydrogels, cells were added about a minute after the H_2O_2. Further exploration into the amount of time that should be waited after the H_2O_2 has been added will enhance methods for cell encapsulation in lower concentration hydrogels.

Stiffness validation by rheometer

AFM was used to measure the single stiffness of the gelatin-HPA hydrogels for this project. However, AFM measures the displacement of a cantilever tip on the surface of the hydrogel and can lead to high variation if the hydrogels are not completely uniform in stiffness. The gelatin-HPA formula is dependent on chemical cross-linking, meaning that if the cross-linker is not thoroughly mixed then the stiffness could vary across the hydrogel. A rheometer could be used in the future to achieve more accurate single-stiffness baseline measurements. The rheometer

destroys each sample that it measures, but it is capable of obtaining a stiffness measurement by coming in contact with the entire hydrogel. Using a rheometer could potentially provide more reproducible results and lower standard of deviations for baseline hydrogels.

Stiffness gradient validation methods

Due to time constraints, the team was unable to obtain stiffness measurements for a gradient-containing hydrogel. However, the team has several suggestions for obtaining consistent measurements across the gradient. AFM is the recommended method for measuring the stiffness along the gradient, because it will enable sampling at specific points without destroying the sample. AFM is typically conducted on hydrogels adhered to glass coverslips. In order to measure the same areas of the gradient on different samples, the team suggests incorporating gridlines on the bottom of the coverslip. This would allow for known incremental measurements to be taken on every hydrogel in designated areas. Developing a standard method for stiffness validation of the gradient is crucial for obtaining reproducible results.

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Glossary

Atomic Force Microscopy	A device used to assess stiffness. AFM consists of a cantilever tip that probes the specimen's surface. Once it makes contact, the deflection is measured as a force.
Conjugate	A chemical compound that is formed by joining two or more compounds.
Cross-link	The covalent bond that forms to create networks within a hydrogel.
Cross-linker	A reagent or process that forms networks within a hydrogel.
Extracellular Matrix	The part of a multicellular structure that provides structural and biochemical support to surrounding cells.
Gelatin	Denatured form of collagen.
Gelation time	The amount of time it takes for a hydrogel to fully cross-link.
Hydrogel	Gel composed of one or more polymers suspended in water.
Hydrophilic	Hydrophilic refers to something that is "water-loving".
In Vitro	Any process or reaction occurring outside of a living organism.
In Vivo	Any process or reaction occurring within a living organism.
Microfluidics	The controlled flowing of liquids at a micro-scale level.
PDMS	Polydimethylsiloxane (PDMS) is the most commonly used silicon-based organic polymer. It is often used to make molds.
Polymer	A substance with a molecular structure consisting of many repeating units bonded together.
Rheometer	A device used to measure the viscosity of samples that cannot be defined by a single value. Measures the response to applied forces.
Stiffness	An object's rigidity or resistance to deformation in response to an applied force.

Appendices

Appendix A: Gantt Chart

	A Term		B Term		Winter Break		C Term		D Term	
Literature review										
Write Introduction chapter										
Practice making hydrogels										
Develop hydrogel formula										
Finalize primary design & alternatives										
Write Background chapter										
Write Methods chapter										
Make hydrogel in lab										
Test hydrogel										
Analyze test results										
Revise design if necessary										
Write results										
Culture cells in hydrogel										
Perform assays										
Analyze cell response to stiffness										
Write Discussion chapter										
Write Final Presentation										

Key: Complete In Progress To Be Completed

Appendix B: 6 Hats Method Exercise

Blue Hat: The purpose of this assignment is to analyze design ideas for our MQP, "3D Hydrogel System with a Stiffness Gradient", by applying various perspectives using the 6 Hats Method. Each perspective is intended to promote the development of new ideas that will lead to success and the generation of innovation. The order of the analytical process for this assignment is as follows: the white hat for objective reasoning, the green hat for creativity, the yellow hat for optimism, the red hat for emotion, and the black hat for critical thinking. All ideas will be recorded for later review and evaluation.

White Hat: It is known that tissue-engineering scaffolds are commonly created using three-dimensional hydrogels because their tissue-like environments are similar to what cells would encounter *in vivo*. Stiffness affects certain cell processes such as differentiation, proliferation, and morphology. Hydrogel scaffolds with stiffness gradients have been developed, but there currently is no method to create cell-encapsulating 3D hydrogels that contain continuous, wide-ranging stiffness gradients. Stiffness within the body ranges from approximately 1kPa to 100kPa, a range that has not yet been completely represented within a single hydrogel.

Green Hat: Various methods for gradient formation have been discussed. Gradients may be formulated using variations of polymer molecular weight or cross-linker concentration. Methods that have been considered include using syringe pumps to control flow rates and microfluidic molds. Particular mechanisms for gradient formation that could be applied to the project design are gravitational forces and diffusion. In research, chemical gradients have been made by physically adjusting test tubes to certain angles and allowing gravity to create a gradient. Perhaps this idea could be applied to develop a method for making precise stiffness gradients. Potentially, a microfluidic mold could be designed to incorporate diffusion of a cross-linker via channels into a polymer.

Yellow Hat: By rapid prototyping a unique microfluidic mold, the appropriate dimensions can be used to allow for diffusion of a cross-linker to successfully penetrate the polymer. Ideally, this would produce a hydrogel, create a continuous stiffness gradient, and have high cell viability. This design would be beneficial by keeping the process of formation simple, and would avoid any conflict of premature polymer/cross-linker mixing. Whether the syringe pumps or the

microfluidic molds are used, a standard operating procedure can be developed to create methods that allow identical hydrogels to be formed each time.

Red Hat: It is most likely that using syringe pumps will be a complicated system. A microfluidic mold would be easier to use in comparison to programming syringe pumps alone. Using a microfluidic mold would be the easiest way to constrain the gradient formation to a highly controlled environment. Designing a unique mold to allow for diffusion to occur allows for more engineering design to be incorporated in the team's project. The best choice would be to create our own mold to fit our desired specifications. It seems like designing the mold for diffusion takes a lot of research into porosity and diffusion coefficients.

Black Hat: There are several complications that could arise during gradient formation. The shear forces of cells moving through a microfluidic mold or through piping from syringe pumps could compromise cell viability. It is possible that if gelation does not occur quickly enough that the cells will settle. If gelation occurs too rapidly, then the variations in either molecular weight or concentration may not mix appropriately for the creation of a continuous gradient to be successful. Diffusion is a difficult process to have precise control over, which could be a complication in developing a completely continuous stiffness gradient.

Appendix C: SOP: Preparing Gelatin-HPA Conjugate

Standard Operating Procedure: Preparing Gelatin-HPA Conjugate (200mL)

Objective: To prepare the Gelatin-HPA conjugate that is used to create stiffness gradient-containing hydrogels. This protocol will yield 200mL of conjugate prior to lyophilization. The procedure takes approximately 7 days to dialyze and lyophilize.

Materials:

60 mL N,N-dimethylformamide (DMF)
53 L MilliQ water
1.66 g (20 mmol) 3,4-Hydroxyphenylpropionic acid (HPA)
1.6 g (27.8 mmol) N-hydroxysuccinimide
1.91 g (20 mmol) 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride
4.688 mg Gelatin
140.256 g sodium chloride
4 L Ethanol
Scale
Weigh boats
Small beaker
pH reader
1 small, 2 medium magnetic stir bars
2 stir/hot plate
2 4 L beakers
1 2 L capped beaker
Parafilm
Spectra/Por 6 dialysis tubing, 1K MWCO, 45mm flat width
4 Standard dialysis tubing closure, 55mm width
Scissors
Spatula
Forceps
Liquid nitrogen bath
Lyophilization flask
Lyophilizer
Autoclave

Procedure:

Day 1

1. Prepare a 75 mL solution of 6.25 wt. % aqueous Gelatin in an autoclave-able container. (To do this, add 4.688 mg of Gelatin to 75 mL of MilliQ water. Do not mix.)

2. Autoclave the Gelatin solution. Set aside for use later in protocol.
3. Autoclave two 4 L glass beakers. Set aside for later use in protocol.
4. Use a graduated cylinder to measure 60 mL of N,N-dimethylformamide (DMF). Carefully empty this amount to a small beaker.
5. Add 90 mL of MilliQ water to the beaker from #4.
6. Mix at medium speed for 3 minutes.
7. Measure 125 mL from the DMF-water solution from #6 and pour into a fresh beaker. Dispose of the remaining original mixture.
8. Add 1.66 g (20 mmol) 3,4-Hydroxyphenylpropionic acid (HPA) to solution from #7.
9. Mix for 4 minutes.
10. Add 1.6 g (27.8 mmol) N-hydroxysuccinimide and 1.91 g (20 mmol) 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride to solution from #9.
11. Mix the solution for 5 hours at room temperature, maintaining a pH of 4.7.
12. Add the 75 mL (6.25 wt.%) aqueous Gelatin solution to the mixture from #11.
13. Stir overnight (~12 hours) at room temperature and maintain a pH of 4.7.

Day 2

14. Prepare two 4 L beakers with 100 mM sodium chloride solution. (To do this, add 23.376 g of sodium chloride to 4 L of MilliQ water. Mix until the sodium chloride dissolves completely.)
15. Cut two 12 in. strips of the Spectra/Por 1000 Da molecular weight cut-off dialysis tubing.
16. Fill a small beaker with distilled water, place the cut tubing inside, place a magnetic stir bar in the beaker, and place the beaker onto a stir plate for 20 minutes.
17. Clamp the bottom of each tubing strip with a dialysis closure piece, leaving about an inch in length from the end of the tubing to the closure.
18. Holding the tubing vertically with the clamp at the bottom, pipette 100 mL of conjugate into each dialysis tube.
19. Clamp the top of the tubing with a second closure, leaving about an inch of empty space on both sides of the clamp. (There should be empty space inside the clamps to allow for influx of liquid.)
20. Place each tube into its own 4 L beaker of sodium chloride solution.

21. Place each 4 L beaker on a stir plate and set to slow speed.
22. Cover the top of the beaker with Parafilm.
23. After 16 hours, carefully remove the dialysis tubing.
24. Empty the 4 L beakers and refill each with 23.376 g of sodium chloride and 4L of MilliQ water.
25. Repeat steps 20-22.
26. After an additional 16 hours, repeat steps 23-25.
27. Continue dialysis for 16 more hours.

Day 4

28. After a total of 48 hours in the sodium chloride solution, remove the dialysis tubes from the 4 L beakers of sodium chloride.
29. Empty the 4L beakers.
30. Rinse the beakers with water.
31. Pour 3 L of MilliQ water into each 4 L beaker.
32. Carefully pour 1 L of ethanol into each 4 L beaker to prepare a mixture of 3:1 water and ethanol.
33. Stir until solution is well mixed.
34. Place each dialysis tube into a 4 L beaker.
35. Place each 4 L beaker on a stir plate and set to slow speed.
36. Cover the top of the beaker with Parafilm.
37. After 16 hours, carefully remove the dialysis tubing from the beakers.
38. Empty the 4 L beakers and refill each with 3L of MilliQ water and 1 L of ethanol.
39. Repeat steps 34-36.
40. Continue dialysis for 16 hours.

Day 5

41. After a total of 32 hours in the ethanol solution, remove the dialysis tubes from the 4 L beakers of water and ethanol.
42. Empty the 4 L beakers.
43. Rinse the beakers with water.

44. Pour 4 L of MilliQ water into each 4 L beaker.
45. Place each dialysis tube into a 4 L beaker.
46. Place each 4 L beaker on a stir plate and set to slow speed.
47. Cover the top of the beaker with Parafilm.
48. After 16 hours, carefully remove the dialysis tubing from the beakers.
49. Empty the 4 L beakers and refill each with 4 L of MilliQ water.
50. Repeat steps 45-47.
51. Continue Dialysis for 16 hours.

Day 6

52. After a total of 32 hours in the MilliQ water, remove the dialysis tubes from the 4 L beakers of water.
53. Empty the 4 L beakers.
54. Rinse the beakers with water.
55. Gently wipe the outsides of the dialysis tubes to remove excess water.
56. Pour the conjugate out of the dialysis tubes and into a lyophilization flask.
57. Wearing gloves for protection, insert the lyophylization flask into the liquid nitrogen bath.
58. Turn the flask so that it is almost horizontal and rotate it so that the conjugate freezes in a thin layer around the entire flask.
59. Continue this process until no liquid conjugate remains within the flask.
60. Attach the lyophilization flask to the lyophilizer.
61. Lyophilize for 24 hours.

Day 7

62. Remove the flask from the lyophilizer.
63. Use a spatula or forceps to remove the lyophilized conjugate from the flask and place into a 2 L beaker for storage.
64. Store the lyophilized conjugate in the -2°C freezer until ready for use.

Appendix D: SOP: Preparing Gelatin-HPA Hydrogels

Standard Operating Procedure for Preparing Gelatin-HPA Hydrogels with Cells

Objective: To prepare hydrogels using the Gelatin-HPA conjugate. This protocol can be followed to create 1, 1.5, and 2% gelatin-HPA gels at various stiffness levels. The hydrogels can be prepared to go in well plates, on coverslips, or in syringes.

Materials:
Lyophilized Gelatin-HPA
10 U/mL horseradish peroxidase (HRP)
DPBS (+)
10X DMEM
H_2O_2 (20, 30, 40, 50, 60, 70 mM)

To prepare hydrogels:

1. Dissolve gelatin-HPA powder in distilled H_2O at 65°C to create a 3% weight stock solution.
2. Pipette (40 µL, 60 µL, or 80 µL) 3% gelatin-HPA stock solution into a microcentrifuge tube.
3. To #2, add 5µL of horseradish peroxidase at a concentration of 10 Units/mL.
4. Add (45 µL, 25 µL, or 5 µL) of DPBS(+) to #3.
5. Add 24 µL of cells suspended in 10X DMEM to #4. If not seeding the gel with cells, use plain 10X DMEM.
6. To #5, add 6µL of H_2O_2 (20 mM, 30 mM, 40 mM, 50 mM, 60 mM, or 70 mM depending on desired final H_2O_2 concentration). For a control without H_2O_2, replace with 6 µL of DPBS(+).
7. Immediately following step #6 pipette the solution up and down 4 times with a 100 µL micropipette to mix. **Be careful not to create bubbles**.
8. If final gel is to be mounted onto a cover slip:
    1) Pipette 63 µL of final solution onto a gluteraldehyde-treated cover slip immediately after mixing.
    2) Place an APTMS treated coverslip on top.
    3) Allow to cross-link for 20 minutes.

4) Pick up coverslips and gently slide the top coverslip apart from the bottom.
5) Hydrogel should stick to the top coverslip.
6) Place the hydrogel on the top coverslip into a petri dish.
7) Cover the hydrogel with DPBS to hydrate.

9. If final gel is to be added to a well plate:
    1) Pipette the appropriate amount per well by gently touching the pipette to the bottom of the well to ensure even spreading.
    2) Gently shake the plate to ensure full coverage of well.
10. If final gel is to be loaded into syringe:
    1) Insert syringe fitted with luer-lock tip into the microcentrifuge tube.
    2) Slowly draw up the gel solution.
    3) Vigorously tap syringe to bring any air bubbles to the surface.
    4) Push air out of the syringe.

Gelatin-HPA Hydrogel Formulas			
Reagents	1%	1.5%	2%
1. Gelatin-HPA (3% Stock)	40 µL	60 µL	80 µL
2. HRP (10U/mL)	5 µL	5 µL	5 µL
3. DPBS (+)	45 µL	25 µL	5 µL
4. 10X DMEM Cell Suspension	24 µL	24 µL	24 µL
5. H_2O_2	6 µL	6 µL	6 µL

H_2O_2 Stock Solutions	
Final H_2O_2 Concentration	Stock Concentration
1 mM	20 mM
1.5 mM	30 mM
2 mM	40 mM
2.5 mM	50 mM
3 mM	60 mM
3.5 mM	70 mM

Appendix E: Microfluidic Gradient Generators Preparation

Standard Operating Procedure for Gradient Generator Preparation

Objective: To prepare microfluidic gradient generators using PDMS. This procedure covers making negative molds from the silicone wafer and making duplicate molds from negatives.

Negative Mold from Silicone Wafer:

1. Weigh 70 g of silicone elastomer base into a weigh boat
2. To #1 add 7 g of silicone elastomer curing agent and mix well.
3. Place weight boat from #2 into a vacuum chamber and vacuum for 90 minutes to remove bubbles.
4. Place silicone microfluidic wafer into a Petri dish.
5. Pour the prepared PDMS around the sides and on top of the silicone wafer.
6. Place the Petri dish from #5 into a 60°C oven for 1 hour.
7. Once curing is complete, cut the PDMS out of the Petri dish in a rectangular shape around the silane-treated mold using a razor blade.
8. Peel the new mold off the silicone wafer and place into a clean Petri dish.

Duplicate Mold from PDMS Negative:

1. Weigh 70 g of silicone elastomer base into a weigh boat
2. To #1 add 7 g of silicone elastomer curing agent and mix well.
3. Place weight boat from #2 into a vacuum chamber and vacuum for 90 minutes to remove bubbles.
4. Place an inverse microfluidic mold into a Petri dish.
5. Add 4 drops of silane to the Petri dish around (but not touching) the mold from #4.
6. Place Petri dish from #5 inside a vacuum chamber set up inside a fume hood. Vacuum for 45 minutes to allow silane to coat the PDMS.
7. Once coated, transfer the silane-treated mold to a new, deeper Petri dish.
8. Pour the prepared PDMS around the sides and on top of the silane-treated mold.
9. Place the Petri dish from #8 into a 60°C oven for 1 hour.

10. Once curing is complete, cut the PDMS out of the Petri dish in a rectangular shape around the silate-treated mold using a razor blade.
11. Peel the new mold of the inverse mold and place into a clean Petri dish.

Appendix F: Conducting AFM on Gtn-HPA Hydrogels

Standard Operating Procedure for Preparing Gelatin-HPA Hydrogels for AFM

Objective: To prepare the Gelatin-HPA hydrogels so that they can be measured for stiffness using AFM. This protocol reviews how to prepare the coverslips, make the hydrogel, and how to take the AFM measurements.

Materials:
25 x 25 mm coverslips
1% APTMS solution
.5% Glutaraldehyde solution
Ceramic boat
Forceps
Aspirator
AFM equipment
Beaker
DI water
Chamber

Preparing Top Coverslips:

1. Oxygen plasma treat coverslips following protocol in Appendix G.
2. Use forceps to place coverslips into ceramic boat.
3. Mix 1.5 mL of APTMS in 150 mL of ethanol in a beaker to make a 1% solution.
4. Place the ceramic boat into the beaker of 1% APTMS.
5. Soak coverslips in APTMS for 3 minutes.
6. Remove ceramic boat from beaker.
7. Let coverslips dry for 3 minutes.
8. Rinse coverslips with DI water.
9. Aspirate the coverslip.
10. Coverslip is ready to use. (Can be stored in refrigerator for a week)

Preparing Bottom Coverslips:

11. Follow steps 1-10 above.
12. Mix 1 mL of 50% Glutaraldehyde in 100 mL of DI water to make a .5% solution.

13. Fill a chamber with 50 mL of the .5% glutaraldehyde solution.
14. Place coverslips into the chamber.
15. Store in refrigerator for at least 4 hours before using.
16. Remove coverslips.
17. Aspirate.
18. Coverslip is ready to use. (Can be stored in refrigerator for a week)

Preparing Coverslips with Hydrogels:

1. Mix together hydrogel precursors following the protocol outlined for the coverslip method in Appendix D.
2. Let the cross-linked hydrogel hydrate in DPBS for 15 minutes.

Conducting AFM:

1. Conduct 3 measurements in 3 different points per hydrogel sample.

Appendix G: Plasma Treating

Standard Operating Procedure for Plasma Treating of Gradient Generator

Objective: To attach a glass slide to a PDMS microfluidic gradient generator using plasma treatment. This will create a tight bond that will allow fluid to flow through the generator

Microfluidic Preparation:

1. Clean any dust off a PDMS gradient generator with a piece of scotch tape.
2. Clean a glass slide using an ethanol then diH_2O then ethanol rinse.
3. Aspirate using air pressure.
4. Remove any further dust using a piece of scotch tape.
5. Place a glass slide so that it covers the well portion of the PDMS microfluidic mold. Leave the gradient tree exposed.

Plasma Treating:

1. Turn on AC red button.
2. Open oxygen.
3. Turn on the vacuum.
4. Open up "plasma closed" valve by turning.
5. Take out the cylindrical chamber gently, do not hit the bulb.
6. Place the gradient generator and glass slide into the cylindrical chamber, then gently return the chamber to the plasma treatment machine.
7. Turn the "level" knob to 50.
8. Turn the "tuning" knob until a violet color appears.
9. Turn the "level" knob to full power, it will cause a noise.
10. Turn the "tuning" knob either direction until the noise stops.
11. Plasma treat the gradient generator and glass slide for 45 seconds.
12. Turn the "level" knob to 0.
13. Turn of the vacuum and wait for the pressure to drop.

14. Remove the cylindrical chamber and use tweezers to remove the microfluidic and the glass slide. **Be careful not to touch the treated surfaces as it could ruin the plasma treatment.**
15. Peel off the glass slide and flip it so that the plasma treated side of the glass slide is flush against the plasma treated gradient tree portion of the PDMS mold.
16. Gently press the glass slide and gradient generator mold together.

Appendix H: Flowing Gradient-Hydrogels

Standard Operating Procedure for Flowing Gradient-hydrogels

Objective: To create a gelatin-HPA hydrogel with a continuous stiffness gradient. This protocol reviews how to prepare the microfluidic gradient generator for use and how to flow the system to create a gradient-hydrogel.

Gradient Generator Preparation:

1. Attach a glass slide to the generator according to the Standard Operating Procedure for plasma treating (Appendix G).
2. Place gradient generator into a Petri dish under a microscope for better visualization.
3. Cut three pieces of tubing to 23 cm long.
4. Place a metal tip into one end of each piece of tubing.
5. Place the tip of a luer-lock into the other end of each piece of tubing.
6. Place the end of each metal tip into the three holes in the gradient generator mold. (Make sure the end of the metal tip reaches all the way down to the glass slide.)
7. Using a syringe, fill each of the three tube and the gradient generator with DPBS(+). (This will remove air bubbles from the system.)

Hydrogel Preparation:

1. Follow the procedure from Appendix D to mix the hydrogel precursors for a final volume of 960 μL for the following concentrations:

Gelatin-HPA Hydrogel Formulas for Gradient Generation			
	Gelatin-HPA Concentration	H_2O_2 Concentration	Dye Concentration
Syringe 1	1.5%	1.5 mM	None
Syringe 2	2%	1.5 mM	Low
Syringe 3	2%	3.0 mM	High

2. **Immediately** after incorporating H_2O_2, load each of the three solutions into a syringe:
    1) Insert syringe fitted with luer-lock tip into the microcentrifuge tube.
    2) Slowly draw up the gel solution.

    3) Vigorously tap syringe to bring any air bubbles to the surface.
    4) Push air out of the syringe.
3. Attach each of the three syringes to the luer-lock tips attached to the gradient generator setup. Arrange them in the following order: 1.5% with 3.0 mM on top, 2% with 1.0 mM in the middle, 2% with 3.0 mM on bottom.
4. Load each of the three syringes into a syringe pump set at 15 µL per minute.
5. Flow the system for 3-5 minutes until gelation occurs in the microfluidic well. Use a microscope for better visualization

Appendix I: Gelation Time Data

	1.0% Gelatin HPA	1.5% Gelatin HPA	2.0% Gelatin HPA
1.0 mM H_20_2	13.0	5.0	4.5
1.5 mM H_20_2	12.0	5.0	4.5
2.0 mM H_20_2	11.0	5.0	3.75
2.5 mM H_20_2	10.5	5.0	3.75
3.0 mM H_20_2	10.0	5.0	3.25
3.5 mM H_20_2	10.0	4.0	3.0

Gelation times of various concentration hydrogels measured in minutes.

Appendix J: Atomic Force Microscopy Data

First Run Baseline AFM Measurements			
	2% Gelatin-HPA		
H_2O_2 Conc. (mM)	0.9	1.35	1.8
Avg Stiffness (Pa)	260,886	424	354
St. Dev. (Pa)	237,402	143	199

Second Run Baseline AFM Measurements			
	1.5% Gelatin-HPA		
H_2O_2 Conc. (mM)	1.0	1.5	2.0
Avg Stiffness (Pa)	1,382,931	285	620
St. Dev. (Pa)	2,548,988	382	308

Third Run Baseline AFM Measurements										
	1.5% Gelatin-HPA					2% Gelatin-HPA				
H_2O_2 Conc. (mM)	1.5	2.0	2.5	3.0	3.5	1.5	2.0	2.5	3.0	3.5
Avg Stiffness (Pa)	277	357	318	482	988	1,753	1,056	1,364	1.108	1,333
St. Dev. (Pa)	140	89	87	452	856	651	560	484	352	531

Fourth Run Baseline AFM Measurements												
	1.5% Gelatin-HPA						2% Gelatin-HPA					
H_2O_2 Conc. (mM)	1.0	1.5	2.0	2.5	3.0	3.5	1.0	1.5	2.0	2.5	3.0	3.5
Avg Stiffness (Pa)	451	378	380	477	464	414	727	1,075	606	1,266	1,520	1,294
St. Dev. (Pa)	84	103	49	215	141	109	428	707	393	214	1,315	181