CREATION OF A MECHANICAL GRADIENT PEG-COLLAGEN SCAFFOLD BY PHOTOMASKING TECHNIQUES

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CREATION OF A MECHANICAL GRADIENT PEG-COLLAGEN SCAFFOLD BY
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Mechanical properties are a critical factor in cell behavior and can signal cells to differentiate, migrate, and proliferate. *In vivo*, the mechanical properties of tissues are complex and heterogeneous. Mechanical gradients are prevalent in the human body as every inter-connective tissue in every organ has gradients across the spatial volume. In addition, pathologies, such as, cancer and myocardial infarction trigger complex physical changes within the tissue. After myocardial infarction, the cardiac tissue hardens creating a mechanical gradient interface between the diseased cells and healthy tissue. Two weeks after myocardial infarction, the tissue hardens creating a gradient from the healthy tissue (16-20 kPa) to infarcted tissue (~50 kPa). In order to mimic the native environment, it is necessary to have a scaffold in which the mechanical properties can be tuned spatially. Photomasking is a method by which one can restrict the amount of UV irradiation intensity depending on the opacity of the photomask. Reducing the irradiation intensity over a fixed duration can selectively crosslink scaffolds which undergo photopolymerization. Polyethylene glycol (PEG) can be functionalized with a diacrylate group to form a hydrogel which can undergo radical photopolymerization. The methods described in this paper analyze how different opacity photomasks (0, 10, 20, 30, and 40%) affect irradiation intensity, and then how the irradiation intensity affects the elastic modulus ($E'$) and swelling ratio of 9% wt/wt PEG hydrogel.
Using the relationship between photomasking and E’ a gradient scaffold was designed resulting in a PEG gradient scaffold between 17-57 kPa. Type I collagen was incorporated within the PEG scaffold at a concentration of 1 mg/mL. The incorporation of the collagen has no significant effect on the E’. A live / dead assay was performed on the PEG collagen scaffold showing high viability (95.91 %) of human mesenchymal stem cells (hMSCs). hMSCs were observed to migrate on the resulting gradient scaffold from the soft (17 kPa) to firm ends (57 kPa) of the scaffold. The methods in this thesis show how using photomasking can create a customized PEG-collagen hydrogel that simulates the E’ of healthy to infarcted myocardial tissue. Additionally, this resulting scaffold can be used as a platform for future studies of cardiac tissue engineering.
DEDICATION

I dedicate this thesis to my mother Donna Patterson-Finch and Larry E. Finch, with special thanks to Thomas Yeager, my childhood mentor, for their love and support throughout my life and college career.
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Cell migration study on a 9% wt/wt PEG-Collagen scaffold with an E’ of 57.77±0.90 kPa. The scaffold was sectioned into 500 by 500 µm squares and the cells within each square counted and graphed in MATLAB. HMSCs were seeded onto the center of the scaffold and observed over 4 days (A-C).
1.1 Introduction

The unique capability of stem cells to differentiate into multiple lineages makes stem cell therapy the most attractive strategy in regenerative medicine. One of the main challenges in stem cell therapy is to direct stem cell differentiation into specific lineages according to the application. *In vivo*, the fate of stem cells is regulated by their surrounding microenvironment or “niche.” The micro-environmental niche is known for cues which are critical for regulating stem cell behavior. The fate of these cells is guided by biochemical signals, such as soluble and insoluble factors that are associated with extracellular matrix (ECM) or neighboring cells [1]. The mechanical signals that stem cells sense in the micro-environment, such as the elastic properties of the environment, are becoming increasingly recognized as playing an important role in stem cell differentiation, spreading, proliferation, and adhesion [1-5].

Mechanical forces play a critical role in regulating the development and function of every tissue within the human body [6, 7]. Cell morphology and cytoskeletal tension can be changed by seeding cells on substrates with different mechanical properties [7]. One example of how mechanical properties can guide stem cell differentiation is when human mesenchymal stem cells (hMSCs) are cultured on hydrogel scaffolds with elasticities
close to native tissues of brain (0.1-1 kPa), muscle (8-17 kPa) and nascent bone (>34 kPa). These cells are found to express key markers of neurogenic, myogenic, and osteogenic lineages even though the culture media remains unchanged [8, 9]. Therefore, the differentiation of stem cells by scaffold mechanics is closely related to the mechanical properties of the native tissues [8, 9]. hMSCs are also strong candidates for clinical applications because they can be isolated from a wide range of autologous sources; some of which are easily accessible, such as bone and adipose tissue [10].

The original investigations of substrate mechanics on stem cell differentiation used scaffolds with homogeneous mechanical properties [9]. *In vivo*, the physical properties of native tissue are heterogeneous; therefore, in order to properly design a scaffold (one that can mimic the natural microenvironment), it is necessary to have a scaffold in which the mechanical properties can be tuned spatially [2, 3, 5]. In each stage of development, physical gradients and stresses exist and will guide cell behavior [6]. Also, spatial mechanical differences are apparent in some pathologies such as cancer, myocardial infarction, atherosclerosis, and wound healing [6]. Therefore, the ability to spatially control the mechanical properties of tissue engineering scaffolds is necessary in order to investigate their effects on stem cell behavior.

The overall objective of this thesis is to explore the feasibility of developing mechanical gradient scaffolds using photomasking techniques and to investigate their physical and biological characteristics. One application of the photomasking techniques is to create a mechanical gradient which mimics the elastic modulus of normal myocardial tissue to infarcted myocardial tissue to serve as an *in vitro* cell culture model. To achieve this goal, the following specific aims will be addressed:
1.1 Specific Aims

Aim 1: Develop a polyethylene glycol (PEG) scaffold which is fully cross-linked and exhibits an elastic modulus (E') of at least 50 kPa and then measure the total amount of ultraviolet (UV) energy required to fully crosslink that hydrogel.

Aim 2: Characterize the feasibility of reducing the total UV energy to the PEG hydrogels through photomasking to create a mechanical gradient.

Aim 3: Characterize cell viability and migration on the developed gradient scaffolds.
2.1 Myocardial Infarction and its Effect on Mechanical Properties

The myocardium has a very limited ability to regenerate after injury. Current stem cell based therapies are being investigated for treatment of myocardial infarction [11]. The damage resulting from myocardial infarction causes complex mechanical alterations in the native tissue resulting in collagen synthesis and scar formation. The damage is caused by a cascade of changes, which typically starts with a blocked artery due to atherosclerosis and results in ischemic cardiac tissue. The ischemia leads to necrotic tissue and inflammation, edema and infiltration of inflammatory cells [12]. At the necrotic cardiac tissue, both intracellular and extracellular collagen synthesis contribute to the scar formation. After the first week of myocardial infarction, re-absorption of necrotic tissue is increased, leading to fibroblast formation in place of dead myocardial cells and deposition of collagen. This process continues throughout the second week causing the cardiac tissue to become increasingly stiff [12].

In each of the stages after myocardial infarction, mechanical changes within the tissue occur and deviate from the normal cardiac E’. Normal rat myocardium has an E’ of about 16-20 kPa compared to infarcted myocardium, which depends on how long the fibrous
scar is allowed to form [13, 14]. The infarcted tissue at 7 days has an $E'$ of $31.38 \pm 0.75$ kPa and at 14 days $53.23 \pm 0.75$ [14]. It is suggested that the optimal window of stem cell treatment for myocardium infarction should be between 7-14 days [14]. The gradient range across a left ventricle section in a C57 mouse was found to be from $21 \pm 2$ (non-infarcted tissue) to $56 \pm 6$ kPa (infarct tissue) over a length of 2 mm [15]. The gradient range was measured 30 days after myocardial infarction [15]. The transition space between normal cardiac tissue and the region of infarcted tissue is not a sudden increase in $E'$, but a gradient [15].

2.2 Poly(ethylene glycol) Hydrogels

Hydrogels are hydrophilic polymers known for their biocompatibility and high water content, and can mimic the mechanical properties of ECM. Synthetic polymer hydrogel systems are advantageous for their ease of synthesis, reproducibility, fabrication, and modification [16]; natural hydrogel scaffolds are advantageous for their unrivaled integration with the host biology [17]. Hybrid hydrogel scaffolds are composed of both synthetic and natural hydrogel components. The unique combination of both materials allows the design of a biomaterial that has the mechanical properties controlled by the synthetic component, and the biological properties through the bioactive component [18, 19]. This benefit has caused hybrid hydrogel systems to become an increasingly popular option in tissue engineering [18, 20, 21].

PEG is a synthetic polymer that has been extensively studied as a tissue engineering scaffold for muscle [22], vasculature [23], cartilage [24] and bone [25]. PEG is a hydrophilic, water soluble, biocompatible polymer that is FDA approved for several
medical devices [5]. PEG hydrogels are highly resistant to protein and cell adhesion, causing the scaffold to be bioinert [5, 26]. PEG can be made bioactive by simply dispersing a cell adhesion protein within the hydrogel prior to crosslinking [27].

PEG hydrogels are typically formed by either chemical crosslinking through the reaction of hydroxyl groups on the ends of PEG or by photocrosslinking. In order for PEG to be photocrosslinkable, the end groups of PEG have to be modified by either a diacrylate or methacrylate end groups [2]. The diacrylate or methacrylated end groups react with a photo-initiator when exposed to UV light. The photo-initiator produces free radicals when exposed to UV light that initiate chain polymerization of the PEG causing it to form the hydrogel [28]. The most common photo-initiators for acrylated monomers are aromatic ketones such as Irgacure 2959 [29].

The physical properties of PEG can be modified depending on the molecular weight and weight percent of the polymer. Varying the molecular weight of PEG and the amount of polymer in solution affects the mesh size as well as the E'; therefore, allowing the polymer to be highly customizable [5, 30]. As the molecular weight of PEG increases, the pore size increases while the compressive modulus, ultimate strain and ultimate strength decreases [30]. The mechanical properties of PEG can also be customized by increasing the weight percent of the PEG within the hydrogel.

2.3 Collagen

Collagens are the most abundant proteins found within the human body. Type I collagen makes up to 90% of all the collagens within the body [31]. The structure of fibril-forming collagens I, II, III, V, XI, XXIV and XXVII consists of three polypeptide chains
which form a right handed triple helix in which the polypeptide chains can have identical or different structure depending on the type of collagen [32]. The fibril-forming collagens have the ability to form large fibrils that are responsible for the tensile strength of tissues [33]. The ECM of the heart consists roughly of 85% of type I collagen, which is arranged into fibers for mechanical support [34]. Type I collagen is considered an important protein for cell adhesion especially for cells having $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin binding sites [31]. The integrin $\alpha_1\beta_1$ in development is predominantly present in the mesenchyme [32]. In adult tissues the integrin $\alpha_1$ subunit is most abundant in vascular and visceral smooth muscle, but it is also expressed in fibroblasts, hMSCs, chondrocytes, neurons, undifferentiated Schwann cells, and numerous types of white blood cells [32].

Due to the abundance of type I collagen in mammalian species, it is relatively inexpensive to use and readily available from multiple sources. Using collagen as an adhesive protein is a well researched and known method for making tissue engineering scaffolds bioactive. The three most common ways of using collagen as an adhesive protein are coating, dispersion, and chemical binding.

Coating collagen is a simple method of introducing a high concentration of the protein to the surface of the biomaterial to let it adsorb onto the surface. The advantage of coating collagen is that it only changes the surface and not the overall bulk of the properties of the material. The amount of protein adsorbed and the structural orientation of the protein will depend on the surface characteristics of the scaffold [36]. The disadvantage of coating collagen is if the material is low fouling, the collagen will absorb poorly onto the surface directly impacting cell adhesion [37].
The dispersion method is mixing collagen into a cross-linkable polymer solution creating a colloid mixture before the polymer undergoes gelation to form a hydrogel. The method essentially tries to entrap the collagen protein within the structure of the hydrogel [38]. The advantage of the dispersion method compared to coating is that it more effectively entraps the collagen within the structure of the polymer resulting in good proliferation, viability and cell attachment [38]. The disadvantage of this method is that the collagen takes up space between the polymer chains, which could potentially interfere with gelation of the polymer resulting in a decrease of E'. Although some of the collagen may be physically entangled within polymer, it is not chemically bound; therefore, the collagen might be capable of release from the hydrogel.

Another method for using collagen is chemically binding the structure to a synthetic polymer like PEG. The amine groups on collagen can be used to covalently bind collagen to synthetic polymers [39-42]. One such method is functionalizing PEG-star with an amine reactive chemistry that can bind to protein/tissue like collagen but does not require photo-polymerization for the reaction [39]. The method can also be done with PEG-DA binding PEG-collagen to create a hydrogel formed by photo-polymerization [40]. The chemically conjugated collagen-PEG has shown to cause a decrease in E’ compared to PEG without collagen [40].

2.4 Mechanical Gradient Creation

Three main methods have been explored to create mechanical gradient scaffolds including photomasking, microfluidic chambers, and mixing devices. Photopolymerization with UV light is a frequent method that consists of radical
polymerization using methacrylate or acrylated functionalized polymers [2]. The use of photomasking can restrict the amount of UV energy, or completely block the UV light from certain regions of the photocrosslinkable polymer; thereby, allowing for selective crosslinking and the creation of mechanical gradients and complex shapes [2, 3].

The amount of crosslinking within the hydrogel is directly related to the E' of the hydrogel. A fully crosslinked hydrogel has the maximum E' possible for that hydrogel. Photomasking reduces the amount of crosslinking from the maximum E', allowing for an increasingly softer hydrogel as the crosslinking density is reduced by limiting the total UV energy to the surface of the hydrogel.

Figure 1 - Visualization of a 0 to 100 % opacity gradient with discrete opacity points.

A mechanical gradient hydrogel could be fabricated by controlling the opacity of the designed photomask. Opacity is the degree of measurement in which light or radiation is blocked, as illustrated in Figure 1. This example shows the opacity as a percentage from 0 to 100%. At 0% opacity the light is transmitted fully and at 100% opacity none of the light is transmitted through the photomask. A continuous gradient photomask will reduce
the UV irradiation intensity from high to low spatially. Therefore, the amount of crosslinking density of the hydrogel will also change spatially creating a mechanical gradient across the hydrogel (Figure 2).

![Diagram showing UV irradiation and photomask effect on hydrogel crosslinking density.]

Figure 2 - The effect of photomasking on the reduction of irradiation intensity and therefore crosslinking density of photopolymerized hydrogels.

Another way of creating a gradient scaffold is by mixing. It has been done by having two chambers which are filled with either a low molecular weight PEG-DA or a higher molecular weight PEG-DA, and both with a photo-initiator premixed in the PEG-DA solution (Figure 3). Fluid flow is controlled by a valve and the gradient outflow is controlled by a peristaltic pump [5]. The outflow of the two molecular weight PEG solutions are mixed by a magnetic stir bar within each reservoir to form a PEG solution with varying concentration. The gradient fluid is then pumped into a glass mold and cross-linked by UV light. The molecular weight of PEG-DA plays a significant role in modulating scaffolds E'. The low molecular weight PEG-DA has a high E' and the high
molecular PEG-DA has low $E'$. A gradient scaffold can be formed by varying the concentration of the mixed PEG-DA solution.

![Image](image.png)

Figure 3 – Illustration of a gradient making device by mixing.

Microfluidic chambers are another method of creating a gradient [43]. Microfluidics creates gradients by precise control of fluids as they pass through the chamber. Typically two PEG-DA solutions with different molecular weights flow into the chamber through the inputs of the microfluidic chamber (Figure 4) [44]. As the PEG solution moves further within the microfluidic chamber it is mixed again and the two different solutions create varying levels of concentration (Figure 4). Each mixed concentration exits to a single outlet and then is exposed to UV light which crosslinks the gradient solution to form a scaffold. The gradient properties are greatly determined by the configuration of the microfluidic chamber and are highly customizable [44].
Figure 4 – Illustration of a microfluidic device which creates a mechanical gradient.

The use of a mixing device or microfluidic chamber for the purpose of the creation of gradients is more complex compared to photomasking. Photomasks can be printed on transparencies by a common LaserJet printer allowing for an inexpensive and easy to adapt method to create a gradient on a hydrogel. The limiting factor in photomasking is the resolution of the printer creating the mask.

2.5 Mechanical Properties of Hydrogels

The mechanical properties of hydrogels are best modeled as a viscoelastic material [45]. Viscoelastic materials build on the viscous and elastic theories. Viscous materials resist shear flow and strain linearly with time when a stress is applied. Elastic materials react to stresses with approximately instantaneous fully reversible deformation. In hydrogels, the $E'$ is almost completely derived from the number of cross-links (crosslinking density) within the system [45]. The higher the crosslinking density in a hydrogel, the larger the
resulting $E'$; likewise, the lower the crosslinking density in the hydrogel the smaller the $E'$. The effect of the swelling of the hydrogel is also related to the $E'$ of the hydrogel (the more the hydrogel swells, the smaller the $E'$). The amount of cross-linker can also play an important role on the $E'$. Decreasing the amount of cross-linker content within the prepolymerized solution has an effect of lowering the crosslinking density of the resulting hydrogel [46]. However, increasing the amount of cross-linker to high concentrations can be cytotoxic to cells [47].

The bulk properties of hydrogels are studied using dynamic mechanical analysis (DMA) and oscillatory shear rheometry both of which apply small oscillatory stress and measures the resulting strain [48]. The primary difference between DMA and shear rheometry is that DMA applies compressive forces and shear rheometry applies shear forces [48]. The compressive storage modulus is denoted as $E'$ and shear storage modulus is denoted as $G'$, but since they are measured by different types of deformation, they are not equal to each other. The correlation between $E'$ and $G'$ is denoted by equation 1 and can be used, with $\mu$ as Poisson's number equal to 0.5 for hydrogels that are isotropic and homogeneous [49]. DMA has a disadvantage compared to shear rheometry due to an artifact called "barreling", which causes an additional shear component when testing hydrogels [48]. Rheometry also has the advantage of testing smaller samples then DMA [48].

$$E' = 2G'(1 + \mu) \quad \text{(Equation 1)}$$

Rheometry and DMA are suitable for measuring bulk properties of hydrogels, but the microstiffness do not always correspond to the bulk properties [47]. This dissimilarity could be due to the change in $E'$ that accompanies the rate of reaction of the UV cross-
linkers [47]. In force mode, atomic microscopy (AFM) has the ability to measure microstiffnesses. Rheometry has the advantage of being a much simpler process of testing mechanical properties.
CHAPTER III

METHODS

3.1 Synthesis of PEG-DA

PEG-DA was synthesized by combining 0.004 moles of dried 3,000 MW PEG (Fluka, 03997), 0.016 moles acryloyl chloride (Aldrich, A2109), and 0.008 moles triethyl amine (Alfa Aesar, A12646) in 40 mL anhydrous dichloromethane (DCM) (Alfa Aesar, 41835) and stirring under argon for at least 12 hours (Figure 5). The resulting solution was washed with 16 mL 2 M K$_2$CO$_3$ (Fisher Chemical, 584-08-7) and allowed to separate into aqueous and DCM phases for at least 3 days. The lower DCM phase was collected and the solution was dried with MgSO$_4$ (Fisher Chemical, 34300). The MgSO$_4$ was removed by filtering the solution (Whatman Filterpaper Grade 2, 1002-500). PEG-DA was precipitated from solution by diethyl ether (EMD, EX0190-4), filtered (Whatman Filter Paper Grade 50, 1450-090) and then left to dry overnight.

![Poly(ethylene glycol) diacrylate synthesis](image)

Figure 5 - Poly(ethylene glycol) diacrylate synthesis
3.2 Mechanical Testing of PEG Hydrogels

PEG hydrogels were made with 5, 9, 7.5, 10, 12.5, 15, and 20% wt/wt PEG-DA, Igracure 2959 solution (10% wt/wt) and DI water. The Igracure 2969 (Ciba, 013670040008) solution was made at a concentration of 0.005 g/mL in DI water. All solutions were sterilized using a filter (Sterile 50ml Disposable Vacuum Filter system, Millipore SE1M179M6). The PEG-DA was gelled under long wave 365 nm UV light (UVP, B-100AP) for 25 minutes. PEG-DA solutions were photo-polymerized under UV light between two Teflon coated glass sheets. PEG hydrogels were photocrosslinked at a UV intensity of 16.6 mW/cm$^2$. A high UV intensity of 16.6 mW/cm$^2$ was chosen to insure the PEG hydrogels were fully cross-linked over the duration of 25 minutes. The hydrogels were allowed to swell overnight in PBS (1X Dulbecco’s phosphate buffered saline, MP 1860454) and then cut into 8 mm rounds.

The $G'$ of the samples from each group was measured via oscillatory shear rheometry (Ares RFS III) by using a parallel plate configuration. The hydrogels were tested in a frequency sweep of 1-50 rad/s at a constant strain of 0.5% in the linear viscoelastic regime for all materials. All samples were tested at a temperature of 25 °C. The rheology data was converted from the $G'$ to the $E'$ by equation 1. The $E'$ was measured for n=5 samples.

3.3 Measuring UV Intensity and Photomask Creation

The UV intensity under the lamp within the exposure box was measured by a UV light meter (Traceable Ultraviolet Light Meter, R5-232) over a square 130.64 cm$^2$ area which was divided into a 4 by 4 grid (each section was 14.51 cm$^2$). The UV light meter was
placed at each of the 4 by 4 grid locations and the UV intensity was recorded. The data was graphed in MATLAB using a matrix (imagesc) plot to help visualize the area where the UV intensity was most uniform within the exposure box.

Photomasks at discrete opacities (0, 10, 20, 30, 35, 40, 45, 50, 60 and 70%) were created in GIMP 2.6 software and printed out on transparencies by a LaserJet printer (HP color Laserjet 4600N). The UV intensity that each photomask blocks was measured by a UV light meter by resting a glass plate with clear Teflon tape over the UV light meter sensor and applying each photomask on top of the plate. The UV intensity for each photomask was determined for n=5 samples.

3.4 Measurement of the Total Input Energy to Fully Crosslink a PEG Hydrogel

PEG-DA 9% wt/wt hydrogels were crosslinked at varying times from 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30 and 60 minutes at a UV intensity of 4.2 mW/cm². The $G'$ of each time interval was measured through oscillatory shear rheometry as previously described in section 3.2. Equation 1 was used to convert the $G'$ to $E'$. The total energy was calculated for each time interval by equation 2 in which the total energy $E_t$ (mJ) is calculated by multiplying the irradiation intensity $I$ (mW/cm²) by the area $A$ (cm²) and time $t$ (seconds). The calculated total energies were 4032, 4284, 4536, 4788, 5040, 5292, 5544, 5796, 6048, and 6300 mJ and the $E'$ was observed for each value for n=8 or greater.

$$E_t = IAt$$  
(Equation 2)
3.5 Characterization of Photomasking on Elastic Modulus

PEG-DA 9% wt/wt hydrogels were crosslinked under photomasks at varying opacities (0, 10, 20, 30, 35 and 40%). The same as previously described in section 3.2, the $G'$ of the PEG hydrogels were calculated by using oscillatory shear rheometry. The duration of crosslinking was fixed at 25 minutes. The amount of irradiation intensity reduced by photomasking was measured by the UV light meter previously mentioned in section 3.3. The resulting irradiation intensities reduced by each photomask (0, 10, 20, 30, 35 and 40%) over a duration of 25 minutes was used to calculated the total input energy using equation 2. The total input energy versus $E'$ was plotted to see how the total input energy given to the hydrogels effects the $E'$. The effect of photomasking on $E'$ was measured for $n=8$ samples.

3.6 Measurement of Swelling Ratio

PEG-DA 9% wt/wt hydrogels were crosslinked under photomasks at varying opacities (0%, 10%, 20%, 30%, and 40%). The duration of crosslinking was fixed at 25 minutes. The amount of irradiation intensity reduced by photomasking was measured by the UV light meter previously mentioned in section 3.3. The PEG hydrogels were allowed to fully swell in PBS for 48 hours. The swelling ratio ($Q$) of the PEG hydrogels were measured and calculated using equation 3, where $W_s$ is the weight of the swollen PEG hydrogel, and $W_d$ is the dry weight of the PEG after it has been lyophilized. The percent of wet weight was calculated by equation 4. The swelling ratio and the percent wet weight was measured for $n=6$ or greater samples.
3.7 Synthesis of PEG-collagen Composite Scaffold

PEG-collagen hydrogels were made with 9% wt/wt PEG-DA, Igracure 2959 solution (10% wt/wt), rat tail collagen type I (Invitrogen, A1048301) and DI water. The collagen concentration was varied at 0, 78, 156, 312.5, 625, 1000, 1248 and 2496 µg/mL. The Igracure 2969 solution was made at a concentration of 0.005 g/mL in DI water. PEG-collagen solutions were photo-polymerized under UV light between two Teflon coated glass sheets. The PEG-collagen was gelled under long wave 365 nm UV light for 25 minutes at a UV intensity of 4.2 mW/cm².

3.8 Cell Attachment on PEG-collagen Hydrogels

Cell attachment was investigated using PEG-collagen composite hydrogels at varying concentrations of collagen (0, 78, 156, 312.5, 625, 1000, 1248 and 2496 µg/mL). PEG-collagen hydrogels were created as previously described in section 3.7. The PEG-collagen hydrogels were washed with fresh medium and placed into the incubator for 15 minutes prior to cell seeding. hMSCs were seeded at a density of 20,000 cells/cm² to each PEG-collagen composite hydrogel. Light microscopy (5x) images were taken at 24 hours after seeding hMSCs.
3.9 Effect of Collagen on Elastic Modulus

Study 3.8 determined that the PEG-collagen composite scaffold with 1 mg/mL of collagen had achieved good cell attachment; therefore, it was necessary to see if the collagen within the PEG hydrogel had an influence on the E’. To determine if the E’ was affected by the incorporation of collagen within the PEG scaffold, the mechanical properties of 9% wt/wt PEG-collagen hydrogels with 1 mg/mL collagen were crosslinked at a total input energy of 3045 and 6030 mJ. The PEG-collagen hydrogels were then compared with 9% wt/wt PEG hydrogel without collagen and crosslinked at a total input energy of 3045 mJ and 6300 mJ. The G’ of each hydrogel was measured through oscillatory shear rheometry as previously described in section 3.2. Equation 1 was used to covert the G’ to E’. Single Factor ANOVA was used to determine if the incorporation of collagen significantly changed the E’. The E’ was measured for n=8 samples.

3.10 Characterization of Viability

The cell viability was calculated on a 9% wt/wt PEG hydrogel without collagen and a 9% wt/wt PEG-collagen hydrogel (1 mg/mL) using a live / dead assay (Invitrogen). The PEG (Section 3.2) and PEG-collagen (Section 3.7) hydrogels were formed the same as previous studies. The PEG and PEG-collagen hydrogels were fully crosslinked at a total input energy of 6300 mJ. Both hydrogels were washed with fresh medium and placed into the incubator for 15 minutes prior to cell seeding. hMSCs were seeded at a density of 15,000 cells/cm² to the surface of each hydrogel and fluorescence images were taken 24 hours after cell seeding. Live cells were stained in green (calcein AM) and dead cells
were stained in red (ethidium homodimer-1). The cell viability for each group was calculated by using equation 5.

\[
Cell\ Viability = \frac{\text{Total number of viable cells}}{\text{Total number of viable + nonviable cells}} \times 100 \quad \text{(Equation 5)}
\]

3.11 Mechanical Gradient Creation

The process of creating a gradient by photomasking is illustrated in Figure 6. All solutions were filter sterilized (Millipore Steriflip, S-29969-26) prior to mixing. The silicone wells and teflon surfaces were sterilized by exposing them to UV light for 30 minutes. Silicone wells were placed on a teflon surface and the PEG-collagen solution was pipetted within the wells. The PEG-collagen solution was made the same as previously described in Section 3.7 with 1 mg/mL of collagen. A glass coverslide was placed on the surface of the well creating a flat surface between the two plates prior to crosslinking the PEG-collagen solution. The photomask with a gradient of 0-40% opacity, which was created in GIMP 2.6 software, was placed on top of the glass slide and then placed under UV light for 25 minutes at a UV intensity of 4.2 mW/cm².
3.12 Cell Migration Study

Cell migration on two different 9% PEG-collagen (1 mg/mL collagen) scaffolds were compared: The first scaffold with a mechanical gradient between 17-57 kPa $E'$, and the second scaffold with a homogeneous $E'$ of 57 kPa. The creation of a gradient scaffold was previously described in section 3.11. The homogeneous scaffold was previously described in section 3.7. Both scaffolds were crosslinked within a 15 mm by 7 mm silicone well. hMSCs were seeded at the center of each hydrogel at a concentration of ~200 cells in 2 µl of hMSC growth medium (Figure 7). The entire scaffolds were imaged by using light microscopy (Axio Observer, Zeiss) with the Mosaic feature in comb mode with a 20% image overlap. The images were taken with a 5x objective at day 1, 2, 3 and 4 for both scaffolds. The microscope images were fit with a 500 by 500 µm grid and the
cells within each square were counted (Figure 7). The cell count within each 500 by 500 µm square was plotted using MATLAB's (R2009b, MathWorks) surf function.

![Figure 7](image)

Figure 7 - Cell migration study diagram showing initial seeding onto gradient/non-gradient scaffolds (A) and showing cell counting by 500 µm by µm grid across entire scaffold (B).

3.13 Statistical Analysis

Results were reported as the mean ± standard error unless otherwise noted. The statistical significance of differences in mechanical properties and swelling between each hydrogel type was determined using ANOVA with Tukey's analysis of variance based on a p ≤ 0.05. ANOVA analysis was performed using SAS software (Version 9.2, SAS). Regression analysis was conducted on the PEG concentrations versus the scaffolds E', total input energy versus E', and opacity versus UV light intensity in Microsoft Excel 2007 to calculate the coefficient of determination.
CHAPTER IV

RESULTS

4.1 PEG Concentration Affects the Scaffold Elastic Modulus

The $E'$ was dependent on the concentration of PEG within the hydrogel, the higher the concentration of PEG the larger the resulting $E'$. The resulting $E'$ for each concentration was as follows: 5% (6.56 ± 0.18 kPa), 7.5% (30.86 ± 0.44 kPa), 10% (62.18 ± 1.56 kPa), 12.5% (171.68 ± 2.86 kPa), 15% (342.44 ± 12.69 kPa) and 20% (697.11 ± 10.05 kPa).

For all the samples, the $G'$ was greater than the loss modulus ($G''$); therefore, all materials were considered to be a hydrogel. Equation 1 was used to convert the $G'$ to $E'$. The relationship of PEG concentration versus $E'$ was fit using exponential regression (Figure 8). The relationship has an exponential curve with a coefficient of determination ($R^2$) of 0.9939.

The $E'$ can be customized by selecting the appropriate weight concentration. In order to mimic the infarcted cardiac tissue, which has an $E'$ of ~50 kPa, the PEG wt/wt percent should be between 7.5% (30.86 ± 0.44 kPa) and 10% (62.18 ± 1.56 kPa). Using the exponential regression equation (Figure 8) a 9% PEG hydrogel should be slightly over 50 kPa. The exponential regression equation should only be used for PEG hydrogels between 5 to 20% wt/wt concentrations.
Figure 8 - $E'$ of PEG hydrogel at different concentrations (wt/wt). The error bars represents standard error (SE) for $n=5$, if the error bar not visible it is to small to visualize on the graph.

4.2 UV Intensity within Exposure Box

The two spots of highest UV intensity were located right under the center of the bulb within the exposure box (Figure 9). The area in light blue on Figure 9 represents a UV intensity of 4.2 mW/cm$^2$ which was the largest area of uniformed UV intensity measuring 0.9 cm by 3.8 cm. The location was marked within the exposure box and used for all further studies involving PEG hydrogel crosslinking.
Figure 9 - UV Light Intensity from the UV Lamp by location within the exposure box.

4.3 Total Energy forFully Crosslinked PEG-DA

The total energy required for completely crosslinking a 9% PEG hydrogel was found to be 6300 mJ or 25 minutes with an $E'$ of 57.77±0.90 kPa (Figure 10). Increasing the total energy after 25 minutes resulted in no statistically significant $E'$ increase. Results indicated a plateau at the 7560 mJ (30 minutes, 56.40±.44 kPa) to 15120 mJ (60 minutes, 57.57±0.80). In order to create a 50 kPa hydrogel, the 9% PEG hydrogel should be given 5544 mJ of total input energy; thereby, resulting in an $E'$ of 50.75±0.98 kPa.
The total input energy and $E'$ between 4032 mJ (26.42±1.12 kPa) to 6300 mJ (57.77±0.90 kPa) has a linear relationship with a coefficient of determination of 0.9543. The linear regression equation (Figure 10) must only be used within the range of 4032 to 6300 mJ. Increasing the total input energy beyond 6300 mJ resulted in no additional $E'$ change due to the hydrogel being fully crosslinked.

![Graph](image)

Figure 10 - $E'$ of 9% PEG hydrogel at different crosslinking times and the total input energy calculated by equation 2. The error bars represent standard error (SE) for n=8 or greater. The error bars are not visible because they are too small to visualize on the graph.

4.4 Opacity Affects UV Light Intensity

The amount of UV intensity was modified by photomasking. The greater the percent of the opacity, the more the UV light intensity was reduced. The amount of UV intensity reduced by different opacity photomasks were as follows: 0% (4.2 mW/cm$^2$), 10% (3.2 mW/cm$^2$), 20% (2.8 mW/cm$^2$), 30% (2.31 mW/cm$^2$), 30% (2.31 mW/cm$^2$), 35% (2.2 mW/cm$^2$).
mW/cm$^2$) and 40% (2.07 mW/cm$^2$). The relationship between UV light intensity and opacity was linear with a $R^2$ of 0.9619. The linear regression equation (Figure 11) should be used between 0 to 70% opacity.

$$y = -0.0443x + 3.7721$$

$$R^2 = 0.9619$$

$0% \leq x \leq 70%$

Figure 11 - UV light intensity compared with opacity of photomasks. Error bars represent SE for n=8.

4.5 UV Intensity Affects Elastic Modulus

Photomasking was able to vary the E' of a 9% PEG hydrogel by varying the amount of the UV light intensity to which the hydrogel was exposed. The higher the UV light intensity, the greater the resulting E' (Figure 12). Photomasking the PEG hydrogels was done at six opacities: 0% (6300 mJ), 10% (4545 mJ), 20% (4155 mJ), 30% (3480 mJ), 35% (3255 mJ), and 40% (3045 mJ) (Table 1). Decreasing the total input energy just slightly from 3480 to 3045 mJ, decreased the E' from 25.78 ± 2.41 to 17.64 ± 0.82 kPa. The difference between 6300 and 3105 mJ was close to triple the E' (57.77 ± 0.91 Pa).
The photomasking technique was used to modify E’ by changing the total input energy that the hydrogel received while crosslinking.

The relationship between the E’ to the total input energy was linear with a coefficient of determination of 0.9738. The linear regression equation (Figure 12) should only be used within the range of 3045 to 6300 mJ. A total input energy greater than 6300 mJ resulted in no further E’ change due to the gel being completely cross-linked. Below 3045 mJ of total input energy, the PEG hydrogels were not fully formed within the silicone well where they were created; thus, resulting in an inability to punch a full 8 mm diameter gel for rheological testing. The resulting gradient from a photomask from 0 to 40% opacity resulted in a mechanical gradient from 17.64 ± 0.82 to 57.77 ± 0.90 kPa.

![Figure 12 - E’ of PEG-DA (9% wt/wt PEG) at different total energies by photomasking. Error bars represent SE for n=6 or greater.](image)

\[ y = 0.0125x - 19.248 \]
\[ R^2 = 0.9738 \]
\[ 3045 \text{ mJ} \leq x \leq 6300 \text{ mJ} \]
Table 1 - Summary of photomasking variables (UV Intensity, total input energy, opacity, and E') over 25 minutes of UV irradiation to a 9% wt/wt PEG hydrogel.

<table>
<thead>
<tr>
<th>Intensity (mW/cm²) ±SE</th>
<th>4.2±0.04</th>
<th>3.03±0.09</th>
<th>2.77±0.10</th>
<th>2.32±0.08</th>
<th>2.17±0.11</th>
<th>2.03±0.06</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Energy (mJ)</td>
<td>6300</td>
<td>4545</td>
<td>4155</td>
<td>3480</td>
<td>3255</td>
<td>3045</td>
</tr>
<tr>
<td>Opacity</td>
<td>0%</td>
<td>10%</td>
<td>20%</td>
<td>30%</td>
<td>35%</td>
<td>40%</td>
</tr>
<tr>
<td>E' (kPa) ±SE</td>
<td>57.77±0.90</td>
<td>41.85±1.14</td>
<td>31.36±1.46</td>
<td>25.78±2.41</td>
<td>19.56±1.73</td>
<td>17.64±0.82</td>
</tr>
</tbody>
</table>

Figure 13 - Swelling ratio of 9% PEG-DA exposed to five different amounts of total energy over 25 minutes (6300, 4545, 4155, 3480 and 3045 mJ). The error bars represent standard error (SE) for n=6 or greater. Note: All hydrogels are significantly different from one another.
Table 2 - Summary of swelling data relating the opacity, total energy over 25 minutes to the swelling ratio and % wet wt of 9% wt/wt PEG-DA.

<table>
<thead>
<tr>
<th>Opacity (%)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Energy (mJ)</td>
<td>6300</td>
<td>4545</td>
<td>4155</td>
<td>3480</td>
<td>3045</td>
</tr>
<tr>
<td>Swelling Ratio ±SE</td>
<td>11.83±0.24</td>
<td>12.37±0.53</td>
<td>13.53±0.32</td>
<td>14.19±0.59</td>
<td>16.04±0.49</td>
</tr>
<tr>
<td>% Wet Wt ±SE</td>
<td>91.51±0.18</td>
<td>91.82±0.33</td>
<td>92.58±0.17</td>
<td>92.87±0.28</td>
<td>93.71±0.18</td>
</tr>
</tbody>
</table>

Changing the total energy by photomasking also directly affected the swelling ratio of the 9% PEG hydrogel. Increasing the total energy decreased the swelling ratio (Figure 13), as expected, since smaller swelling ratios denote increased E’ agreeing with the photomasking results when exposed at the same amount of total energy (Figure 13). The percent of the wet weight of a fully crosslinked (6300 mJ) 9% hydrogel was 91.51±0.18% (Table 2). The lowest total energy amount 3045 mJ had the highest swelling ratio of 16.04±0.49, with a wet weight percent of 93.71±0.18%, which made it similar to the E’ of a 6.29% PEG hydrogel. All samples represented the swelling ratio at equilibrium swelling.

4.6 Total Energy

The amount of total input energy which PEG hydrogel received was changed in this study by reducing the irradiation intensity by photomasking, and altering the duration of exposure. Despite the method in altering the total energy, the effect of the total input energy on changing E’ when both data sets were plotted together had a high correlation coefficient of 0.9769 (Figure 14). The linear regression equation (Figure 14) has the same restrictions as it had in section 4.5 and should only be used over the range of 3045 to
6300 mJ. The equation indicated (Figure 14) that if the amount of the total input energy was the same, the same E' would result.

Figure 14 - E' of PEG-DA (9% wt/wt PEG) at different total energies by photomasking and exposure time (16-25 minutes). Error bars represent SE for n=6 or greater.
4.7 Cell Attachment

Figure 15 - Cell attachment after 24 hours on the 9% wt/wt PEG scaffolds at different concentrations of collagen at 0 (A), 78 (B), 156 (C), 312 (D), 624 (E), 1000 (F), 1248 (G), and 2496 (H) µg/mL mixed within the hydrogel prior to crosslinking. Scale bar indicates 100 µm.

The concentration of collagen that was mixed within the PEG hydrogel was investigated for attachment of hMSCs to the surface of the hydrogel. The PEG hydrogel without collagen resulted with hMSCs exhibiting a rounded morphology loosely connected to the surface of the hydrogel (Figure 15 A), as expected. At lower amounts of collagen 78, 156 and 312 µg/mL (Figure 15 B-D) the cells had some spread morphology, but many of the cells were rounded. At 624 µg/mL concentration of collagen most of the cells were attached (Figure 15 E). High concentrations of collagen (1000 and 1248 µg/mL) resulted
in nearly all of the cells spreading across the hydrogels surface (Figure 15 F,G). The highest concentration of collagen 2496 µg/ml showed round morphology of the hMSCs (Figure 16 H). All studies below used 1 mg/mL concentration of collagen within the 9% PEG hydrogel.

4.8 The Effect of Collagen on Elastic Modulus

The $E'$ of fully crosslinked 9% PEG hydrogel with the addition of 1 mg/mL collagen (56.12±1.07 kPa) was not statistically significant different from 9% PEG hydrogel without collagen (57.77±0.90 kPa). Likewise, when the 9% PEG hydrogels were exposed to 3105 mJ of total energy with collagen (18.01±1.36 kPa) and without (17.64±0.82 kPa) collagen, they showed no statistically significant difference. The addition of 1 mg/mL of collagen within 9% PEG hydrogels had no effect on the $E'$. 
Figure 16 - Comparision of E’ (E’) between 9% wt/wt PEG hydrogels with and without 1000 µg/mL of collagen at two different total energies 6300 and 3105 mJ. The error bars represent standard error (SE) for n=8.

4.9 Cell Viability

A live / dead assay was conducted to test the cell viability of the PEG-collagen hydrogel (9% wt/wt PEG, 1000 µg/mL collagen) compared with the PEG (9% wt/wt) hydrogel without collagen at 24 hours (Figure 17) after cell seeding. After 24 hours, the cells have spread across PEG-collagen hydrogel (Figure 17 A). At this point, higher cell viability was observed on PEG-collagen scaffold (95.91 %) as compared to PEG (34.78%) (Figure 17 B). The low viability on PEG was expected due to PEG's poor cell attachment and protein adsorption.
Figure 17 - A live / dead assay was conducted to determine cell viability on the surfaces of different scaffolds. The hMSCs were seeded onto the surfaces of PEG-Collagen hydrogels (A) and PEG hydrogels (B). Live cells were labeled with calcein-AM (green) and dead cells were labeled with ethidium homodimer-1 (red). Scale bar indicates 100 µm.

4.10 Cell Migration

Cell migration on PEG-collagen hydrogel with a mechanical gradient (Figure 18) and a homogeneous surface (Figure 19) was analyzed on days 1-4 by counting the cells across the entire scaffold. The location of the cells was tracked by placing a 500 by 500 µm grid across the scaffolds. Cells were counted within each square and then compared over each day. The gradient scaffold had an E' change from 17.64±0.82 to 57.77±0.90 kPa with the gradient going from left (low E') to right (high E'). Between day one and day two (Figure 18 A) on the gradient scaffold (Figure 18 B), the cells started to migrate toward the side of the scaffold with the higher E'. The cell migration was most apparent when comparing day one (Figure 18 A) with day four (Figure 18 C) where most of the cells had shifted toward the side with the higher E'. The homogeneous scaffold represents a scaffold with an E' of 57.77±0.90 kPa. The homogeneous scaffold exhibited cell migration spreading out in a radial pattern from day 1 (Figure 19 A) through day 4 (Figure 19 B-C). The non-gradient scaffold was seeded with slightly more cells initially (245 cells) when compared
to the gradient scaffold (155 cells). The variance can be attributed to error in attempting to seed such a small volume of cells (~200 cells in 2 µl).

Figure 18 - Cell migration study on a 9% wt/wt PEG-Collagen gradient scaffold from 17.64±0.82 to 57.77±0.90 kPa (E') (Left to Right). The scaffold was sectioned into 500 by 500 µm squares and the cells within each square was counted and graphed in MATLAB. HMSCs were seeded onto the center of the scaffold and observed over 4 days (A-C).
Figure 19- Cell migration study on a 9% wt/wt PEG-Collagen scaffold with an E’ of 57.77±0.90 kPa. The scaffold was sectioned into 500 by 500 µm squares and the cells within each square counted and graphed in MATLAB. HMSCs were seeded onto the center of the scaffold and observed over 4 days (A-C).
CHAPTER V

DISCUSSION

5.1 Methodology of Photomasking and Gradient Creation

The goal of this thesis was to create a mechanical gradient on a PEG-DA hydrogel using photomasking. The mechanical gradient was designed to mimic the physiological gradient range from healthy myocardium to infarcted myocardium (~16 to 50 kPa E') [50, 14, 15]. Together gradient strength [51] and gradient range (kPa/mm) are important for mimicking the myocardial infarction gradient [50, 52]. The physiological gradient strength of healthy to infarcted tissue varies from 0.6 to 8.5 kPa/mm [51]. The gradient strength of the developed PEG collagen hydrogel in this thesis has a gradient strength of 5.73 kPa/mm which is within the physiological range [51]. The gradient range possible for 9% PEG hydrogel was found to be 17.64±0.82 to 57.77±0.90 kPa which results in a hydrogel which contains both gradient range and gradient strength from healthy myocardial to infarcted. Previous studies have mimicked the gradient strength on a PEG hydrogel but not both gradient strength and range [53]. Other gradient strengths besides 5.73 mm/kPa were not investigated in this thesis but with this system it is possible to change the gradient strength by changing the strength the opacity over distance of the photomask. The limiting factor for increasing the gradient strength will be the resolution
of the printer printing the photomask and the limiting factor of decreasing the gradient range will depend on the size of the UV light and photomask.

In previous published studies, photomasking has been shown to be an effective method for creating mechanical gradients on photopolymerized hydrogels [2, 5, 54]. The ability to make mechanical gradient scaffolds is important in mimicking disease conditions like myocardial infarction as well as normal healthy tissue. *In vivo*, gradients were observed within the different cell layers [53]. Each cell layer may possess different cell types or phenotypes which are different from other tissue layers within the organ resulting in different mechanical properties from layer to layer [53]. Studying the cell response to gradient hydrogels can learn the behind mechanisms and use them to direct cells to behave in certain ways such as proliferation, migration, anchorage, and outgrowth [54]. The ability to study these aspects give insight into how a biomaterial scaffolds should be designed to control various cell responses.

In order to study how photomasking can create a mechanical gradient within the target physiological range (~16 to 50 kP E’) the following variables were investigated: PEG concentration, opacity of the photomask, UV irradiation intensity, duration of UV exposure, total input energy, wt/wt concentration of PEG, and E’. The concentration of PEG-DA was varied to find the E’ which best mimiced infarcted myocardial tissue. The results of this study using rheology to measure the G’ of PEG-DA at varying (5%, 7.5%, 10%, 15% and 20% wt/wt) concentrations were consistent with the previous studies [55]. The calculation of total input energy was important because each UV lamp will output different irradiation intensity and can possibly dim over time. Knowing the total energy required for a specific E’ PEG hydrogel made it possible to calculate the exposure time
with equation 2. Additionally, knowing the relationship of the opacity transparencies to irradiation intensity made it possible to figure out the duration of exposure for a specific E’ within the E’ range for the PEG-DA hydrogel. The E’ range for a PEG-DA hydrogel depends on the upper limit where the hydrogel is cross-linked completely at a given wt/wt percent. The method used in this study to find the total input energy of a fully cross-linked hydrogel was to fix the UV intensity and change the exposure duration by minute intervals until the E’ remains the same (57.77±0.90 kPa) and no longer increased with further exposure time (Figure 10).

The lower limit (17.64 ± 0.82 kPa) was found by decreasing the total energy by photomasking at 10% intervals of opacity until the 9% wt/wt PEG hydrogel no longer was able to form a whole hydrogel within the entire silicone molding (Figure 6). At 50% opacity (2430 mJ) the PEG hydrogel would not completely form within the mold. The coefficient of determination (R^2 = 0.9696) between the total input energy and E’ by photomasking was linear, which highly suggested that the data can be used to create a continuous mechanical gradient between 17.64 ± 0.82 kPa to 57.77±0.90 kPa (Figure 6). Knowing the relationship between E’ and total input energy created the ability to make two scaffold ranges for mimicking the mechanical properties of myocardial infarcted tissue after 7 and 14 days. Normal myocardial tissue E' was between 16-20 kPa [13,14]. After myocardial infarction at day 7, the myocardium has an E’ of 31.38 ± 0.75 kPa, and at day 14 an E’ of 53.23 ± 0.75 kPa [14]. The 9% PEG-DA hydrogel was able to cover these gradients from 17-32 kPa and 17-54 kPa. Future studies should directly test these gradients with atomic force microscopy.
Photomasking has been previously used to change the swelling ratio spatially to create complex anisotropic swelling patterns on hydrogels [56]. The results in this thesis are consistent with previous photocrosslinked hydrogels by the lower the total energy the less crosslinking and higher swelling is exhibited [57]. On the developed gradient scaffold the swelling ratio range is ~12-16 meaning the photomask created a slight slope across the gel from lower E’ to higher E’. The swelling ratio of a fully crosslinked 9% hydrogel (6300 mJ) has a swelling ratio of ~12 which is consistent with previously reported PEG-DA hydrogels [58]. Swelling studies give insight to the affect of photomasking on the content of PEG within the hydrogel after crosslinking at different total input energies (Table 2). The wet weight percent shows how much water the hydrogel contains; additionally, it indicates how much solid polymer (PEG) material remains after swelling to equilibrium. The wet weight percent of a fully crosslinked 9% wt/wt PEG hydrogel was 91.51±0.18%, leaving about ~8.48% PEG after crosslinking. Additionally, decreasing the total input energy by photomasking increases the amount of water concentration in the hydrogel; thus, increasing the swelling ratio and decreasing the concentration of PEG left within the hydrogel. The swelling studies showed that the amount of PEG incorporated into the gel decreased with decreasing total energy, as expected based on the literature [5]. The results indicated that photomasking does reduce the crosslinking density of PEG resulting in less material.

The original investigation in to differentiation by mechanoinduction of hMSCs was prepared on homogeneous E’ polyacrylamide gels coated with collagen [59].

Polyacrylamide gels are able to make mechanical gradients with a linear gradient range of ~1 to 240 kPa (E’) [60]. The gradient range possible for polyacrylamide gels is within
the possible range for mimicking myocardial infarction. The disadvantage of polyacrylamide gels is that the monomer acrylamide is toxic to cells [61, 62]. The cytotoxicity of acrylamide prevents the ability to encapsulate cells within the acrylamide solution prior to gelation which led researchers previously using polyacrylamide for 2D cell culture to switch other hydrogels when going toward 3d cell culture [63]. PEG-based hydrogels are highly tunable and have well characterized mechanical properties like polyacrylamide gels but they also lack the cytotoxicity for 3D cell culture making it a more suitable material for both 2d and 3d culture [63]. 3D PEGDA gradient scaffolds have been recently used successfully in an in vitro model for neovascularization with endothelial and smooth muscle cells [64]. The in vivo “niche” is better mimicked by 3D cell culture giving the developed scaffold in this thesis a good platform for future studies. The creation of mechanical gradients by photomasking is not a new method [2, 5, 65]; nor is using photomasks for making hydrogels with homogeneous mechanical properties [5]. However, as far as the author is aware, the method of relating photomasking opacity to intensity, total input energy, and thus E’, has not been published.

5.2 Cell Attachment, Viability and the Effect of Collagen on Elastic Modulus

The cell attachment to PEG-DA was achieved by incorporation of collagen type I into the PEG solution prior to crosslinking (Figure 6) at a concentration of 1 mg/mL. The ECM of the myocardium overwhelmingly consists of (~85%) type I collagen [34]. In order to mimic the natural myocardium, collagen was used for cell adhesion to the PEG hydrogel. However, unlike in vivo, the collagen in the gels studied here would not be oriented into fibrous structures like natural ECM. Additionally, unlike in vivo, the collagen does not provide mechanical support. The mechanical support was totally reliant on the PEG-DA
hydrogel (Figure 16) with collagen concentration having no significant effect on the E'. The 1 mg/mL concentration of collagen achieved good cell attachment with hMSCs and showed no statistical difference between the E' with or without the incorporation of collagen into the hydrogel (Figure 16). The PEG-collagen hydrogel displayed excellent cell viability (95.91%) and cell attachment after 24 hours (Figure 17). The round morphology of the hMSC's on the 9% PEG hydrogel with 2.496 mg/mL of collagen was unexpected. hMSC's have been cultured sucessfully on 100% collagen gels at a concentration of 2.5 mg/ml with good cell viability [66]. One possible explanation would be it is difficult for high concentration of collagen (2.496mg/ml) to evenly dispersed within the 9% PEG resulting in poor cell attachment.

5.3 Cell Migration

One method to help confirm that a gradient was created across the PEG-collagen hydrogel was to track cell durotaxis on the mechanical gradient. In previous studies, cell durotaxis was a very prevalent behavior of hMSCs [2], vascular smooth muscle cells [54] and human foreskin fibroblasts (HFFs) [47] from regions of low E' to regions of higher E’. hMSCs experience higher proliferation, confluence and spread morphology on firmer areas of the mechanical gradient hydrogels [2]. The cell migration study was designed to seed a small amount of cells (~200 cells) at the center of gradient and non-gradient scaffolds and track the hMSCs durotaxis over a period of four days. The reasoning for using such a small number of cells was so that within the period of four days the cells would not proliferate too quickly and cover the entire hydrogels surface. After crosslinking, both the gradient (13,000 by 4,500 µm) and non-gradient (13,000 by 6,000 µm) hydrogels were slightly smaller than the silicone mold (15,000 by 7,000 µm) in
which the PEG-collagen hydrogels were formed. The author's experience in crosslinking the hydrogels was that the resulting hydrogels were always smaller than the mold. The size difference of the gradient scaffold after crosslinking should be accounted for in future studies, so that the gradient mask and the mold will fit the length of the resulting hydrogel.

The cell migration study showed the migration of hMSCs across the entire PEG-collagen hydrogel over a period of four days. The results were consistent with previous studies [2] where hMSCs migrated from regions of low $E'$ to regions of higher $E'$ on the mechanical gradient hydrogel. The cell migration on the gradient hydrogel was noticeable after the first day toward the region of high $E'$ (Figure 18). Whereas, the non-gradient hydrogel cell migration was more of a radial growth from the center seeding point (Figure 19).
Aim 1: Develop a polyethylene glycol (PEG) scaffold which is fully cross-linked and exhibits an elastic modulus (E') of at least 50 kPa and then measure the total amount of ultraviolet (UV) energy required to fully crosslink that hydrogel.

To accomplish this aim a series of characterization studies were performed. These were a series of rheology studies at different PEG-DA weight concentrations to measure the G' and calculate E'. The data series was plotted and a curve was developed which found a 9% hydrogel will give an E' of at least 50 kPa. The output of UV intensity from the UV lamp was recorded and graphed spatially by a UV light meter to find the most uniform UV intensity area. The total energy was found by changing the exposure time of the 9% PEG hydrogel by minute intervals while keeping the UV intensity fixed (4.2 mW/cm²). The E’ of the 9% PEG hydrogel was found to be 57.77±0.90 kPa and the total energy required to crosslink the gel was 6300 mJ.

Aim 2: Characterize the feasibility of reducing the total UV energy to the PEG hydrogels through photomasking to create a mechanical gradient.

This aim involved characterizing photomasks and the effect they have on the mechanical properties on a 9% PEG-DA hydrogel. The amount of UV intensity able to pass through
each photomask was measured by a UV light meter. The results show a linear relationship between opacity and UV intensity with a $R^2$ value of 0.9619. The discrete photomasks were used to change the UV intensity at a fixed UV exposure time of 25 minutes on the 9% PEG gel. The PEG hydrogels mechanical properties were characterized through rheology and swelling studies. Photomasking over a 9% PEG hydrogel was shown to be able to create a gradient range between 17.64 to 57.77 kPa with a linear relationship between total energy and $E'$ ($R^2$ 0.9738). Reducing the total energy further than 3045 mJ was not able to further reduce the $E'$. The gradient range on the developed hydrogel is able to mimic the $E'$ of the physiological range of infarcted to health cardiac tissue. In addition, the gradient strength is able to mimic the infarcted to healthy cardiac tissue at 5.73 mm/kPa.

Aim 3: Characterize cell viability and migration on the developed gradient scaffolds.

To accomplish this aim a series of characterization studies were performed including: collagen concentration in PEG on cell attachment, effect of collagen concentration on $E'$, live/dead assay and cell migration study. The 9% PEG hydrogels with 1000 µg/mL of collagen had good cell attachment with 95.91% viability. The addition of 1000 µg/mL of collagen showed no significant different on the $E'$ compared to without collagen across the gradient range 17.64 to 57.77 kPa. Cell migration was tracked over 4 days on a homogeneous 9% and a gradient hydrogel between 17.64 to 57.77 kPa with a gradient strength of 5.73 mm/kPa. Migration on the gradient scaffold caused hMSC’s to move to
the stiffer end of the scaffold whereas on the homogeneous scaffold the cells dispersed evenly across the surface.
REFERENCES


[57] Scott A. Zawko, Shalu Suri, Quan Truong, Christine E. Schmidt "Photopatterned anisotropic swelling of dual-crosslinked hyaluronic acid hydrogels" Acta Biomaterialia 1 5 14-22 (2009)


