Biomaterials Science

Intra-Articular TSG-6 Delivery from Heparin-based Microparticles Reduces Cartilage Damage in a Rat Model of Osteoarthritis

Journal:	*Biomaterials Science*
Manuscript ID	BM-ART-01-2018-000010.R2
Article Type:	Paper
Date Submitted by the Author:	07-Mar-2018

Complete List of Authors:	Tellier, Liane; Georgia Institute of Technology and Emory University, Biomedical Engineering Treviño, Elda; Georgia Institute of Technology and Emory University, Biomedical Engineering Brimeyer, Alexandra; Georgia Institute of Technology and Emory University, Biomedical Engineering Reece, David; Georgia Institute of Technology and Emory University, Biomedical Engineering Willett, Nick; Georgia Institute of Technology and Emory University, Biomedical Engineering; Emory University, Orthopedics; Atlanta Veterans Affairs Medical Center; Georgia Institute of Technology, Petit Institute for Bioengineering and Bioscience Guldberg, Robert; Georgia Institute of Technology, Mechanical Engineering; Georgia Institute of Technology, Petit Institute for Bioengineering and Bioscience Temenoff, Johnna; Georgia Institute of Technology and Emory University, Biomedical Engineering; Georgia Institute of Technology, Petit Institute for Bioengineering and Bioscience

Intra-Articular TSG-6 Delivery from Heparin-based Microparticles Reduces Cartilage Damage in a Rat Model of Osteoarthritis

Liane E. Tellier,[1] Elda A. Treviño,[1] Alexandra L. Brimeyer,[1] David S. Reece,[1] Nick J. Willett, PhD[1,2,3,4] Robert E. Guldberg, PhD[4,5] Johnna S. Temenoff, PhD[1,4]

[1]W.H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA

[2]Department of Orthopedics, Emory University, Decatur, GA

[3]Atlanta Veterans Affairs Medical Center, Decatur, GA

[4]Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA

[5]Department of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA

Abstract

As a potential treatment for osteoarthritis (OA), we have developed injectable and hydrolytically degradable heparin-based biomaterials with tunable sulfation for the intra-articular delivery of tumor necrosis factor-alpha stimulated gene-6 (TSG-6), a protein known to inhibit plasmin which may degrade extracellular matrix within OA joints. We first assessed the effect of heparin sulfation on TSG-6 anti-plasmin activity and found that while fully sulfated (Hep) and heparin desulfated at only the N position (Hep^{-N}) significantly enhanced TSG-6 bioactivity *in vitro,* fully desulfated heparin (Hep-) had no effect, indicating that heparin sulfation plays a significant role in modulating TSG-6 bioactivity. Next, TSG-6 loaded, degradable 10 wt% Hep^{-N} microparticles (MPs) were delivered via intra-articular injection into the knee at 1, 7, and 15 days following

medial meniscal transection (MMT) injury in a rat model. After 21 days, cartilage thickness, volume, and attenuation were significantly increased with soluble TSG-6, indicating degenerative changes. In contrast, no significant differences were observed with TSG-6 loaded MP treatment, demonstrating that TSG-6 loaded MPs reduced cartilage damage following MMT injury. Ultimately, our results indicate that Hep^{-N} can enhance TSG-6 anti-plasmin activity and that Hep^{-N}-based biomaterials may be an effective method for TSG-6 delivery to treat OA.

1. Introduction

Osteoarthritis (OA) affects more than 60% of Americans over 65 years of age and is characterized by significant articular cartilage degeneration including cartilage fibrillation, fissures, and loss of proteoglycan and collagen content within the cartilage extracellular matrix (ECM).[1–3] While OA is a multi-factorial disease, at stages of disease progression both cartilage-resident chondrocytes and synoviocytes in the joint capsule have been found to secrete increased levels of soluble factors including interleukins, tumor necrosis factor-alpha (TNF-α), and matrix metalloproteinases (MMPs), among others, which are known to promote or be directly involved in cartilage degradation.[3–5] Additionally, components of the plasminogen activation pathway including plasminogen and plasminogen activators, receptors, and inhibitors[6–9] have also been shown to be upregulated in OA joints when compared to healthy patients.[8,10] As plasmin, the active form of plasminogen, has been shown to activate MMPs and degrade ECM,[11,12] it is postulated that the plasminogen activation pathway plays a significant role in the cartilage degeneration exhibited in OA joints.[7,9] Thus, plasmin-inhibiting therapeutics may be a promising method to ameliorate cartilage degeneration in the context of OA.

TNF-α-stimulated gene-6 (TSG-6) is a positively charged 35 kDa protein with anti-

plasmin and anti-inflammatory properties.[11] In particular, TSG-6 has been studied extensively for its ability to potentiate inter-alpha-inhibitor (IalphaI)-mediated inhibition of plasmin[11,12] and more recently, its ability to suppress the response of chondrocytes to inflammatory factors such as interleukin-1 and TNF-α.[13] In the context of OA, while little constitutively expressed TSG-6 has been observed in healthy patients, TSG-6 protein expression was found to be upregulated in OA joints and greater TSG-6 levels were observed in patients where OA symptoms had advanced over a three year period compared to non-progressing OA patients.[14–16]

Despite the increased production of endogenous TSG-6, however, tissues within OA joints continue to degenerate, leading to studies on the effect of adding exogenous TSG-6 on arthritis progression. In rheumatoid arthritis (RA) mouse models, soluble TSG-6 treatment led to a significant improvement in joint swelling[17] and cartilage damage,[17,18] assessed via joint diameter and histology, respectively; but these effects were often short-lived, and by day 12[17] or day 35[18] no differences were observed between treated and untreated animals. Most recently, soluble TSG-6 treatment was also investigated in a rat OA model induced by anterior cruciate ligament and meniscus transection.[13] In this work, a portion of the TSG-6 molecule was delivered via intra-articular injection weekly up to 21 days following injury. After 28 days, histology indicated that cartilage fibrillation and ulceration were significantly diminished with TSG-6 treatment compared to untreated controls, indicating that TSG-6 or TSG-6 derivatives may be an effective OA treatment strategy.[13]

Despite these promising results, one drawback to soluble treatments are the high doses often required and short retention due to rapid clearance from the joint space.[1,19] Therefore, in this study we explored the use of heparin, a naturally derived and highly sulfated GAG that can bind to a myriad of positively charged proteins including TSG-6,[20–25] as an injectable biomaterial

carrier. Heparin is of particular interest in this application because previous work has demonstrated that soluble complexes of heparin and TSG-6 resulted in enhanced TSG-6 anti-plasmin activity.[12,26] Although desulfated heparin derivatives have been explored as a safer *in vivo* therapeutic delivery strategy due to their diminished anti-coagulant properties compared to fully sulfated heparin,[20,22,27,28] the effect of heparin desulfation on TSG-6 binding and bioactivity has yet to be determined. Therefore, investigating the ability for desulfated heparin derivatives to maintain or enhance TSG-6 bioactivity is important to the development of an efficacious delivery strategy for TSG-6.

In this work, we first assessed the ability for desulfated heparin derivatives ranging from 0 to 100% total sulfation to enhance TSG-6 bioactivity *in vitro*. Next, though heparin-based hydrogels have been utilized extensively for protein delivery,[28–34] hydrogel volume may be prohibitive for drug delivery to joint spaces,[1]. Therefore, we have developed hydrolytically degradable heparin-based microparticles (MPs) based on our *in vitro* results and delivered TSG-6 loaded heparin-based MPs via intra-articular injection following medial meniscal transection (MMT) injury in a rat model. We hypothesized that heparin of greater total sulfation would enhance TSG-6 bioactivity and, ultimately, that TSG-6 loaded on MPs with the appropriate sulfation level and delivered via intra-articular injection would reduce cartilage damage following MMT injury significantly more than soluble TSG-6 treatment.

2. Materials and Methods

2.1. Heparin modifications

N-desulfated (Hep^{-N}) and fully desulfated (Hep-) heparin were prepared as described previously, whereby 10 mg/mL heparin sodium salt (Hep) from porcine intestinal mucosa (Sigma) was dissolved in dH_2O and passed through Dowex 50WX4 resin (mesh size 100-200, Sigma).[20,35] Pyridine was added until the heparin solution reached pH 6, after which time excess dH_2O and pyridine were removed via rotatory evaporator (Buchi), flash frozen, and lyophilized. For Hep^{-N}, the heparin pyridinium was then dissolved at 1 mg/mL in 9:1 v/v dimethyl sulfoxide (DMSO)/ dH_2O at 50°C for 2 hours.[36,37] For Hep-, heparin pyridinium was dissolved at 10 mg/mL in 9:1 v/v N-methylpyrrolidone (NMP, Acros Organics)/ dH_2O at 100°C for 24 hours.[38] Subsequently, Hep^{-N} and Hep- were precipitated with 95% ethanol saturated with sodium acetate, collected via centrifuge, dissolved in dH_2O, dialyzed, lyophilized, and stored at -20°C.

Hep^{-N} methacrylamide (Hep^{-N} MAm) functionalization was performed as described previously.[39] Briefly, 1.1 mM Hep^{-N}, 83.0 mM N-hydroxysulfosuccinimide (sulfo-NHS, Sigma), 101.0 mM N-(3-Aminopropyl) methacrylamide hydrochloride (APMAm, Polysciences Inc.), and 156.0 mM (N-3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Sigma) were combined in 10 mL phosphate buffer saline (PBS, Teknova). After stirring on ice for 6 hours, Hep^{-N} MAm was dialyzed, lyophilized, and stored at -20°C.

2.2. Poly (ethylene glycol) diacrylate synthesis

Poly (ethylene glycol) (PEG, 3.4 kDa, Sigma) was reacted in a 8:1 molar ratio of acryloyl chloride (AcCl, Sigma) to PEG in dichloromethane (DCM) solution.[33] Next, a 1:1 molar ratio of triethylamine (TEA, Sigma) to AcCl was added as a catalyst resulting in linear PEG-diacrylate (PEGDA).

2.3. Proton nuclear magnetic resonance

Proton nuclear magnetic resonance (^{1}H NMR) was performed whereby 10 mg/mL Hep^{-N} MAm and PEGDA samples were each dissolved in deuterated H_2O (Sigma), run on a Bruker Avance III spectrometer at 400 Hz, and analyzed using iNMR software.[35] Percent modification was determined by dividing the integral of the methacrylamide peak by the heparin peak for Hep^{-N} MAm and the acrylate peaks by the PEG peak for PEGDA.

2.4. Plasmin inhibition assay

Plasmin inhibition via TSG-6 was determined as described previously.[12] Briefly, clear 96-well plates were blocked for 1 hour with 0.5 wt% bovine serum albumin (BSA, Thermo) PBS solution. For TSG-6 with soluble heparin derivatives, 108 nM TSG-6 was added with 108 nM of Hep, Hep^{-N} or Hep- in a pH 7.4 buffer of 10 mM HEPES, 150 mM NaCl, and 0.02% v/v Tween20 in dH_2O for 30 mins at 37°C. Next, 24 nM IalphaI (Athens Research & Technology) was added and allowed to incubate for an additional 30 mins at 37°C. Finally, 3.4 nM plasmin (Sigma) and 197 μM plasmin substrate (N-p-Tosyl-Gly-Pro-Lys 4-nitroanilide acetate salt, Sigma) were added and incubated for 20 mins at RT and 20 mins at 37°C, at which time the plate was analyzed on a plate reader at 405 nm every ~40 mins for a total of 180 mins; n = 3.

2.5. Microparticle fabrication

To fabricate 10 wt% Hep^{-N} MPs, 50.0 mg PEGDA and 1.61 mg dithiothreitol (DTT, 35 mM, Sigma) were added to 273 μL 10 wt% BSA PBS solution and incubated at 37°C for 30 mins to allow for Michael Type addition between PEGDA and DTT. Then, 5.6 mg Hep^{-N} MAm was added and the aqueous solution was incubated again at 37°C for 30 mins.

Next, an oil phase of 5 mL mineral oil (Amresco) with 3.0-3.2 μL Span80 (TCI) was placed under a homogenizer (Polytron PT 3100, Kinematica) set to 4000 RPM. After adding 27 μL of 0.05 wt% Irgacure 2959 photoinitiator (Ciba) to the aqueous phase, the solution was added drop-wise to the oil phase and the water-and-oil emulsion was allowed to homogenize for 5 mins. Subsequently, the water-and-oil emulsion was nitrogen purged for 1 min and crosslinked under UV (~15 mW/cm^2) via free radical polymerization between PEGDA and Hep^{-N} MAm. MPs were then washed through 3 iterations of the following procedure: MPs were combined with 35 mL dH_2O, centrifuged at 4000 RPM for 5 mins, and the supernatant consisting of water and oil was removed. In the final wash, MPs were pipetted through 40 μm cell strainers to remove MPs under 40 μm in diameter. To sterilize MPs, each MP batch was incubated with 70% ethanol on rotary platform at 4°C for 30 mins, followed by 3 30-min washes in sterile PBS. Phase microscopy and ImageJ software were used to image and determine the size distribution of each MP batch. MPs were stored in sterile PBS at 4°C until use.

2.6. TSG-6 loading and release from microparticles

To load TSG-6 onto MPs, 1.0 μg human TSG-6 (R&D Systems) was added to 0.6 mg MPs in 50 μL 0.1 wt% BSA solution. TSG-6 and MPs were incubated for 2 hours at 4°C, after which time MPs were rinsed by adding an additional 450 μL 0.1 wt% BSA solution. The MPs were centrifuged for 3 mins at 10,000 RCF and 495 μL supernatant was removed. For all *in vitro* and *in vivo* studies, MP loading was completed on the day of the experiment to reduce variability and avoid potential protein denaturation prior to the start of the experiment.

For *in vitro* TSG-6 release studies, the removed supernatant was replaced with 495 μL fresh 0.1 wt% BSA solution and samples were incubated at 37°C. MPs were centrifuged and

495 μL supernatant was removed and replaced 3 hours, 1, 3, 7, and 10 days following TSG-6 loading until MPs degraded. TSG-6 protein levels were quantified using a human TSG-6 sandwich ELISA with the following steps: 10 μg/mL monoclonal capture antibody (Santa Cruz Biotechnology) overnight, 100-10,000 pg/mL recombinant human TSG-6 used as standards and samples for 2 hours, 0.5 μg/mL biotinylated secondary detection antibody (R&D Systems) for 2 hours, streptavidin-horseradish peroxidase enzyme (R&D Systems) for 20 mins, substrate solution (R&D Systems) for 20 mins, and lastly a stop solution of 2 N sulfuric acid (Ricca) that was read at 450 nm; n = 3-5.

To assess TSG-6 bioactivity after release from MPs, the plasmin inhibition assay protocol was followed with day 1 TSG-6 release supernatant (11 ng/mL) and compared to a soluble TSG-6 control at the same concentration (11 ng/mL); n= 3-5.

2.7. Medial meniscal transection model

Animal use was reviewed and approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee which follows the "Public Health Service Policy on Humane Care and Use of Laboratory Animals" and uses the National Guidelines outlined in the "Guide for the Care and Use of Laboratory Animals, 8th Ed." as its basis. In addition, experiments followed all institutional guidelines, personnel were enrolled in the Georgia Institute of Technology Occupational Health & Safety Program and experiments were reviewed by Environmental Health & Safety and Radiation Safety Committee. Medial meniscal transection (MMT) injury was induced using a similar method to previously established protocols with male Sprague-Dawley rats (11 total animals used for all *in vivo* experiments, 250-300 g initial weight, 8-10 weeks old from Charles River).[2] Prior to surgery animals were anesthetized by 5%

isoflurane (Isothesia), followed by 2-3% isoflurane during surgery and were administered sustained release buprenorphine as an analgesic. Next, a small incision was made through the skin on the medial aspect of the left femoro-tibial joint. The medial collateral ligament was exposed by blunt dissection and transected to visualize the joint space and medial meniscus. The meniscus was then transected completely at its narrowest point. The skin was sutured with 4.0 silk sutures (Ethicon) and then closed using wound clips.

2.8. *In vivo* TSG-6 delivery

For TSG-6 loaded MP treatment, MPs were loaded as described above but 3.6 mg MPs were resuspended in a total volume of 50 μL sterile 0.1 wt% BSA PBS solution and subsequently loaded into sterile syringes. 1, 7, and 15 days following MMT injury, MPs were delivered via intra-articular injection through the infrapatellar ligament and into the stifle joint of the left leg. For soluble TSG-6 controls, 16.7 μg TSG-6 was dissolved in a total volume of 50 μL sterile 0.1 wt% BSA PBS solution, loaded into sterile syringes, and delivered using the same method as TSG-6 loaded MPs. Overall, *in vivo* experimental groups included TSG-6 loaded MPs (18 μg TSG-6 loaded onto 10.8 mg MPs delivered over 3 time points; n = 4), soluble TSG-6 (50 μg TSG-6 delivered over 3 time points; n = 4) or injury only (no TSG-6 or MPs; n = 3). The same animals were used for all subsequent outcome measures. Contralateral tibiae served as uninjured controls.

2.9. MicroCT analysis

21 days following MMT injury, rats were euthanized and tibiae were harvested and fixed in 10% neutral buffered formalin (EMD Chemicals) for 7 days. Tibiae were then immersed in a 30%

Hexabrix (Covidien) PBS solution at 37°C for 30 mins and scanned using a μCT40 (Scanco Medical) at 45 kVp, 177 μA, 200 ms integration time, and 16 μm voxel size.

For cartilage assessment via μCT, Equilibrium Partitioning of an Ionic Contrast agent-μCT (EPIC-μCT) and Scanco evaluation software (Scanco μCT evaluation software V6.5-3) was used as described previously.[40] First, raw scan data were automatically reconstructed to 2D grayscale tomograms, which were subsequently rotated to sagittal sections. Next, cartilage (gray in images) was manually contoured on the head of the tibiae to separate it from trabecular bone (white in images) and surrounding area (black in images). Fixed threshold values were used to separate cartilage from air and bone: a lower threshold of 458 mg HA/cm^3 was used to separate cartilage from air and a higher threshold of 635 mg HA/cm^3 was used to separate cartilage from bone. The threshold values were globally applied for both the left and right tibiae of all animals. After manually defining the cartilage region of interest (ROI), Scanco evaluation software was used to measure cartilage thickness (distance transformation of stacked 2D images), volume (# of voxels * voxel volume), attenuation (inversely proportional to proteoglycan content), osteophyte volume, and lesion volume within the medial third of the medial tibial plateau, which is the characteristic region of damage in MMT injuries; n = 3-4.[2,41,42]

2.10. Histology

Following μCT, tibiae were decalcified in Cal-Ex II (Fisher) for 10 days, then processed for frozen histology sectioning as described previously.[43] Sections were stained in 0.1% fast green (Sigma) and 0.25% safranin-O solution (Sigma) and imaged at 20x magnification with a Nikon Eclipse 80i; n = 3-4.

2.11. Statistical analysis

All data are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey's post hoc multiple comparison test (significance value of $p \leq 0.05$) were run using Prism software.

3. Results

3.1. Materials characterization

^{1}H NMR indicated that PEGDA was ~55% functionalized while Hep^{-N} MAm was between 20-28% functionalized (Figure S1).

3.2. Plasmin inhibition assay with soluble heparin derivatives

Hep and Hep^{-N} both significantly enhanced TSG-6 anti-plasmin activity (57.3 ± 1.2% and 66.1 ± 1.7% plasmin activity compared to plasmin control, respectively) compared to TSG-6 alone (74.6 ± 0.2%), though Hep enhanced TSG-6 activity significantly more than Hep^{-N}. In contrast, Hep- had no significant effect (69.6 ± 1.2%) compared to TSG-6 alone (Figure 1).

3.3 Microparticle fabrication, loading, and release

Degradable 10 wt% Hep^{-N} MPs were found to be 80 ± 60 μm in diameter (Figure 2) and have previously been shown to degrade within 10-16 days *in vitro*.[43] *In vitro*, 6.0 μg TSG-6 was loaded onto MPs and over 1-3 days ~1 μg TSG-6 was released (Figure 3A). Therefore, over 3 injections *in vivo*, 18.0 μg TSG-6 was originally added to MPs and ~3 μg TSG-6 was released. In comparison, for soluble TSG-6 treatment, 16.7 μg TSG-6 was delivered per injection, resulting in a total dosage of 50.0 μg soluble TSG-6 over three injections. Importantly, TSG-6

released from MPs after 1 day exhibited significantly greater anti-plasmin activity (65.7 ± 3.0% plasmin activity compared to plasmin control) than soluble TSG-6 (96.4 ± 3.1%) at the same concentration (Figure 3B).

3.4. MicroCT analysis

μCT images 21 days following MMT injury were quantified to assess changes in cartilage thickness, volume, and attenuation (Figure 4E-H). After 21 days following MMT injury, cartilage thickness (1.9 ± 0.4X compared to uninjured control) and volume (2.0 ± 0.6X) increased significantly compared to uninjured contralateral controls (Figure 4I-J). Soluble TSG-6 treated samples also exhibited significantly increased cartilage thickness (1.5 ± 0.2X) and volume (1.9 ± 0.5X) as well as increased attenuation (1.6 ± 0.2X) (Figure 4I-K) compared to uninjured controls. In contrast, neither cartilage thickness, volume, nor attenuation were increased in the TSG-6 loaded MP group compared to uninjured controls (Figure 4I-K). Osteophyte volume, focal lesion volume, and surface roughness were not significantly different between each experimental group or compared to respective contralateral controls (data not shown).

3.5. Histology

Safranin-O staining of GAG within cartilage was observed in TSG-6 loaded MP treated tibiae (Figure 4C) and in uninjured controls (Figure 4D). Qualitatively, less intense safranin-O staining was observed in the medial one-third of the injured and soluble TSG-6 treated tibiae (Figure 4A-B).

4. Discussion

The objectives of this work were to elucidate the effect of heparin sulfation on TSG-6 bioactivity and, utilizing this information, to develop heparin-based biomaterials for the intra-articular delivery of TSG-6. To this end, soluble heparin derivatives including Hep, Hep^{-N}, and Hep-, found to have 100%, ~80% and ~0% sulfation compared to natively sulfated heparin,[20,35] were incubated with TSG-6 and results indicated that Hep and Hep^{-N} significantly enhanced TSG-6 anti-plasmin activity compared to soluble TSG-6 alone, whereas Hep- had no effect (Figure 1). For Hep specifically, these results parallel previous work, and it is hypothesized that the ability for Hep to maintain TSG-6 in a "closed" conformation upon binding, unlike other GAGs such as HA, may have contributed to the enhanced anti-plasmin activity observed.[26,44] Secondly, as Hep can bind to both TSG-6 and IalphaI, it is possible that Hep increased the proximity of the two proteins, thereby enabling TSG-6 to potentiate IalphaI-mediated plasmin inhibition.[26]

In comparison to Hep, Hep^{-N} enhanced TSG-6 activity to a significantly less degree, whereas Hep- showed no enhancement over soluble TSG-6, indicating that heparin desulfation reduced heparin-mediated enhancement of TSG-6 anti-plasmin activity (Figure 1). One possible explanation may be that the sulfate groups removed from Hep^{-N} and Hep-, the N sulfate group for Hep^{-N} as well as the 2O and 6O sulfate groups for Hep-, are necessary for complete heparin binding to TSG-6 and the subsequent enhancement of TSG-6 bioactivity. In a study with derivatives of another GAG, chondroitin sulfate, with varying sulfation patterns, it was found that chondroitin-4-sulfate (C4S) could bind to TSG-6 while chondroitin-6-sulfate (C6S) could not,[45] suggesting that specific sulfate groups may significantly affect GAG binding to TSG-6. Alternatively, as Hep- is ~0% sulfated, it is possible that Hep- bound to TSG-6 in a similar

manner as HA, a naturally non-sulfated GAG which binds to TSG-6 at a distinct and non-overlapping binding site from heparin, and which did not enhance TSG-6 bioactivity in previous work.[26,44]

Finally, more sulfated heparin derivatives have also been shown to protect proteins from denaturation, providing an additional mechanism by which Hep and Hep^{-N} may have maintained TSG-6 bioactivity in this study. For example, when Hep, Hep^{-N}, and Hep- were incubated with bone morphogenetic protein-2 (BMP-2) and exposed to 65°C heat treatment for 15 mins, Hep was significantly more effective in protecting BMP-2 than all other desulfated heparin derivatives.[20,35] As our *in vitro* studies were conducted at 37°C over ~4 hours, it is possible that Hep and Hep^{-N}, due to binding with TSG-6, could protect TSG-6 from denaturation over this time course, thereby protecting TSG-6 bioactivity. In summary, these findings indicate that Hep and Hep^{-N} can enhance TSG-6 anti-plasmin activity, potentially due to interactions with specific sulfate groups which may enable binding to TSG-6, through the protection of TSG-6 from denaturation when bound to heparin, or through a combination of mechanisms. Ultimately, given its diminished anti-coagulant properties compared to Hep, Hep^{-N}-based MPs were chosen as the biomaterial carrier for intra-articular delivery of TSG-6.

To determine the ability for Hep^{-N}-based MPs to enhance TSG-6 treatment *in vivo*, we first assessed the effect of soluble TSG-6 and injury alone via EPIC-μCT, an analysis technique which utilizes a contrast agent to better distinguish cartilage from bone in μCT images.[40,46] Our results following injury alone parallel previous findings whereby cartilage thickness, volume, and attenuation (which is inversely proportional to proteoglycan content) were all significantly increased after 21 days following MMT injury in rats (Figure 4I-K).[41] Furthermore, these results also parallel findings from human OA joints, where chondrocytes have been shown to

proliferate, produce ECM, and undergo hypertrophy potentially resulting in increased cartilage thickness and volume, whereas the upregulation of proteinases in OA patients is known to cause cartilage proteoglycan loss.[2,41,47,48]

However, our results also indicate that soluble TSG-6 was unable to improve cartilage degeneration following injury (Figure 4B,F,I-K). In contrast, in a recent study the intra-articular injection of soluble TSG-6 link modules, a portion of TSG-6 that has been shown to retain certain TSG-6 anti-inflammatory properties,[49] over 3 weeks within a rat OA model led to a significant reduction in cartilage fibrillation and ulceration when analyzed via histology.[13] It is important to note, however, that several differences exist between this study and our work, including the OA animal model, which included both anterior cruciate ligament transection and MMT compared to MMT alone in our work, and the methods for analyzing cartilage degeneration, which included only histology. Furthermore, the doses of TSG-6 used in the study were not reported, which may be an explanation for the results of soluble TSG-6 treatment observed in this case.

In our experiments, in contrast to injury and soluble TSG-6, EPIC-μCT revealed that neither cartilage thickness, volume, nor attenuation were increased in the TSG-6 loaded MP group compared to uninjured controls (Figure 4G,I-K), indicating that cartilage hypertrophy and proteoglycan loss were reduced by TSG-6 loaded MP treatment. Histological analysis similarly showed that cartilage safranin-O staining remained qualitatively similar to uninjured controls, again suggesting that proteoglycan loss was reduced by TSG-6 loaded MP treatment (Figure 4C). As TSG-6 treatment was only effective when delivered with Hep^{-N}-based MPs, even with a 3X lower dose than soluble TSG-6, our results indicate that the Hep^{-N}-based carrier played a significant role in the efficacy of TSG-6 *in vivo*. Combining these findings with our *in vitro*

studies, it is possible that Hep^{-N} MPs enhanced TSG-6 anti-plasmin activity, thereby reducing plasmin-mediated MMP activation and ECM degradation, or protected TSG-6 against denaturation, further improving the efficacy of TSG-6 treatment. Importantly, our findings indicate that Hep^{-N}-based MPs may reduce the amount of TSG-6 required for therapeutic effect by at least 3-fold, providing a more efficacious approach to TSG-6-mediated OA treatment.

However, in our system TSG-6 is still released from MPs over a relatively short period of time, within 1-3 days after loading (Figure 3A). Moreover, given the relatively fast degradation rate of MPs (between 9-16 days *in vitro*[43]), we delivered TSG-6 loaded MPs in weekly injections which is unsuitable for direct clinical translation. Thus, future optimization of this carrier may include extending the degradation rate of MPs over several weeks by decreasing the concentration of DTT within MPs,[20,22,43] potentially extending the release of TSG-6 from MPs. Furthermore, in other work, by increasing the heparin content within heparin-PEG hydrogels from 3 to 6 wt%, a ~20% reduction in the amount of protein released after the first 7 days was observed,[50] suggesting that increasing heparin content may provide another mechanism to prolong the release of TSG-6 from our MPs.

Ultimately, in this system, TSG-6 loaded onto Hep^{-N}-based MPs was significantly more chondroprotective than a 3X dosage of soluble TSG-6. While, based on our soluble GAG experiments, TSG-6 anti-plasmin activity is likely one potential mechanism through which TSG-6 displayed a chondroprotective effect following MMT injury, TSG-6 has also been shown to interact with a number of cell populations potentially found within the injured joint space, including leukocytes,[49] neutrophils,[12] macrophages,[51] and MSCs.[52,53] Thus, in the future, additional studies designed to fully elucidate which functions of TSG-6 are responsible for the

results seen in these experiments and how Hep^{-N} may enhance these capabilities may further improve TSG-6-mediated OA treatment.

Conclusions

In this work, we assessed the effect of heparin sulfation on TSG-6 bioactivity to inform the development of heparin-based MPs for the intra-articular delivery of TSG-6 following MMT injury. More sulfated heparin derivatives (Hep and Hep^{-N}) significantly enhanced TSG-6 anti-plasmin activity *in vitro,* whereas fully desulfated heparin (Hep-) had no effect, indicating that heparin sulfation plays a significant role in modulating TSG-6 bioactivity. Based on this data, hydrolytically degradable TSG-6 loaded Hep^{-N}-based MPs were delivered via intra-articular injection following MMT injury. After 21 days, EPIC-μCT analysis indicated that TSG-6 loaded MPs reduced cartilage damage following MMT injury, whereas a 3X higher dose of soluble TSG-6 did not. These results suggest that Hep^{-N} can enhance TSG-6 bioactivity *in vivo* and, ultimately, that Hep^{-N}-containing MPs may be an effective method for delivery of TSG-6 for OA treatment in the future.

Acknowledgements

We would like to acknowledge the Petit Institute Core Facilities (Histology and microCT) for their services and shared resources that enabled us to produce this publication. This research was funded by NSF Stem Cell Biomanufacturing IGERT (DGE 0965945). This research was also supported by a Georgia Tech/Emory University Immunoengineering Center Seed Grant and Georgia Tech Petit Institute Faculty Fellow funds.

References

1. Gerwin N, Hops C, Lucke A. Intraarticular drug delivery in osteoarthritis. *Adv Drug Deliv Rev*. 2006;58(2):226-242. doi:10.1016/j.addr.2006.01.018.
2. Willett NJ, Thote T, Lin AS, Moran S, Raji Y, Sridaran S, Stevens HY, Guldberg RE. Intra-articular injection of micronized dehydrated human amnion/chorion membrane attenuates osteoarthritis development. *Arthritis Res Ther*. 2014;16(1):R47. doi:10.1186/ar4476.
3. Goldring MB. Osteoarthritis and cartilage: the role of cytokines. *Curr Rheumatol Rep*. 2000;2(6):459-465. http://www.ncbi.nlm.nih.gov/pubmed/15480070.
4. Pearle AD, Warren RF, Rodeo SA. Basic science of articular cartilage and osteoarthritis. *Clin Sports Med*. 2005;24(1):1-12. doi:10.1016/j.csm.2004.08.007.
5. Goldring MB, Otero M. Inflammation in Osteoarthritis. *Curr Opin Rheumatol*. 2011;23(5):471-478. doi:10.1097/BOR.0b013e328349c2b1.
6. Kummer JA, Abbink JJ, DeBoer J, Roem D, Nieuwenhuys EJ, Kamp AM, Swaak TJG, Hack CE. Analysis of intraarticular fibronolytic pathways in patients with inflammatory and noninflammatory joint diseases. *Arthritis Rheum*. 1992;35(8):884-893.
7. Brommer P, Dooijewaard G, Dijkmans B, Breedvelf F. Plasminogen activators in synovial fluid and plasma from patients with arthritis. *Ann Rheum Dis*. 1992;51:965-968.
8. Belcher C, Fawthrop F, Bunning R, Doherty M. Plasminogen activators and their inhibitors in synovial fluids from normal , osteoarthritis , and rheumatoid arthritis knees. *Ann Rheum Dis*. 1996;55:230-236.
9. Ronday HK, Smits HH, Van Muijen GN, Pruszczynski MS, Dolhain RJ, Van Langelaan EJ, Breedveld FC, Verheijen JH. Difference in expression of the plasminogen activation

system in synovial tissue of patients with rheumatoid arthritis and osteoarthritis. *Br J Rheumatol*. 1996;35(5):416-423.

10. Busso N, Peclar V, So A, Sappino A-P. Plasminogen activation in synovial tissues: differences between normal, osteoarthritis, and rheumatoid arthritis joints. *Ann Rheum Dis*. 1997;56(9):550-557.
11. Wisniewski HG, Vilček J. TSG-6: An IL-1/TNF-inducible protein with anti-inflammatory activity. *Cytokine Growth Factor Rev*. 1997;8(2):143-156. doi:10.1016/S1359-6101(97)00008-7.
12. Wisniewski HG, Hua JC, Poppers DM, Naime D, Vilcek J, Cronstein BN. TNF/IL-1-inducible protein TSG-6 potentiates plasmin inhibition by inter-alpha-inhibitor and exerts a strong anti-inflammatory effect in vivo. *J Immunol*. 1996;156:1609-1615.
13. Day AJ, Drummond SP, Anand S, Bartnik E, Milner CM. A novel chondroprotective property of TSG-6 has therapeutic potential for OA. *Osteoarthr Cartil*. 2016;24(2016):S19-S20. doi:10.1016/j.joca.2016.01.064.
14. Bayliss MT, Howat SLT, Dudhia J, Murphy JM, Barry FP, Edwards JCW, Day AJ. Up-regulation and differential expression of the hyaluronan-binding protein TSG-6 in cartilage and synovium in rheumatoid arthritis and osteoarthritis. *Osteoarthr Cartil*. 2001;9(1):42-48. doi:10.1053/joca.2000.0348.
15. Wisniewski H, Colón E, Liublinska V, Karia RJ, Stabler T V, Attur M, Abramson SB, Band PA, Kraus VB. TSG-6 activity as a novel biomarker of progression in knee osteoarthritis. *Osteoarthr Cartil*. 2014;22(2):235-241. doi:10.1016/j.joca.2013.12.004.
16. Chou C, Wisniewski H, Band P, Huebner J, Stabler T, Lattermann C, Kraus VB. TSG-6 activity reflects severity of inflammation in knee osteoarthritis and acute joint injury.

Osteoarthr Cartil. 2016;24(2016):S81-S82. doi:10.1016/j.joca.2016.01.174.

17. Bárdos T, Kamath R V., Mikecz K, Glant TT. Anti-Inflammatory and chondroprotective effect of TSG-6 (Tumor Necrosis Factor-α-Stimulated Gene-6) in murine models of experimental arthritis. *Am J Pathol*. 2001;159(5):1711-1721. doi:10.1016/S0002-9440(10)63018-0.
18. Glant TT, Kamath R V., Bárdos T, Gál I, Szántó S, Murad YM, Sandy JD, Mort JS, Roughley PJ, Mikecz K. Cartilage-specific constitutive expression of TSG-6 protein (product of tumor necrosis factor α-stimulated gene 6) provides a chondroprotective, but not antiinflammatory, effect in antigen-induced arthritis. *Arthritis Rheum*. 2002;46(8):2207-2218. doi:10.1002/art.10555.
19. Owen SG, Francis HW, Roberts MS. Disappearance kinetics of solutes from synovial fluid after intra-articular injection. *Br J Clin Pharmacol*. 1994:349-355.
20. 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.
21. Ogle ME, Krieger JR, Tellier LE, McFaline-Figueroa J, Temenoff JS, Botchwey EA. Dual affinity heparin-based hydrogels achieve pro-regenerative immunomodulation and microvascularremodeling. *ACS Biomater Sci Eng*. 2017. doi:10.1021/acsbiomaterials.6b00706.
22. Peng Y, Tellier LE, Temenoff JS. Heparin-based hydrogels with tunable sulfation & degradation for anti-inflammatory small molecule delivery. *Biomater Sci*. 2016;4(9):1371-1380. doi:10.1039/C6BM00455E.
23. Sadir R, Imberty A, Baleux F, Lortat-Jacob H. Heparan sulfate/heparin oligosaccharides

protect stromal cell-derived factor-1 (SDF-1)/CXCL12 against proteolysis induced by CD26/dipeptidyl peptidase IV. *J Biol Chem*. 2004;279(42):43854-43860. doi:10.1074/jbc.M405392200.

24. Anderson EM, Kwee BJ, Lewin SA, Raimondo T, Mehta M, Mooney DJ. Local delivery of VEGF and SDF enhances endothelial progenitor cell recruitment and resultant recovery from ischemia. *Tissue Eng Part A*. 2015;21(7-8):1217-1227. doi:10.1089/ten.tea.2014.0508.
25. Xu X, Jha AK, Duncan RL, Jia X. Heparin-decorated, hyaluronic acid-based hydrogel particles for the controlled release of bone morphogenetic protein 2. *Acta Biomater*. 2011;7(8):3050-3059. doi:10.1016/j.actbio.2011.04.018.
26. Mahoney DJ, Mulloy B, Forster MJ, Blundell CD, Fries E, Milner CM, Day AJ. Characterization of the interaction between tumor necrosis factor-stimulated gene-6 and heparin: Implications for the inhibition of plasmin in extracellular matrix microenvironments. *J Biol Chem*. 2005;280(29):27044-27055. doi:10.1074/jbc.M502068200.
27. Roy S, Lai H, Zouaoui R, Duffner J, Zhou H, P Jayaraman L, 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):1194-1205. doi:10.1093/glycob/cwr053.
28. Freudenberg U, Zieris A, Chwalek K, Tsurkan M V., Maitz MF, Atallah P, Levental KR, Eming SA, Werner C. Heparin desulfation modulates VEGF release and angiogenesis in diabetic wounds. *J Control Release*. 2015;220:79-88. doi:10.1016/j.jconrel.2015.10.028.
29. Baumann L, Prokoph S, Gabriel C, Freudenberg U, Werner C, Beck-Sickinger AG. A

novel, biased-like SDF-1 derivative acts synergistically with starPEG-based heparin hydrogels and improves eEPC migration in vitro. *J Control Release*. 2012;162(1):68-75. doi:10.1016/j.jconrel.2012.04.049.

30. Nakamura S, Ishihara M, Obara K, Masuoka K, Ishizuka T, Kanatani Y, Takase B, Matsui T, Hattori H, Sato T, Hidemi Hattori, 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;81:364-371. doi:10.1002/jbm.a.

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):6054-6067. doi:10.1016/j.biomaterials.2005.03.012.

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. *J Biomater Sci Polym Ed*. 2006;17(1-2):187-197. doi:10.1163/156856206774879090.

33. Zieris A, Dockhorn R, Ro A, Zimmermann R, Mu M, Welzel PB, Tsurkan M V, Sommer J, Freudenberg U, Werner C. Biohybrid networks of selectively desulfated glycosaminoglycans for tunable growth factor delivery. *Biomacromolecules*. 2014;15:4439-4446.

34. Wood MD, Borschel GH, Sakiyama-elbert SE. Controlled release of glial-derived neurotrophic factor from fibrin matrices containing an affinity-based delivery system. *J Biomed Mater Res Part A*. 2008:909-918. doi:10.1002/jbm.a.32043.

35. 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(2t):286-293. doi:10.1021/bc500565x.

36. Inoue Y, Nagasawa K. Selective N-desulfation of heparin with dimethyl sulfoxide containing water or methanol. *Carbohydr Res*. 1976;46:87-95. doi:10.1016/S0008-6215(00)83533-8.

37. Nagasawa K, Inoue Y, Kamata T. Solvolytic desulfation of glycosaminoglycuronan sulfates with dimethyl sulfoxide containing water or methanol. *Carbohydr Res*. 1977;58:47-55.

38. 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. *Carbohydr Res*. 1998;308:381-388. doi:10.1016/S0008-6215(98)00097-4.

39. Rinker TE, Philbrick BD, Temenoff JS. Core-shell microparticles for protein sequestration and controlled release of a protein-laden core. *Acta Biomater*. 2016;56:91-101. doi:10.1016/j.actbio.2016.12.042.

40. Xie L, Lin ASP, Levenston ME, Guldberg RE. Quantitative assessment of articular cartilage morphology via EPIC-??CT. *Osteoarthr Cartil*. 2009;17(3):313-320. doi:10.1016/j.joca.2008.07.015.

41. Thote T, Lin ASP, Raji Y, Moran S, Stevens HY, Hart M, Kamath R V., Guldberg RE, Willett NJ. Localized 3D analysis of cartilage composition and morphology in small animal models of joint degeneration. *Osteoarthr Cartil*. 2013;21(8):1132-1141. doi:10.1016/j.joca.2013.05.018.

42. Willett NJ, Thote T, Hart M, Moran S, Guldberg RE, Kamath R V. Quantitative pre-clinical screening of therapeutics for joint diseases using contrast enhanced micro-

computed tomography. *Osteoarthr Cartil*. 2016;24(9):1604-1612. doi:10.1016/j.joca.2016.04.021.

43. Tellier LE, Krieger JR, Sellick AL, Coogan AC, Falis AA, Rinker TE, Schudel A, Thomas SN, Jarrett CD, Willett NJ, Botchwey EA, Temenoff JS. Localized SDF-1 delivery increases pro-healing none marrow-derived cells in the supraspinatus muscle following severe rotator cuff injury. *Acta Biomater*. 2017:Submitted.
44. Blundell CD, Mahoney DJ, Almond A, DeAngelis PL, Kahmann JD, Teriete P, Pickford AR, Campbell ID, Day AJ. The link module from ovulation- and inflammation-associated protein TSG-6 changes conformation on hyaluronan binding. *J Biol Chem*. 2003;278(49):49261-49270. doi:10.1074/jbc.M309623200.
45. Parkar AA, Day AJ. Overlapping sites on the link module of human TSG-6 mediate binding to hyaluronan and chondroitin-4-sulphate. *FEBS Lett*. 1997;410(2-3):413-417. doi:10.1016/S0014-5793(97)00621-2.
46. Palmer AW, Guldberg RE, Levenston ME. Analysis of cartilage matrix fixed charge density and three-dimensional morphology via contrast-enhanced microcomputed tomography. *Proc Natl Acad Sci U S A*. 2006;103(51):19255-19260. doi:10.1073/pnas.0606406103.
47. Wancket LM, Baragi V, Bove S, Kilgore K, Korytko PJ, Guzman RE. Anatomical localization of cartilage degradation markers in a surgically induced rat osteoarthritis model. *Toxicol Pathol*. 2005;33(4):484-489. doi:10.1080/01926230590965364.
48. Goldring MB. Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Ther Adv Musculoskelet Dis*. 2012;4(4):269-285. doi:10.1177/1759720X12448454.

49. Cao T V, La M, Getting SJ, Day AJ, Perretti M. Inhibitory effect of TSG-6 link module on leukocyte-endothelial cell interactions in vitro and in vio. *Microcirculation*. 2004;(40):615-624. doi:10.1080/10739680490503438.

50. Nie T, Baldwin A, Yamaguchi N, Kiick KL. Production of heparin-functionalized hydrogels for the development of responsive and controlled growth factor delivery systems. *J Control Release*. 2007;122(3):287-296. doi:10.1016/j.jconrel.2007.04.019.Production.

51. Choi H, Lee RH, Bazhanov N, Oh JY, Prockop DJ. Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2 / NF-kB signaling in resident macrophages. *Immunobiology*. 2016;118(2):330-339. doi:10.1182/blood-2010-12-327353.

52. Wang N, Li Q, Zhang L, Lin H, Hu J, Li D, Shi S, Cui S, Zhou J, Ji J, Wan J, Cai G, Chen X. Mesenchymal stem cells attenuate peritoneal injury through secretion of TSG-6. *PLoS One*. 2012;7(8). doi:10.1371/journal.pone.0043768.

53. Torihashi S, Ho M, Kawakubo Y, Komatsu K, Nagai M, Hirayama Y, Kawabata Y, Takenaka-Ninagawa N, Wanachewin O, Zhuo L, Kimata K. Acute and temporal expression of tumor necrosis factor-alpha-stimulated gene 6 product, TSG-6, in mesenchymal stem cells creates microenvironment required for their successful transplantation into muscle tissue. *J Biol Chem*. 2015;290(37):22771-22781. doi:10.1074/jbc.M114.629774.

Figure Captions

Figure 1. Degree of heparin sulfation affects TSG-6 bioactivity *in vitro*. (A-B) Fully sulfated and N-desulfated heparin significantly enhanced TSG-6 anti-plasmin activity whereas (C) fully desulfated heparin had no effect on TSG-6 activity compared to soluble TSG-6 controls. *Significantly different than all other groups; $p \leqq 0.05$. #Not significantly different from each other but significantly different than all other groups; $p \leqq 0.05$; n = 3; data shown as mean ± SD.

Figure 2. Degradable, 10 wt% N-desulfated heparin microparticles were fabricated via Michael type addition and free radical polymerization. (A) Microparticles were fabricated with 10 wt% N-desulfated heparin methacrylamide, 90 wt% linear poly (ethylene glycol) diacrylate and 35 mM dithiothreitol (DTT). (B) Phase image analysis indicated that microparticles were 80±60 μm in diameter; black arrows indicate microparticles; scale bar is 100 μm.

Figure 3. 10 wt% Hep^{-N} MPs released TSG-6 over ~3 days and enhanced TSG-6 bioactivity. (A) Over 5 days, ~1.0 μg TSG-6 was released from MPs; n = 3-4; data shown as mean ± SD. (B) TSG-6 released from MPs exhibited significantly more anti-plasmin activity than soluble TSG-6 at the same concentration. *Significantly lower than all other groups; $p \leq 0.05$; n = 3-4; data shown as mean ± SD.

Figure 4. TSG-6 loaded MPs reduce cartilage damage 3 weeks following MMT injury. (A-D) Safranin-O stained coronal sections of tibiae 3 weeks following injury and treatment indicated that GAG loss was observed in the (A) injured and (B) soluble TSG-6, but not in the (C) TSG-6 loaded MP group. (E-H) Contrast-enhanced μCT imaging of the same samples indicated that cartilage fibrillation was present in the (E) injured and (F) soluble TSG-6 groups but was not present in the (G) TSG-6 loaded MP group. Scale bars are 500 μm; n = 3-4; data shown as mean ± SD. (I-K) Quantified evaluation of articular cartilage indicated that (I) cartilage thickness and (J) volume were significantly increased compared to uninjured controls after injury and soluble TSG-6 treatment, and (K) cartilage attenuation was significantly increased after soluble TSG-6 treatment. In contrast, no significant increase in cartilage thickness, volume, or attenuation was observed in the TSG-6 loaded MP group compared to uninjured controls. *Significantly different than contralateral control; $p \leqq 0.05$; n = 3-4; data shown as mean ± SD.

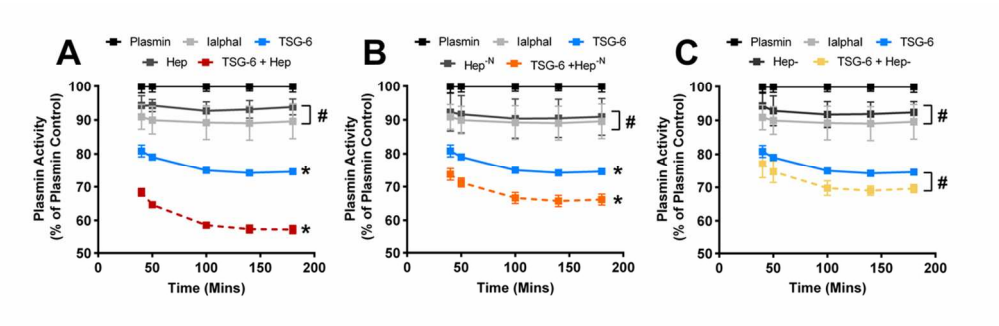


Figure 1. Degree of heparin sulfation affects TSG-6 bioactivity in vitro. (A-B) Fully sulfated and N-desulfated heparin significantly enhanced TSG-6 anti-plasmin activity whereas (C) fully desulfated heparin had no effect on TSG-6 activity compared to soluble TSG-6 controls. *Significantly different than all other groups; p ≦0.05. #Not significantly different from each other but significantly different than all other groups; p ≦0.05; n = 3; data shown as mean ± SD.

52x17mm (600 x 600 DPI)

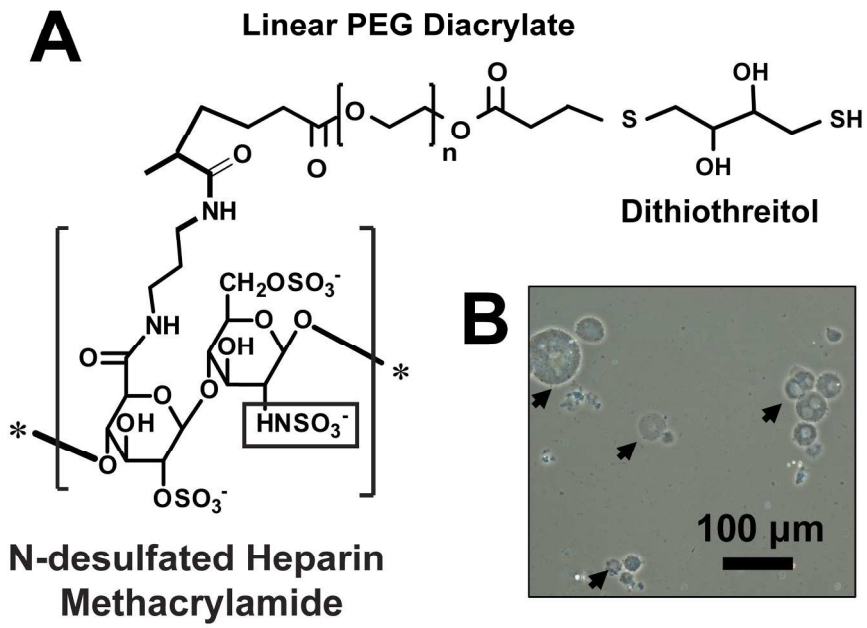


Figure 2. Degradable, 10 wt% N-desulfated heparin microparticles were fabricated via Michael type addition and free radical polymerization. (A) Microparticles were fabricated with 10 wt% N-desulfated heparin methacrylamide, 90 wt% linear poly (ethylene glycol) diacrylate and 35 mM dithiothreitol (DTT). (B) Phase image analysis indicated that microparticles were 80±60 µm in diameter; black arrows indicate microparticles; scale bar is 100 µm.

207x218mm (300 x 300 DPI)

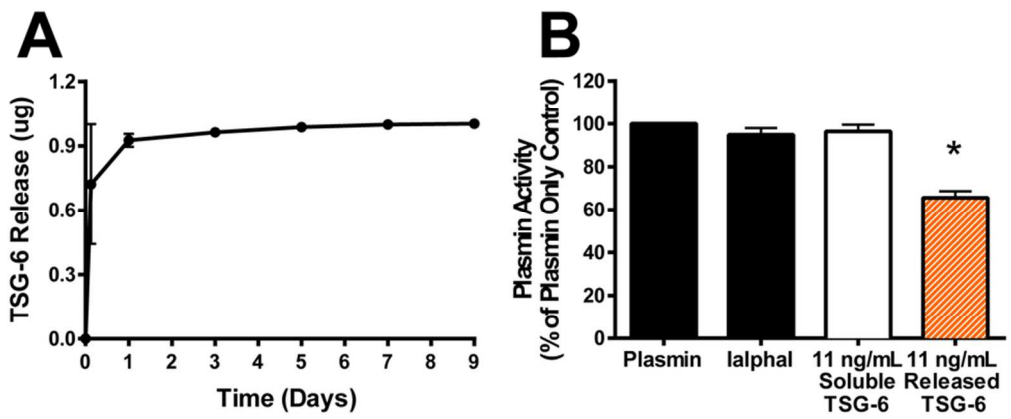


Figure 3. 10 wt% Hep-N MPs released TSG-6 over ~3 days and enhanced TSG-6 bioactivity. (A) Over 5 days, ~1.0 μg TSG-6 was released from MPs; n = 3-4; data shown as mean ± SD. (B) TSG-6 released from MPs exhibited significantly more anti-plasmin activity than soluble TSG-6 at the same concentration. *Significantly lower than all other groups; p 0.05; n = 3-4; data shown as mean ± SD.

81x34mm (300 x 300 DPI)

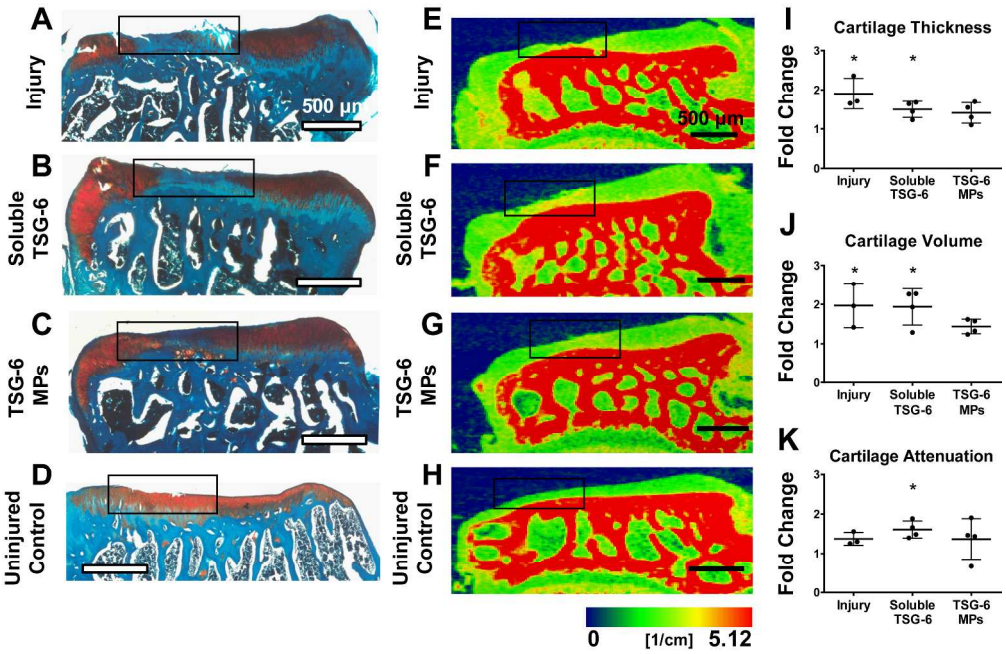
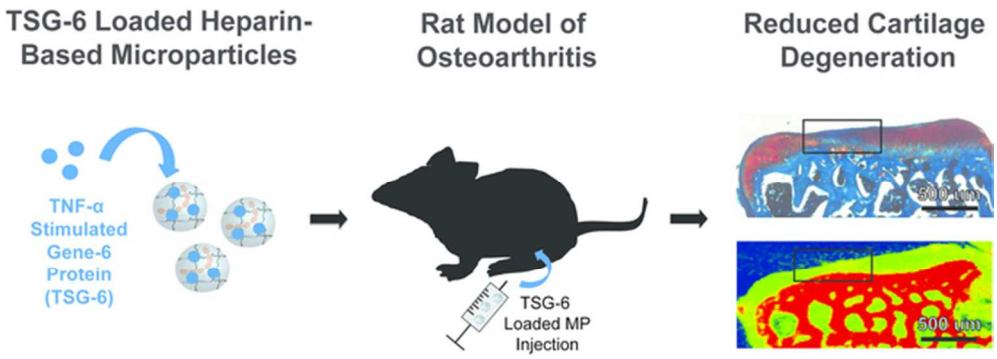


Figure 4. TSG-6 loaded MPs reduce cartilage damage 3 weeks following MMT injury. (A-D) Safranin-O stained coronal sections of tibiae 3 weeks following injury and treatment indicated that GAG loss was observed in the (A) injured and (B) soluble TSG-6, but not in the (C) TSG-6 loaded MP group. (E-H) Contrast-enhanced µCT imaging of the same samples indicated that cartilage fibrillation was present in the (E) injured and (F) soluble TSG-6 groups but was not present in the (G) TSG-6 loaded MP group. Scale bars are 500 µm; n = 3-4; data shown as mean ± SD. (I-K) Quantified evaluation of articular cartilage indicated that (I) cartilage thickness and (J) volume were significantly increased compared to uninjured controls after injury and soluble TSG-6 treatment, and (K) cartilage attenuation was significantly increased after soluble TSG-6 treatment. In contrast, no significant increase in cartilage thickness, volume, or attenuation was observed in the TSG-6 loaded MP group compared to uninjured controls. *Significantly different than contralateral control; $p \leqq 0.05$; n = 3-4; data shown as mean ± SD.

280x182mm (300 x 300 DPI)



58x20mm (300 x 300 DPI)