TUNABLE RELEASE OF BMP-2 FROM THIOL-ENE CLICK HYDROGELS

An Undergraduate Research Scholars Thesis

by

ASHLEY TUCKER

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ABSTRACT

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Ashley Tucker
Department of Biomedical Engineering
Texas A&M University

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

With over 6.3 million fractures that occur in the United States each year, autogenic and allogenic bone sources are becoming a dwindling resource [1]. Bone Morphogenetic Protein-2 (BMP-2) has been shown to induce osteoblast differentiation, but uncontrolled release of these growth factors can cause potentially life threatening complications to occur [2]. This issue provides motivation for the development of synthetic polymer based drug delivery systems, more specifically hydrogels, due to their tunability of the mesh size, rate of degradation, and ability to incorporate different growth factor-binding chemical moieties, such as bisphosphonates [3]. Bisphosphonate is an affinity ligand that has been shown to electrostatically interact with BMP-2 and can be tethered into hydrogel matrices in order to non-covalently control release and maintain the bioactivity of the growth factor [4]. In this study, we compare controlled release of osteogenic growth factor BMP-2 from tunable poly(ethylene glycol) hydrogels that were functionalized with varying amounts of the bisphosphonate sodium alendronate. By characterizing our hydrogel system through storage modulus and swelling ratio measurements and monitoring growth factor release rate through a rhBMP-2 specific ELISA assay, we found significant differences in release over a period of one week due to varying incorporation of bisphosphonate. These results indicate the need for further investigation to explore the release and tunability of our platform.

DEDICATION

To John, the provider of queso, chips, brisket, lasers, laughs, love, and one completed undergraduate thesis.

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I would like to thank my principle investigator, Dr. Alge, for offering me guidance and support throughout the course of this research. I would also like to thank Faraz Jivan for mentoring me throughout the entire year-long process and promoting me to think critically every time I worked in the lab or on my thesis.

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Finally, I'd like to thank my friends and peers for supporting me through the program and providing me the encouragement I needed to finish the project.

NOMENCLATURE

BMP-2	Bone Morphogenic Protein-2
BP	Bisphosphonate
BP-SH	Thiolated Bisphosphonates
hMSC	Human Mesenchymal Stem Cells
NMR	Nuclear Magnetic Resonance spectroscopy
PBS	Phosphate Buffered Saline
PEG	Poly(ethylene glycol)
PEG-NB	Poly(ethylene glycol) norbornene
PEG-BP	Poly(ethylene glycol) bisphosphonates
rhBMP-2	Recombinant Bone Morphogenic Protein-2
SH	Thiol

CHAPTER I

INTRODUCTION

Affecting over 6.3 million individuals in the United States each year, fractures are arguably the most common orthopedic problem in the world [1]. With an estimated 2 fractures incurred within an average person's lifetime, the sheer amount of fractures cost the medical industry up to $12.2-17.9 billion dollars in 2002[1,5]. On average, it takes 6-12 weeks for complete bone remodeling, but this can be impacted by the location of the trauma, lifestyle, certain medications, and diseases [6]. Common treatments include cast immobilization, open fixation, and external fixation, but these methods can only be utilized when the fracture can regrow [3]. For patients suffering from osteosarcoma and conditions that cause non-union bone fractures, individuals require surgical treatments such as bone grafts and growth factor delivery to heal [5].

Considered the gold standard in bone grafting due to their predictability and reliability in packing bioactive tissues with a lack of immune rejection into irregular shaped fractures, autografts are commonly used in treating non-union bone fractures [7]. Nevertheless, the patient must endure multiple invasive surgeries, which increases medical costs and can potentially cause donor site morbidity and chronic pain from the donation site [7,8]. Furthermore, there is a limitation on how much bone can be harvested from the patient without causing further complications, which is why many have turned to allografts. Allogenic sources of bone are ideal for patients that have large non-union fractures, but like autogenic sources they have several drawbacks such as a potential for immune rejection, lower chance of bone fusion to the graft, and a risk of disease transmission [9]. With all current methods of treating non-union long bone

fractures having drawbacks that could prolong the immobilization of the patient, other options, such as the usage of growth factors, are being explored to synthetically repair or stimulate bone growth.

Growth factors are biomolecules naturally produced in the body and can be used to stimulate cellular growth and differentiation. BMP-2, which is a part of the TGF-β superfamily, is a growth factor that stimulates the development of bone and cartilage, and induces osteoblast differentiation [10,11]. Medtronic, the world's largest standalone medical technology development company, developed the Medtronic INFUSE product, a collagen based sponge soaked in BMP-2, to treat lower lumbar spinal injuries [2]. However, due to the high concentration of BMP-2 released from the device, patients with the implanted device experienced ectopic bone growth, uncontrolled bone growth around their vital organs requiring emergency secondary surgeries. Unfortunately, many patients died due to the inability to receive treatment in time. This critical need to control release rate of BMP-2 into the body has paved the way for synthetic biocompatible drug delivery devices that can be tuned to control release to prevent unwanted, and potentially lethal, side effects.

Hydrogels, highly water-swollen polymer networks, have shown much promise as drug delivery vehicles, especially with sensitive growth factors, due to their advantageous properties. Click chemistry, a highly specific chemistry, has been used in polymer synthesis and can be utilized to protect the bioactivity of growth factors [12]. PEG, a commonly used hydrogel polymer, is bio-inert and easily tunable through mesh size, degradation, and chemical conjugation. Through directly changing the mesh size of the polymer via crosslinking density, which is modeled by the Flory-Rehner equation, we can directly affect the diffusion properties of the hydrogel. Although this feature is easily tunable, this is not a feasible way to prolong release

as the rate of diffusion of the loaded drug is determined the mesh size. If the mesh size is too small in comparison to the protein size, there will be no release, while if the mesh size is too large all the protein would release into the surrounding tissue. This means that without any means of slowing release, the BMP-2 would passively release through the device, inhibiting the drug delivery devices ability to be sustainable over long periods of time. Other options include tethering of growth factors to the numerous functional handles of the hydrogel through chemical conjugation. This is not ideal as it can hinder the amount of drug loading due to the need to create a stable hydrogel, can disrupt bioactivity of the tethered growth factor through conformational restraint and exposure to conjugation chemistries, and requires the use of a degradable hydrogel or tethered linker to be released into the environment. Thus, non-covalent methods for controlling growth factor release are desirable.

Affinity ligands are molecules capable of binding with high affinity to moiety specific molecules. Due to their ability to preserve the protein structure of the growth factor, affinity ligands such as bisphosphonates have been explored as a method to control release of BMP-2 in hydrogels [4]. By tuning the number of bisphosphonates that are chemically tethered to the hydrogel mesh, it has been shown that greater control of release of BMP-2 is achieved [16]. Additionally, affinity systems have been developed and are utilized through reversible association-dissociation reactions between the therapeutic and the affinity ligand, but require careful tuning and changing of the strength of the affinity interaction [13,14]. By developing a PEG hydrogel system using thiol-ene click chemistry, it is possible to produce a BMP-2 compatible environment with the appropriate release rate [15,16,17].

In this study, we developed PEG hydrogels that were functionalized with bisphosphonates using thiol-ene click chemistry. Through dropwise addition of BMP-2, we

loaded and tested the release rate of BMP-2 from our photo-polymerized hydrogels. In addition, we characterized the physical properties of our PEG-based system by measuring the storage modulus and swelling ratio in order to determine that release was only affected by the concentration of tethered bisphosphonate in the system. By monitoring the release rate of the hydrogels, we observed that high concentrations of bisphosphonates in our system may result in denaturation of BMP-2, since BMP-2 release was significantly hindered under these conditions.

CHAPTER II

METHODS

Thiolation of Bisphosphonate Sodium Alendronate (BP-SH)

Bisphosphonate thiolation was performed using standard procedure [18]. Briefly, sodium alendronate (working concentration 9.370 mM) and 2-iminothiolane (working concentration 46.65mM) were mixed and adjusted to pH 7-8.5 with 1M NaOH. Solution was allowed to react at room temperature for 1 hour. Thiolation was then quantified using Ellman's Assay and can be seen in Figure 1 [18].

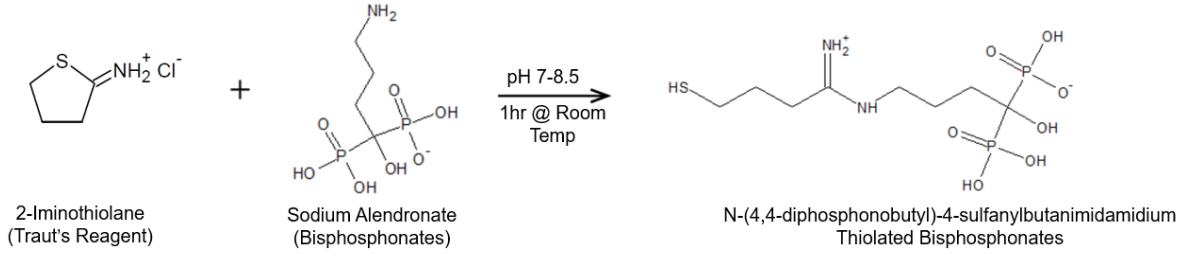

Figure 1. Thiolation of bisphosphonates sodium alendronate. 2-Iminothiolane, Traut's Reagent was added to Sodium Alendronate at a pH of 7-8.5 at room temperature for one hour to produce N-(4,4-diphosphonobutyl)-4-sulfanylbutanimidamidium, thiolated bisphosphonate (BP-SH).

Following standard procedure, cysteine standards and thiolated bisphosphonate samples were prepared in 1X Sodium Borate for the Ellman's Assay. Samples were placed on the shaker plate for 3 minutes at 300rpm. Absorbance readings were then taken at 405nm (TECAN Infinite M200 Pro).

PEG-NB Conjugation with Thiolated Bisphosphonates (PEG-BP)

20kDa, 4-arm PEG-NB (working conc. 20wt%), SH-BP (thiol:ene ratio: 0.5), and LAP (working conc. 2mM) were photoreacted under 365nm ultraviolet light source (Omnicure s2000) at an intensity of 10mW/cm^2 for 10 minutes. This can be seen in Figure 2. PEG-NB functionalized with thiolated bisphosphonates (PEG-BP) was dialyzed (10kDa MWCO) against DI water for 48 hours. PEG-BP was frozen at -80C and lyophilized to obtain solid product. PEG-BP conjugation was confirmed via ^{1}H NMR.

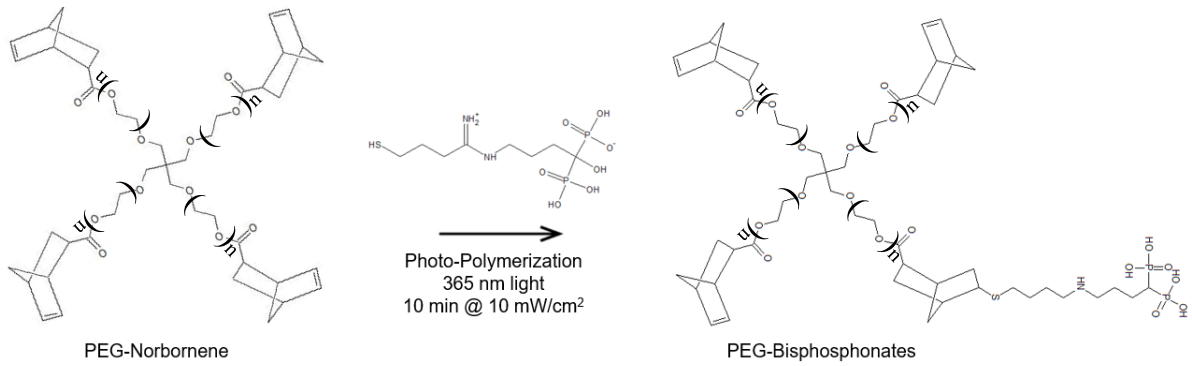

Figure 2. Conjugation of BP-SH to PEG-NB. Under a free radical thiol addition, thiolated bisphosphonates linked to PEG-NB in a photopolymerization reaction at 365nm light for 10minutes at 10mW/cm^2.

Photopolymerization of PEG-Bisphosphonate Thiol-ene Hydrogels

30 µL pre-polymer solutions containing PEG-NB (working conc. 20wt%), DTT (working conc. 10 mM), LAP (working conc. 2mM), and varying amounts of PEG-BP (working conc. 0-4 wt%) in PBS were loaded into 1mL uncapped syringe-tip mold. Solutions were polymerized under 365nm ultraviolet light (Omnicure s2000) at an intensity of 10mW/cm^2 for 2 minutes. Newly formed hydrogels (n = 3 hydrogels/experimental group) were placed in a polystyrene 24 well plastic plate (Corning) and hydrated overnight in PBS prior to use. Schematic of photo-polymerization can be seen in Figure 3.

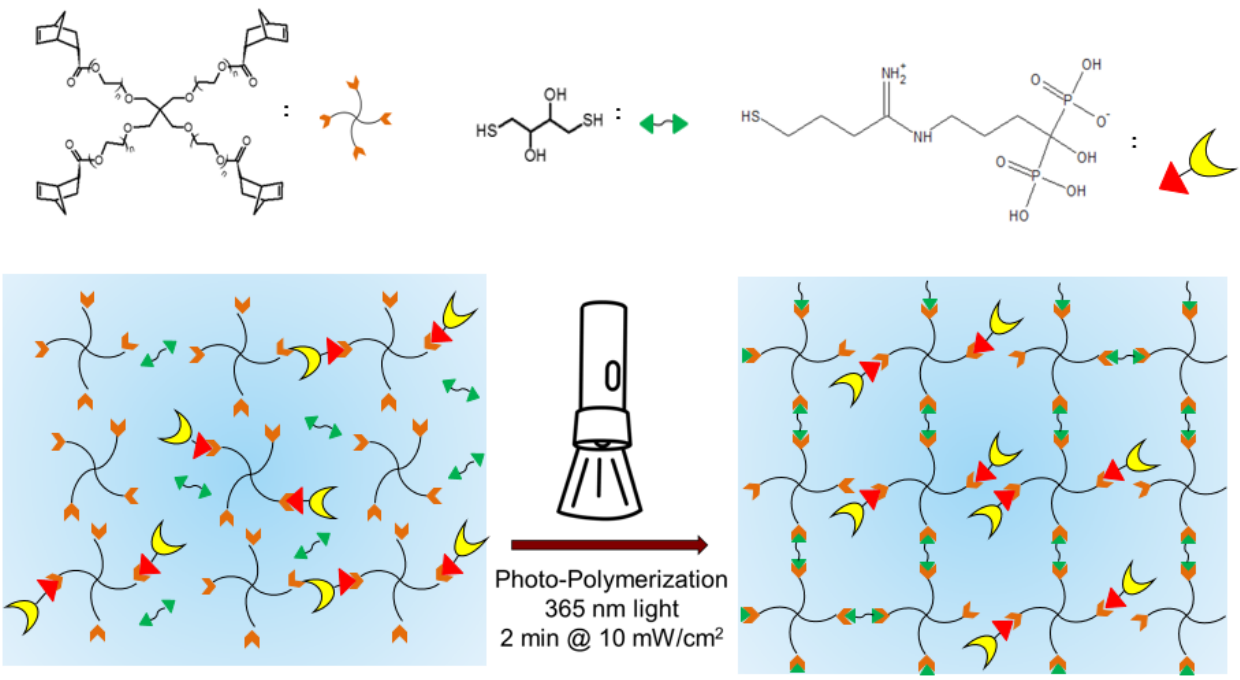

Figure 3. Fabrication and Loading of Hydrogels from Thiol-ene Click Chemistry. Bisphosphonate tethered, 4-arm PEG-NB hydrogels were photopolymerized at 365nm light for 2 minutes at 10mW/cm^2.

Storage Modulus of PEG-Bisphosphonate Thiol-ene Hydrogels

Fabricated hydrogels were swollen in PBS for 24 hours. Hydrogels (n = 3 hydrogels/experimental group) were placed on a rheometer (Anton Paar) , and a time-sweep (1% strain, 1 rad/sec angular frequency) was performed to obtain storage modulus of PEG-Bisphosphonate thiol-ene hydrogels. One way ANOVA was performed in order to test for statistical significance between fabricated groups.

Swelling Ratio of PEG-Bisphosphonate Thiol-ene Hydrogels

Photo-polymerized hydrogels (n = 3 hydrogels/experimental group) were dehydrated for 24 hours and subsequently weighed. Hydrogels were then swelled in a 1 mL PBS sink and reweighed after a 24-hour soaking period. Swelling ratio was calculated through usage of Equation 1.

$$Swelling\ Ratio = \frac{W_s - W_d}{W_d}$$

Equation 1. Swelling ratio equation.

BMP-2 Release Studies from Hydrogels

After 24 hour drying period, dehydrated hydrogels were loaded dropwise with 10 µL of 2.0 µg/ml of rhBMP-2 diluted in PBS. Hydrogels were allowed to absorb solution for 24 hours in a 24 well plate (Corning) before being used for release studies. Loading is depicted in Figure 4.

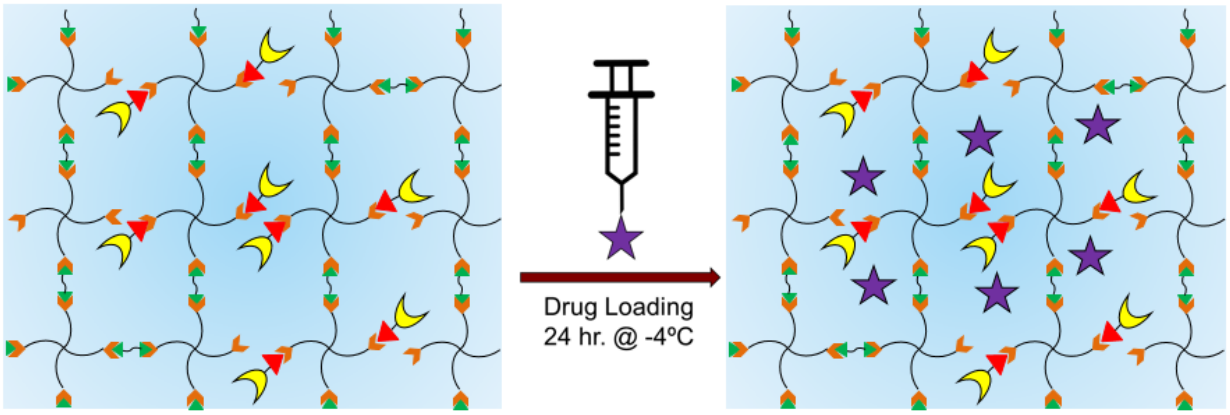

Figure 4. rhBMP-2 loading into PEG-Bisphosphonate thiol-ene hydrogels. Loading occurred through the addition of 10µL of rh-BMP-2 at 2.0 µg/mL. Hydrogels were allowed to absorb rh-BMP-2 for 24 hrs at 4ºC

Hydrogels were then added to 2 mL PBS sinks, and 1mL samples (n = 3 samples/experimental group) of the PBS were retrieved at various time points over the course of 10 days. Release rate was monitored through the usage of a rhBMP-2 specific sandwich ELISA assay following Peprotech's protocol.

Briefly, a high affinity 96 well plates (Corning) was used to tether rhBMP-2 capture antibodies, the plates with the capture antibodies were allowed to incubate overnight at room temperature. Well plates were then aspirated 4 times and block buffer solution was added to the

plates, plates were allowed to incubate for 2 hours at room temperature, were aspirated 4 times, and subsequently loaded with known rhBMP-2 standards and collected samples and allowed to incubate for 2 hours at room temperature. Wells were aspirated 4 times, and detection body was added to the wells, an incubation period of 1 hour followed at room temperature, thereafter the wells were aspirated 4 times and Streptavidin-HRP Conjugate was added to the wells for an incubation period of 30 minutes. Wells were aspirated 4 times, and TMB Liquid Substrate was added to the wells for 20 minutes. TMB Stop Solution (1 M HCL) was then added to each of the wells. Absorbance readings at 450 and 620 nm were taken of all the wells.

CHAPTER III

RESULTS

PEG-NB Conjugation with Thiolated Bisphosphonates (PEG-BP)

As show in Figure 5 and Figure 6, tethering of thiolated bisphosphonate was successful and proven through the usage of ^{1}H NMR. By showing the reduction in the two peaks located in the 5.8-6.5 ppm region, which correspond to the alkenes of the norbornene group in PEG-NB, we were able to quantify the tethering of the thiolated bisphosphonates to the PEG-NB. By comparing the integrals under the alkene proton peaks, we were able to conclude that the PEG-NB was functionalized with bisphosphonate at 80%, meaning that roughly 3 arms out of the 4 were theoretically tethered with bisphosphonate.

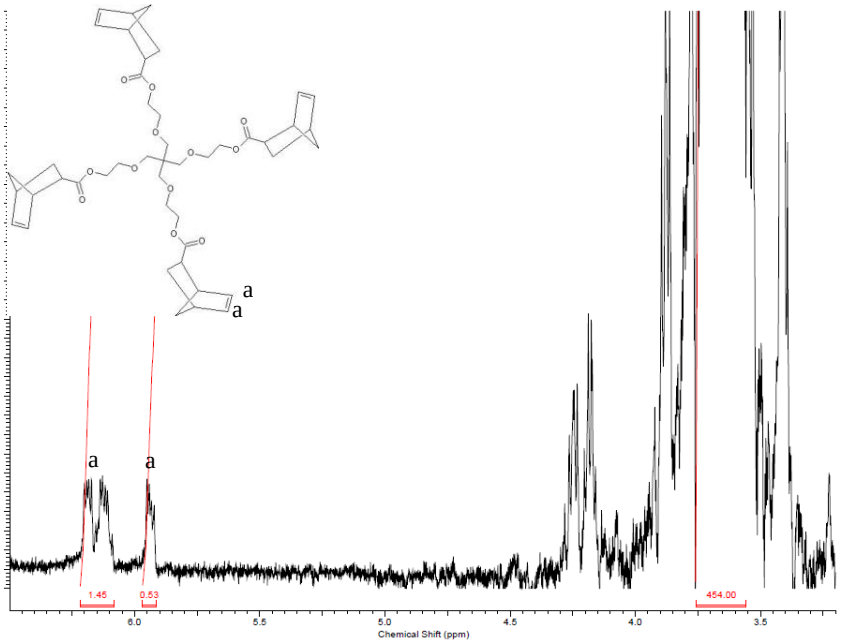

Figure 5. ^{1}H NMR of PEG-NB. NMR shows that PEG-NB is characterized by the 2 peaks located in the 5.8-6.5 ppm region. These peaks symbolize the alkene peaks that are in the molecule.

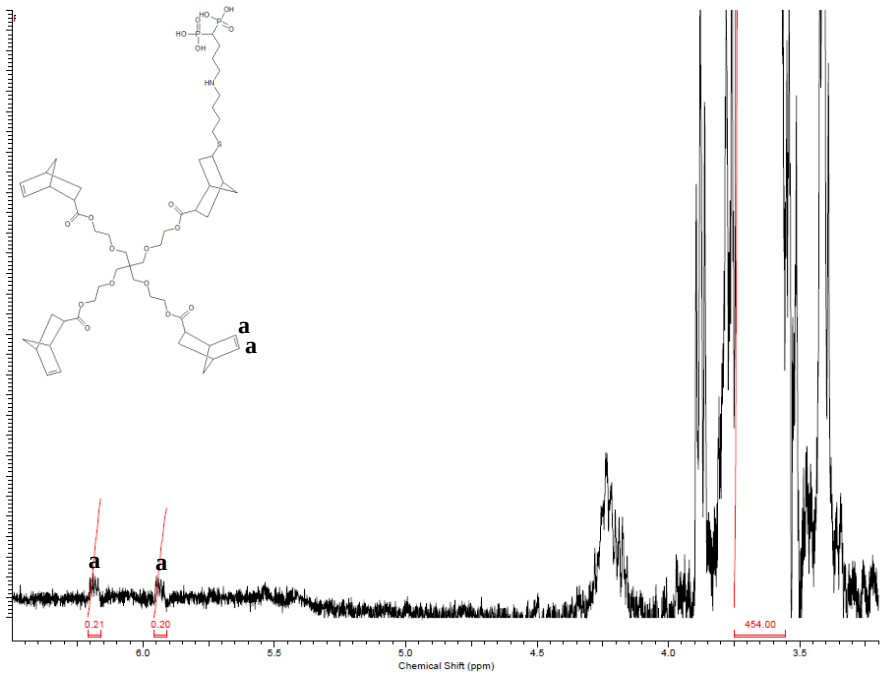

Figure 6. ^{1}H NMR of Bisphosphonate functionalized PEG-NB (PEG-BP). Decrease in the two peaks in the 5.8-6.5 ppm region indications consumption of alkene groups from the 4-arm PEG-NB and successful tethering of BP-SH to the molecule.

Storage Modulus of PEG-Bisphosphonate Thiol-ene Hydrogels

Crosslinking density and mesh size are important factors relating to drug release rate. They directly correlate to the elastic modulus of the hydrogel. Thus, mechanical characterization of the hydrogels was used to show crosslinking equivalence between PEG-BP experimental groups and indirectly compare mesh size this can be seen in Figure 7. Through one way ANOVA, storage modulus of varying weight percentage of bisphosphonate-functionalized PEG hydrogels showed no statistical difference between groups.

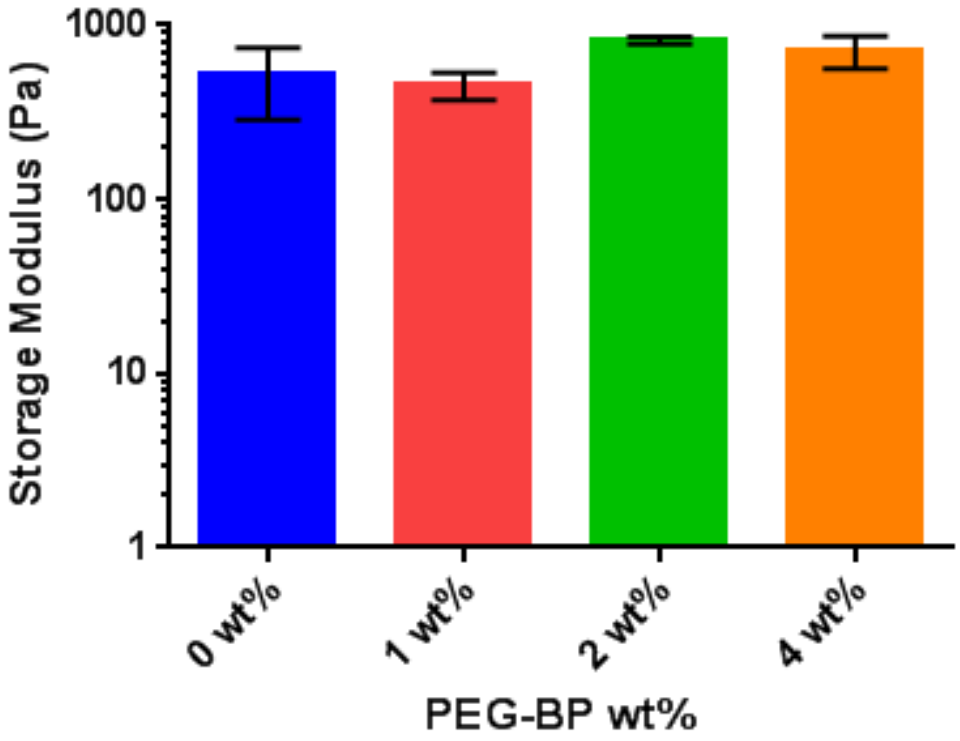

Figure 7. Storage Modulus of Varying Weight Percent of Bisphosphonate-Functionalized PEG Hydrogels. One way ANOVA proved no statistical significance between tested groups (p=0.05).

Swelling Ratio of PEG-Bisphosphonate Thiol-ene Hydrogels

Swelling ratio is another physical property that correlates to hydrogel crosslinking and mesh size. Through one way ANOVA, swelling ratio of varying weight percentage of bisphosphonate-functionalized PEG hydrogels showed no statistical difference between tested groups. Shown in Figure 8, swelling ratio did inversely correlate with storage modulus, as expected indicating that the mesh size between all groups was the same, meaning that only the tethered bisphosphonates would influence the rate of release.

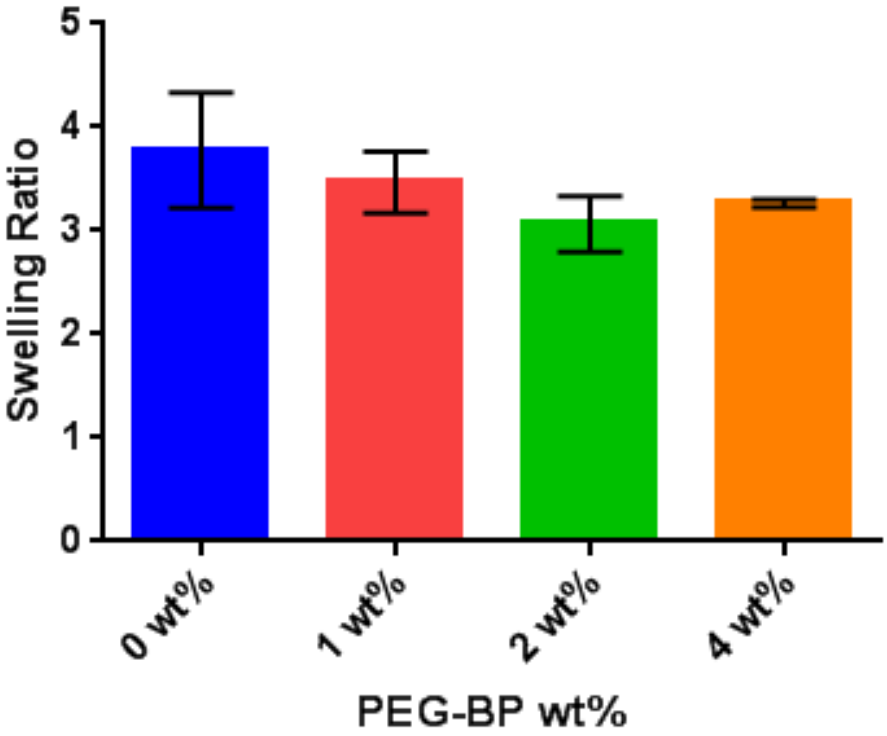

Figure 8. Swelling Ratio of Varying Weight Percent of Bisphosphonate-Functionalized PEG Hydrogels. One way ANOVA proved no statistical significance between tested groups (p=0.05), showing that the mesh size would not affect the rate of release of BMP-2 out of the fabricated hydrogel systems.

BMP-2 Release Studies from Hydrogels

After a 10-day release study, a rhBMP-2 ELISA assay was performed on the 30 minutes, 1, 2, 8, 10, 24, 24, 72, 120, 192, and 240 hour time points. The rate of release was reduced as the concentration of bisphosphonate increased in the hydrogels, which is shown in Figure 9. When evaluating the percentage released out of the hydrogel, it was found that BMP-2 release was significantly reduced in all tested hydrogels compared to previous works, which showed higher percentage of BMP-2 release at earlier timepoints [16].

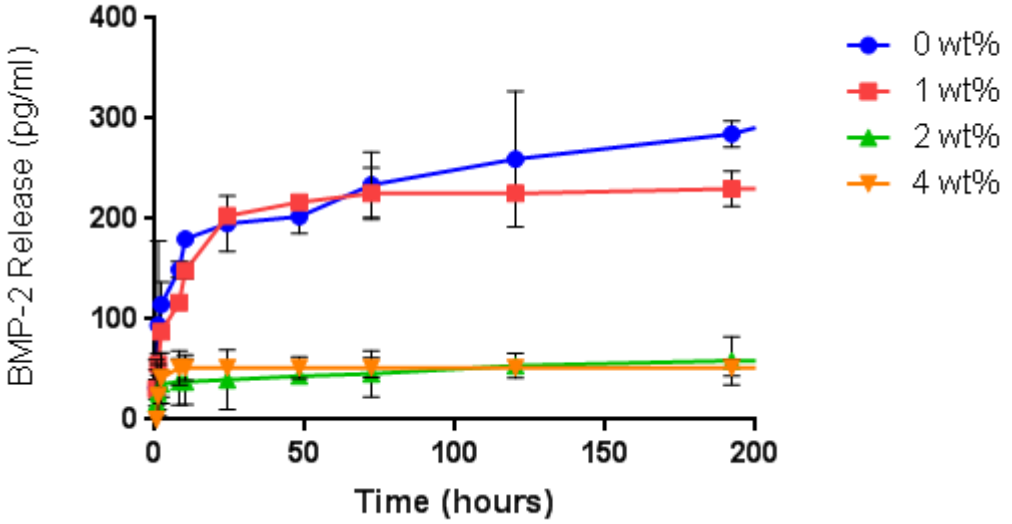

Figure 9. Cumulative BMP-2 Release out of Varying Weight Percent of Bisphosphonate-Functionalized PEG Hydrogels. Higher weight percent hydrogels showed slowed to no release rate out of the hydrogel over time.

CHAPTER IV

DISCUSSION

In this study, we aimed to design a PEG based hydrogel functionalized with bisphosphonates in order to tune release of BMP-2. Although we achieved reduced release rate through increasing the weight percentage of BP-SH, release rate was considered too low throughout all tested hydrogel groups, in comparison to previous works, which showed at least 20 % release within 100 hours of release when testing hydrogels that were incorporated with 0-2µg of BP-SH [16]. Possibilities of potential errors are described below.

From NMR, we determined a BP-SH functionality of 80%. Knowing this, it is possible that the high incorporation of this bisphosphonate-functionalized PEG sequestered the BMP-2 due to its presence in the hydrogel matrix causing the produced results. Looking at previous works, hydrogels incorporated with BP-SH at a functionality of 15% showed release of 20% or higher within the first 100 hours of performing a release study [16]. Although this is a possible issue, this does not address our 0wt% group, which did not incorporate our bisphosphonate-functionalized 4-arm PEG, meaning that rhBMP-2 should have passively diffused out of the hydrogel mesh into the PBS sink over time. This suggests that the hydrogels might need an active source of diffusion, such as a microplate shaker, to stimulate rhBMP-2 to be released out of the hydrogels. Not only could this potentially improve our results, it would simulate the environment that the drug delivery device would be exposed to when implanted into an animal model or patient.

BMP-2 can have a half-life of minutes to hours depending on the application [19]. It is possible due to the extent of the release study, which lasted over 10 days, and the continual change in temperature caused by removing and placing the samples in the fridge caused the released BMP-2 that was taken slowly to denature over time causing the ELISA assay to be unable to accurately detect how much BMP-2 was released from our drug delivery systems. This would explain why the BMP-2 standards were detected with relative ease while the samples showed little to no detection, and shows a necessary need to perform the ELISA over time or to store the samples in such a way that they are not exposed to changing temperatures that could possibly denature them.

CHAPTER IV

CONCLUSION

In this study, we successfully incorporated bisphosphonates into our PEG hydrogel system through the usage of thiol-ene click chemistry, which was verified by NMR. Through one way ANOVA analysis of storage modulus and swelling ratio, we demonstrated that the mesh size of all tested hydrogels was the same. This was critical for ensuring that the only variable that would affect BMP-2 release would be the concentration of bisphosphonate in the hydrogel. Although the release rate of BMP-2 out of all tested hydrogels was significantly lower than what was shown in previous works, there was a reduction in release as the concentration of tethered bisphosphonates increased, supporting the possibility of controlling BMP-2 release with this system. Future work should investigate decreasing the number of bisphosphonates tethered per PEG molecule and preventing BMP-2 denaturation. If these pitfalls can be avoided, this new delivery system could be promising for treating nonunion bone fractures.

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