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14. ABSTRACT

We have successfully synthesized and characterized an injectable hydrogel biomaterial with tunable thermosensitivity and the capability for covalent attachment of therapeutic peptides. This new material can be tuned and tested with the purpose of delivery a gel to the injured brain that becomes responsive to the injury environment. This work resulted in a publication in the Journal of Controlled Release (J Control Release. 2015 Jun 28;208:76-84).

15. SUBJECT TERMS

prevalence, trauma, hydrogel, stem cell therapy, regeneration

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A unifying event common to all traumatic or vascular insults to the brain and spinal cord is the extravasation of blood. Extravasated blood is a principal toxin that leads to neuronal and glial cell death, inflammation and permanent cavitations that are not spontaneously repaired. Early after injury, blood enters the central nervous system (CNS) and directly kills brain cells but also orchestrates the formation of an inflammatory zone that is never regenerated. This region becomes surrounded by reactive glial and migrating stem cells that are induced to form a permanent scar. In turn, the therapeutic potential of transplanted stem cells is restricted by extravsated blood and ensuing inflammation. **Here we hypothesize that inhibition of select components of the blood coagulation cascade is both neuroprotective but also necessary to unlock the full therapeutic value of stem cell-based regenerative therapies.** The present proposal takes advantage of a long-standing, cross-disciplinary collaboration between the Horner and Pun laboratories. We combine our molecular insight in to the mechanism of thrombin damage with a state-of-the art bioengineered hydrogel for the simultaneous delivery of neural stem cells and therapeutic agents. Due to the challenges of sustained and directed drug delivery to the spinal cord, we will incorporate a novel hydrogel system developed in the Pun lab that we have shown is safe when delivered acutely following cervical spinal cord injury. These studies will establish the preclinical feasibility of anti-thrombin therapy to both protect the acutely injured spinal cord and improve the therapeutic capacity of stem cell therapy.

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1. Introduction

Our central objectives are to establish thrombin as a clinical target for preventing scarring and inflammation following CNS trauma in order to insulate and augment the effectiveness of stem cell transplant therapy. Our first aim is to engineer a tissue-responsive, injectable hydrogel to inhibit thrombin and thereby lessen the formation of scar after spinal cord injury. Our second aim is to promote host-transplant integration and regeneration by human induced pluripotent stem cell (hiPSC) transplants by co-injecting a biomaterial containing neural stem cells derived from induced-pluripotent stem cells.

In year 3 we completed formulation and characterization of our hydrogel and completed one small scale efficacy test in spinal cord-injured rats. For our no-cost extension year, we are completing a larger scale evaluation in injured rats.

2. Keywords

human induced pluripotent stem cell, spinal cord injury, gliosis, hydrogel, cell therapy, thrombin

3. Overall Project Summary

MAJOR TASK 1

Subtask 1: Synthesize panel of thermosensitive oligoethyleneglycol -based polymers and characterize by gel permeation chromatography, 1H-nuclear magnetic resonance, Fourier transform infrared and ultraviolet spectroscopy. Synthesize bivalirudin-membrane-

metaloproteinase-9 linker peptide and characterize by mass spec and high pressure liquid chromatography. May require iteration and fine-tuning based on characterization studies.

Subtask 1 Progress: Complete as reviewed in Year 1 Report. This work resulted in a publication in the Journal of Controlled Release (J Control Release. 2015 Jun 28;208:76-84).

Subtask 2: Generation and characterization of neuralized, human induced-pluripotent stem cells

Subtask 2 Progress: We have completed this subtask and results Year 1 and 2 Annual reports.

Subtask 3: Synthesize bivalirudin-conjugated polymers and test for bivalirudin release kinetics.

Subtask 3 Progress: This task is completed and reported in Year 2 Annual report and our 2015 publication (J Control Release. 2015 Jun 28;208:76-84).

Subtask 4: Evaluate the mechanical properties and lower critical solution temperature of polymers

Subtask 4 Progress: This task is complete. Cloud point measurements were completed and reported in Year 2 annual report. We performed additional rheological testing in Year 3 to characterize gelation behavior and mechanical properties. Gelation occurs, as shown by increases in storage modulus (G') with low loss modulus (G''). The gelation rate increases with temperature, polymer concentration and catalyst concentration (Fig 1A-C). Peptide crosslinkers slightly reduces gelation rate, but gelation occurs within 10 min.

.Subtask 5: Evaluate biocompatibility of neuralized, human induced-pluripotent stem cells with polymers with mitotic indices and immunofluorescence assessment.

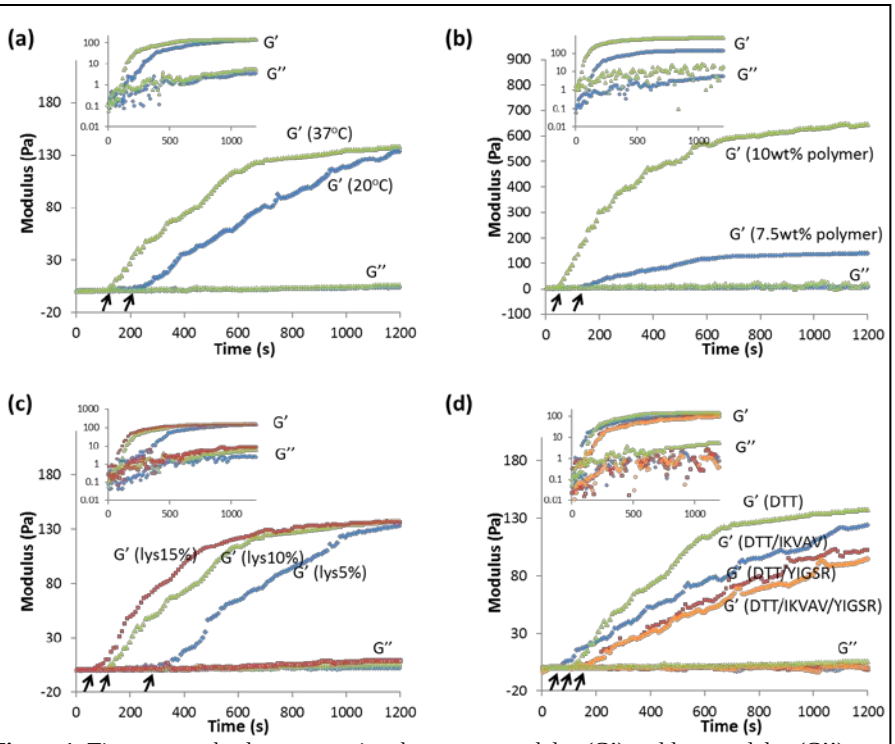

Figure 1. Time-sweep rheology measuring the storage modulus (G') and loss modulus (G'') as a function of time after polymer crosslinking. (a) Effect of temperature on gelation (b) Effect of polymer solution concentration on gelation (c) Effect of catalyst (lysine) concentration on gelation, and (d) Effect of peptide crosslinkers on gelation.

Subtask 6: Evaluate neuralized, human induced-pluripotent stem cell migration from hydrogels via transwell assay

Subtask 5 & 6 Progress These subtasks are complete and reported in our Year 2 annual report and in our recent publication (2017 Biomacromolecules v18:2723).

MAJOR TASK 2:

Subtask 1: Obtain IACUC and ACURO approval for all procedures involving animals

Subtask 1 Progress: This is completed and IACUC protocol is approved. ACURO approval has been obtained - SC130249.

In Year 2, we synthesized our bivalirudin-conjugated polymers. Bivalirudin is a peptide inhibitor of thrombin and we hypothesize that localized delivery of bivalirudin at the injury site will reduce glial scar formation. In Year 3, we characterized bivalirudin activity from our materials. An MMP-3 substrate peptide sequence was included between the hydrogel and

bivalirudin (Fig 2A). We showed previously that MMP-3 is upregulated after spinal cord injury. The MMP3 peptide sequence is cleaved specially by MMP-3 (Fig 2B). We then tested bivalirudin activity through a thrombin activity assay. In our first assay, we incubated hydrogel with MMP-3 to release bivalirudin and measured thrombin inhibition in the supernatant due to released bivalirudin. Thrombin inhibition was observed at similar levels to our previously-reported biomaterial (Fig 2C). We then tested thrombin inhibition from conjugated bivalirudin on the hydrogel and showed that conjugated bivalirudin is also active (Fig 2D). Thus, bivalirudin in our formulation should be active both after MMP3 cleavage and release and in the immediate location of the hydrogel.

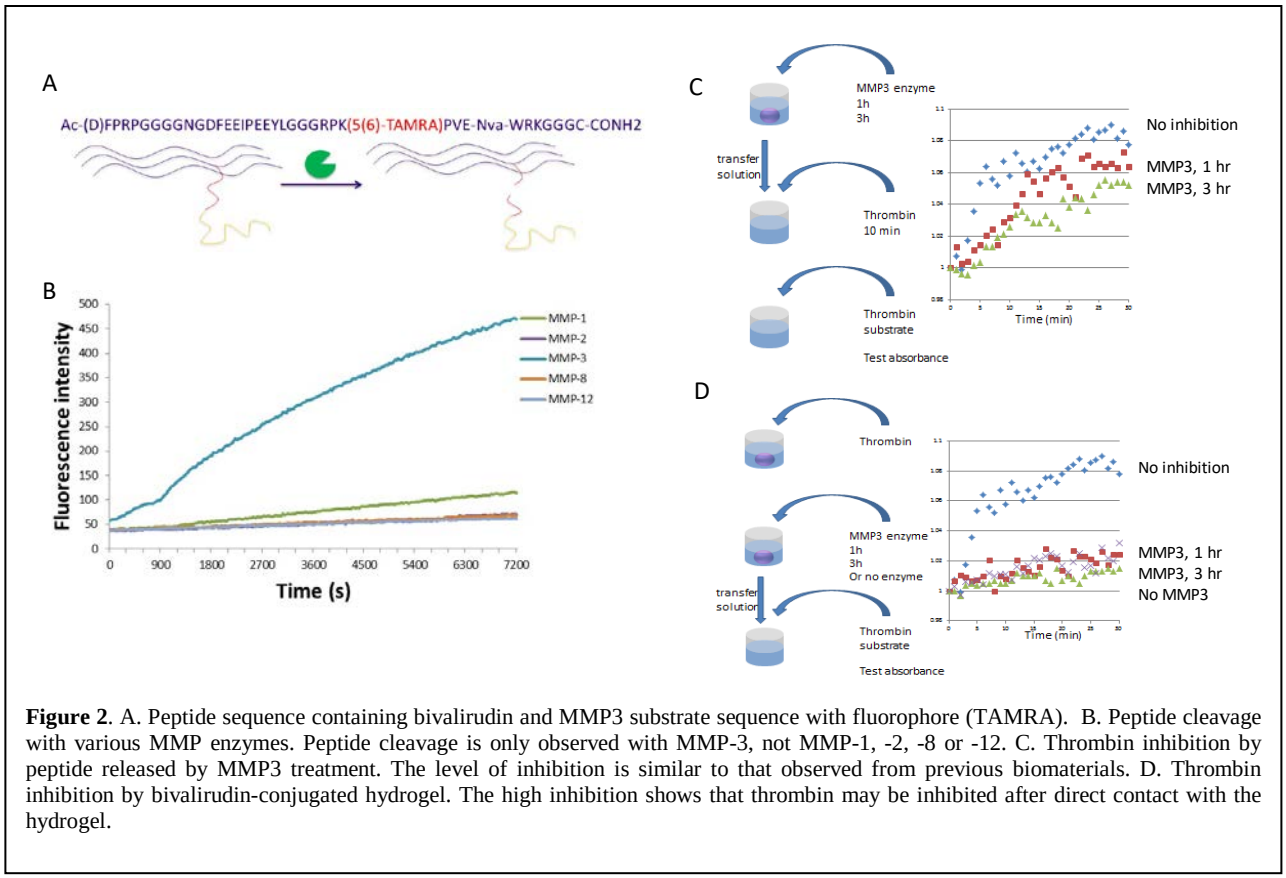

Figure 2. A. Peptide sequence containing bivalirudin and MMP3 substrate sequence with fluorophore (TAMRA). B. Peptide cleavage with various MMP enzymes. Peptide cleavage is only observed with MMP-3, not MMP-1, -2, -8 or -12. C. Thrombin inhibition by peptide released by MMP3 treatment. The level of inhibition is similar to that observed from previous biomaterials. D. Thrombin inhibition by bivalirudin-conjugated hydrogel. The high inhibition shows that thrombin may be inhibited after direct contact with the hydrogel.

Subtask 2: Synthesize labeled polymers and evaluate bivalirudin release and hydrogel resorption in rat spinal cord after contusion injury by fluorescence imaging.

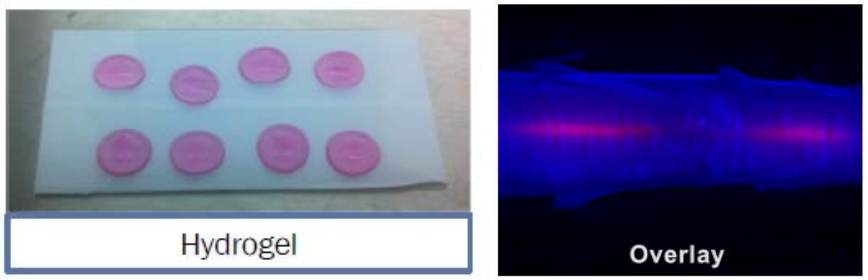

Figure 3. (Left) Fluorescently-labeled hydrogel used for pilot studies. (Right) Hydrogel injected into spinal cord. Bivalirudin peptide is labeled with rhodamine. The gel and peptide (red) is retained in the central canal of the spinal cord (blue).

Substask 2 Progress: We have synthesized rhodamine-labeled bivalirudin and incorporated the peptide into our hydrogel formulation. We show that the material is retained in the central canal of the spinal cord after injection (Fig 3).

Subtask 2: Synthesize control polymers for in vivo studies. We have synthesized control hydrogel that do not contain active bivalirudin for our pilot studies.

Subtask 3 : Implantation of bivalirudin and control hydrogels in rat SCI model. We have completed a pilot study with 12 injured rats treated with control hydrogel, bivalirudin hydrogel and bivalirudin hydrogel with NiPSCs. The experimental timeline is shown in Figure 4A.

Subtask 4: Behavioral analysis of rats. We analyzed behavior by the catwalk gait analysis and the forearm reaching test at 1 week post injury, 1 week post-implant and 2 weeks post-implant. Preliminary results are shown in Figure 4B for the catwalk gait analysis. Based on these preliminary results, increasing the assessment beyond two weeks and increasing animal number may reveal a significant difference in function.

Subtaask 5: Collection of tissue for immunohistochemical analysis. We have collected spinal cords for analysis of gliosis at the injury site. A sample histology image is shown in Figure 4C. We are currently completing analysis and quantification for this pilot study while planning for our larger scale animal study for the No Cost Extension Year.

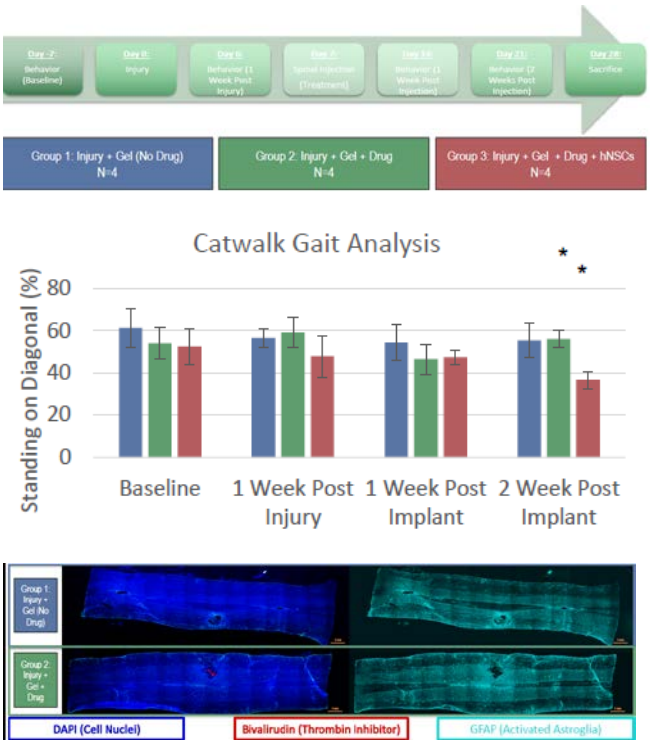

Figure 4. A. Experimental timeline for pilot study in injured rats treated with our hydrogel formulations. B. Preliminary catwalk gait analysis (Blue: control hydrogel; Green: bivalirudin-hydrogel; Red: bivalirudin-hydrogel with NhiPSCs. C. Sample histology fo spinal cords treated with control hydrogel (top) and bivalirudin-hydrogel (bottom). Bivalirudin (red) can be observed at the injection site. We are using these IHC slides to evaluate gliosis after injury.

4. Key Research Accomplishments

- Produced a new material based on cyclized vinyl polymers, synthesized by RAFT polymerization and shown this material is not toxic to neural progenitor cells.
- Conjugated and optimized the cyclized vinyl polymer hydrogesl for therapeutic delivery of bivalirudin.
- Proven that a metalloproteinase sensitive material can be applied to locally delver bivalirudin in the injured spinal cord.
- Hydrogel material is well tolerated in spinal cord and bivalirudin is localized and retained at the injury site.

5. Conclusion

There are multiple barriers that prevent the optimal delivery of biologics and cells to the injured nervous system. A significant problem is the formation of scar tissue that has a negative and long lasting impact on recovery but also limits the introduction of new nerve cells. Thermo-sensitive hydrogels offer a promising approach to develop a material that can integrate into the soft tissue of the nervous system. In this research we have modified hydrogels to become biologically responsive to the negative cues that occur after injury. In particular we have created a material that contains a natural inhibitor of scar formation; bivalirudin. The innovative aspect of this research is the development of a 'linker' in the material that will only releases bivalirudin when a scar-associated enzyme is activated. This reduces off-target effects and makes the material bio-responsive thereby delivering only the dose that is needed and only in the microenvironment that it can do the most benefits. In our first year we had tremendous success implanting materials that reduced scarring in the injured central nervous system. However, we noted significant toxicity to cultured cells. In year 2 we completed reformulated our material. We have produced a cyclized vinyl polymer and dithiol linker system that can serve as a universal template on which biomolecules and their combination can be applied to study the 3D cell-biomaterial interactions and drug release. We now demonstrate MMP3-selective release of either a fluorescent marker or bivalirudiin peptide from the newly synthesized material. In our third year, we completed an in initial evaluation of this material in injured rats. We showed that the material is retained at the injury site, is well tolerated, and we see some modest improvement in behavior. Moving forward, we will confirm these findings in a larger scale animal evaluation in our no cost extension year.

6. Publications, Abstracts, and Presentations

There are five publications and two meeting abstracts associated with this grant.

1. Lay press – nothing to report

2. Peer reviewed publications.

Publication 1: Elias PZ, Liu GW, Wei H, Jensen MC, Horner PJ, Pun SH. A functionalized, injectable hydrogel for localized drug delivery with tunable thermosensitivity: synthesis and characterization of physical and toxicological properties. J Control Release. 2015 Jun 28;208:76-84. doi: 10.1016/j.jconrel.2015.03.003. Epub 2015 Mar 4. PubMed PMID: 25747144.

Publication 2: Chu DS, Sellers DL, Bocek MJ, Fischedick AE, Horner PJ, Pun SH. MMP9-sensitive polymers mediate environmentally-responsive bivalirudin release and thrombin inhibition. Biomater Sci. 2015 Jan;3(1):41-5. doi: 10.1039/C4BM00259H. PubMed PMID: 25589953; PubMed Central PMCID: PMC4289632.

Publication 3: Sellers DL, Kim TH, Mount CW, Pun SH, Horner PJ. Poly(lactic-co-glycolic) acid microspheres encapsulated in Pluronic F-127 prolong hirudin delivery and improve functional recovery from a demyelination lesion. Biomaterials. 2014 Oct;35(31):8895-902. doi: 10.1016/j.biomaterials.2014.06.051. Epub 2014 Jul 23. PubMed PMID: 25064804; PubMed Central PMCID: PMC4136545.

Publication 4: Tianyu Zhao, Drew L. Sellers, Yilong Cheng, Philip J. Horner, Suzie H. Pun.

Tunable, injectable hydrogels based on peptide-crosslinked, cyclized polymer nanoparticles for neural progenitor cell delivery. *Biomacromolecues* v18:2723-2731.

3. Invited Articles

Publication 5: James-Kevin Y. Tan, Drew L. Sellers, Binhan Pham, Suzie H. Pun and Philip J. Horner. (2016) Non-Viral Nucleic Acid Delivery Strategies to the Central Nervous System. Frontiers in Molecular Medicine, v9:108.

4. Abstracts

Abstract 1: T. Zhao, D. L. Sellers, P. J. Horner and S. H. Pun, Injectable Hydrogel from Synthetic Cyclic Vinyl Polymers for Cell Therapy, July 17–20, 2016, The 43rd Annual Meeting & Exposition of the Controlled Release Society, Washington State Convention Center, Seattle, Washington, U.S.A.

Abstract 2: Hogan, M.K., Zhao, T.Y., Kondiles, B.R., Sellers, D.L., Pun, S.H., and Horner, P.J. (2018) Potential therapeutic effects of injectable hydrogel for controlled release of thrombin inhibitor following spinal cord injury. AANS.

7. Inventions, Patents and Licenses

Nothing to report.

8. Reportable Outcomes

Development of a biomaterial prototype for bio-responsive delivery of bivalirudin to the injured nervous system via direct injection.

9. Other Achievements

N/A

10. Appendices