Published in final edited form as:

Biomater Sci. 2016 August 16; 4(9): 1371–1380. doi:10.1039/c6bm00455e.

Heparin-based Hydrogels with Tunable Sulfation & Degradation for Anti-Inflammatory Small Molecule Delivery

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Abstract

Sustained release of anti-inflammatory agents remains challenging for small molecule drugs due to their low molecular weight and hydrophobicity. Therefore, the goal of this study was to control the release of a small molecule anti-inflammatory agent, crystal violet (CV), from hydrogels fabricated with heparin, a highly sulfated glycosaminoglycan capable of binding positively-charged molecules such as CV. In this system, both electrostatic interactions between heparin and CV and hydrogel degradation were tuned simultaneously by varying the level of heparin sulfation and varying the amount of dithiothreitol within hydrogels, respectively. It was found that heparin sulfation significantly affected CV release, whereby more sulfated heparin hydrogels (Hep and Hep^{-N}) released CV with near zero-order release kinetics (R-squared values between 0.96-0.99). Furthermore, CV was released more quickly from fast-degrading hydrogels than slow-degrading hydrogels, providing a method to tune total CV release between 5-15 days while maintaining linear release kinetics. In particular, N-desulfated heparin hydrogels exhibited efficient CV loading (~90% of originally included CV), near zero-order CV release kinetics, and maintenance of CV bioactivity after release, making this hydrogel formulation a promising CV delivery vehicle for a wide range of inflammatory diseases.

Introduction

Acute inflammation as a result of trauma or biomaterial implantation is thought to occur over approximately 1 week, but unresolved chronic inflammation can occur over weeks to months for a variety of conditions from arthritis to inflammatory bowel disease.¹⁻³ Although treatment would ideally span the entire time course of inflammation, the sustained release of small molecule anti-inflammatory drugs in particular remains a challenge. Due to their low molecular weight, small molecules can rapidly diffuse from the drug delivery system, often resulting in a bolus release of drug.^{4,5} Previous methods to fabricate small molecule delivery systems have included layer-by-layer films, microparticles, nanoparticles, and hydrogels.^{4,6,7}

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].
See DOI: 10.1039/x0xx00000x

Multi-month small molecule release has been demonstrated from poly (L-glutamic acid)-based layer-by-layer films.⁴ However, though promising, drawbacks to layer-by-layer films include a time-consuming fabrication process and poor small molecule entrapment resulting in exponential drug release kinetics.^{8,9} Furthermore, although several methods exist for the incorporation of small molecules into microparticles and nanoparticles, small molecule release is dependent on the amount of surface-associated drug compared to the amount encapsulated within the particles, and it is therefore difficult to achieve linear release in such systems.^{6,10,11} Thus, an efficient method for loading and releasing small molecules, such as small molecule anti-inflammatories, in a sustained, linear manner would be extremely useful.

Hydrogels are a promising drug delivery vehicle for small molecules, in part because of their simple and rapid fabrication. In our laboratory and others, hydrogel crosslinking methods such as free radical polymerization and click chemistry have frequently been implemented and allow for hydrogel gelation to occur within minutes.¹²⁻¹⁴ Hydrogels also provide a means for localized drug delivery to the site of injury, thereby increasing drug efficacy and avoiding harmful side effects often encountered with systemic administration.¹⁵ Despite these advantages, a majority of current hydrogel-based systems rely on hydrophobic interactions or physical entrapment to load small molecules of interest, which hinders the overall tunability of subsequent small molecule release.^{16,17} To improve drug loading and to control release more effectively, electrostatic interactions between charged biomaterials and oppositely-charged small molecules have been utilized.^{5,18,19} These include N-isopropylacrylamide and acrylic acid,⁵ low molecular weight gelators,¹⁸ and chondroitin 4-sulphate¹⁹ for the delivery of bupivacaine,⁵ 8-aminoquinoline,¹⁸ and vasopressin, respectively.¹⁹ However, depending on the choice of carrier, burst release can still occur⁵ or the entire drug cargo may be released within hours.¹⁸ Thus, a hydrogel-based system that can electrostatically bind charged small molecules to effectively control loading and sustain release over the course of weeks has yet to be fully achieved.

In this study, we have developed a tunable, negatively-charged hydrogel system for the delivery of a cationic small molecule, crystal violet (CV). Though historically used as a stain to identify gram-positive bacteria,²⁰ CV has also shown to have anti-bacterial, anti-fungal and more recently, anti-inflammatory properties in part by acting as a NADPH oxidase and angiopoietin-2 (ang-2) inhibitor.^{21,22} NADPH oxidase is a primary reactive oxygen species (ROS)-producing enzyme in inflammation and regulates ang-2 production.^{23,24} In turn, ang-2 is also pro-inflammatory and is required to induce the acute inflammatory response *in vivo*.^{23,25,26} Compared to CV's long history as a topical anti-bacterial and anti-fungal treatment,²³ CV has only recently been recognized as a promising anti-inflammatory small molecule and, to our knowledge, has never before been released from a hydrogel-based drug delivery system. Thus, the development of a tunable hydrogel system for the delivery of CV may be a novel method for the treatment of many inflammatory conditions.

In these studies, we employed two methods to tune the release of CV from our hydrogels, including the incorporation of (1) heparin, a highly sulfated glycosaminoglycan (GAG), and (2) dithiothreitol (DTT). The introduction of DTT results in increased hydrolytic degradation of the resulting acrylated PEG–DTT network, likely due to the proximity of the thiol group

and the ester linkage in the final polymer.^{27,28} Heparin, with its high degree of negative charge along its backbone, has been previously utilized as a component in hydrogels to provide sustained release of a wide variety of positively-charged growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and members of transforming growth factor family (TGF, e.g. BMPs), among others.²⁹⁻³² Following from these results, in previous work from our laboratory and others, to further tune interactions between the cargo and the carrier, heparin derivatives of varying sulfation levels ranging from fully sulfated to fully desulfated were synthesized and crosslinked within hydrogels and microparticles.^{12,33-39} Positively-charged proteins such as stromal cell-derived factor-1 (SDF-1), bone morphogenetic protein-2 (BMP-2), and VEGF were then loaded on or into these biomaterials for controlled release based on electrostatic affinity.^{12,33-39}

Similarly, we believe that interactions between the negatively-charged heparin molecules and the positively-charged small molecule CV will result in sustained release of CV. Specifically, we hypothesized that systematically reducing the degree of heparin sulfation within hydrogels would reduce CV binding and, in turn, promote faster CV release kinetics. In addition, by varying the concentration of DTT within hydrogels we hypothesized that the CV release would be further enhanced as the hydrogel degraded. Overall, we hypothesized that altering heparin sulfation and the degradation rate of hydrogels would enable us to control the release of CV, allowing for potential future treatment of sustained inflammatory conditions.

Results

Heparin characterization

Four heparin derivatives were prepared through solvolytic desulfation, including Hep, Hep^{-N}, Hep^{-N,-6O}, and Hep- with 100, ~80, ~20, and 0% total sulfation, respectively (Figure 1).³⁴ Furthermore, ¹H NMR indicated that the heparin derivatives were MAm-functionalized with 13, 11, 8, and 8% functionalization per saccharide unit for Hep, Hep^{-N}, Hep^{-N,-6O}, and Hep-, respectively (Figure S1).

CV binding to soluble heparin derivatives

Using a UV-vis spectrometer, unbound CV showed a characteristic absorption peak at 590 nm and when incubated with heparin, an absorption peak at 490 nm formed, which was attributed to the binding of CV and heparin.⁴⁰ Thus, the ratio between absorption peaks at 490 and 590 nm (490/590 absorption ratio) was used to measure the binding between CV and heparin (Figure 2).

Except for Hep-, as each soluble heparin or heparin-MAm derivative was added in solution with CV, the 490/590 absorption ratio increased dramatically between 0 to 100 µg/mL heparin. Additionally, maximum 490/590 absorption ratios correlated to the level of heparin sulfation, where the most sulfated heparin derivative, Hep, exhibited the highest 490/590 absorption ratio, and the most desulfated heparin derivative, Hep-, exhibited the lowest. For most heparin derivatives, however, no further increase in the 490/590 absorption ratio was observed with heparin concentrations above 200 µg/mL.

Hydrogel degradation

Hydrogels were formed via free radical polymerization with 10 wt% heparin-MAm, 90 wt% PEGDA, and varying concentrations of DTT. Gelation kinetics, fold swelling, and storage moduli for each formulation are included in the supplemental information (Figure S1-S6 and Table S2-S3). Slow-degrading hydrogels, which contained 30 mol% DTT, required 9-13 days for degradation, whereas fast-degrading hydrogels, which contained 42 mol% DTT, required only 3-9 days (Table 1 and Figure S2-S3). Furthermore, more sulfated heparin hydrogels (Hep and Hep^{-N}) required more time for degradation than less sulfated heparin hydrogels (Hep^{-N,-6O} and Hep-) with the same DTT concentration.

CV loading and release from hydrogels

All Hep and Hep^{-N} hydrogels loaded ~90% of the originally included CV, significantly more than Hep^{-N,-6O} and Hep-, which loaded only 40-80% of the originally included CV (Figure 3).

Regardless of heparin sulfation, however, all non-degradable heparin hydrogels loaded significantly more CV than PEGDA-only hydrogels. Thereafter, CV release was examined over 3-15 days and most Hep and Hep^{-N} hydrogels exhibited near-zero order release kinetics of CV with R-squared values between 0.96-0.99 after linear regression analysis (Figure 4 and Table 2).

In contrast, significantly more burst release was observed from the more desulfated heparin (Hep^{-N,-6O} and Hep-) and PEGDA-only hydrogels at day 1, followed by a plateau in release over time. The total release from the PEGDA-only hydrogels (averages over 100% in some cases) is attributed to the large variance and very low amounts released per time point after day 3. In terms of linearity of release, the R-squared values for Hep and Hep^{-N} were greater than all Hep^{-N,-6O} and Hep- R-squared values, though non-degradable and slow-degrading Hep^{-N,-6O} hydrogels still maintained R-squared values between 0.88-0.90. Heparin sulfation also affected the rate of CV release, whereby more sulfated heparin hydrogels (Hep and Hep^{-N}) required more time to release an equivalent amount of CV as the more desulfated heparin hydrogels (Hep^{-N,-6O} and Hep-) (Table 3-5).

Moreover, for non-degradable gels, total cumulative release from Hep and Hep^{-N} hydrogels ranged between 50-65% of loaded CV, whereas cumulative release from Hep^{-N,-6O} and Hep- hydrogels ranged between 70-100% of loaded CV, again suggesting that CV release was slower from the more sulfated heparin hydrogels.

Finally, comparisons between the slow- and fast-degrading hydrogels revealed that hydrogel degradation also affected CV release, whereby fast-degrading hydrogels required less time to release an equivalent amount of CV as slow-degrading hydrogels with the same heparin derivative (Table 4-5). For example, fast-degrading Hep hydrogels required 5 days to release ~60% of the loaded CV, whereas slow-degrading Hep hydrogels required 13 days.

CV bioactivity after hydrogel loading and release

Given that CV has previously shown to inhibit bEnd.3 cell ang-2 gene expression, hydrogel supernatant was incubated with bEnd.3 cells to assess the bioactivity of CV after hydrogel

loading and release.²² Hep^{-N} hydrogels were chosen for this experiment as they showed the most linear, long-term release of CV and, due to their diminished anti-coagulation properties compared to fully sulfated heparin, may be a safer alternative for *in vivo* therapies.^{41,42} Furthermore, only the supernatant from the last time point prior to hydrogel degradation was used. After 6.5 hours incubation, ang-2 gene expression was significantly more inhibited in the 20 μ M soluble CV and Hep^{-N} hydrogel supernatant groups, which also contained $\sim$ 20 μ M CV, than in the no treatment control (Figure 5). In addition, ang-2 gene expression in Hep^{-N} hydrogel supernatant groups was not significantly different from the 20 μ M soluble CV control, indicating that CV maintained its bioactivity after loading and release from hydrogels.

Discussion

In this work, heparin/PEGDA hydrogels were fabricated as a novel approach to treat sustained inflammatory conditions through the release of CV, a small molecule recently shown to possess anti-inflammatory properties.²² Unlike many current small molecule delivery systems that rely on hydrophobic interactions and physical entrapment,^{16,17} electrostatic interactions between negatively-charged heparin and positively-charged CV were used to enhance control of CV release kinetics. Two approaches were used to further tune CV release including, (1) altering the degree of heparin sulfation to vary CV binding to hydrogels and (2) altering the concentration of DTT to vary hydrogel degradation. Ultimately, hydrogel formulations included non-degradable, slow-degrading, and fast-degrading hydrogels with PEGDA and one of four heparin derivatives (Figure 1).

We first analyzed how the degree of heparin sulfation affected CV binding by comparing absorption peaks of CV in solution with each heparin derivative. Overall, as heparin sulfation decreased, CV binding to each heparin derivative decreased as well, likely due to the diminished electrostatic interactions between the positively-charged CV and the negatively-charged sulfate groups of heparin (Figure 2). It is well known that heparin can bind to a range of positively-charged growth factors, and the electrostatic interactions between heparin and CV have also been documented.^{40,43,44} Thus, in accordance with previous work, heparin was found to electrostatically bind cationic CV and, furthermore, altering the level of heparin sulfation provided a means to alter CV binding within our heparin-based hydrogels.

Next, hydrogels were fabricated via free radical polymerization with 0, 30, or 42 mol% DTT for non-degradable, slow, and fast-degrading hydrogels, respectively (Figure 1). Full gelation occurred within 5-7 minutes and was similar for all formulations. For all hydrogels, CV loading efficiency was found to be highest in the Hep and Hep^{-N} hydrogels ($\sim$ 90% of originally included CV), which was expected given the greater CV binding to these soluble heparin derivatives compared to more desulfated heparin (Figure 3). In addition to the electrostatic interactions between heparin and CV, however, the hydrophobicity of CV likely contributed to the loading efficiency of CV as well. With less swelling at day 0 in the Hep and Hep^{-N} hydrogels compared to the Hep^{-N,-6O} and Hep- hydrogels (Figure S2 and S3), the hydrophobic CV molecules were more likely to remain within the more sulfated heparin hydrogels, ultimately encouraging CV loading. Regardless of heparin sulfation, all heparin-

based hydrogels loaded significantly more CV than PEGDA-only hydrogels, indicating that the heparin within hydrogels significantly contributed to the loading of CV. In previous work, one study using low molecular weight gelators observed 100% loading of an uncharged small molecule, primarily through physical entrapment, although it was quickly released within 40 minutes.¹⁸ In contrast, when using a charged small molecule, only 45% loading efficiency was reported, and this decreased efficiency was attributed to the increased acid-base interactions between the hydrogel and small molecule.¹⁸ Overall, our results indicate heparin-based hydrogels are an efficient system for loading CV.

For the degradable formulations, fast-degrading hydrogels required between 3-9 days to degrade whereas slow-degrading hydrogels of the same heparin derivative required at least 4 additional days for complete degradation (Table 1). It should be noted that, in general, as more DTT was added to the network for each heparin derivative, there was greater day 0 swelling and reduced storage modulus (Figures S1-S6), suggesting a lower crosslinking density, which would further enhance degradation.^{45,46} Furthermore, for both types of degradable hydrogels, Hep and Hep^{-N} hydrogels required at least 2 additional days to degrade than Hep^{-N,-6O} and Hep- hydrogels, possibly due to the decreased functionalization in the more desulfated heparin derivatives (Figure S1). With fewer methacrylamide groups to bind Hep^{-N,-6O} and Hep- to PEGDA, it is possible that more DTT was able to bind to PEGDA instead, resulting in more DTT within the hydrogel network and subsequently faster degradation. It is important to note, however, that there are likely several factors at play that may affect gel degradation, including the overall charge of the heparin derivative utilized.³⁴ Overall, while these results suggest that to achieve a desired degradation time, DTT amounts may need to be selected based on the particular heparin derivative used, overall, varying the DTT concentration within hydrogels allowed for the fabrication of hydrogels that fully degraded over a range of 3 to 13 days. In contrast, a small molecule-releasing hydrogel composed of chondroitin 4-sulphate, which is a GAG similar to heparin,⁴⁴ was non-degradable,¹⁹ which is usually undesirable when implanting such drug delivery vehicles *in vivo*.

It was found that CV release kinetics from both degradable and non-degradable Hep and Hep^{-N} hydrogels were near zero-order over 5-15 days with R-squared values between 0.96-0.99 (Figure 4 and Table 2). For the Hep and Hep^{-N} hydrogels, the greater electrostatic interactions between heparin and CV likely encouraged the retention of CV, causing significantly less burst release compared to the more desulfated heparin hydrogels. Furthermore, as the degradable Hep and Hep^{-N} hydrogels exhibited significantly less swelling than the Hep^{-N,-6O} and Hep- hydrogels, the hydrophobic CV molecules may have been more easily retained, again contributing to the more sustained, linear release observed (Figure S3). Total CV release also appeared to be affected by heparin sulfation, whereby Hep and Hep^{-N} hydrogels released lower total fractions of loaded CV than Hep^{-N,-6O} and Hep- hydrogels regardless of rate of degradation (Figure 4). For the non-degradable hydrogels, though release time points were discontinued after 15 days, it is expected that the Hep and Hep^{-N} hydrogels would continue to release additional CV in a linear manner until 100% of loaded CV was released.

For the degradable hydrogels, degradation was defined as the loss of three-dimensional hydrogel integrity, though small fractions of the hydrogel still remained in solution, which is a potential explanation as to why 100% cumulative CV release was not observed. Varying the amount of DTT within hydrogels altered CV release; at least 2 additional days were required to release ~60% of loaded CV from slow-degrading hydrogels as fast-degrading hydrogels with the same heparin derivative (Table 3-5). While increasing the amount of DTT within hydrogels for a given heparin derivative did, in many cases, reduce the storage modulus compared to the non-degradable formulations (Figure S4-S6), it is important to note that, within a single DTT concentration the storage moduli were statistically similar between most heparin types, suggesting that the differences in release profiles observed were dictated more by affinity to the heparin than mechanical properties of the gel. Combining these results, it is likely that heparin-CV electrostatic interactions played a large role in determining linearity of release kinetics, although degradation of the hydrogel matrix affected the overall time course of drug delivery.

In previous work, other hydrogel systems have also exhibited linear small molecule release, although in one case the small molecule was rapidly released within hours,¹⁶ and in another case, the small molecule dexamethasone was conjugated to peptide amphiphiles prior to hydrogel formation to achieve linear release over at least 1 month.⁴⁷ In studies most similar to our system, where electrostatic interactions were utilized to further control the loading and release of small molecules, various materials have been used, including N-isopropylacrylamide and acrylic acid,⁵ low molecular weight gelators,¹⁸ peptides,⁴⁸ and chondroitin 4-sulphate.¹⁹ For many of these materials, while linear release was obtained over some period of time, complete release occurred in less than 90 minutes,¹⁸ or release was tracked only on the order of hours¹⁹ to 1 week.⁴⁸

Moreover, whereas previous systems investigated model small molecules and dyes^{5,16,48} or commonly used drugs such as dexamethasone,⁴⁷ to our knowledge no prior research has investigated the controlled loading and release of CV. Using a cell-based bioactivity assay, CV maintained its anti-inflammatory activity after release from Hep^{-N} hydrogels, even after remaining within the hydrogel for 9-15 days (Figure 5). Similarly, other small molecules such as dexamethasone have shown to retain their bioactivity after release from hydrogels, due in large part to the greater stability of small molecules compared to larger biologics.^{47,49} In previous studies from our laboratory using desulfated heparin-based biomaterials with growth factors such as BMP-2, BMP-2 denatured rapidly and made it difficult to elucidate the effect of heparin sulfation on BMP-2 release kinetics.^{34,50} Using a small molecule, however, it is evident that heparin sulfation within the hydrogel matrix significantly affected small molecule loading and release, and that the more sulfated heparin hydrogels were able to release bioactive CV with near zero-order release kinetics over at least two weeks. Finally, as desulfated GAGs have shown to have diminished anti-coagulation properties over fully sulfated heparin,^{41,42} Hep^{-N} hydrogels may be a safer alternative for future *in vivo* therapies.

Experimental

Preparation of heparin derivatives

N-desulfated heparin (Hep^{-N}), 6O,N-desulfated heparin (Hep^{-N,-6O}), and fully desulfated heparin (Hep⁻) were prepared as described previously.⁵⁰ Briefly, heparin sodium salt from porcine intestinal mucosa (Sigma Aldrich, St. Louis, MO) was reconstituted at ~10 mg/mL in water and passed through Dowex 50WX4 resin (mesh size 100-200, Sigma). Pyridine was added drop-wise to the desalted heparin until the pH of the solution was ~6.0. Excess water and pyridine were removed on a rotary evaporator (Buchi). The heparin pyridinium salt solution was flash frozen in liquid nitrogen, lyophilized to a powder, and stored at -20°C.

For Hep^{-N} preparation, heparin pyridinium salt was dissolved at 1 mg/mL in 90% v/v dimethyl sulfoxide (DMSO)/10% water and mixed at 50°C for 2 hours.^{51,52} For Hep^{-N,-6O}, heparin pyridine was dissolved at 10 mg/mL in 90% v/v N-methylpyrrolidone (NMP, Acros Organics, Belgium)/10% water and maintained at 90°C for 48 hours.⁵³ Hep⁻ was prepared under identical conditions to Hep^{-N,-6O} but the reaction proceeded at 100°C for 24 hours. Following each reaction, each heparin solution was cooled on ice and precipitated with 95% ethanol saturated with sodium acetate. The heparin precipitates were stirred for 2 hours on ice and centrifuged to remove excess ethanol and water. The resulting material was dissolved in water, dialyzed for 3 days, lyophilized to a powder, and stored at -20°C.

To functionalize heparin derivatives with methacrylamide (MAM) groups, 200 mg heparin, 180 mg N-hydroxysulfosuccinimide (sulfo-NHS, Sigma), 180 mg N-(3-Aminopropyl) methacrylamide hydrochloride (APMAM, Polysciences Inc.), and 300 mg *N*-Ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma) were dissolved in 10 mL phosphate buffer saline (PBS) solution. After stirring on ice for 6 hours, the heparin-MAM was dialyzed for 2 days, lyophilized to a powder, and stored at -20°C. After synthesis, each derivative was analyzed via proton NMR (¹H NMR) to determine extent of functionalization (see Supplemental Information.)

Preparation of poly (ethylene glycol) diacrylate

Poly (ethylene glycol) (PEG, 3.4 kDa, Sigma) was reacted with acryloyl chloride (AcCl, Sigma) in an 8:1 AcCl to PEG molar ratio in dichloromethane (DCM) solution.⁵⁴

Triethylamine (TEA, Sigma) was added drop-wise in a 1:1 TEA to AcCl molar ratio as a catalyst to yield linear PEG-diacrylate (PEGDA).

CV binding to soluble heparin derivatives

In 1 mL of PBS solution, 250 µg/mL CV was incubated with 0-1000 µg/mL of each of the heparin derivatives before and after the addition of methacrylamide groups. The absorbance was then assessed at 490 and 590 nm using a UV-vis spectrometer (SpectraMax M2, Molecular Device), where absorbance at 490 nm was characteristic of CV bound to heparin and absorbance at 590 nm was characteristic of unbound CV (n = 3).⁴⁰

Hydrogel fabrication and degradation

To 800 μ L distilled water, 90 mg PEGDA (~ 0.03 mmol) along with 0, 30, or 42 mol% dithiothreitol (DTT, Sigma) relative to PEGDA, were added for non-degradable, slow, and fast-degrading hydrogels, respectively. After incubating at 37°C for 2 hours, 20 mg CV (25 mg/mL) was dissolved into the PEGDA solution. Next, 30 μ L of the CV/PEGDA solution, 3.3 μ L of each heparin derivative (10 mg/mL), 3.3 μ L 1 M ammonium persulfate (APS, Sigma), and 3.3 μ L 1 M N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma) were added to cylindrical Teflon molds (6 x 1 mm). Finally, hydrogel molds were incubated at 37°C for 10 minutes to ensure complete crosslinking, resulting in 10 wt% heparin/90 wt% PEGDA hydrogels. Hydrogel degradation was monitored at 37°C over the time course of CV release, and complete degradation was defined as the loss of three-dimensional hydrogel integrity where only small hydrogel fragments remained in solution ($n = 3$).

CV loading and release from hydrogels

Once fabricated, each hydrogel was incubated at 37°C in 1 mL PBS solution. After 3 hours incubation, the supernatant was removed and replaced with 1 mL fresh PBS solution. Assuming that the CV released over the initial 3 hours was never well incorporated within the hydrogel, the CV in the supernatant at 3 hours was subtracted from the initial CV included (750 μ g CV total) to determine the amount of CV loaded into the hydrogel. Following this loading procedure, the hydrogels continued to incubate at 37°C and the supernatant was collected every 2 days from day 1 until the hydrogel degraded. CV release was quantified using a UV-vis spectrometer (SpectraMax M2, Molecular Device) at 590 nm ($n = 3$). Linear regression analysis was performed and R-squared values were recorded with Microsoft Excel software under the assumption that at 0 hours, no CV had released and therefore, the data point (0,0) was included when creating linear regressions.

CV bioactivity after hydrogel loading and release

CV has previously shown to inhibit bEnd.3 cell (ATCC CRL 2299) ang-2 gene expression in a dose-dependent manner.²² Therefore, to assess CV bioactivity bEnd.3 cells were grown to confluency in 24-well plates with 1 mL media consisting of 4.5 g/mL glucose Dulbecco's Modified Eagle Medium (DMEM, Cellgro, Manassas, VA), 10% v/v fetal bovine serum (FBS, Atlanta Biologics, Atlanta, GA), 1.4% v/v L-glutamine (Cellgro), 1.4% v/v antibiotic-antimitotic, and 0.1% vascular endothelial growth factor (VEGF). Once confluent, cells were treated with 20 μ M soluble CV or release supernatant from non-degradable, slow, and fast-degrading Hep^N hydrogels from the final release time point prior to hydrogel degradation. Cells treated with media only were used as no treatment controls ($n = 4$).

All groups were incubated for 6.5 hours at 37°C and 95% O₂, 5% CO₂, after which the cells were lysed with 350 μ L RLT lysis buffer (Qiagen, Hilden, Germany). The cell lysates were further filtered using a QIAshredder tissue homogenizer and mRNA was extracted with an RNeasy kit (Qiagen). cDNA was generated using SuperScript III Reverse Transcriptase (Invitrogen) with Oligo(dT)15 primers and dNTP Mix/PCR nucleotide mix (Promega). The gene expression was analyzed using quantitative PCR amplification performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) in the presence of SYBR Green/ROX master mix (Life Technologies, Carlsbad, California). The raw fluorescence

data were first analyzed using LinRegPCR software to more accurately determine individual PCR efficiency and mRNA starting concentration. Custom-designed primers were used for ang-2 and the endogenous control, 40S ribosomal protein S18 (RSP18), and primer sequences can be found in the Supplementary Information (Table S1). Ang-2 gene expression was normalized to RSP18 expression levels and to the untreated control group to obtain relative expression values ($n = 4$).

Statistical analysis

All data are presented as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) and Tukey's post hoc multiple comparison test with a significance value set at $p = 0.05$ were used to identify significant differences. Statistical analysis was performed with Minitab (v15.1).

Conclusions

Hydrolytically degradable heparin/PEGDA hydrogels were fabricated for the ultimate purpose of loading and releasing a novel small molecule anti-inflammatory drug, CV. It was demonstrated that negatively-charged heparin hydrogels can load positively-charged CV, with Hep and Hep^{-N} hydrogels exhibiting ~90% CV loading efficiency. Moreover, both electrostatic interactions and hydrogel degradation can be tuned to control CV release kinetics and CV release rates from hydrogels over time. Most strikingly, our results indicate that Hep and Hep^{-N} hydrogels exhibit near-zero order release kinetics of CV over a range of 5-15 days. Ultimately, Hep and Hep^{-N} hydrogels could be employed in the future as efficient, cationic small molecule delivery vehicles with linear release kinetics for a wide range of inflammatory injuries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to acknowledge The Arbiser Lab from Emory University School of Medicine and Atlanta VA Medical Center for their assistance with the bEnd.3 cell *in vitro* assay and Chris Johnson of the García Lab from Georgia Tech for his assistance with the rheometry experiments. We would also like to acknowledge our funding sources, NSF Stem Cell Biomanufacturing IGERT (DGE 0965945) and NIH (R01 AR063692).

References

1. Temenoff, JS.; Mikos, AG. Biomaterials: The Intersection of Biology and Materials Science. Pearson Prentice Hall; Upper Saddle River, New Jersey: 2008. p. 504
2. Ratner, BD.; Hoffman, AS.; Schoen, FJ.; Lemons, JE. Biomaterials Science: An Introduction to Materials in Medicine. 3rd ed. Academic Press; 2012. p. 1576
3. Fiocchi C. Inflammatory Bowel Disease: Etiology and Pathogenesis. Gastroenterology. 1998; 115(1):182–205. [PubMed: 9649475]
4. Hsu BB, Park MH, Hagerman SR, Hammond PT. Multimonth controlled small molecule release from biodegradable thin films. Proc Natl Acad Sci U S A. 2014; 111(33):12175–80. doi: 10.1073/pnas.1323829111. [PubMed: 25092310]

5. Sivakumaran D, Maitland D, Hoare T. Injectable microgel-hydrogel composites for prolonged small-molecule drug delivery. *Biomacromolecules*. 2011; 12(11):4112–20. doi: 10.1021/bm201170h. [PubMed: 22007750]
6. Whittlesey KJ, Shea LD. Delivery systems for small molecule drugs, proteins, and DNA: the neuroscience/biomaterial interface. *Exp Neurol*. 2004; 190(1):1–16. doi: 10.1016/j.expneurol.2004.06.020. [PubMed: 15473976]
7. Goldberg M, Langer R, Jia X. Nanostructured materials for applications in drug delivery and tissue engineering. *Journal of Biomaterials Science, Polymer Edition*. 2007; 18(3):241–268. doi: 10.1163/156856207779996931. [PubMed: 17471764]
8. Shukla A, Fuller RC, Hammond PT. Design of multi-drug release coatings targeting infection and inflammation. *J Control Release*. 2011; 155(2):159–66. doi: 10.1016/j.jconrel.2011.06.011. [PubMed: 21699932]
9. Moskowitz JS, Blaisse MR, Samuel RE, Hsu HP, Harris MB, Martin SD, Lee JC, Spector M, Hammond PT. The effectiveness of the controlled release of gentamicin from polyelectrolyte multilayers in the treatment of *Staphylococcus aureus* infection in a rabbit bone model. *Biomaterials*. 2010; 31(23):6019–30. doi: 10.1016/j.biomaterials.2010.04.011. [PubMed: 20488534]
10. Wischke C, Schwendeman SP. Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. *International Journal of Pharmaceutics*. 2008; 364(2):298–327. doi: 10.1016/j.ijpharm.2008.04.042. [PubMed: 18621492]
11. Raman C, Berkland C, Kim K, Pack DW. Modeling small-molecule release from PLG microspheres: effects of polymer degradation and nonuniform drug distribution. *Journal of Controlled Release*. 2005; 103(1):149–158. doi: 10.1016/j.jconrel.2004.11.012. [PubMed: 15773062]
12. Krieger JR, Ogle ME, McFaline-Figueroa J, Segar CE, Temenoff JS, Botchwey EA. Spatially localized recruitment of anti-inflammatory monocytes by SDF-1 α -releasing hydrogels enhances microvascular network remodeling. *Biomaterials*. 2016; 77:280–90. doi: 10.1016/j.biomaterials.2015.10.045. [PubMed: 26613543]
13. Tibbitt MW, Kloxin AM, Sawicki L, Anseth KS. Mechanical Properties and Degradation of Chain and Step Polymerized Photodegradable Hydrogels. *Macromolecules*. 2013; 46(7) doi: 10.1021/ma302522x.
14. McKinnon DD, Brown TE, Kyburz KA, Kiyotake E, Anseth KS. Design and characterization of a synthetically accessible, photodegradable hydrogel for user-directed formation of neural networks. *Biomacromolecules*. 2014; 15(7):2808–16. doi: 10.1021/bm500731b. [PubMed: 24932668]
15. Bhattarai N, Gunn J, Zhang M. Chitosan-based hydrogels for controlled, localized drug delivery. *Adv Drug Deliv Rev*. 2010; 62(1):83–99. doi: 10.1016/j.addr.2009.07.019. [PubMed: 19799949]
16. Sutton S, Campbell NL, Cooper AI, Kirkland M, Frith WJ, Adams DJ. Controlled release from modified amino acid hydrogels governed by molecular size or network dynamics. *Langmuir*. 2009; 25(17):10285–91. doi: 10.1021/la9011058. [PubMed: 19499945]
17. Koutsopoulos S, Unsworth LD, Nagai Y, Zhang S. Controlled release of functional proteins through designer self-assembling peptide nanofiber hydrogel scaffold. *Proc Natl Acad Sci U S A*. 2009; 106(12):4623–8. doi: 10.1073/pnas.0807506106. [PubMed: 19273853]
18. Friggeri A, Feringa BL, van Esch J. Entrapment and release of quinoline derivatives using a hydrogel of a low molecular weight gelator. *J Control Release*. 2004; 97(2):241–8. doi: 10.1016/j.jconrel.2004.03.012. [PubMed: 15196751]
19. Jensen M, Hansen PB, Murdan S, Frokjaer S, Florence AT. Loading into and electro-stimulated release of peptides and proteins from chondroitin 4-sulphate hydrogels. *European Journal of Pharmaceutical Sciences*. 2002; 15:139–148. [PubMed: 11849910]
20. Claus D. A standardized Gram staining procedure. *World Journal of Microbiology and Biotechnology*. 1992; 8:451–452. [PubMed: 24425522]
21. Bragulat MR, Abarca ML, Bruguera MT, Cabanes FJ. Dyes As Fungal Inhibitors: Effect on Colony Diameter. *Applied and Environmental Microbiology*. 1991; 57(9):2777–2780. [PubMed: 1768153]
22. Perry BN, Govindarajan B, Bhandarkar SS, Knaus UG, Valo M, Sturk C, Carrillo CO, Sohn A, Cerimele F, Dumont D, Losken A, Williams J, Brown LF, Tan X, Ioffe E, Yancopoulos GD, Arbiser JL. Pharmacologic blockade of angiopoietin-2 is efficacious against model hemangiomas

- in mice. *J Invest Dermatol.* 2006; 126(10):2316–22. doi: 10.1038/sj.jid.5700413. [PubMed: 16741507]
23. Maley AM, Arbiser JL. Gentian violet: a 19th century drug re-emerges in the 21st century. *Exp Dermatol.* 2013; 22(12):775–80. doi: 10.1111/exd.12257. [PubMed: 24118276]
 24. Dang PM, Stensballe A, Boussetta T, Raad H, Dewas C, Kroviarski Y, Hayem G, Jensen ON, Gougerot-Pocidallo MA, El-Benna J. A specific p47phox-serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites. *J Clin Invest.* 2006; 116(7):2033–43. doi: 10.1172/JCI27544. [PubMed: 16778989]
 25. Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzenzrath M, Rosseau S, Suttorp N, Sobke A, Herrmann M, Preissner KT, Vajkoczy P, Augustin HG. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. *Nat Med.* 2006; 12(2):235–9. doi: 10.1038/nm1351. [PubMed: 16462802]
 26. Fiedler U, Augustin HG. Angiopoietins: a link between angiogenesis and inflammation. *Trends Immunol.* 2006; 27(12):552–8. doi: 10.1016/j.it.2006.10.004. [PubMed: 17045842]
 27. Hudalla GA, Eng TS, Murphy WL. An Approach to Modulate Degradation and Mesenchymal Stem Cell Behavior in Poly(ethylene glycol) Networks. *Biomacromolecules.* 2008; 9:842–849. [PubMed: 18288800]
 28. Qiu Y, Lim JJ, Scott L Jr, Adams RC, Bui HT, Temenoff JS. PEG-based hydrogels with tunable degradation characteristics to control delivery of marrow stromal cells for tendon overuse injuries. *Acta Biomater.* 2011; 7(3):959–66. doi: 10.1016/j.actbio.2010.11.002. [PubMed: 21056127]
 29. Edelman ER, Mathiowitz E, Langer R, Klagsbrun M. Controlled and modulated release of basic fibroblast growth factor. *Biomaterials.* 1991; 12:619–626. [PubMed: 1742404]
 30. Sakiyama-Elbert SE. Incorporation of heparin into biomaterials. *Acta Biomater.* 2014; 10(4):1581–7. doi: 10.1016/j.actbio.2013.08.045. [PubMed: 24021232]
 31. Cai S, Liu Y, Zheng Shu X, Prestwich GD. Injectable glycosaminoglycan hydrogels for controlled release of human basic fibroblast growth factor. *Biomaterials.* 2005; 26(30):6054–67. doi: 10.1016/j.biomaterials.2005.03.012. [PubMed: 15958243]
 32. Tae G, Scatena M, Stayton PS, Hoffman AS. PEG-cross-linked heparin is an affinity hydrogel for sustained release of vascular endothelial growth factor. *Journal of Biomaterials Science, Polymer Edition.* 2006; 17(1-2):187–197. doi: 10.1163/156856206774879090. [PubMed: 16411608]
 33. Seto SP, Casas ME, Temenoff JS. Differentiation of mesenchymal stem cells in heparin-containing hydrogels via coculture with osteoblasts. *Cell Tissue Res.* 2012; 347(3):589–601. doi: 10.1007/s00441-011-1265-8. [PubMed: 22083260]
 34. Tellier LE, Miller T, McDevitt TC, Temenoff JS. Hydrolysis and sulfation pattern effects on release of bioactive bone morphogenetic protein-2 from heparin-based microparticles. *J. Mater. Chem. B.* 2015; 3(40):8001–8009. doi: 10.1039/c5tb00933b.
 35. Nakamura S, Ishihara M, Obara K, Masuoka K, Ishizuka T, Kanatani Y, Takase B, Matsui T, Hattori H, Sato T, Kariya Y, Maehara T. Controlled release of fibroblast growth factor-2 from an injectable 6-O-desulfated heparin hydrogel and subsequent effect on in vivo vascularization. *J Biomed Mater Res A.* 2006; 78(2):364–71. doi: 10.1002/jbm.a.30688. [PubMed: 16673389]
 36. Zieris A, Dockhorn R, Rohrich A, Zimmermann R, Muller M, Welzel PB, Tsurkan MV, Sommer JU, Freudenberg U, Werner C. Biohybrid networks of selectively desulfated glycosaminoglycans for tunable growth factor delivery. *Biomacromolecules.* 2014; 15(12):4439–46. doi: 10.1021/bm5012294. [PubMed: 25329425]
 37. Freudenberg U, Zieris A, Chwal ek K, Tsurkan MV, Maitz MF, Atallah P, Levental KR, Eming SA, Werner C. Heparin desulfation modulates VEGF release and angiogenesis in diabetic wounds. *J Control Release.* 2015; 220(Pt A):79–88. doi: 10.1016/j.jconrel.2015.10.028. [PubMed: 26478015]
 38. Ratanavaraporn J, Tabata Y. Enhanced osteogenic activity of bone morphogenetic protein-2 by 2-O-desulfated heparin. *Acta Biomater.* 2012; 8(1):173–82. doi: 10.1016/j.actbio.2011.09.035. [PubMed: 22005328]
 39. Takada T, Katagiri T, Ifuku M, Morimura N, Kobayashi M, Hasegawa K, Ogamo A, Kamijo R. Sulfated polysaccharides enhance the biological activities of bone morphogenetic proteins. *J Biol Chem.* 2003; 278(44):43229–35. doi: 10.1074/jbc.M300937200. [PubMed: 12912996]

40. Sun W, Ding Y, Jiao K. Electrochemical studies on the interaction of heparin with crystal violet and its analytical application. *Journal of Analytical Chemistry*. 2006; 61(4):359–364. doi: 10.1134/s1061934806040113.
41. Roy S, Lai H, Zouaoui R, Duffner J, Zhou H, L PJ, Zhao G, Ganguly T, Kishimoto TK, Venkataraman G. Bioactivity screening of partially desulfated low-molecular-weight heparins: a structure/activity relationship study. *Glycobiology*. 2011; 21(9):1194–205. doi: 10.1093/glycob/cwr053. [PubMed: 21515908]
42. Shipp EL, Hsieh-Wilson LC. Profiling the sulfation specificities of glycosaminoglycan interactions with growth factors and chemotactic proteins using microarrays. *Chem Biol*. 2007; 14(2):195–208. doi: 10.1016/j.chembiol.2006.12.009. [PubMed: 17317573]
43. Gandhi NS, Mancera RL. The Structure of Glycosaminoglycans and their Interactions with Proteins. *Glycobiology*. 2009; 19(10):1103–15. doi: 10.1093/glycob/cwp101. [PubMed: 19643843]
44. Miller T, Goude MC, McDevitt TC, Temenoff JS. Molecular engineering of glycosaminoglycan chemistry for biomolecule delivery. *Acta Biomater*. 2014; 10(4):1705–19. doi: 10.1016/j.actbio.2013.09.039. [PubMed: 24121191]
45. Temenoff JS, Athanasiou KA, LeBaron RG, Mikos AG. Effect of poly(ethylene glycol) molecular weight on tensile and swelling properties of oligo(poly(ethylene glycol) fumarate) hydrogels for cartilage tissue engineering. *J Biomed Mater Res*. 2002; 59(3):429–37. [PubMed: 11774300]
46. Canal T, Peppas NA. Correlation between mesh size and equilibrium degree of swelling of polymeric networks. *Journal of Biomedical Materials Research*. 1989; 23:1183–1193. [PubMed: 2808463]
47. Webber MJ, Matson JB, Tamboli VK, Stupp SI. Controlled release of dexamethasone from peptide nanofiber gels to modulate inflammatory response. *Biomaterials*. 2012; 33(28):6823–32. doi: 10.1016/j.biomaterials.2012.06.003. [PubMed: 22748768]
48. Nagai Y, Unsworth LD, Koutsopoulos S, Zhang S. Slow release of molecules in self-assembling peptide nanofiber scaffold. *J Control Release*. 2006; 115(1):18–25. doi: 10.1016/j.jconrel.2006.06.031. [PubMed: 16962196]
49. Fu K, Klivanov AM, Langer R. Protein stability in controlled-release systems. *Nature Biotechnology*. 2000; 18:24–25.
50. Seto SP, Miller T, Temenoff JS. Effect of selective heparin desulfation on preservation of bone morphogenetic protein-2 bioactivity after thermal stress. *Bioconjug Chem*. 2015; 26(2):286–93. doi: 10.1021/bc500565x. [PubMed: 25621929]
51. Inoue Y, Nagasawa K. Selective N-Desulfation of Heparin with Dimethyl Sulfoxide Containing Water or Methanol. *Carbohydrate Research*. 1976; 46:87–95. [PubMed: 1248016]
52. Nagasawa K, Inoue Y, Kamata T. Solvolytic Desulfation of Glycosaminoglycuronan Sulfates with Dimethyl Sulfoxide Containing Water or Methanol. *Carbohydrate Research*. 1977; 58:47–55. [PubMed: 144018]
53. Baumann H, Scheen H, Huppertz B, Keller R. Novel regio- and stereoselective O-6-desulfation of the glucosamine moiety of heparin with N-methylpyrrolidinone-water or N,N-dimethylformamide-water mixtures. *Carbohydrate Research*. 1998; 308:381–388. [PubMed: 9711830]
54. Hahn MS, Taite LJ, Moon JJ, Rowland MC, Ruffino KA, West JL. Photolithographic patterning of polyethylene glycol hydrogels. *Biomaterials*. 2006; 27(12):2519–24. doi: 10.1016/j.biomaterials.2005.11.045. [PubMed: 16375965]

Figure 2.

Crystal violet binding to soluble heparin derivatives. The UV absorption ratio between 490 and 590 nm with crystal violet and (A) heparin or (B) heparin methacrylamide derivatives indicated that more crystal violet binding occurred with more sulfated heparin derivatives and that CV binding was relatively unaffected by methacrylamide-functionalization; $n = 3$.

Figure 3.

Crystal violet loading (% of CV included during crosslinking). Crystal violet loading was significantly greater in (A) non-degradable, (B) slow-degrading, and (C) fast-degrading hydrogels with more sulfated heparin. *Significantly greater than all other hydrogel formulations; $p < 0.05$. **Significantly different than any other hydrogel formulation; $p < 0.05$; $n = 3$.

Figure 4.

Crystal violet release from hydrogels. Near-zero order release kinetics were observed from (A) non-degradable, (B) slow-degrading, and (C) fast-degrading Hep and Hep^N hydrogels. &Significantly lower than Hep^{N,6O}, Hep- and PEGDA-only hydrogels at day 1; $p < 0.05$. +Significantly lower than PEGDA-only hydrogels at day 1; $p < 0.05$; $n = 3$. The letter 'D' indicates hydrogel degradation.

Figure 5. bEnd.3 cell angiopoietin-2 gene expression. Crystal violet bioactivity was maintained after loading and releasing from Hep^N hydrogels. *Significantly higher than all other groups; $p < 0.05$; $n = 4$.

Table 1

Degradation time (days) for all degradable hydrogel formulations.

	Hep	Hep^{-N}	Hep^{-N,-60}	Hep-
Slow-degrading hydrogels	13	13	9	9
Fast-degrading hydrogels	5	9	3	3

Table 2

Linear regression R-squared values of cumulative crystal violet release.

	Hep	Hep^{-N}	Hep^{-N-60}	Hep-	PEGDA-only
Non-degradable hydrogels	0.96	0.97	0.88	0.70	0.68
Slow-degrading hydrogels	0.97	0.97	0.90	0.70	N/A
Fast-degrading hydrogels	0.97	0.99	N/A	N/A	N/A

Table 3

Crystal violet release kinetics from non-degradable hydrogels. Time (days) required to release ~30%, 60% and 100% loaded crystal violet was slower for more sulfated heparin hydrogels than more desulfated heparin and PEGDA-only hydrogels.

	Hep	Hep ^{-N}	Hep ^{-N,-60}	Hep-	PEGDA-only
~30%	5	5	1	1	<1
~60%	15	11	3	3	1
~100%	N/A	N/A	7	5	3

Table 4

Crystal violet release kinetics from slow-degrading hydrogels. Time (days) required to release ~30%, 60% and 100% loaded CV was slower for more sulfated heparin hydrogels than more desulfated heparin hydrogels.

	Hep	Hep ^{-N}	Hep ^{-N,-60}	Hep ⁻
~30%	5	7	1	1
~60%	13	N/A	5	3
~80%	N/A	N/A	9	7

Table 5

Crystal violet release kinetics from fast-degrading hydrogels. Time (days) required to release ~30%, 60% and 100% loaded crystal violet was slower for more sulfated heparin hydrogels than more desulfated heparin hydrogels.

	Hep	Hep ^{-N}	Hep ^{-N,-60}	Hep-
~30%	<1	5	1	<1
~60%	5	9	3	1
~100%	N/A	N/A	3	3