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Protein Release Study using Heparin Modified Methacrylamide Chitosan Hydrogels

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Executive Summary

Problem Statement

The study proposed in this technical report was performed to improve the release of proteins from natural polysaccharide hydrogels in order to increase their effectiveness in treating central nervous system (CNS) injuries. This study is specifically looking into the effects of modifying, with the use of heparin, the release profile of chitosan-based hydrogels to determine if the release kinetics of a test protein from a hydrogel can be improved, i.e., released over a longer period of time more slowly and uniformly. This type of release is desired because hydrogels, loaded with a drug or protein, that are used to treat human diseases or improve tissue regeneration may diffuse out quickly with an initial burst reducing its long-term availability for the healing process. Based on previous research into the use of hydrogels for delivery[1], additives to hydrogels, such as heparin, can extend and improve the release of drugs or proteins due to the interactions the additives have with said drugs and proteins, such as electrostatic interactions, chemical bonding, and van der Waals forces. Thus, a look into how an additive such as methacrylated heparin affects the release profile of an already established hydrogel (methacrylamide chitosan (MAC) hydrogel) would provide important information as to how the gels can be improved as a delivery vehicle for proteins.

Quantitative Results

In order for heparin to be added to the MAC hydrogels through crosslinking, methacrylation of the heparin had to be carried out. The extent of methacrylation of the heparin, 33%, was determined through hydrogen nuclear magnetic resonance spectroscopy (^{1}H NMR). Rheology was performed on the fabricated gels in order to determine their complex modulus, which is a good

indication of the stiffness of the gels. The complex modulus of the hydrogels containing 10 wt%, 20 wt%, and 30 wt% methacrylated heparin was determined to be 5328±230.3 dyn/cm^2, 2983±32.91 dyn/cm^2, and 3874±297.8 dyn/cm^2 respectively (mean ± SD). Finally, a release study using a model protein, stromal cell-derived factor 1α (SDF-1α), was carried out where the protein was allowed to release into a solution until equilibrium was reached. It was proposed that SDF-1α be used due to its ability to recruit neural stem cells for the repair of central nervous system injuries. The concentration of SDF-1α at time points was determined and then used to find the amount and percentage of the protein that was released. After one week from the start of the release study, the percentages of SDF-1α released from the 0 wt%, 10 wt%, 20 wt%, and 30 wt% methacrylated heparin hydrogels was calculated to be 44.3±7.58%, 59.8±9.34%, 29.7±7.74%, and 33.0±9.92% respectively. These results show that the 20 wt% methacrylated heparin showed a slower release rate than the other gels. The final percentages of SDF-1α released from each hydrogel were eventually determined and are as follows: pure MAC gels released 52.2±10.1%; gels with 10 wt% methacrylated heparin released 61.2±10.9%; gels with 20 wt% methacrylated heparin released 33.8±9.62%; gels with 30 wt% methacrylated heparin released 197±27.1%. The high percentage of SDF-1α released from the hydrogels containing 30 wt% methacrylated heparin was an error caused by degradation after day 8. Before the degradation of the hydrogels occurred, the amount of the protein released was 34.5±10.3%.

Conclusions

First, we were able to conclude that heparin can be successfully methacrylated and subsequently blended in various weight percentages with MAC to form stable hydrogels. From the results obtained from the rheology performed on the modified hydrogels, gels that were fabricated with 20

wt% methacrylated heparin had a more similar complex modulus to pure MAC hydrogels when compared with the gels containing 10 wt% and 30 wt% heparin. These differences in modulus could be due to experimental differences, such as different crosslinking times or very slightly higher amounts of photoinitiator being used for the gels. Both of these differences would lead to stiffer gels which would subsequently lead to a higher complex modulus. Lastly, although less of the SDF-1α was released, the hydrogels containing 20 wt% methacrylated heparin showed a slower release rate of the protein than the pure MAC gels. For eight days, the gels with 30 wt% methacrylated heparin also showed a slower rate, but these gels started to degrade after this time. These slower release rates could be due to an increase in electrostatic interactions between the methacrylated heparin and the SDF-1α as a result of the higher amount of heparin in the hydrogels. Due to its polar nature, heparin interacts electrostatically with proteins, primarily growth factors, as well as acting as a storage unit for these molecules, both of which allow for heparin to bind to proteins and slow their release[1]. As such, the hydrogels fabricated with 20 wt% methacrylated heparin were shown to have a more similar complex modulus to unmodified gels than the other modified hydrogels as well as having a more desired release profile, one with a small or nonexistent initial burst of loaded protein and a slower release rate of the protein.

Implications of Work

Throughout the experience of working on this project, several technical skills and personal improvements were obtained. Technical skills included creating an experimental procedure from personal knowledge and through research, carrying out previously unfamiliar procedures, including rheometry and enzyme-linked immunosorbent assays, and learning was how to research top-

ics effectively through the use of different media, such as online sources and the knowledge and experiences of others.

Personal improvements made during the project were increased confidence while working independently in a lab setting, an increase in ease of asking for help from others when needed, and learning how to work alongside others of varying backgrounds and educational levels.

The results obtained from this study show that use of additives in the formation of hydrogels can lead to improvements in sustained protein release. If further development of hydrogel modification is carried out, the creation of a gel with a uniform and sustained release profile with desired properties such as being nontoxic and not causing damage to tissue around the injection site or having release characteristics that are pH or thermally dependent is possible. This type of gel would lead to great improvements in the healing and restoration of those in society who have suffered severe injuries through the efficient and effective release of proteins and pharmaceuticals in the damaged areas.

Recommendations

Based upon the results of the release study, methacrylated heparin improved the release profile on methacrylated chitosan hydrogels. In regards to future work, additional release studies involving heparin modified MAC hydrogels should be performed in order to solidify these findings. Due to the results found from the study, further work with methacrylated heparin should be pursued in order to determine if improvements can be made to hydrogels containing heparin such that an initial burst is nonexistent, the release profile is more linear, and the amount of protein released is closer to the amount that was loaded into the gels. Using other proteins in the study in order to test the effects of various protein properties on release kinetics is also proposed. Such

effects that could be tested are the molecular weight and the isoelectric point of proteins as compared to SDF-1α. To test the effects of a higher molecular weight, the proteins β-nerve growth factor and bone morphogenetic protein 2A could be used. Higher and lower isoelectric points can also be tested by using fibroblast growth factor 2 and transforming growth factor β-1, respectively[2]. In addition, studies utilizing different additives should be explored in order to determine if a better modification of the hydrogels exists to improve their release profile. One such additive to consider would be silk fibroin as it has been shown to have the ability to create hydrogels that maintain a sustained release and release a majority of a loaded drug[3].

Introduction

This study was performed to improve the release of proteins from hydrogels in order to increase their effectiveness in treating spinal cord injuries. Heparin, which has the ability to bind to proteins and slow their release, underwent methacrylation, was added to prepared methacrylamide chitosan (MAC) solutions with a model protein (SDF-1α) and photopolymerized to create MAC-heparin hydrogels. Varying weight percentages of methacrylated heparin were tested in this study in order to determine an optimal amount of heparin to be added to improve the release profile of the hydrogels. With pure MAC hydrogels, as well as the gels that contained 10 wt% methacrylated heparin, a rapid release of the SDF-1α occurred, while those gels that contained 20 wt% and 30 wt% heparin had a relatively slower release of the protein. The four variants of hydrogels had relatively uniform release profiles after the initial release for the duration of three weeks, excluding the gel with 30 wt% heparin which degraded after eight days as the amount of SDF-1α released from this variant increased significantly only one day after and continued to do so for the remainder of the study. These results show that, in certain amounts, methacrylated heparin can be used to positively affect the release of proteins from MAC hydrogels, greatly decreasing the amount released initially and maintain a steady and uniform release for an extended period.

Background

Hydrogels are polymeric networks that consist primarily of water or a biological fluid and mimic the behavior of living tissue relatively well in softness, flexibility, and biocompatibility compared to other synthetic biomaterials. Because of this mimicry, hydrogels have been utilized for biomedical purposes, such as protein or drug delivery into living organisms, wound and damaged

tissue repair and healing, and contact lenses[4]. Looking specifically at the release of proteins and pharmaceuticals, unlike injections or drugs in capsule form, hydrogels can offer an improved release of healing agents into the body. Injections and capsules offer a onetime "burst", so to speak, of an agent, which can be beneficial and effective in some instances. However, a more effective method of release would be one that does not have a "burst" of the drug or protein, but has a controlled, slow release. Hydrogels are able to offer this type of release by using certain polymers as building blocks and modification by way of additives. Hydrogels are able to offer more desired release profiles and degradation properties, such as those based on the pH or the temperature of the surrounding environment. Looking into the modification of hydrogels, the following study was conducted to determine the effects using methacrylated heparin as an additive to methacrylamide chitosan hydrogels had on the release of the protein SDF-1α. Heparin was selected as the additive for this study due to its polar nature which allows it to interact electrostatically with proteins and subsequently bind and slow their release from hydrogels[1]. The rationale for selection SDF-1α as the model protein was that this protein aids in the recruitment of neural stem cells. These stem cells are used after an injury to the central nervous system occurs in order to replenish dead cells and repair the damage. Thus, by using SDF-1α the results from this study can be applicable to *in vitro* and *in vivo* studies performed to determine the effectiveness of the MAC:heparin hydrogels on the recruitment of neural stem cells and the repair of central nervous system injuries.

Experimental Methods

Synthesis of methacrylamide chitosan and methacrylated heparin

Methacrylamide chitosan was synthesized by first adding methacrylic anhydride (Sigma-Aldrich, St. Louis, MO, USA) to liquid chitosan (NovaMatrix, Sandvika, Norway) (3 wt% (w/v) in acetic acid) in a 1/0.4 molar ratio (chitosan/methacrylic anhydride). Vials of the solution were vortexed and placed on a stir plate for approximately three hours at room temperature with the solution being vortexed every 15 minutes. The solution was then dialyzed against diH_2O until the distilled water had been exchanged nine times. Once dialyzed, the solution was placed in an -80°C freezer until frozen, and then placed in a lyophilizer (FreeZone 4.5; Labconco, Kansas City, MO, USA) for two days to remove any water from the solution. The MAC samples were subsequently dissolved in diH_2O to achieve a weight percent of 2%. A 10-fold concentration of phosphate buffered saline (PBS) was then added to the existing buffer of the samples.

Methacrylated heparin was prepared using a previous method[5] with some modifications. Heparin (sodium salt from porcine intestinal mucosa, MW ~ 16kDa; Sigma-Aldrich) was first dissolved in diH_2O to obtain a 2% (w/v) solution. The solution was then reacted with a 5-fold molar excess of methacrylic anhydride. The pH of the reaction mixture was adjusted to 8.5 using 1M NaOH and then placed on a stir plate for approximately 3 hours at room temperature with the pH being checked every 15 minutes. Additional 1M NaOH was added as needed to the mixture to ensure the pH was maintained at 8.5. Following the 3 hours of pH adjustment, the reaction was allowed to proceed overnight on the stir plate. The mixture was then dialyzed (1000MW cutoff dialysis tubing; Spectrum Labs, Rancho Dominguez, CA, USA) against diH_2O for 48 hours with four distilled water exchanges. Once dialyzed, the solution was placed in an -80°C freezer until frozen and subsequently placed in a lyophilizer for two days to remove any water

from the solution. The methacrylated heparin samples were then dissolved in a 10-fold concentration of PBS to achieve a weight percent of 2%.

Nuclear magnetic resonance (NMR)

A Varian Mercury 300 spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used to perform ^{1}H NMR to determine the amount of methacrylation of the synthesized methacrylated heparin. Following a previous method[6], the obtained ^{1}H NMR spectrum (*Figure 1*) was analyzed using an NMR processor program (ACD/NMR Processor Academic Edition; Advanced Chemistry Development, Inc., Toronto, Ontario, Canada) in order to determine the area under the peaks that represent the methacrylate vinyl protons (δ 6.1-6.4 and δ 5.6-5.9) and the protons on the repeating disaccharide unit of heparin (δ 3.0-4.6). The percent methacrylation of the heparin was then determined using the found areas and the equation:

$$Percent\ methacyrlation\ of\ heparin\ = \frac{\frac{1}{2}(I_1 + I_2)}{\frac{1}{9}(I)} \times 100\%$$

Where I is the area of the disaccharide unit peak and I_1 and I_2 represent the areas of the two methacrylate vinyl proton peaks. The utilization of the equation is located in the appendix.

Fabrication of heparin modified methacrylamide chitosan hydrogels

Four different sets of hydrogels were fabricated. The 2 wt% methacrylated heparin and 2 wt% MAC solutions that were prepared previously were mixed in ratios of 0:100, 10:90, 20:80, and 30:70 (heparin:MAC). The 0, 10, 20, and 30 in the mixing ratios are in reference to the weight percent of methacrylated heparin that is contained in each modified gel, thus 0 wt%, 10 wt%, 20 wt%, and 30 wt% of the total weight of the gels are made of heparin. Using the 10 wt% hydro-

gel as an example, if there was one gram total of gel solution then 100 mg of that total mass is methacrylated heparin and the remaining 900 mg is MAC. Prior to crosslinking, 75 µL/mL of gel mixture of a 2000 ng/mL solution of Human SDF-1α in nanowater were added to each set of hydrogels. These gels were crosslinked by first adding 6 µL/g of gel solution of a 300 mg/mL solution of Irgacure 184 (Sigma-Aldrich) in 1-vinyl-2-pyrrolidinone (NVP; Sigma–Aldrich). The gel solution was then mixed at 3000 RPM for three minutes using a speedmixer (SpeedMixer DAC 150 FVZ; Hauschild Engineering, Hamm, Germany). Once mixed, the solution was placed under a UV lamp (365 nm) for three minutes to be crosslinked. The resulting products were 2 wt% gels.

Rheometry of methacrylamide chitosan hydrogels

To determine the rheological properties, primarily the elastic and viscous moduli and subsequently the complex modulus, of the modified hydrogels, a slab (approximately 2.5 cm × 2.5 cm × 0.3 cm in size) of each of the hydrogel types was prepared using the previously described method. Several small discs (roughly 0.6 cm in diameter), cut from the hydrogel slabs, were then measured using an Ares RFS-III rheometer (Rheometric Scientific, Piscataway, NJ, USA) to determine their complex moduli.

Human SDF-1α Release Study Procedure

An SDF-1α release study was performed using a previous method[1] with modifications. 100 µL hydrogels from each ratio set were first prepared in quadruplicate. These gels were then placed in a PBS buffer solution and the SDF-1α protein was allowed to release from the gels into the solution at 37°C. At hours 1.5, 3, 6, 12, 24, then once daily for 10 additional days, and then once

weekly for three weeks, the release solution was removed and an equal volume of fresh solution was added to maintain the total volume of release medium. The removed release solutions were then placed into a -80°C freezer until measurements were taken.

The amounts of SDF-1α released were determined with sandwich enzyme-linked immunosorbent assay (ELISA) using a purchased development kit (PeproTech Human SDF-1α Mini ELISA Development Kit, Cat. # 900-M92; PeproTech, Rocky Hill, NJ, USA). Briefly, 100 μL/well of 2.0 μg/mL capture antibody (antigen-affinity purified rabbit anti-hSDF-1α; PeproTech) was immobilized on a clear flat-bottom 96-well plate by incubation overnight at room temperature. Once incubation had been completed the wells were aspirated and washed four times with 300 μL/well of wash buffer (0.05% Tween-20 in PBS; PeproTech). 300 μL of block buffer (sterile filtered 1% BSA in PBS; PeproTech) was then added to each well and the plate was allowed to incubate for one hour at room temperature. The wells were aspirated and washed four times with wash buffer. SDF-1α solutions in release media from each gel were thawed and diluted 1:10 in diluent (sterile filtered 0.05% Tween-20, 0.1% BSA in PBS) for a total volume of 400 μL. Two samples of 100 μL of diluted SDF-1α solutions from each gel were added to the plate along with a dilution series of known concentrations of human SDF-1α in dilutent to act as standards. The plate was then incubated at room temperature for two hours. After incubation, the plate was aspirated and washed four times followed by the addition of 100 μL/well of 0.5 μg/mL detection antibody (biotinylated antigen-affinity purified rabbit anti-hSDF-1α; PeproTech) and an incubation of two hours at room temperature. The plate was then aspirated and washed four times. After aspiration and washing, 100 μL/well of a 1:2000 dilution of Avidin-HRP Conjugate (PeproTech) was added and the plate was incubated for 30 minutes at room temperature. Following the incubation, the plate was aspirated and washed four times. 100 μL of ABTS liquid substrate (Pepro-

Tech) was added to each well and the plate was incubated at room temperature for color development. Color development was monitored with an ELISA plate reader (Tecan Infinite M200; Tecan, Männedorf, Switzerland) at 405 nm with a wavelength correction set at 650 nm in order to determine the SDF-1α concentration present in each SDF-1α solution from each gel. To determine the mass of SDF-1α released at time i (M_i), a formula used in a previous study[7] was utilized:

$$M_i = C_i V + \sum C_{i-1} V_s$$

Where C_i is the concentration of SDF-1α in the release solution at time i, V is the total volume of release solution (0.5 mL) and V_s is the sample volume of release solution (0.5 mL). An example of using this formula is located in the appendix.

Data and Results

Nuclear magnetic resonance

Methacrylic anhydride was reacted with heparin in order to induce methacrylation of the compound. The extent of the methacrylation was then determined using ^{1}H NMR analysis. From the analysis, approximately 33% of the heparin was methacrylated. The NMR spectrum that was analyzed to determine the extent of methacrylation of the heparin can be seen in *Figure 1*.

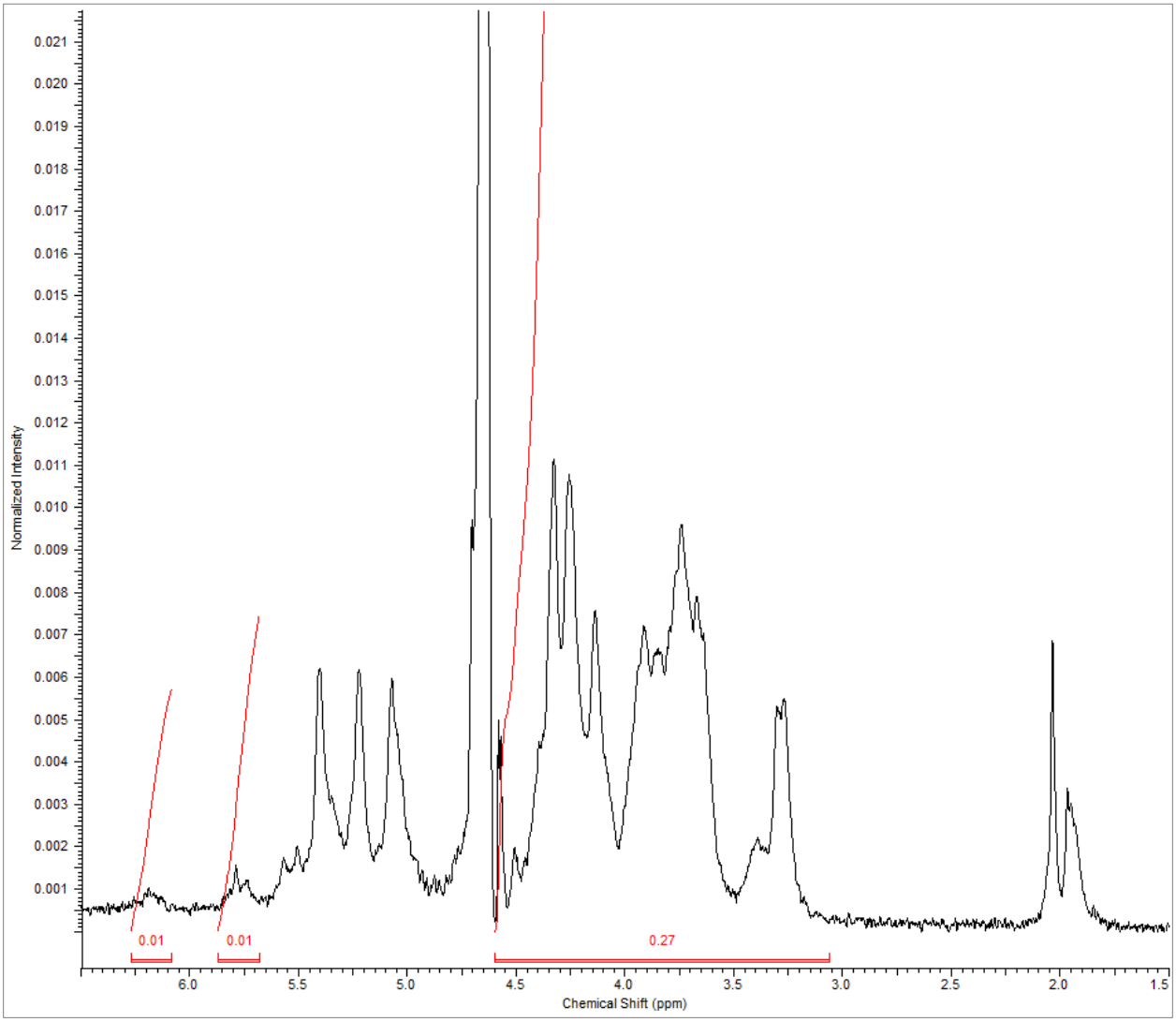

Figure 1: ^{1}H NMR of methacrylated heparin in D_2O.

Rheometry of methacrylamide chitosan hydrogels

A rheometer was used in order to determine the elastic and viscous properties, primarily the complex modulus, of the fabricated hydrogels. The complex modulus is the overall resistance of a material to deformation, be it recoverable or non-recoverable, and it is a good indicator of the stiffness or flexibility of the hydrogels[8]. These obtained data were then compared with the properties of previously made MAC gels without any modifications. The purpose of measuring the complex modulus of the gels and comparing it to unmodified MAC is to confirm whether the modified hydrogels underwent crosslinking and were in fact gels and to verify that heparin could be incorporated into the gels without dramatically altering the stiffness of the MAC hydrogels.

From the use of a rheometer, the complex modulus (denoted by G*) of each modified hydrogel was determined. The complex modulus of the gels having 10 wt%, 20 wt%, and 30 wt% were found to be 5328±230.3 dyn/cm^2, 2983±32.91 dyn/cm^2, and 3874±297.8, respectively. From a previous study[9], the complex modulus of pure MAC hydrogels was found to be 1890 dyn/cm^2. Thus, the hydrogels containing 20 wt% methacrylated heparin have a more similar complex modulus to pure MAC gels when compared to the gels containing 10 wt% and 30 wt%. Due to this similar modulus it can be said that the 20 wt% gels have a relatively unaffected stiffness compared to unmodified gels and therefore have a more favorable modulus compared to the other modified hydrogels. This observation is shown graphically in *Figure 2* below.

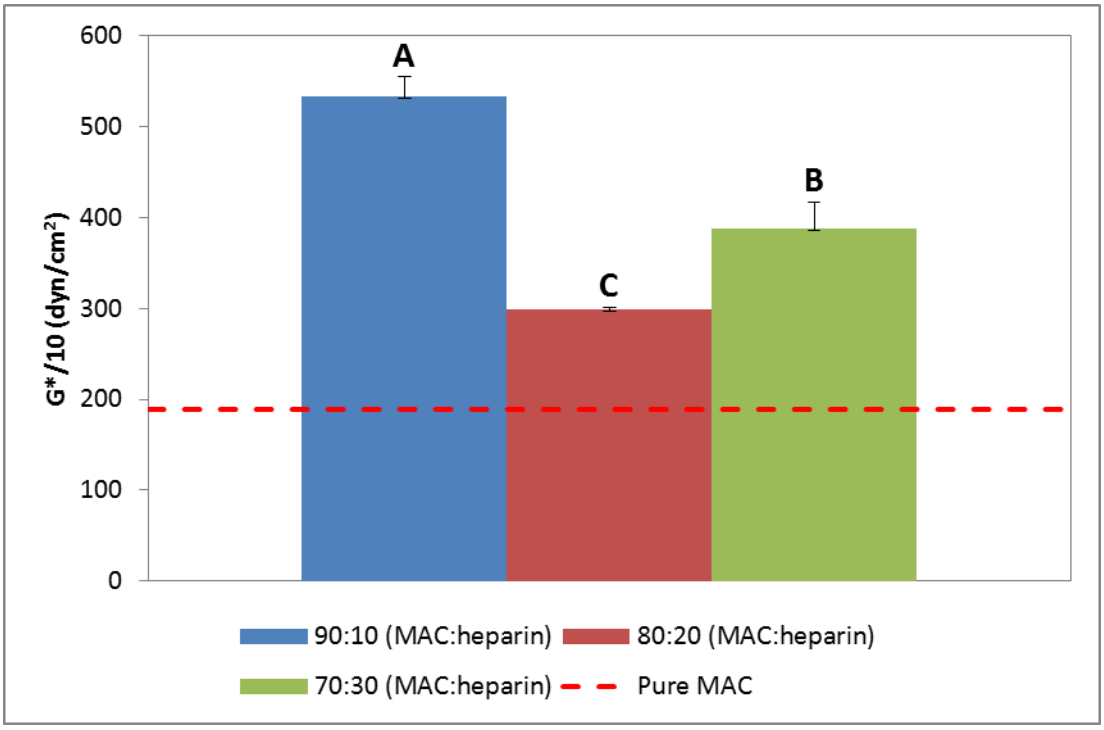

Figure 2: Complex moduli of the hydrogels containing 10 wt%, 20 wt%, and 30 wt% methacrylated heparin. The dashed red line is the complex modulus of a pure MAC hydrogel obtained from the previous study. Letters denote significance by single factor ANOVA with Tukey's *post hoc* analysis ($p<0.01$). Mean ± SD with n = 1.

SDF-1α Release Study

The release of SDF-1α into buffered solutions from the fabricated hydrogels was allowed to proceed for three weeks. Samples were taken from the solutions at specified time points and analyzed using an ELISA to determine the amount of the protein released from the gels at each time point. A graphical representation of the amount released from each type of hydrogel at each time point can be seen in *Figure 3*. The percent of SDF-1α released from each gel at each time point is provided in *Figure 4* and the percentages at each time point were determined considering the fact that each hydrogel was loaded with 15 ng of SDF-1α. From the provided figures, it is observed that the hydrogels that contained 20 wt% and 30 wt% methacrylated heparin had a slower and relatively more uniform release rate during the first eight days of the study when compared to the pure MAC gels and the gels with 10 wt% methacrylated heparin. After the first eight days,

the release of SDF-1α from the hydrogels containing 30 wt% methacrylated heparin increased dramatically. This increase was due to the fact that the hydrogels were beginning to degrade at this point, which led to the amount of the protein in each sample of buffer solution to be much higher than what was truly released from the gels, eventually leading the calculated final amount released to be impossibly high. Overall, 7.82±1.69 ng (52.2±10.1%), 9.18±1.76 ng (61.2±10.9%), 5.07±1.57 ng (33.8±9.62%), and 29.5±4.22 ng (197±27.1%) of the SDF-1α was released from the hydrogels containing 0 wt%, 10 wt%, 20 wt%, and 30 wt% methacrylated heparin, respectively. Before the degradation of the hydrogels containing 30 wt% methacrylated heparin occurred, the amount of the protein released was 5.17±1.70 ng (34.5±10.3%).

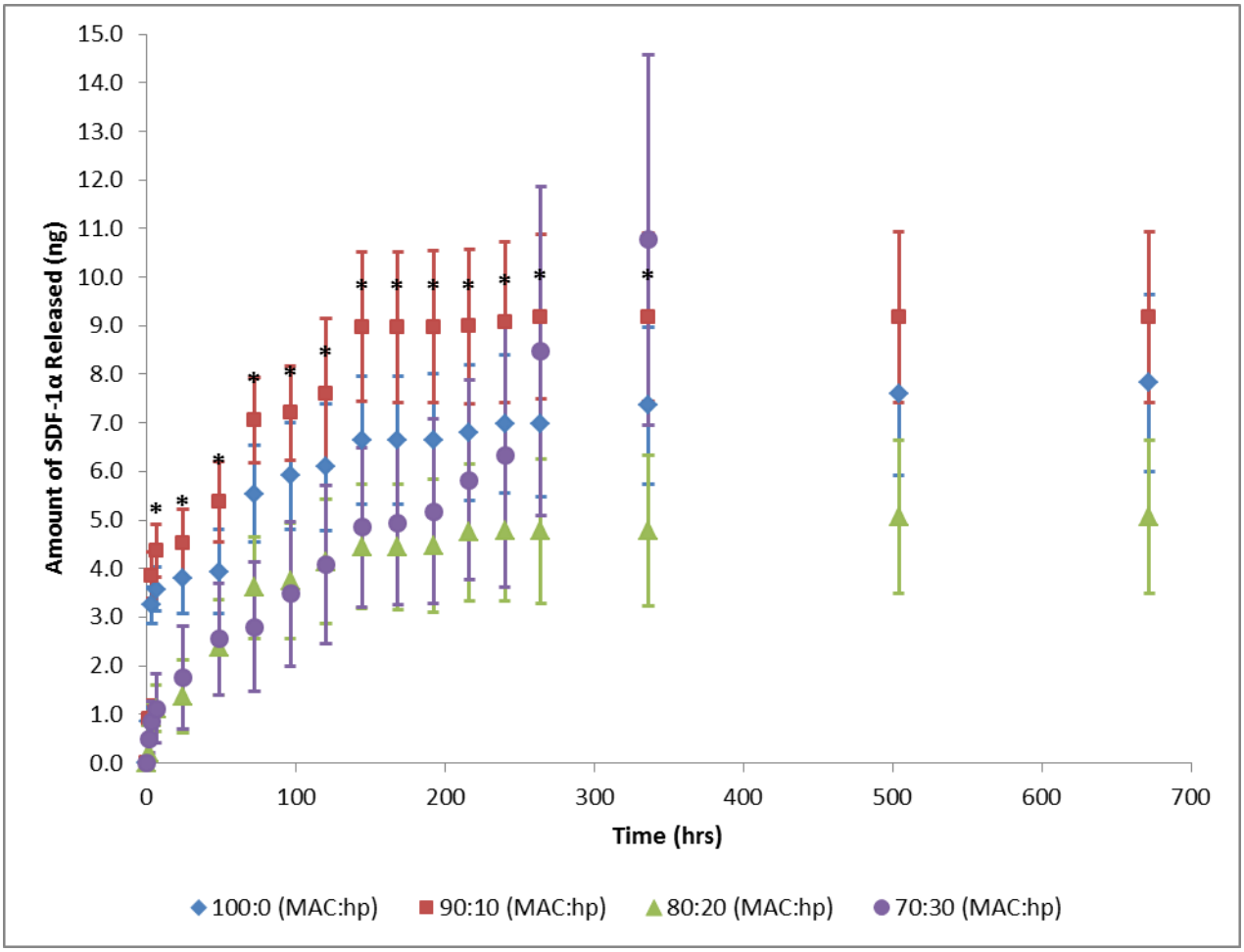

Figure 3: Amount of SDF-1α released from each type of hydrogel at each time point. *denotes significance for each hydrogel at that time point by single factor ANOVA with Tukey's *post hoc* analysis ($p<0.01$). Mean ± SD with n = 4.

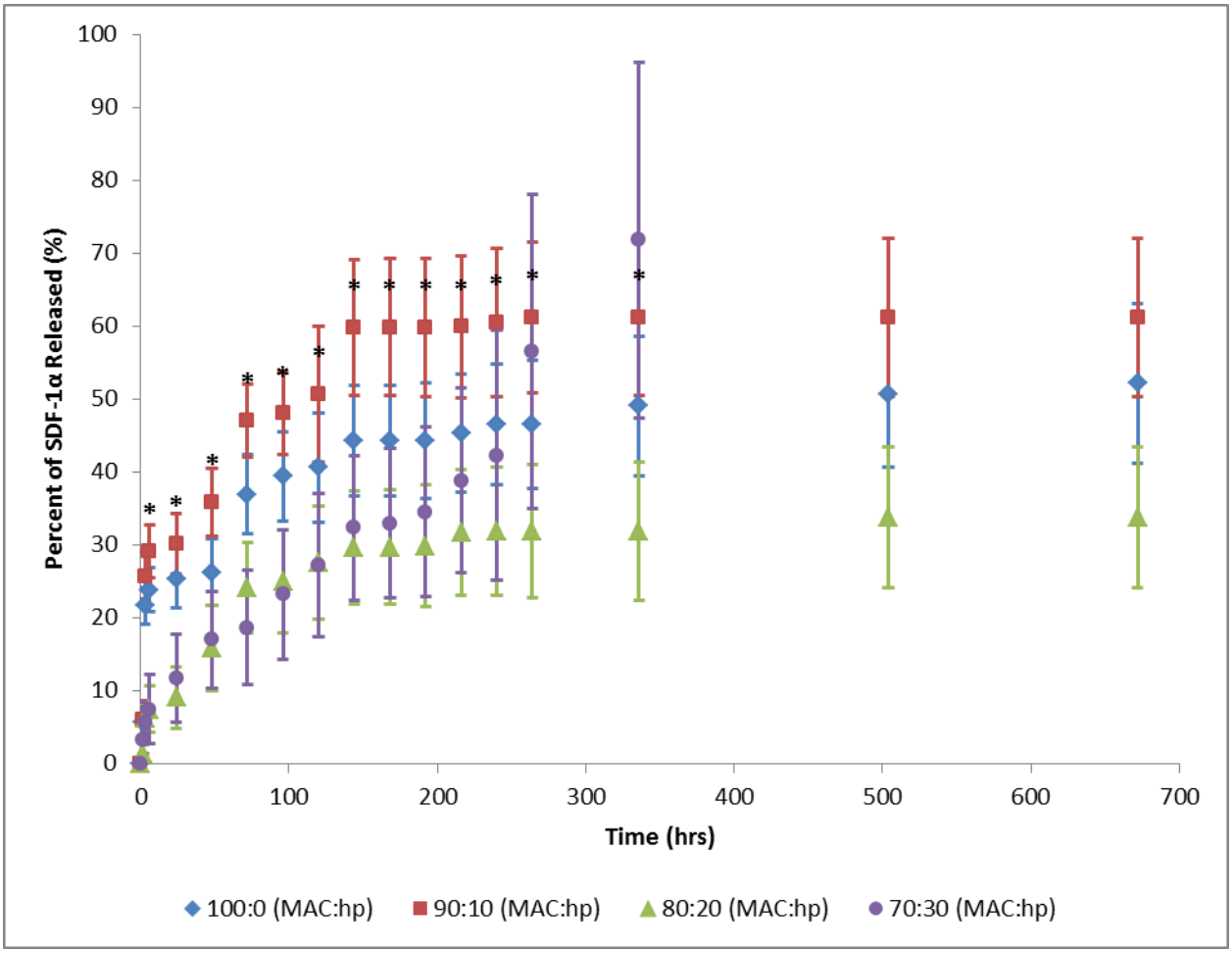

Figure 4: Percent of SDF-1α released from each type of hydrogel at each time point. *denotes significance for each hydrogel at that time point by single factor ANOVA with Tukey's *post hoc* analysis ($p<0.01$). Mean ± SD with n = 4.

Discussion/Analysis

The goal of this study was to determine if the release of proteins from hydrogels can be improved through the use of additives to increase hydrogel effectiveness in treating CNS injuries. It was proposed that a model protein, SDF-1α, be used due to its ability to recruit neural stem cells for the repair of central nervous system injuries and thus apply results from this study to future studies looking at MAC:heparin in the recruitment of stem cells and repair of nervous system injuries. It was also proposed that heparin be used as the additive in the study due to its ability to bind and slow the release of proteins as a result of its polar nature and subsequent electrostatic interactions with said proteins. The findings obtained from this study have shown that the modification of hydrogels can be successfully accomplished through the use of additives as well as showing that additives can be used to improve the release profile of hydrogels. Due to these results, this study can benefit from further improvements, including the use of different additives or gelation techniques, in order to develop a more effective hydrogel for healing properties.

From the study performed, it was shown that heparin can successfully undergo methacrylation as well as blend with methacrylamide chitosan to form modified MAC hydrogels. 33% of the heparin was shown to undergo methacrylation through the use of a ^{1}H NMR analysis. A study that was previously performed that also looked into the use of methacrylated heparin[5] achieved an average of 6% and 22% methacrylation of heparin. The differences in the amount of methacrylation are to be expected due to variations in the methods of the two studies. Also, as can be seen, the differences in the methacrylation found in the previous study were larger than the differences between this study and the previous one.

The rheometry that was performed on the three variations of the modified MAC hydrogels gave complex moduli of 5328±230.3 dyn/cm^2, 2983±32.91 dyn/cm^2, and 3874±297.8 dyn/cm^2 for the

gels containing 10 wt%, 20 wt%, and 30 wt% methacrylated heparin, respectively. From a previous study[9], pure MAC hydrogels were found to have an average complex modulus of 1890 dyn/cm^2. It was found in a prior study[10] that the native CNS displayed an elastic modulus in the range of 5,000 to 10,000 dyn/cm^2. Comparing this modulus to those of the modified hydrogels, the 10 wt% methacrylated heparin gel was in this range while the 20 wt% and 30 wt% gels were below this range. From another study[11] it was stated that the mechanical properties, specifically stiffness, of the hydrogel scaffolds had a significant impact on the differentiation of neural stem cells into the cell types that would be used to restore function to the CNS. In this study it was also stated that neural stem cells respond and differentiate when the stiffness of the gel is in the range of 1,000 to 100,000 dyn/cm^2. Thus, from the prior findings the modified hydrogels would likely induce differentiation of the stem cells when tested *in vivo* and *in vitro* due to their moduli being within the appropriate response range.

From the release study that was performed, the four variations of MAC hydrogels displayed varying release profiles of the SDF-1α that was loaded into each gel as well as releasing varying amounts of the protein overall. All hydrogels showed an initial burst of the protein during the first few hours of the study, however, both the gels containing 20 wt% and 30 wt% methacrylated heparin showed a smaller burst than the gels containing 0 wt% and 10 wt%. The 20 wt% and 30 wt% also showed a slower release of SDF-1α during the first eight days of the study. After this period of eight days the hydrogels containing 20 wt% methacrylated heparin continued showing a slow release of the protein compared to the other gels, however, the gels containing 30 wt% began to degrade resulting in the dramatic increase in the release of the protein. At the conclusion of the study the overall amounts of SDF-1α released were 7.82±1.82 ng, 9.18±1.76 ng, 5.07±1.57 ng, and 29.5±4.22 ng for the hydrogels containing

0 wt%, 10 wt%, 20 wt%, and 30 wt% methacrylated heparin, respectively. Before the degradation of the 30 wt% methacrylated heparin hydrogels occurred, the amount of the protein released was 5.17±1.70 ng.

From the results of both the rheometry and the release study, the hydrogels containing 20 wt% methacrylated heparin was shown to have a complex modulus relatively close to that of unmodified MAC hydrogel. The gels containing 20 wt% heparin also showed a slower release rate as well as a smaller initial burst of the SDF-1α when compared to the other three hydrogels, however the amount of the protein released overall was smaller than the other gels. As such, the hydrogels fabricated with 20 wt% methacrylated heparin were shown to have relatively unaffected stiffness compared to unmodified gels and therefore have a more favorable modulus compared to the other modified hydrogels. These gels were also shown to have a more desired release profile, one with a small or nonexistent initial burst of loaded protein and having a slower release rate of the protein.

A few recommendations are posed in regards to future work due to the results found from the study. One such recommendation is performing additional release studies involving heparin-modified MAC hydrogels in order to solidify the study findings. Another suggestion is conducting further work with methacrylated heparin in order to determine if improvements can be made to hydrogels containing heparin such that the an initial burst is nonexistent, the release profile is more linear, and the amount of protein released is closer to the amount that was loaded into the gels. Using other proteins in the study in order to test the effects of various protein properties on release kinetics is also proposed. Such effects that could be tested are the molecular weight and the isoelectric point of proteins as compared to SDF-1α (10,666 Da and 9.92 respectively). To test the effects of a higher molecular weight, the proteins Beta-nerve growth factor and bone

morphogenetic protein 2A could be used which have molecular weights of 26,959 Da and 44,702 Da, respectively. Higher and lower isoelectric points can also be tested by using fibroblast growth factor 2 and transforming growth factor beta-1, which have an isoelectric point of 11.18 and 8.83, respectively[2]. A final proposal is exploring different additives in order to determine if a better modification of the hydrogels exists to improve their release profile. One such additive to consider would be silk fibroin as it has been shown to have the ability to create hydrogels that produce a sustained release and disperse a majority of a loaded drug[3]. Different processing and gelation techniques should also be explored to create gels with uniform and sustained release profiles with desired properties such as being nontoxic and not causing damage to tissue around the injection site or having release characteristics that are pH or thermally dependent. Such techniques and processing have been explored in previous studies[12][13] and have been shown to slow the release of loaded drugs. One of these techniques is to graft a thermosensitive, pH-sensitive or other stimuli sensitive film onto an existing hydrogel. Typically these films have one structure at an environmental condition, such as expanding at a low temperature, and another structure at a different condition, such as shrinking at a high temperature[14]. This dependence on environmental conditions allows for the limiting of drug or protein release at certain conditions. This technique has been performed and shown to control the drug release rate as a function of the release solution temperature in a previous study using a poly(N-isopropylacrylamide) film grafted onto a poly(hydroxyethyl methacrylate) hydrogel[12]. Another technique is to embed microparticles (or other delivery vehicles) with a loaded drug or protein into a pre-crosslinked hydrogel. Once crosslinked, the hydrogels provide not only an additional physical barrier for the drug or protein but also a stimuli sensitive barrier. By implementing an additional barrier the release rate of either compound would be slowed, the initial burst typically seen in hydrogels possibly

eliminated, and the surrounding tissue near the composite hydrogel potentially protected from any toxic effects caused by the microparticles. The stimuli sensitivity of the hydrogel would also limit the release of the drug or protein at certain conditions[14]. This technique has been investigated and proven to work using poly(lactic-co-glycolic acid) microparticles loaded with 5-fluorouracil embedded in a thermosensitive chitosan hydrogel[13].

Conclusions

First, we were able to conclude that heparin can be successfully methacrylated and subsequently blended in various weight percentages with MAC to form stable hydrogels. From the results obtained from the rheology performed on the modified hydrogels, gels that were fabricated with 20 wt% methacrylated heparin had a more similar complex modulus to pure MAC hydrogels when compared with the gels containing 10 wt% and 30 wt% heparin. These differences in modulus could be due to experimental differences, such as different crosslinking times or very slightly higher amounts of photoinitiator being used for the gels. Both of these differences would lead to stiffer gels which would subsequently lead to a higher complex modulus. Lastly, although less of the SDF-1α was released, the hydrogels containing 20 wt% methacrylated heparin showed a slower release rate of the protein than the pure MAC gels. For eight days, the gels with 30 wt% methacrylated heparin also showed a slower rate, but these gels started to degrade after this time. These slower release rates could be due to an increase in electrostatic interactions between the methacrylated heparin and the SDF-1α as a result of the higher amount of heparin in the hydrogels. Due to its polar nature, heparin interacts electrostatically with proteins, primarily growth factors, as well as acting as a storage unit for these molecules, both of which allow for heparin to bind to proteins and slow their release[1]. As such, the hydrogels fabricated with 20 wt% methac-

rylated heparin were shown to have a more similar complex modulus to unmodified gels than the other modified hydrogels as well as having a more desired release profile, one with a small or nonexistent initial burst of loaded protein and a slower release rate of the protein.

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Appendix

Percent Methacrylation of Heparin Calculation:

$$Percent\ methacyrlation\ of\ heparin\ = \frac{\frac{1}{2}(I_1 + I_2)}{\frac{1}{9}(I)} \times 100\%$$

$$I_1 = 0.01$$

$$I_2 = 0.01$$

$$I = 0.27$$

$$Percent\ methacrylation\ of\ heparin\ = \frac{\frac{1}{2}(0.01 + 0.01)}{\frac{1}{9}(0.27)} \times 100\%$$

$$Percent\ methacrylation\ of\ heparin = \frac{0.01}{0.03} \times 100\% = 33.3\%$$

Mass of SDF-1α Released at Time i Calculation:

For the mass of SDF-1α released after 3 hours for pure MAC hydrogels:

$$M_i = C_i V + \sum C_{i-1} V_s$$

$$M_{3hrs} = C_{3hrs} V + (C_{1.5hrs} V_s + C_{0hrs} V_s)$$

$$C_{0hrs} = 0\ ng/\mu L$$

$$M_{3hrs} = C_{3hrs} V + C_{1.5hrs} V_s$$

$$C_{1.5hrs} = 1.72\ ng/\mu L$$
$$V_s = 0.5\ \mu L$$
$$C_{3hrs} = 4.78\ ng/\mu L$$
$$V = 0.5\ \mu L$$

$$M_{3hrs} = 4.78\ ng/\mu L(0.5\ \mu L) + 1.72\ ng/\mu L(0.5\ \mu L)$$
$$M_{3hrs} = 3.25\ ng + 0.86\ ng$$
$$M_{3hrs} = 3.25\ ng$$