COMPARISON OF THIOL-ENE AND TETRAZINE CLICK CROSSLINKING OF GELATIN HYDROGELS FOR TISSUE ENGINEERING

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Comparison of Thiol-ene and Tetrazine Click Crosslinking of Gelatin Hydrogels for Tissue Engineering

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Chemically crosslinked gelatin hydrogels are popular biomaterials applied for uses such as 3D matrices for tissue engineering applications. Gelatin hydrogels can be chemically crosslinked using various reactions that fall under the "click" chemistry paradigm. Click chemistry is an incredibly powerful tool to alter different hydrogel properties in fast, simplistic way. Here, we compare two impactful click reactions: a radical mediated, thiol-ene–norbornene reaction and an inverse electron demand Diels-Alder (IEDDA) tetrazine–norbornene reaction. The radical mediated reaction resulted in chemical crosslinks throughout the hydrogel while the IEDDA reaction resulted in not only chemical crosslinks, but physical crosslinks as well due to various secondary interactions. To truly understand how these different click reactions impact hydrogel properties, experiments were performed to characterize hydrogel modulus, swelling, degradability, and cytocompatibility. Based upon the contrasting aspects of the two gel formulations, it can be concluded that these two different chemistries have diverse applications due to their differences in modulus, swelling ratio, and degradability.

DEDICATION

I dedicate this thesis to all those that have been by my side throughout my undergraduate degree from the start and to those who have inspired me to do better for others.

ACKNOWLEDGMENTS

I would like to thank my research advisor Dr. Alge for his guidance and patience throughout the course of this project. It truly means a lot to have someone help guide me in the beginning so that I may finish strong.

Also, I would like to thank my friends and colleagues for supporting me throughout this experience as well. Without them pushing me, I would not be able to move forward with such confidence as they raise my spirits.

Finally, I would like to thank my mother and father for their support as well. They have pushed me to be the best version of myself since the beginning and the best part is, I am just now getting started.

NOMENCLATURE

IEDDA	Inverse Electron Demand Diels-Alder
GelNB	Gelatin Norbornene
PEG-di-SH	Poly(ethylene glycol)-di-thiol
PEG-di-Tz	Poly(ethylene glycol)-di-tetrazine
LAP	Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
hMSC	Human Mesenchymal Stem Cells
PBS	Phosphate-buffered saline
DI water	Deionized water
Pa	Pascals

CHAPTER I

INTRODUCTION

Chemically crosslinked gelatin hydrogels are popular biomaterials for use as a 3D matrices for tissue engineering (Maitra 2014). Gelatin can be chemically crosslinked using a variety of reactions, including "click" chemistry reactions. Click chemistry has been recognized as a powerful tool, and reactions fitting this paradigm are particularly attractive due to the superior control they offer over hydrogel properties. One example of a click reaction is the radical-mediated thiol-norbornene click reaction, which can be implemented in gelatin hydrogels by reacting norbornene-functionalized gelatin (GelNB) with a di-thiol crosslinker such as poly(ethylene glycol)-di-thiol (PEG-di-SH) (Yu 2015). GelNB can also be crosslinked via inverse electron demand Diels-Alder click reactions with di-tetrazine molecules, such as poly(ethylene glycol)-di-tetrazine (PEG-di-Tz) (Knall 2013). However, it is unknown how these different click reactions affect the properties of the hydrogel and their utility in different applications. Therefore, the objective of this study was to compare the effects of thiol-ene and tetrazine click crosslinking on the material properties and cytocompatibility of gelatin hydrogels.

Hydrogels

Hydrogels are crosslinked polymers that are either linear or branched that utilize covalent, physical, or both types of crosslinking(Schoenmakers 2018). These bonds allow the polymers to create a dimensionally stable three-dimensional network. Chemically crossed linked networks result in thermosets which lead to having a stronger bonding network. Physically crosslinked hydrogels result in thermoplastic hydrogels which contain non-covalent crosslinks which are stabilized by hydrogen bonds and secondary interactions ("Thermoplastic and

Thermosetting Polymers"). These thermoplastics are naturally found, playing a part in DNA as well as carbohydrates. A visual representation of the two different types of crosslinking can be found in **Figure 1** where it possible to see how the different types of crosslinks will create two completely different type of mesh network.

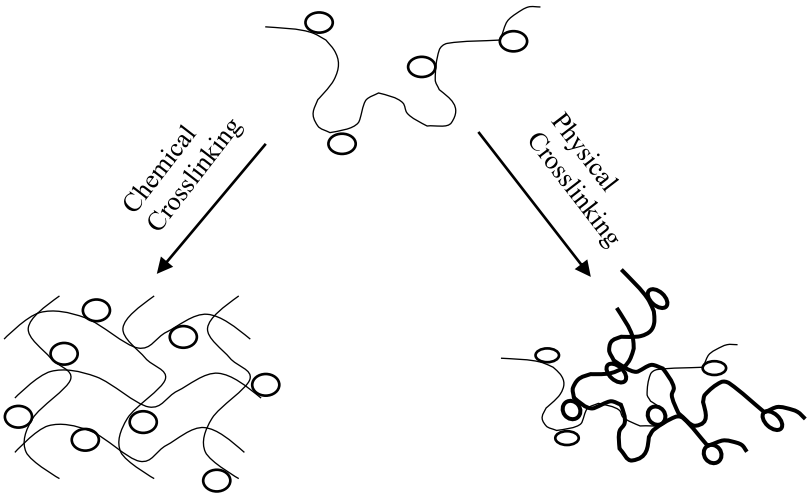

Figure 1. The matrices formed by chemical crosslinking and phyiscal crosslinking are compared.

Hydrogels themselves are lightly crosslinked, water swollen polymer networks which can be assumed by the prefix hydro-. Hydrogels exist naturally in nature and the human body include collagen, alginate, and the everyday snack, Jell-O. In this scope of appearances is applications of hydrogels in biomedical engineering specifically (Ahmed 2015). These biocompatible gels have the capability to be used in various ways such as tissue fillers, drug delivery systems, dental impressions, and more.

Mechanically, hydrogels contain a tunable crosslink density which will affect the modulus of the overall gel. The modulus, stiffness, of the hydrogels play an important part because this usually will dictate the application of the said gel. The modulus will also guide the cells in differentiation where the stiffer the gel, the stem cells will have the inclination to

differentiate into an osteogenic cell line to mimic the stiffness that can be seen in bone (Engler 2006). Not only will modulus play an important part but so will the swell-ability of the gel. This will affect the protein interactions because of the surface hydrophilicity, the ability to draw in water. In drug delivery systems, the gels' ability to swell will govern how the drug and water will diffuse through the system, playing an important deciding factor of application. Specialized hydrogels can be created and formed in order to be stimuli-responsive (Zhang 2010). Due to the versatility of hydrogels, they have begun to be expanded upon further for a multitude of disciplines.

Click Chemistry

Click Chemistry was brought to light in 2001 between azide as well as alkyne groups. Click chemistry itself is simplistic, stable and are stereospecific. Chemistries that fall into this category must result in high yield at which the byproducts from said reaction can be simply removed. This removal does not need the use of chromatographic methods. Varieties of reagents and "building blocks" can be used when performing click chemistry which is why this type of chemistry is so versatile across the scope of science with its ability to work in not only small- but also large-scale applications (Kolb 2001). Due to the large scope of reactions that fall into this category, curiosity of the different outcomes from these reactions is a topic for tissue engineering and their possible applications for cell matrices.

Radical Mediated Thiol-ene Click Reaction

Thiol-ene reactions are reactions between a thiol and an alkene group and have been of common knowledge since the early 1900s. This reaction takes places through two mechanisms: photoinitiated free-radical addition and catalyzed Michael addition reactions. This reaction falls under the click-chemistry paradigm, as it is a rapid, stable, high yielding reaction. This specific

reaction has been utilized in polymer network modification (Sun 2018). This radical-initiated reaction undergoes photo-mediated, reversible cleavage with the presence of a photo-initiator such as lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) which creates an unable radical on the pendant so that the reaction can take place there. This quick process, seen in **Figure 2**, occurs and results in a stable gel when all the pendant groups have been used and the polymer network has been created.

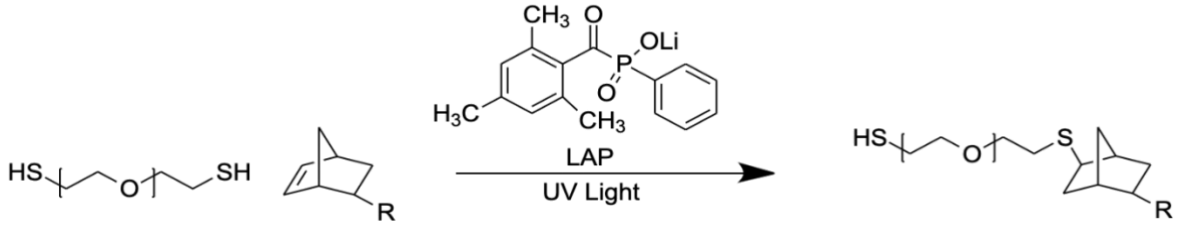

Figure 2. Radical Mediated thiol-ene click reaction

Although radicals have been seen to decrease cytocompatibility, this reaction specifically creates a biocompatible environment with the presence of the photo-initiator which is pertinent in this area of work.

Inverse Electron Demand Diels-Alder (IEDDA) Tetrazine Reaction

IEDDA's are emerging as novel click-chemistry reactions for their various applications throughout chemistry. The reaction itself falls under the click-chemistry paradigm but this is a relatively unexplored area for scientist in biomaterials. IEDDAs are characterized by having rapid kinetics, stereochemistry specificity, and biocompatibility (Alge 2013) . This biorthogonal reaction takes place specifically with a tetrazine pendant group in order to create not only chemical but physical bonds to form a stable cellular matrix for a means for a three-dimensional cell culture (Oliveira 2017) that can be seen in **Figure 3**.

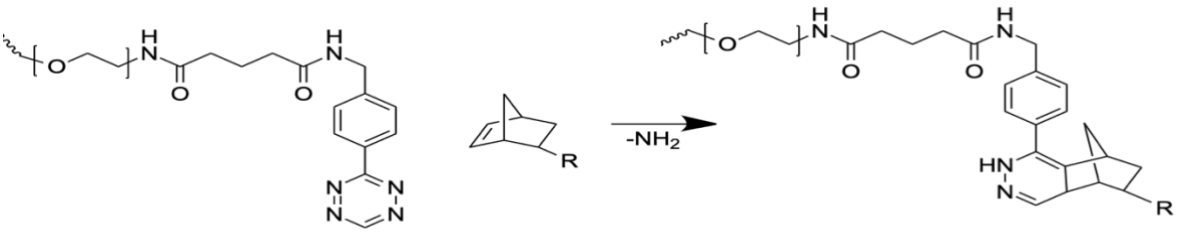

Figure 3. Inverse Electron Demand Diels-Alder tetrazine click reaction

This reaction contrasts with other click reaction being compared due to the physical bonds created as well as the chemical bonds. This novelty of the reaction changes the material characterizations and potentially the biocompatibility and these aspects will be assessed.

Human Mesenchymal Stem Cells

Human mesenchymal stem cells (hMSCs) are characterized by three different properties: they are unspecialized cells, they have the ability to proliferate for long periods, and they have the capacity to differentiate into specialized cells ("Human Mesenchymal Stem Cells (HMSC)"). Initially, hMSCs are unspecialized cells which means that they do not have the ability to perform a specific function. This allows the cells to proliferate for a prolonged period of time, dividing multiple times to produce millions of viable cells. These viable cells then have the ability to differentiate into specialized cells such as bone, cartilage and fat cells (Ullah 2015). All of these properties are dependent on the hMSCs' microenvironment. These microenvironments will determine their proliferation status as well their differentiation. Adult tissues, such as bone marrow, contain hMSCs, which can be isolated and from this be differentiated not only into bone marrow tissue but other mesenchymal tissues (Bernardo 2007). Due to their ability for constant self-renewal, hMSCs have gain traction in tissue engineering as a promising platform for clinical applications. These cells have been studied in-depth, ranging from their cell morphology to their

motility based upon various aspects they are introduced to such as contrasting environments (Winer 2009). For example, if the environment mimics the stiffness of brain tissue, the cells will start to proliferate and differentiate into neuron-like cells. Based upon the specificity and versatility of bone-marrow derived hMSCs, these cells are the ideal test subjects to analyze the biocompatibility of varying three-dimensional cell matrices.

CHAPTER II

MATERIALS AND METHODS

Gelatin Norbornene Functionalization

Gelatin was functionalized with 5-norbornene-2-carboxylic acid using succinimidyl ester chemistry. This can be seen in **Figure 4**.

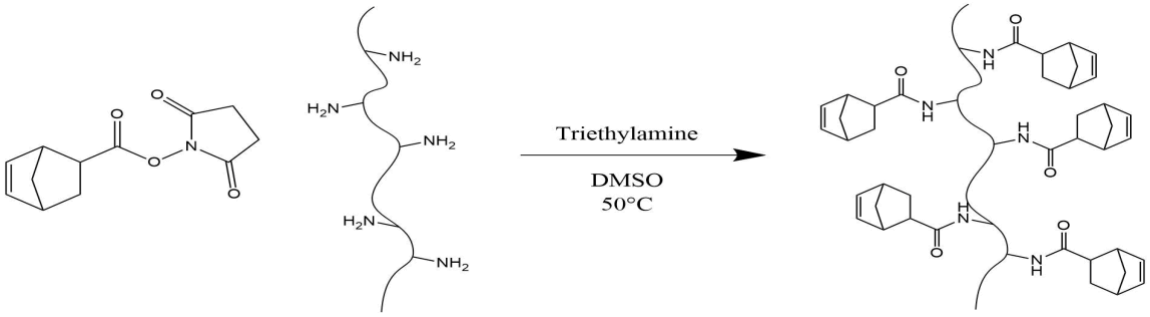

Figure 4. Gelatin norbornene being functionalized through succinimidyl ester chemistry.

Percent functionalization with norbornene groups was analyzed and quantified with NMR.

PEG-Di-SH Preparation

Pure PEG-di-SH was weighed and then sterilized under UV-light for 30 minutes. After, the pure PEG-di-SH was diluted with sterile PBS in order to make a sterile stock solution of 20% w/v. For non-sterile stock solutions, a sterile stock solution was taken and used on the bench top and noted as non-sterile from that point forward.

PEG-Di-Tz Functionalization

This process was begun with partially functionalized PEG-di-Tz. To re-functionalize the PEG-di-Tz, 3.4 kDa linear PEG-NH_2/Tz, Tetrazine carboxylic acid (Tz-COOH), and N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl) uranium hexafluorophosphate (HBTU) were warmed to

room temperature and then weighed. 0.875g of 3.4 kDa linear PEG-Tz was added to a round bottom flask which was then purged with argon gas. To dissolve this, 10 mL of N-methylpyrrolidone (NMP) was added followed by 51 µL of triethylamine (TEA) which was mixed at room temperature for 15 minutes. To prepare the tetrazine, 3 equivalents of Tz-COOH (165.4 mg) and HBTU (209 mg) were added then purged with argon gas. This mixture was then dissolved in NMP for 5 minutes at room temperature. Next, the two separate mixtures were added together using a Pasteur pipette. The activated Tz-COOH was added to the PEG mixture and then washed with 1 mL of NMP. This reacted for 15 hours. Post reaction, the mixture in the round bottom flask was transferred into a beaker of 40 mL of cold diethyl ether. These precipitant mixtures were then even distributed into 4 conical tubes and centrifuged at 4500 rpm for 1 minute. This step removed the salt byproducts. After this separation, the diethyl ether was discarded and vacuum dried in the desiccator for 1 hour. After the 1 hour, the product was going to undergo dialysis. The dialysis tubing of 1 kDa was prepared in ultrapure water. Before dialysis, the product was weighed. Separate ultrapure water was chilled and a hot plate with a magnetic stirrer were placed in the fridge due to the temperature dependence of the reaction. The products were then dissolved in 10 mL of chilled ultrapure water. The solution was centrifuged and the liquid sample was added to the dialysis tubing. The tubing was then added to the beaker placed on the hot plate in the fridge and dialysis began. After 2 hours, the ultrapure water was changed and then was changed every 12 hours for the next 48 hours. After dialysis, the sample was placed into a conical tube and was frozen overnight in the -80 °C freezer. After the product was frozen, it was lyophilized for 3 days, until the sample was dry. In order to confirm the functionalization of the PEG-di-Tz, NMR spectroscopy was performed and analyzed.

Gel Formation

Gel formation occurred simultaneously. As the PEG-di-Tz gels were crosslinking, the PEG-di-SH gels were undergoing UV-polymerization. This was in order to create a standard approach for both gel formations. The gel formula changed minimally when the gels were made for cellular investigation. The main change is the addition of cells to the formula. These cells were suspended in PBS prior to being placed in gel molds so the change between cellular experiments and material characterization experiments lies heavily in the use of hMSCs.

GelNB Crosslinked with PEG-Di-Thiol

To create a gel that consists of GelNB crosslinked with PEG-Di-Thiol, the proper components are necessary to create a precursor solution prior to exposure to UV-light. It is important to note that the gelatin norbornene was placed in a bead bath set to 40 °C in order to create a liquid consistency. To create this precursor solution on the bench, a working 5% w/v gelatin norbornene, 2% w/v of PEG-di-SH, 2 mM of LAP, and PBS were combined. Once the precursor solution was made, it was placed back into the bead bath. During the time the precursor solution was in the bead bath, the syringe molds were prepared as well as the Omnicure. The Omnicure emits UV-light at various intensities as well as for varying lengths of time. The Omnicure was primed to emit a 5 mW/cm^2 intensity for 5 minutes. This was confirmed with a radiometer. Once the Omnicure was prepared as well as the molds, the precursor solution was pipetted from the main precursor solution to each mold, allowing the gels to be 30 µL. When the solution was pipetted into the molds, three molds would be exposed to UV-light at the same time. Each experiment called for 3 gels in order to perform each experiment in triplicate. Post UV-curing, the gels were placed in their respective solutions depending on which experiment was being performed on the gels. If the experiment required

hMSCs, the same procedure would apply with the addition of 30,000 hMSCs in the precursor solution. This amount of cells broke down to 1,000 cells per µL which was sufficient to interpret the data from the experiment. This was because there were ample amount of cells to analyze for each time point.

GelNB Crosslinked with PEG-Di-Tetrazine

To create this gel that consists of GelNB crosslinked with PEG-Di-Tetrazine, the proper precursor solution must be formed prior to gelatin formation. To create the precursor solution, 5% w/v gelatin norbornene and PBS were added together to create this precursor solution and placed in a bead bath to reach the temperature of 40 °C. The syringe molds were prepared during this time and the PEG-di-Tz was also ready. As shown in **Figure 5**, the precursor solution was first added to the syringe molds followed by the addition of PEG-di-Tz.

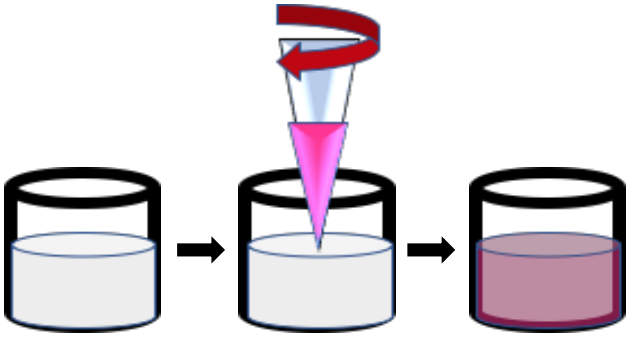

Figure 5. Visual depiction of the addition of PEG-di-Tz to the precursor solution

The reaction between the GelNB and PEG-di-Tz occurs quickly at first and therefore must be added at different steps. Once the PEG-di-Tz was added to the mold, it was quickly pipetted together followed by stirring in a clock-wise motion with the precursor solution to ensure a homogeneous gel. After about 15 minutes, the gels were formed and then placed in their respective solution which was dictated by the experiment.

Material Characterization

In order to properly compare the two click chemistries effectively, various material properties had been compared. The techniques to gather quantitative data such as modulus, degradation rate, swelling ratio, and gel fraction.

Storage Modulus

The gels post-polymerization were placed in DI water for 24 hours in order to swell properly. In order to obtain the storage modulus of the two gels, the gels were placed individually on a rheometer. Each gel underwent a time sweep of 1 rad/second for 60 seconds at 1% strain. This particular strain was applied as it is within the linear viscoelastic regime for hydrogels. The output data consisted of the storage modulus of each individual gel. Then for each group, the data was averaged together to compare the two types of gels.

Swelling Ratio

Post-polymerization, the gels were swollen to equilibrium in DI water in a 24 well-plate for 24 hours. The weight after 24 hours was taken, this was considered to the mass of the swollen gel (W_s). These masses were recorded. After recording the masses, the gels were then dried in a desiccator in uncapped Eppendorf tubes overnight until they were dried. This time varied between 12-18 hours. After the gels were dried, the mass was obtained and considered to the mass of the dried gels (W_d). These values were recorded. Using these values, the swelling ratio was calculated using **equation 1.**

$$Swelling\ Ratio = \frac{Mass\ of\ swollen\ gel - Mass\ of\ dry\ gel}{Mass\ of\ dry\ gel} \qquad (1)$$

These swelling ratios were calculated and compared between the two gel formulations.

Gel Fraction

Post-polymerization, the samples were moved to Eppendorf tubes and dried in the desiccator for 24 hours. The mass of the dried samples were obtained. This mass consists of the sol fraction (M_s) plus the gel fraction (M_g). Therefore, this mass is M_{total}. After recording the M_{total}, the samples were rehydrated with DI water in a 24 well plate. The plate was then placed on a plate shaker for 24 hours in order to remove the sol fraction. After 24 hours, the gels were removed from the water and were placed again in Eppendorf tubes. These Eppendorf tubes remained uncapped and were placed in the desiccator to dry for 48 hours. The dried gels were then retrieved again and the mass of the remaining crosslinked gel fraction were recorded. By using **equation 2** the gel fraction was calculated and evaluated.

$$Gel\ Fraction = \frac{Mass\ of\ remaining\ crosslinked\ gel\ fraction}{Mass\ of\ dried\ sample} \qquad (2)$$

Degradation Study

Post-polymerization, the gels were swollen overnight in DI water. Cell-strainers were obtained and modified in order to fit within a 6 well-plate. These modified cell strainers would hold the gel as it degrades. The goal of the modified cell strainer was to ease the process of weighing the degrading gel and obtain the true mass that was left. The day before the collagenase digestion study, the collagenase B solution was prepared. The lyophilized stock of the protein was obtained and was made into a solution with a concentration of 0.4 mg/mL in PBS. Prior to beginning the study, the mass of the modified cell strainers were obtained and recorded. The gels were then placed in the cell strainers and the mass overall was taken and recorded after the gels were dried by blotting it and exposing them to an air vent. The

experiment began when the gels and cell strainers were submerged in the collagenase solution. The masses were recorded at time points 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90 minutes and 24 hours. Between time points, the gels were incubated at 37 °C. The masses were recorded and pictures were taken in order to obtain quantitative and qualitative data. To get the exact gel mass, the mass of each cell strainer was subtracted from their respective overall mass with the corresponding gel. These masses were then plotted and analyzed.

Cytocompatibility Studies

Cytocompatibility is an important aspect when the intended use is for tissue engineering. The gels underwent a live/dead assay to assess the viability of the hMSCs. This was in order to see if the method of polymerization could have potential cytotoxic effects. If this is the case, that particular method of polymerization would be deemed unsatisfactory for the application of tissue engineering.

Live/Dead Assay

During the cell encapsulation, the methodology to create the gels stayed the same except that everything is sterilized and the hMSCs are added. After polymerization, the gels were placed in their own well with 2 mL of media in a 24 well plate. Each time point was made in triplicate. The time points that viability was assessed were days 1, 5, and 8. At each time point, the gels that corresponded to that day were stained using Calcein AM and Ethidium homodimer. The stain formula consisted of these two stains and PBS. Prior to staining, the gels were washed 3 times with PBS, each time lasting 5 minutes. After the last wash, the stain was added to the gels and the 24 well plate was encased with aluminum foil. The plate was placed on a plate shaker for 45 minutes. After the 45 minutes, the gels were washed twice, each time lasting 5 minutes on the plate shaker. Once the gels were washed and all solution was removed, they were

analyzed under a fluorescent microscope using the Zeiss program. Pictures were taken with a 10x lens and exported onto ImageJ where they were analyzed both qualitatively and quantitatively.

CHAPTER III

RESULTS AND DISCUSSION

Gelatin Norbornene Functionalization

The gelatin norbornene was 91.77% functionalized according to the NMR analysis. The percent functionalization was used to determine how much crosslinker is needed. Within the gel formulation, it was key to maintain a 1:1 norbornene:thiol/tetrazine ratio. The gelatin norbornene was then suspended into PBS in order to reach a 5% w/v working ratio.

PEG-Di-Tz Functionalization

The PEG-di-Tz was 77% functionalized based upon the NMR spectra and suspended in PBS to reach a working functionalization that ensured the correct 1:1 norbornene:tetrazine stochiometric ratio.

Material Characterization

Material characterization of each gel was crucial to draw any conclusions that were pertinent to the hypothesis of these experiments. There is a direct correlation between material behavior and the way the cells interact with their environment. Being that the application of these chemistries are in tissue engineering, it is vital to have exceptional cell integration within the material. Material properties such as the modulus, swelling ratio, and susceptibility to enzymatic degradation aid this cell migration within the gel. By comparing the radical-mediated and the IEDDA method, it is expected to have variance in material properties due to the bonds created in each reaction. Their intrinsic properties were reviewed separately and then compared against each other in order to analyze the properties in respect to each other. In order to investigate these properties, these materials were characterized and are explained further.

Storage Modulus

The shear storage modulus was obtained by the use of an Anton-Paar Rheometer. The storage modulus of hydrogels are low given their composition of hydrophilic polymers and water. For reference, the storage modulus of hydrogels have been reported to range from 368 Pa to 5 kPa (Hoch 2012). From the frequency sweeps, the gel formulation of GelNB with PEG-di-SH exhibited a storage modulus of 1,677.57 ± 314.59 Pa. These gels had polymerized into a flexible gel that was soft and more resistant to tearing. This can be explained through the low percentage of GelNB used as well as the covalent crosslinks that were formed during the polymerization of these gels under the UV-light. The storage modulus of GelNB crosslinked with PEG-di-Tz was 5869.72 ± 190.15 Pa. These gels presented themselves as stiffer, brittle hydrogels which were more susceptible to damage. This qualitative analysis can be explained by the raised modulus. The modulus of the GelNB crosslinked with PEG-di-Tz was higher which was expected because during fabrication, not only covalent bonds are created but physical bonds as well. The bicyclic rings have the possibility to create attractive, non-covalent pi-pi stacking. This interaction, the covalent bonds, as well as the physical crosslinking is expected to create a gel with a higher storage modulus. In **Figure 6,** both the storage modulus of the thiol-ene and tetrazine gels are compared side-by-side.

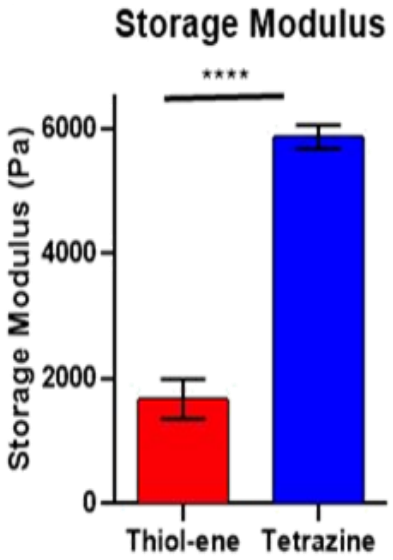

Figure 6. Plot of the average shear storage moduli of both gelatin compositions. This test was replicated 3 times with a P-value of <0.0001.

A t-test was used to statistically analyze the significance between the two compositions. The p-value obtained from the t-test was <0.0001 which leads to a significant difference between the two groups. This significant difference between the two can lead to two separate applications. The PEG-di-SH gel with a modulus close to 1.5 kPa can be used in tissue engineering applications that involve neural tissue because the tissue in that area in the body closely mimics the storage modulus in this particular gel. Likewise, the PEG-di-Tz mimics the modulus that can be found in soft cartilage (Engler 2006). However, both have the capabilities to be used in tissue engineering revolving around skin regeneration.

Swelling Ratio

The swelling ratio in this experiment was calculated for both polymerization methods. The goal of finding the swelling ratio is to possibly deduce an idea of the mesh size formed through the various chemical reactions. In **Figure 7**, it can be seen two varying mesh sizes.

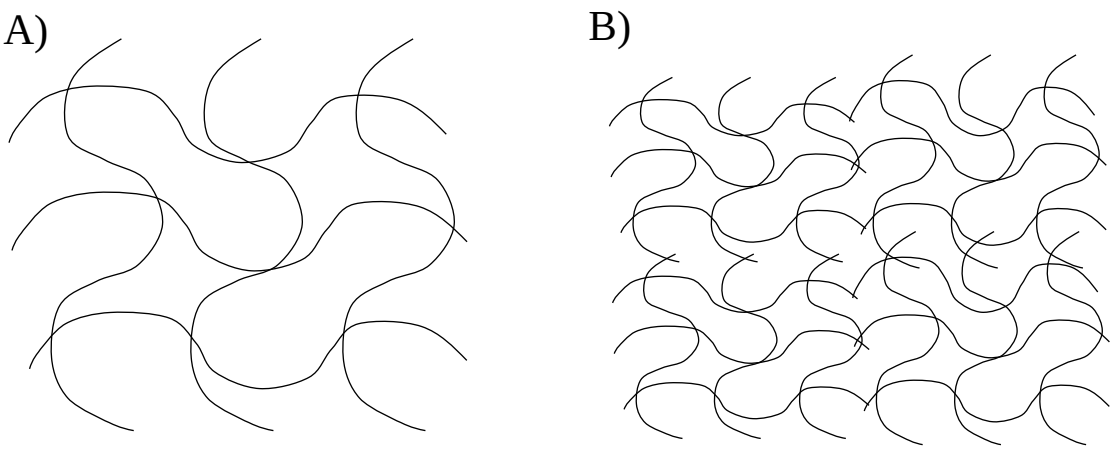

Figure 7. Mesh A on the left with fewer crosslinks compared to Mesh B on the right

Mesh A on the left has a larger mesh size which will correlate to a larger swelling ratio. This is due to the water being able to penetrate and be absorbed into the polymer network which is a characteristic of a hydrogel. Mesh B, also a hydrogel, has a higher crosslink density and results in a smaller mesh size. This will correspond to a lower swelling ratio. The swelling ratio is also pertinent to the mass transport throughout the gel. This is a critical aspect in order for nutrients to be delivered throughout the gel. The PEG-di-SH gel resulted in a swelling ratio of 24.62 ± 1.04 based off the measured mass of the dried and swollen state. The PEG-di-Tz produced of a swelling ratio of 14.74 ± 1.26. This was also based off of the mass measured before, in its dried state, and after the gels had the ability to swell to completion. A graphical comparison is shown in **Figure 8**.

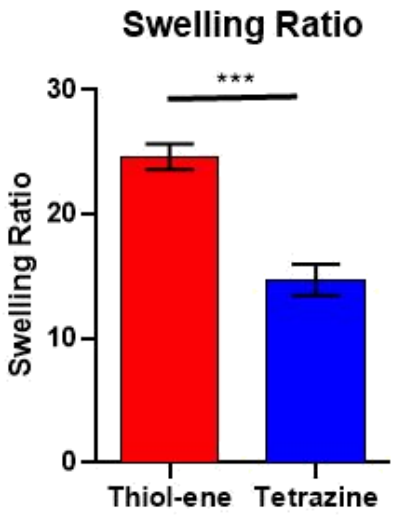

Figure 8. Graphical comparison between the swelling ratio of the thiol-ene and the tetrazine gels. This was completed 3 times with a resulting P-value of 0.0006.

To compare **Figure 7** and **Figure 8**, the thiol-ene gel would look more like mesh A while the tetrazine gel would mimic closer to mesh B. This can be explained due to the bonds found in the PEG-di-Tz gel. As explained previously, the PEG-di-Tz has essentially more bonds that create this gel that is stiffer. Swelling ratio and mesh size have a direct correlation where the lower the swelling ratio, the smaller the mesh size. This can also be applied towards the modulus of a gel. If the swelling ratio of a hydrogel is lower, it can be assumed that the modulus will be higher. Therefore, the swelling ratio has an indirect correlation to the modulus of the gel. Due to the small mesh size, this concludes that there are possible differences in the gel formation. This also suggests that cell growth and integration within a gel with a smaller mesh size will vary from the larger mesh size. This test confirms that a viability study needs to be performed to investigate the environment the cells will be placed in. A t-test was run in order to see if there was a significant difference between the two formulations. The t-test resulted in a P

value of 0.0006 which is less than 0.05, confirming that there was a significant difference between the swelling ratios of the two gel compositions.

Gel Fraction

The gel fraction was calculated in order to calculate the amount of polymer that was crosslinked. It is important to quantify this data in order to make sure that the stoichiometry in the formulations are correct and to also make sure that the polymer network is forming without issues that could skew the data. The gel fraction calculated for the PEG-di-SH gels was 77.55 ± 1.98 %. The gel fraction calculated for the PEG-di-Tz gels was 79.79 ± 2.43%. To show these values of the thiol-ene and tetrazine gels compared to each other, a bar graph in **Figure 9** can utilized for a visual representation.

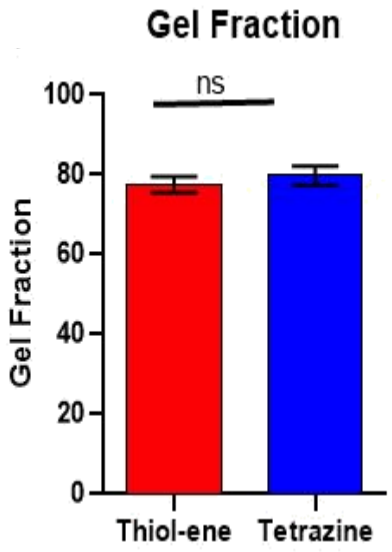

Figure 9. Plot exhibiting the insignificance between the two gels' gel fraction. This experiment was completed 3 times and resulted in a P-value of 0.29.

A t-test was run to statistically compare these to values in order to find a significance if there was one. When analyzed, the P value between these two test groups was 0.29 which is

greater than 0.05, leading to the conclusion that there was no significance between the two groups. This is an expected result because the click chemistry used in both reactions are known to be highly efficient as high efficiency is a characteristic of click chemistry (Hein 2009). Noting their similar gel fractions is significant because it confirms that the differences between the two gels are based upon their formulations. Furthermore, it would explain that the differences are due to the additional secondary interactions associated in the IEDDA tetrazine-norbornene click reaction. This does not only affect the gel fraction but also characteristics such as the modulus and the swelling ratio as stated earlier. The gel fractions reported are lower compared to most crosslinked polymers, however not for gelatin hydrogels. It has been reported before that the range can be from 84-95% depending on the components and method of polymerization (Bukhari 2015). Therefore, the lower gel fraction of the hydrogels is acceptable taking into consideration that these gels are only 5% w/v of gelatin along with their respective components. The take away from this is that while the gel fractions are low, they are in an acceptable range. More importantly, there is no statistical significance between the two groups leading to no differences in the crosslinking abilities overall.

Degradation Study

A crucial material characteristic in tissue engineering is the ability to degrade. This is pertinent because based upon the application of the material, one may want degradation to occur while others may not. Based off of the different chemistries being applied, it was questionable on how each gels were to degrade. By using collagenase, the gels underwent an accelerated degradation to study the rate of degradation. Cells naturally secrete collagenases. Therefore these conditions in place during the degradation study was to mirror the conditions that the gels would undergo if they were exposed to cells, just at a higher concentration. The thiol-ene gels

degraded completely at the end of the study compared to the tetrazine gels that degraded 64% as seen in **Figure 10**.

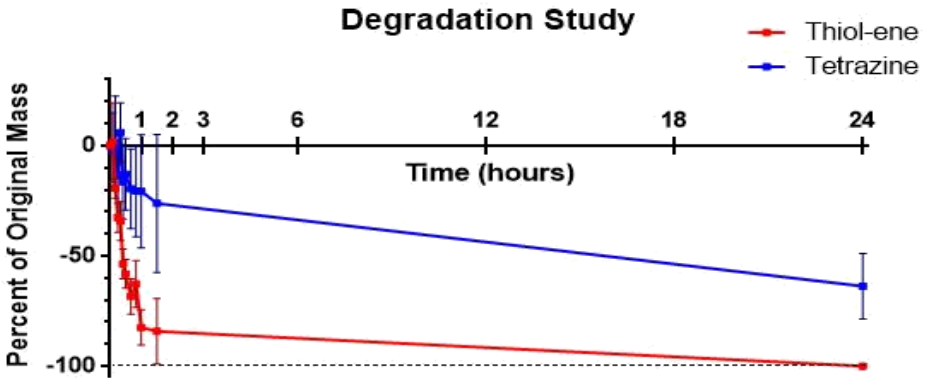

Figure 10. Percent of original mass remaining after various time points

After 24 hours, the thiol-ene gel had degraded completely while the tetrazine gel had 36% of it's original mass left. One of the contributing factors to the degradation study was the gelatin component. This component is susceptible to bond cleavage from the enzyme used because the enzyme mimics matrix metalloproteinases. These enzymes have the ability to cleave the extracellular matrix proteins (Chung 2004). The extracellular matrix is made of up collagen and gelatin is the denatured form of collagen. This leads to why the gelatin is able to degrade during this study.

This then leads to the question as to why both gels did not degrade at the same rate. This has to do with the bonds that are present in the tetrazine gels. These physical bonds that are present cannot be chemically cleaved. It should also be noted that due to the decreased mesh size, there could have been a decreased diffusion of collagenous throughout the gel. The reasoning that tetrazine gels degraded from the beginning is that the gelatin in the gels are hydrolyzed and degraded down. This will then break down the polymer network and the gel will

start to collapse. This can be expected to be seen in a cell culture study since the cells will release these matrix metalloproteinases.

Cytocompatibility Studies

To test the way that hMSCs react in these different environments, a cytocompatibility study was executed. For tissue engineering, it is important to note how cells integrate themselves within the gel. In order to analyze the cytocompatibility, a live/dead stain kit was used in order to visually see the live cells versus the dead cells.

The live/dead assay performed on the gels were analyzed at 24 hours, 5 days, and 8 days. This was in order to visually interpret the viability of the cells within the gels over time. The images in **Figure 11** were imaged using epifluorescence microscopy.

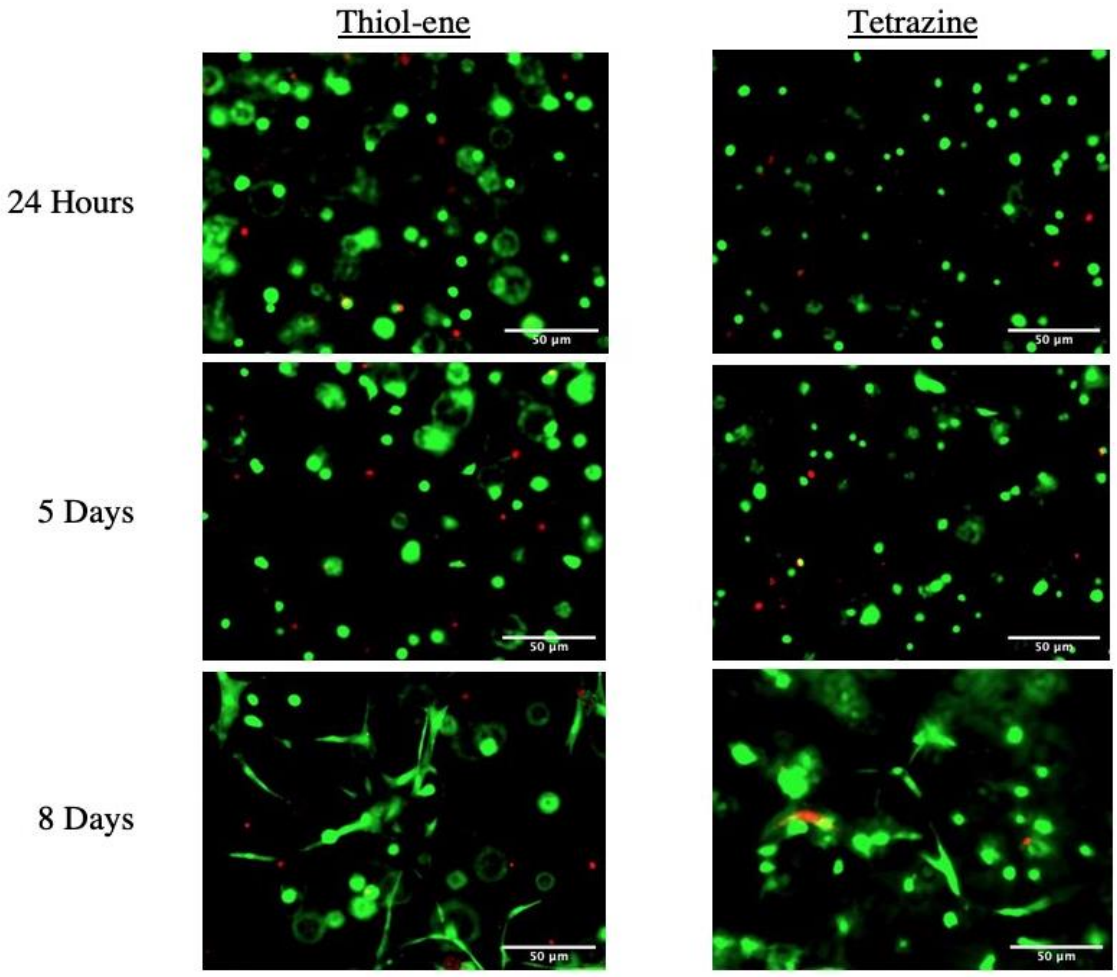

Figure 11. Day 1-8 live/dead assay on hMSCs with a 50 µm scale bar. The cells shown in green are live cells while the cells exhibiting the red color are dead cells.

The cells were stained using calcein AM and ethidium homodimer. Calcein AM is a membrane-permeant stain that is cleaved by esterase in live cells. These live cells will yield a cytoplasmic green fluorescence. When the cells are dead, the plasma membrane is damaged. This allows the membrane-impermeant ethidium homodimer to stain the nucleus of the dead cells. This stain will dye the cells a red fluorescence because it enters the cells. It should be noted that is why the dead cells appear smaller compared to the live cells. After being processed

by ImageJ, it was concluded that both gels exhibited ~90% viability at day 8 with no significance between the two types of gel. High viability suggests that both click chemistry platforms are promising for tissue engineering applications. Therefore, based on the qualitative analysis of day 8, there is no significance in cell viability between the thiol-ene or tetrazine gels. However, there is a difference within the cell spreading. As seen on day 8, the hMSCs encapsulated in the thiol-ene gels are exhibiting a higher spreading compared to the cells in the tetrazine gels. This could be a possibility that the thiol-ene gels are undergoing a more rapid cell-mediated degradation, a conclusion that would be supported by the results from the degradation study.

CHAPTER IV

CONCLUSION

The marked differences in modulus, swelling ratio, and susceptibility to collagenase degradation observed between thiol-ene and tetrazine click crosslinked gelatin hydrogels suggest that the tetrazine click reaction results in the formation of additional physical crosslinks. This leads to the conclusion that IEDDA click chemistry hydrogels are a viable hydrogel option with different material properties. This difference can be useful throughout tissue engineering where a hydrogel with additional bonds are needed. This will include applications that call for hydrogels with similar characteristics as observed in the tetrazine gels.

Although the material characteristics varied, the cell viability was comparable between the two gel types. This proves that the IEDDA click chemistry in the tetrazine gels is similar to the radical-mediated click chemistry in the thiol-ene gels in terms of cell viability. However, this data does not include information on the proliferation or differentiation of the hMSCs.

Future studies include further investigation into the mechanisms leading to the differences in gel properties. For example, the degradation study should be extended out until the tetrazine gel is also completely degraded. By isolating the mechanisms, the gels and their capabilities will be understood further. Also, studies done on the hMSC's quantitative viability, proliferation, and differentiation should be performed. This would give comprehension to the true effects of the varying click chemistries on the stem cells as well as the environment that they are encapsulated in.

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