HYDROGELS STABILIZED VIA NOVEL CLICK CHEMISTRY-ENABLED SECONDARY INTERACTIONS FOR CELL CULTURE

An Undergraduate Research Scholars Thesis

by

ISABELLE AGURCIA[1], JULIO ARROYO[1], AUSTEN FRICKS[1], and EMILY POUX[2]

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ABSTRACT

Hydrogels Stabilized via Novel Click Chemistry-enabled Secondary Interactions for Cell Culture

Isabelle Agurcia[1], Julio Arroyo[1], Austen Fricks[1], and Emily Poux[2]
Departments of Biomedical Engineering[1] and Chemical Engineering[2]
Texas A&M University

Research Advisor: Dr. Daniel Alge
Department of Biomedical Engineering
Texas A&M University

Hydrogels, water-imbibing crosslinked polymer networks, are useful for 3D cell culture because of their tissue-like mechanical properties and high water content. A variety of "click chemistry" reactions have been utilized to crosslink (covalently bond) the polymer network and incorporate different bio-instructive peptides. Until now, there has been an assumption that the click reaction used does not significantly alter the overall hydrogel properties. However, our lab has found that the tetrazine-norbornene click reaction results in additional non-covalent supramolecular interactions, which increase gel stiffness and decrease enzymatic degradability. Our project aims to leverage these novel non-covalent intermolecular interactions resulting from the Inverse Electron Demand Diels-Alder tetrazine-norbornene click cycloaddition products to develop a user-controlled, dynamically stiffening hydrogel platform that could more accurately recapitulate mechanical characteristics of fibrotic diseases or cancerous conditions in vitro. Although there has been previous research done using thiol-ene reactions to form hydrogel networks and the use of tetrazine-functionalized poly(ethylene glycol) molecules within hydrogels to incorporate bio-instructive materials into the gels, there is limited literature utilizing pendant groups on polymers to control secondary interactions. Therefore, this project aims to explore how different concentrations of pendant tetrazine-norbornene cycloaddition products (TNCPs) influence the mechanical properties of PEG hydrogels.

ACKNOWLEDGMENTS

We would like to thank our research advisor Dr. Daniel Alge and graduate student Samantha Holt for their guidance and support throughout the course of this research. It would not have been possible without their dedication and assistance.

Additional gratitude to the Bio-instructive Materials Lab, in particular Amanda Rakoski and Marissa Heintschel, for their support to our team and always being available to assist whenever needed. Lastly, thank you to LAUNCH for providing our team with the incredible opportunity to grow and develop new skills as we prepare to embark on our careers, and to Tawfik Hussein for recommending we apply to the program.

NOMENCLATURE

PEG	Poly(ethylene glycol)
mPEG-SH	methoxyPEG-thiol
PEG-di-SH	PEG-di-thiol
mPEG-Tz	methoxyPEG-Tetrazine
KCGPQ-W	KCGPQGIWGQCK
KCGPQ-A	KCGPQIAGQCK
LAP	Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
TNCP	Tetrazine norbornene cycloaddition product

CHAPTER I

INTRODUCTION

Hydrogels

Hydrogels are water-imbibing crosslinked polymer networks with various applications ranging from cell culture to contact lenses. They are often classified according to the nature of the polymer from which they are constituted. These polymers are grouped into two classes, natural and synthetic. Natural polymers are high molecular weight compounds found in nature, such as collagen and starch [1]. Collagen in particular can be used to produce gelatin, a natural polymer often used in cell culture. Natural polymers are biocompatible and biodegradable, which makes them useful for biomedical applications. Natural polymers, however, tend to possess poor mechanical properties [1], which scientists have attempted to remedy using different methods. For example, double hydrogel networks and interpenetrating networks have been developed; this causes an increase in the crosslinking and therefore an increase in the strength and rigidity. [1] An additional method is through the introduction of nanomaterials such as nano fibers and nanorods. For example, gold nanorods (GNR) in combination with gelatin methacrylate(GelMA) gel. Due to the electrostatic interaction between the positive GNR and negatively charged amino group GelMA, there is an increase in stiffness [1]. In contrast to natural polymers, synthetic polymers can have far better mechanical properties, are easily reproducible, and can be tailormade, but often suffer from poor biodegradability and potential toxicity, thus limiting their use [1].

The hydrogel platform can also be classified according to the means by which the polymer chains are crosslinked together into a network. In physically crosslinked gels, such as calcium alginate and some commonly used gelatin gels, the network is bound together with non-covalent physical interactions such as ionic, hydrogen bonding, or hydrophobic interactions [2]. These secondary intermolecular interactions occur across the polymer backbone, and are not localized to specific points. Researchers who use calcium alginate gel platforms use alginate-poly(l-lysine)

to stabilize the hydrogel and reinforce the secondary interactions [2]. Without the extra stability, these hydrogels can be disrupted by changes in the physical environment such as pH, temperature, and physical stresses [2]. Chemically crosslinked gels, also called 'permanent' due to the irreversible nature of the covalent bonds that connect the network, are gels produced by chemically linking many polymer chains together. Due to their stability, chemical crosslinks are more commonly used. One attempt at combining the effects of both types of crosslinks was the development of chemical-physical double network zwitterionic hydrogels to improve the poor mechanical properties of purely chemical or physical versions [3]. This "double network" was a chemically crosslinked hydrogel network interpenetrated with physically crosslinking polymer chains, and exhibited improved mechanical properties [3]. Our hydrogel, in contrast, chemically links pendant molecules (not crosslinked into the polymer network) to the network that exhibit secondary intermolecular interactions [4] in an attempt to produce a physical crosslinking effect via interactions between these molecules.

Chemical crosslinking polymerization reactions for hydrogels can be further categorized into chain-growth or step-growth polymerizations. Chain-growth polymerization is a reaction in three distinct phases: initiation, propagation, and termination. During propagation, a chain grows through the addition of individual monomers until it encounters a species that terminates the chain-growth. These types of reactions are useful primarily for their simplicity [5]. However, chain-growth polymerization produces networks of lesser strength and strain tolerance compared to step-growth polymerization reactions, likely due to a more even distribution of crosslink junctions in step-growth polymers [6]. Step-growth polymerization occurs when polymer chains can also combine as they grow, because the reactions that grow them can occur between chains, instead of only through the addition of monomer units like in chain-growth polymerization. For our purposes, step-growth polymerization reactions were chosen for their increased homogeneity, strength, and efficiency over chain-growth reactions [6][7]. The step-growth polymerization reactions chosen for this application are "click chemistry" reactions. Click chemistry reactions are reactions that are modular, wide in scope, high yield, generate inoffensive byproducts, and occur under "simple re-

action conditions" [8]. Simple reaction conditions require readily available materials and reagents, a benign solvent or none at all, simple product isolation, and product stability at physiologic conditions [8]. Click chemistry was chosen primarily for its ease of use and biocompatibility. This work utilizes two click chemistry reactions for step-growth polymerization, thiol-norbornene and tetrazine-norbornene reactions, which have been used in other hydrogels for cell culture [9][10].

3D Cell Culture

In order to make a gel suitable for 3D cell culture, several requirements must be met. One of the most crucial is that the cross-linking mechanism used to form the gel must be biocompatible; that is, it must not produce any cytotoxic byproducts that could compromise the viability of cells seeded within the gel. In addition, the gels must be enzymatically degradable to more accurately mimic the extracellular matrix (ECM) and to allow the cells to grow and spread throughout the gel [11]. PEG hydrogels with poly(lactic acid) (PLA) tri-block copolymers have been utilized previously to encapsulate chondrocytes utilizing click chemistry to crosslink the polymer network. With the lack of cytotoxic byproducts from the click chemistry reaction and the PLA's ability for degradability, cartilage from encapsulated chondrocytes was observed to be viable within these early gels [12]. Lastly, gels must be reasonably easy to make using reagents that are easy to attain and/or cost-efficient. Past work has seen the utilization of strain promoted alkyne-azide cycloadditions(SPAAC) as a type of click reaction used to covalently bond hydrogels together but has seen it as an expensive alternative to other click reactions such as inverse electron demand Diels Alder(IEDDA) [10]. The approach we are proposing, after covalently crosslinking our hydrogels with thiol-ene click chemistry, utilizes the IEDDA reaction through our tetrazine-norbornene click cycloaddition products (TNCPs) which enables cell viability through producing no cytotoxic byproducts and potentially allows for enzymatic degradation, which will be explored further in this work.

Dynamic Stiffening

Dynamic stiffening, or stiffening over time, is a valuable characteristic for cell culture platforms because it better mimics the mechanical environment during certain biological processes

(e.g. the formation of some cancers [13], fibrotic disease [14]). Several different methods of creating stiffening hydrogels have already been established. For example, "Double network" hydrogels consisting of multiple interpenetrating networks (IPNs) have been made successfully [1], such as the sodium alginate and poly(N,N-dimethylacrylamide) gels created by Bai et. al. that demonstrated superior strength relative to semi-IPN gels made of chitosan and poly(N,N-dimethylacrylamide) [15]. Other gels made of alginate have also been utilized [16]. Stowers et. al. used alginate gels stiffened with calcium that provided spatial and temporal control, were cyto-compatible, and had reversible crosslinks [16]; however, the gels we propose in this paper stiffen over a shorter period of time. Young and Engler developed a thiolated-hyaluronic acid gel that stiffened via a slow crosslinking reaction. In this reaction, the stiffness of the gels was able to be adjusted by changing the molecular weight of the poly(ethylene glycol) diacrylate crosslinker [17]. Although hyaluronic acid gels can be advantageous due to their ease of modification [17], the Michael addition that was proposed as the cause of stiffening in the hyaluronic acid gels does not provide for as much spatial and temporal control [9].

Although dynamically stiffening gels have been used for cell culture, many platforms have limited room for cell spreading and penetration through the gel matrix due to the multiple gel networks being closely interwoven in the same space, as they rely on an increase in polymer density to produce their stiffening effects. These same platforms also need a specific activation mechanism that is able to control the stiffness of the hydrogel matrix. Gels comprised of alginate that utilized irradiated gold nano rods to release calcium saw stiffening over time, but this method saw that increasing stiffness decreased the porosity of the gel, inhibiting the cells from spreading through the gel and left the cells with a round morphology [16]. Other dynamically stiffening platforms such as nanocomposite gels require activation from outside magnets, heat source or sound waves [1] to activate stiffening properties, in contrast to our proposed method.

A recent discovery by the Bioinstructive Materials Lab has demonstrated potential for an improved dynamically stiffening 3D cell culture platform through leveraging secondary interactions in pendant (attached at one end) tetrazine molecules. When used in covalent crosslinking,

tetrazine binds with norbornene under physiologic conditions to produce tetrazine-norbornene cycloaddition products, which form the polymer network of the gels [10]. These products have been shown to experience strong intermolecular secondary interactions in these covalently crosslinked gels [4]. These interactions were so strong that these gels did not exhibit degradation, either hydrolytically or enzymatically, in accelerated degradation conditions over a period of 24 hours, in contrast to controls crosslinked with radical-mediated thiol-ene click chemistry, which degraded completely after 15 minutes. While this stability is impressive, it creates problems for cell culture applications because it inhibits the ability of cells to grow and proliferate through the gel [11].

Our work aims to develop an improved hydrogel platform for 3D cell culture that leverages these secondary interactions in a novel "pendant" (not crosslinked into the polymer network) configuration, to still allow for degradation as needed. We accomplished this by chemically linking monofunctional tetrazines into a polymer network composed of poly(ethylene glycol) molecules end functionalized with norbornene and crosslinked with PEG-di-SH or an enzymatically degradable crosslinker, producing pendant tetrazine-norbornene cycloaddition products. This configuration is expected to allow the pendant tetrazine molecules to contribute the stiffening effects of their secondary, physical interactions, while producing a gel which exhibits degradability and supports cell viability.

CHAPTER II

MATERIALS AND METHODS

Two reactions are central to this work. The first is the radical mediated thiol-ene click reaction, shown in Figure 1. This reaction is used to covalently crosslink the gel network with di-thiol molecules, and to covalently attach mPEG-SH molecules to free norbornene sites at the ends of the arms of the PEG-NB molecules. This step is done through photopolymerization, prior to the gels being swelled in mPEG-Tz, and is done with mPEG-SH to control the amount of free norbornene molecules that are available for mPEG-Tz to bond to.

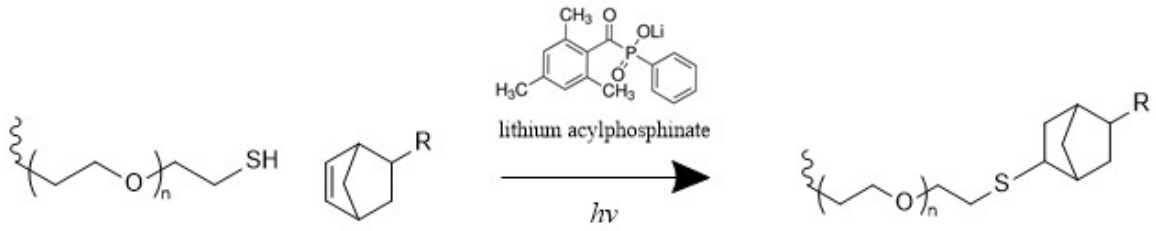

Figure 1: From left to right: mPEG-SH, norbornene, LAP, thiol-ene product. This is a radical-mediated reaction; radicals are produced when LAP is exposed to UV light, initiating the thiol-ene click reaction.

The second reaction, illustrated in Figure 2, is the Inverse Electron Demand Diels Alder Click Reaction (IEDDA). This cycloaddition reaction proceeds spontaneously at room temperature and was produced in our lab through swelling gel samples overnight in solutions containing concentrations of mPEG-Tz ranging from 1 mM to 5.32 mM. An overview of the reagents used and a representative schematic of the gel network can be found in Figure 3 down below.

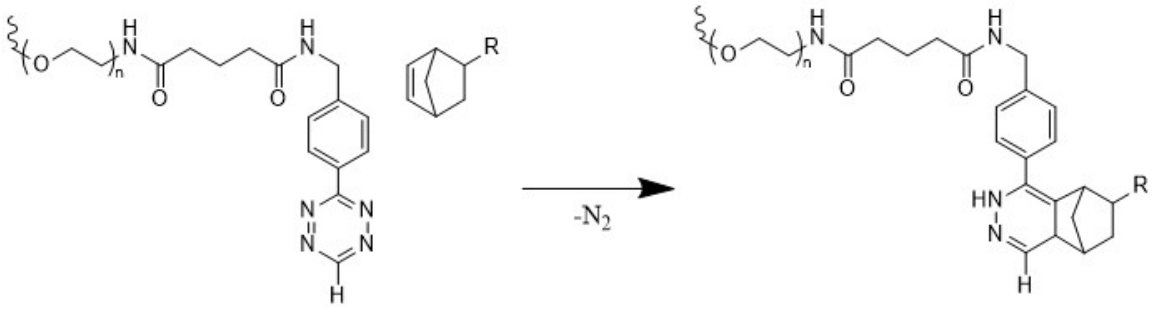

Figure 2: From left to right: tetrazine, norbornene, TNCP. This reaction produces N_2 gas as a byproduct.

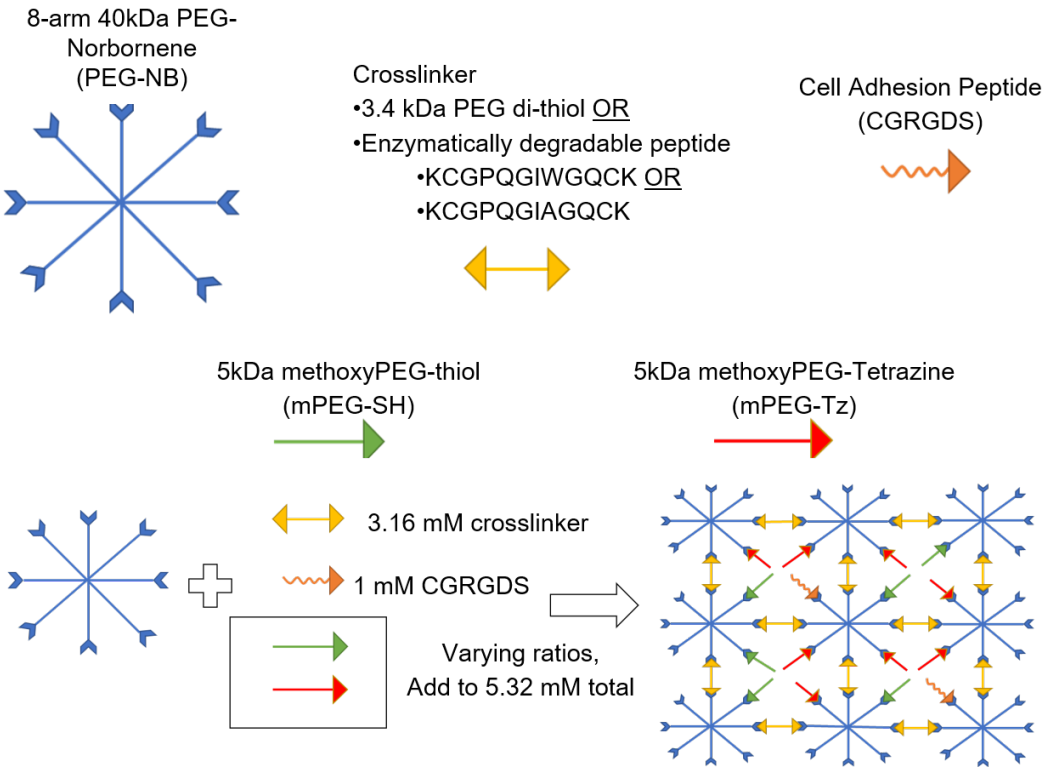

Figure 3: This figure provides an overview of the reagents used and includes a representation of the composition of the gel network.

Gel Preparation

Gels were created from a prepolymer solution containing 1.8 mM 40 kDa 8-arm, 7.5 wt% PEG-NB (synthesized via a modification of the 4-arm PEG-norbornene synthesis protocol in [18]), 2 mM LAP (synthesized according to the protocol outlined in [19]), 1 mM CGRGDS (synthesized using solid-phase Fmoc peptide synthesis), PBS, and 3.16 mM PEG-dithiol (Laysan Bio) that was UV photopolymerized to form the crosslinked hydrogel network at a 1:2 thiol-ene crosslinker ratio. All reagents were stored at -20° C until thawed at room temperature for use. Each reagent was vortexed for 10 seconds to ensure the reagent was homogenous before use. In order to control the available free norbornene sites, prepolymer solutions also incorporated mPEG-SH (Laysan Bio) of concentrations ranging from 0 mM to 5.32 mM. The prepolymer solution was vortexed for 10 seconds, and the contents pipetted into three separated 8mm x 1mm silicone molds affixed via digital pressure to slides treated with Sigmacote®. An Omnicure® was then turned on and warmed up, and a radiometer was used to verify that the light intensity was 20 mW/cm^2. The glass slide was then placed into the Omnicure® to be UV polymerized under 365 nm light for 300 seconds. After polymerization, each of the gels was gently removed from the mold, placed in a 24-well plate, and swelled overnight in excess PBS or mPEG-Tz (synthesized using the protocol outlined in [10]) of concentrations ranging from 0 mM to 5.32 mM on an orbital shaker. The gels that were swelled in mPEG-Tz were then transferred to new wells and rinsed in excess PBS overnight on the orbital shaker plate at 300 rpm to wash out any unreacted mPEG-Tz.

Rheological Characterization

After the gels were rinsed in PBS, each sample was cut using a round 8 mm biopsy punch. An Anton-Parr Physica MCR 301 with 8 mm parallel plate geometry was used to perform time sweeps on each cut gel sample at 22° C, 1 rad/s, and 1% strain to obtain storage (G') moduli for each individual gel every 5 seconds for 60 seconds. Reported moduli are an average over these 12 time points.

Modulus Evolution Over Time

Gel samples were prepared according to the Gel Preparation section above using a PEG-di-SH crosslinked 5 mM mPEG-Tz (no mPEG-SH) formulation, omitting the mPEG-Tz swell-in step. The gels were swelled overnight in excess PBS, and the storage modulus was recorded on an Anton Paar Physica MCR 301 rheometer using time sweeps that lasted 60 s with a frequency of 1 rad/s and 1% strain at 37° C . The gels were then swelled in a 5 mM mPEG-Tz solution for one hour, rinsed for 5 minutes in PBS on an orbital shaker, and the storage modulus was again tested and recorded on the rheometer. The swelling in mPEG-Tz, rinsing in PBS, and testing on the rheometer was repeated until 6 total hours of mPEG-Tz swell-in was achieved.

Swelling Ratios

Excess PBS was removed from the swollen gel samples, and the mass of each sample was measured and recorded. The samples were then placed in a vacuum desiccator for approximately 24 hours. The dried samples were weighed, and swelling ratios were calculated according to Eq. 1 below.

$$Swelling\ Ratio = \frac{mass\ of\ swollen\ gel - mass\ of\ dried\ gel}{mass\ of\ dried\ gel} \qquad \text{(Eq. 1)}$$

Degradable Gels - Rheology and Collagenase Digestion

Gel samples for collagenase digestion were produced using the methods described above in the Gel Preparation section. Negative controls were crosslinked with PEG di-SH as normal, but degradable samples had this replaced with KCGPQ-W or KCGPQ-A, shown in Figure 4, one set each. Rheology was then performed on the degradable gels as described in the Rheology section above.

KCGPQ-W

KCGPQ-A

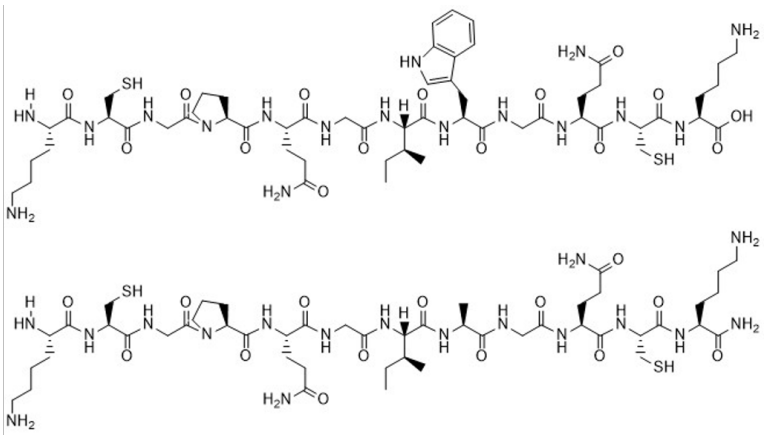

Figure 4: The two enzymatically degradable crosslinkers used in this study. Notice the benzene ring structure present in the KCGPQ-W that is absent in the KCGPQ-A.

For digestion a solution of 0.2 mg/mL of collagenase B in PBS was prepared. The previously cut gels were then weighed and transferred to 1.5 mL microtubes. The microtubes were then filled with 1 mL each of the collagenase B solution and placed in a bead bath at 37° C. Gels were then temporarily removed from the microtubes, lightly dried on a gloved hand, and weighed at intervals of 5 min for the first 30 min, then 30 minute intervals until 90 total had elapsed, and 60 minute intervals thereafter until the gels had completely degraded.

Cell Encapsulations

To investigate the cell viability in 7.5 wt% mPEG-Tz experimental gels, 3T3 fibroblasts were suspended and polymerized within two separate pre-polymer solutions. The pre-polymer solutions both contained 8-arm, 40 kDa PEG-NB with a 7.5% weight per volume(w/v), 100 mM KCGPQ-W, 2 mM LAP, and 1 mM CGRGDS. One pre-polymer solution was then given mPEG-SH at a 5.32 mM concentration while the other was given regular cell media consisting of Dulbecco's Modification of Eagle's Medium (DMEM 1X) infused with 10% fetal bovine serum (FBS) and 1X Pen-Strep (P/S) to correct for the missing volume. The 3T3 fibroblasts were cultured on tissue culture polystyrene flasks in cell media and were passaged every two to three days until collection for cell encapsulation experiments. The collected 3T3 fibroblasts were allocated to both pre-polymer solutions and efficiently mixed within the solution. The pre-polymer solution con-

taining the 3T3 fibroblasts was then pipetted onto sterile cut syringe molds with 20 μL of solution placed onto each syringe. The syringe molds were made by sawing the tips off of normal 1 mL syringes to create a larger round hole for the rubber stopper to push through. The pre-polymer solution was then photopolymerized utilizing 5 mW/cm^2 (365 nm) of UV light for 3 minutes and transported to sterile 24-well plates. The gels polymerized with the mPEG-SH were submerged in 450 μL of regular cell media while the gels given regular media were then treated with media that had been supplemented with mPEG-Tz to reach a final concentration of 5.32 mM. The mPEG-Tz media was sterile filtered to aliquot 450 μL of the solution to swell the experimental gels. After 4 hours post-encapsulation, 1 mL of regular media was provided to each sample to focus on the viability of the 3T3 media beyond the addition of the pendant groups.

Evaluation of Cell Viability

The viability of the encapsulated 3T3 fibroblasts was evaluated at the two separate time points of 1 day and 3 days using fluorescence microscopy. The sets of samples were monitored utilizing Live/Dead staining with Calcein AM (green) for live cells and Ethidium Homodimer (red) for dead cells. A direct light source was placed beneath the well-plates when being handled to provide guidance when washing the media from the gels before the stain was introduced. The stained gels were then imaged at 5x magnification and analyzed with ImageJ.

CHAPTER III

RESULTS AND DISCUSSION

Rheology

Increased concentration of mPEG-Tz in the swelling solution generally corresponded with a higher storage modulus (G'), as shown in Figure 5, which is indicative of a stiffer gel. The storage moduli for all-mPEG-SH, 1 mM mPEG-Tz, 3 mM mPEG-Tz, and 5 mM mPEG-Tz gels were 703.23 $\pm$ 66.04 Pa, 501.59 $\pm$ 182.04 Pa, 1236.18 $\pm$ 181.87 Pa, and 1571.03 $\pm$ 160.56 Pa, respectively. The differences in storage moduli between the all-mPEG-SH and 5 mM mPEG-Tz ($0.001 < p < 0.01$), all-mPEG-SH and 3 mM mPEG-Tz ($0.01 < p < 0.01$), and 1 mM mPEG-Tz and 3 mM mPEG-Tz gels ($0.001 < p < 0.05$) were all significant.

This is in line with expectations because the secondary interactions in the TNCPs were anticipated to stiffen the gels. Although the gels swelled in 1 mM mPEG-Tz had a statistically equivalent G' to the all-SH gels, this is perhaps due to an as-yet-uninvestigated lower threshold for the concentration of pendant TNCPs that needs to be crossed in order for the secondary interactions to be effective in stiffening the gels.

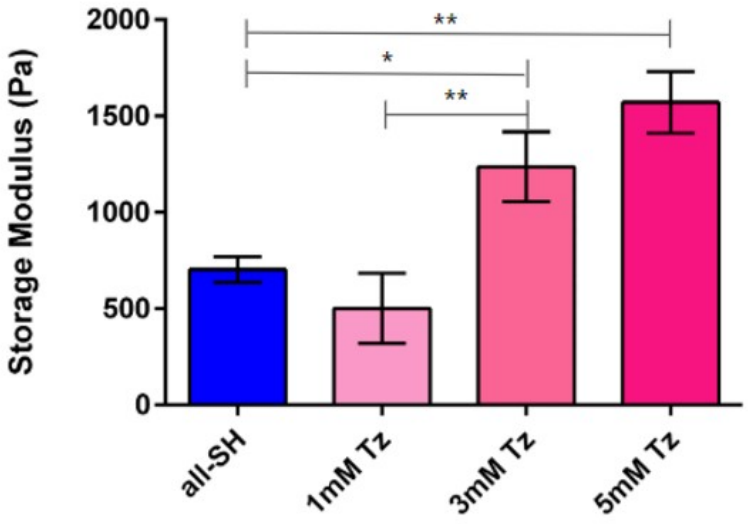

Figure 5: Gels swelled in higher concentrations of mPEG-Tz exhibited a higher storage modulus.

Modulus Evolution Over Time

The storage modulus of the gels increased with the time spent swelling in the 5 mM mPEG-Tz solution, as shown in Figure 6. Over a period of 6 hours, the storage modulus of the gel samples more than doubled from 502 $\pm$ 109 Pa to 1151 $\pm$ 89 Pa, a 229% increase. This supports our hypothesis that the gels are dynamically stiffening.

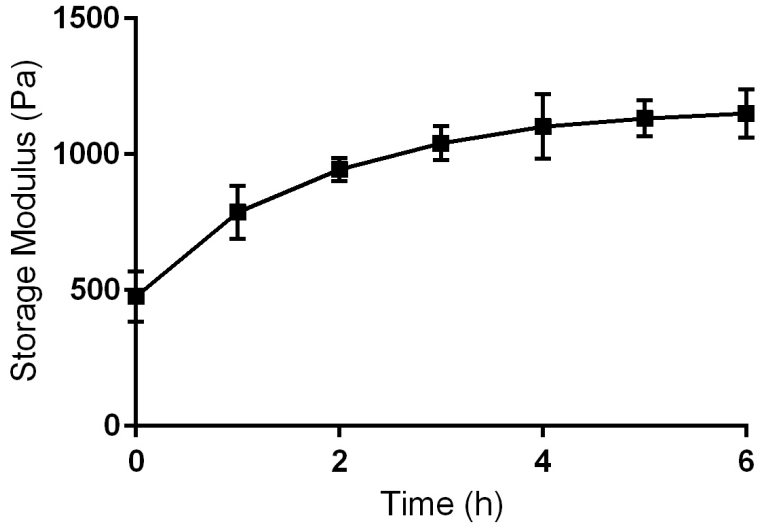

Figure 6: When swelled in an mPEG-Tz solution and tested periodically, the gel samples exhibited an increase in storage modulus over a period of several hours.

Swelling Ratios

Gels swelled in 3 mM and 5 mM solutions of mPEG-Tz had smaller swelling ratios compared to gels swelled in 1 mM mPEG-Tz and gels containing all-mPEG-SH, as shown in Figure 7. The swelling ratios of the all-mPEG-SH, 1 mM mPEG-Tz, 3 mM mPEG-Tz, and 5 mM mPEG-Tz gels were 36.3 ± 1.57, 37.84 ± 0.39, 27.29 ± 0.13, and 25.77 ± 0.42, respectively. The differences in swelling ratios between the all-mPEG-SH and 3 mM mPEG-Tz ($0.01 < p < 0.05$), all-mPEG-SH and 5 mM mPEG-Tz ($0.001 < p < 0.01$), 1 mM mPEG-Tz and 3 mM mPEG-Tz ($0.0001 < p < 0.001$), and 3 mM mPEG-Tz and 5 mM mPEG-Tz ($0.01 < p < 0.05$) were all significant.

This was in line with expectations because as the secondary interactions pull the gel network together, there will be less space for water; therefore, the grams of water that each gram of dehydrated gel network is able to absorb will be lower.

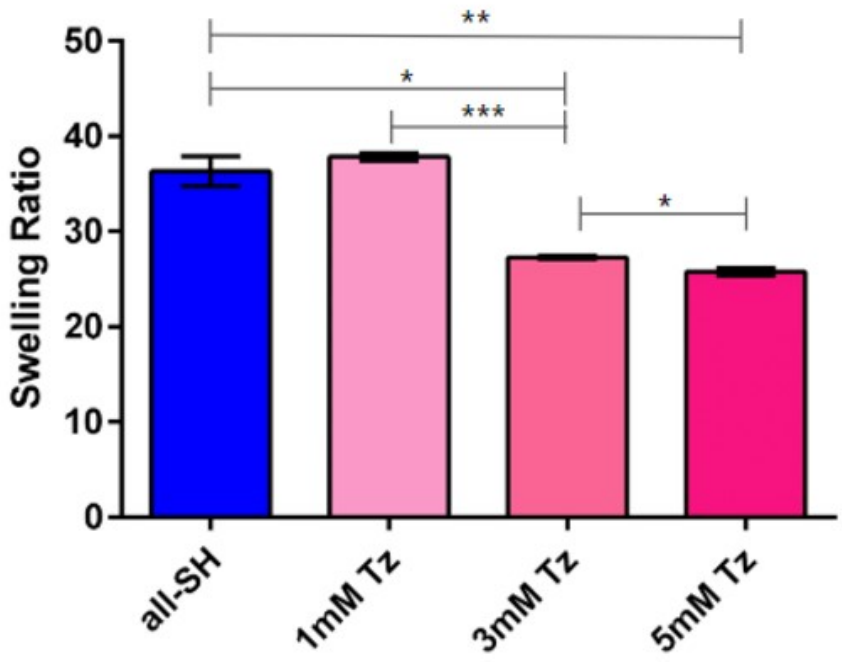

Figure 7: Gels swelled in a higher concentration of mPEG-Tz exhibited lower swelling ratios (grams of water per gram absorbed per gram of dry gel network).

Degradable Gels - Rheology and Collagenase Digestion

As in the PEG-di-SH crosslinked gels, higher pendant TNCP concentrations correlated with an increased storage modulus (G') in the enzymatically degradable gels, as shown in Figure 8. The KCGPQ-A gels had a storage modulus of 308.68 $\pm$ 185.35 Pa and 582.78 $\pm$ 341.43 Pa, for the 0 and 5.32 mM mPEG-Tz samples, respectively. The KCGPQ-W gels had storage moduli of 1869.69 $\pm$ 459.96 Pa and 2839.06 $\pm$ 65.89 Pa, for the 0 and 5.32 mM mPEG-Tz samples respectively. The KCGPQ-W gels were also significantly stiffer than their KCGPQ-A counterparts, by an order of magnitude.

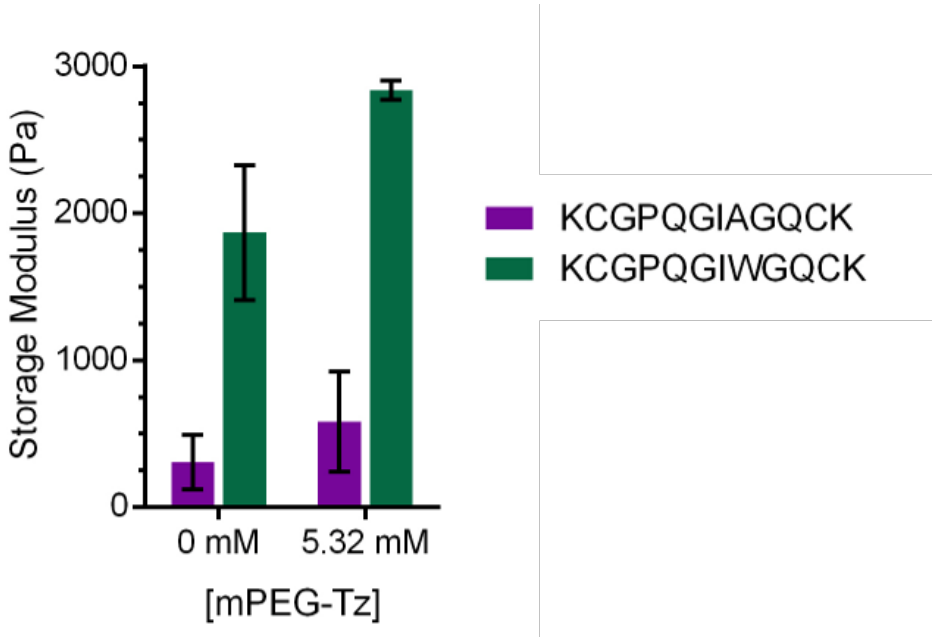

Figure 8: Rheology performed on the degradable gel samples to confirm similar trends to non-degradable samples, as well as to measure the differences between KCGPQ-W and KCGPQ-A crosslinked gels.

In order to evaluate the enzymatic degradability of the gels an accelerated collagenase digestion was performed on four gel formulations in triplicate. These gel formulations and their degradation profiles are shown in Figure 9. The formulations crosslinked with KCGPQ-W took 3 times longer to degrade than their KCGPQ-A counterparts. This could be due to unforeseen variations in crosslink density, but it is thought that this is due to the benzene ring in the KCGPQ-W contributing to pi-pi stacking secondary inter-molecular interactions, and increasing gel stability. The 5.32 mM mPEG-Tz containing gels took 9 times longer to degrade than their 0 mM mPEG-Tz counterparts and exhibited apparent increases in mass, due to increased swelling of the gels as the polymer network degraded. This subset of gels also appears to have undergone bulk, instead of surface, degradation because the gels became mechanically unstable before decreasing significantly in size. This is what led to the large standard deviations seen in Figure 9, since as the gels degraded they became more difficult to handle without damaging them.

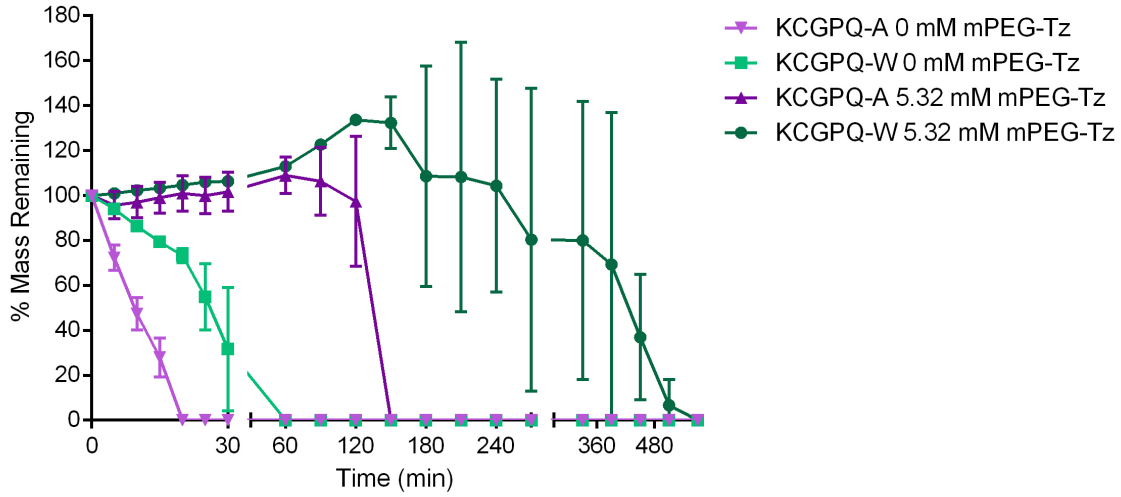

Figure 9: The change in measured mass plotted against time throughout the collagenase digestion.

Cell Viability

When assessing the cell viability of the 3T3 fibroblasts encapsulated in mPEG-Tz and mPEG-SH, the 1 day samples were expected to indicate cytocompatibility of the cells with the gel components, particularly with the mPEG-Tz while the 3 day samples should demonstrate if the gels are enzymatically degradable by the 3T3's and allow for cell expansion throughout the gel. Evaluating our results, shown in Figure 10, both sets of gels showed cytocompatibility with the polymer components after 1 day, but a higher cell viability within the mPEG-Tz gels was observed to have an average live cell amount to be $70.64 \pm 3.81\%$, compared to the mPEG-SH gels with an average live cell amount to be $54.65 \pm 10.65\%$($0.0001 < p < 0.001$ for day 1). This trend stayed consistent when evaluating the encapsulated cells on day 3, with the mPEG-Tz showing more cell spreading throughout the gel with an average live amount of cells to be $73.24 \pm 4.50\%$ compared to the control mPEG-SH gels with an average live amount of cells of $50.20 \pm 4.80\%$($p < 0.0001$ for day 3). Fluorescence microscopy images of the gel samples at day 1 and day 3 are shown in Figure 11.

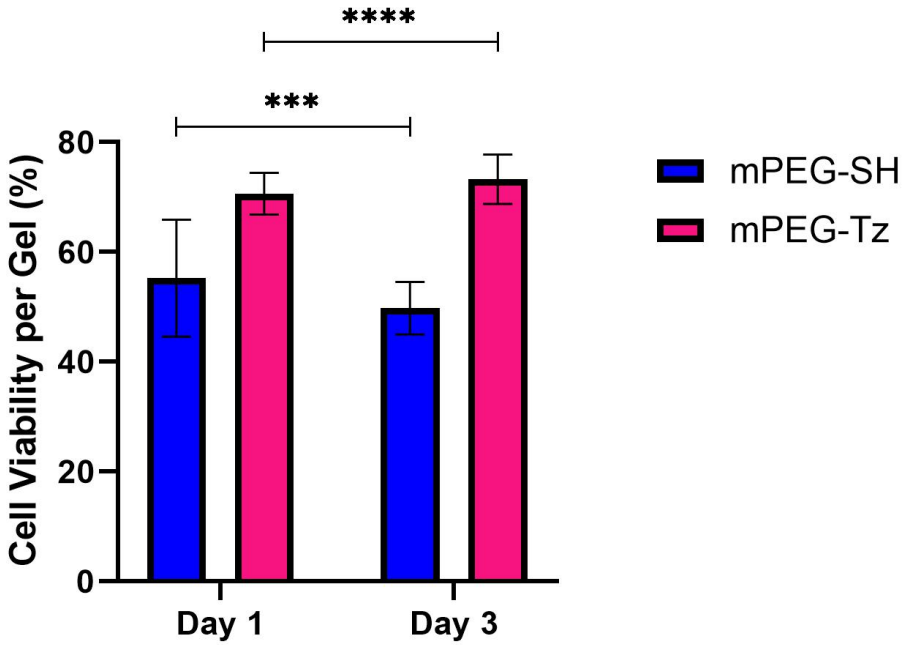

Figure 10: Cell Viability was measured by the % live cells per gel which not only confirmed cytocompatibility and cell growth in the mPEG-Tz gels but a higher cell viability compared to the control mPEG-SH gels.

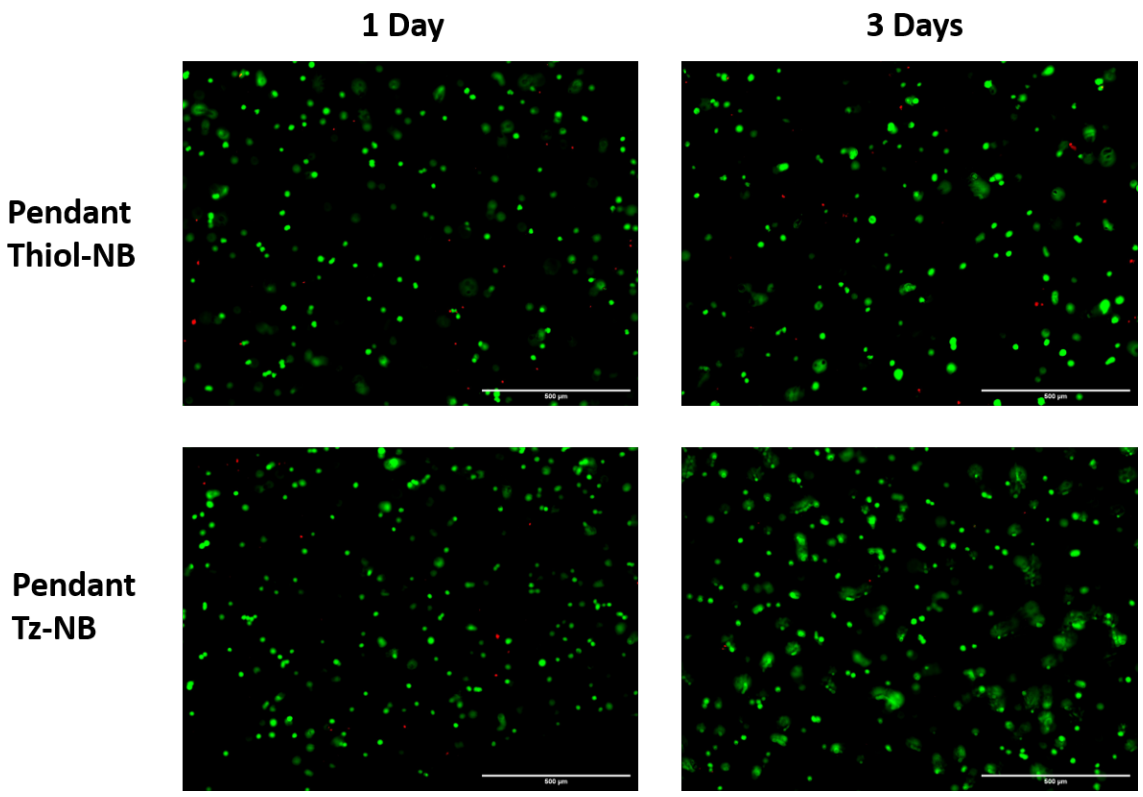

Figure 11: 3T3 fibroblasts were encapsulated and evaluated using fluorescence microscopy at 24 hours and 3 days. The mPEG-Tz gels exhibit increasing cell viability and cell spreading compared to the control mPEG-SH gels.

CHAPTER IV

CONCLUSION

This gel platform exhibits the properties necessary for 3D cell culture while allowing for dynamic stiffening via click cycloaddition products. It exhibits enzymatic degradability, high cell viability, and the capacity to dynamically stiffen as it is swelled in mPEG-Tz containing solutions. This potentially offers a user-friendly method for in vitro modeling of dynamic biomechanical environments such as in fibrotic diseases, tissue morphogenesis, and cancer as the mPEG-Tz can be combined with cell culture media to stiffen the gel over time. Opportunities for future study include user-controllability of the dynamic stiffening, quantifying a critical concentration at which bulk property effects emerge, evaluating this method's applicability to other commonly used hydrogel platforms for cell culture, and determining potential applications in drug delivery.

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