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TITLE: Bioengineered Hydrogel to Inhibit Post-Traumatic Central Nervous System Scarring

PRINCIPAL INVESTIGATOR: Dr. Philip Horner

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| 6. AUTHOR(S)<br><br>Philip J. Horner and Suzie H. Pun<br><br>Email: pjhorner@houstonmehtodist.org/ spun@uw.edu                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                  |                          |                                      | 5d. PROJECT NUMBER                            |                                            |
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| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                  |                          |                                      |                                               |                                            |
| 14. ABSTRACT<br><br>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<br>prevalence, trauma, hydrogel, stem cell therapy, regeneration                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                  |                          |                                      |                                               |                                            |
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| a. REPORT<br>U                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | b. ABSTRACT<br>U | c. THIS PAGE<br>U        |                                      |                                               | 19b. TELEPHONE NUMBER (include area code)  |

[SF298]

**Note: An abstract is required to be provided in Block 14**

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 extravasated 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 2 we focused our efforts on completing the engineering of the hydrogel. In year 1 we discovered some cellular toxicity that could have limited the benefits of our approach in vivo. This year we re-developed our material and have created a non-toxic and flexible platform that is ready for in vivo testing. A second major achievement is the optimization of the material to release a therapeutic in a bio-responsive mechanism. These successes bring us on target to complete our in vivo testing and efficacy analysis in the trauma model in year 3.

#### **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, <sup>1</sup>H-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 are detail in Year 1 Report. We have successfully induced neural stem and progenitor cells from an IMR90, fetal lung fibroblast-derived embryonic stem cells. We have used retinoic acid to induce neural stem cells from the spinal cord that subsequently differentiate into all three major neural cell subtypes. We have also engrafted these cells in to the injured spinal cord and published these studies in the journal Experimental Neurology (Exp Neurol. 2013 Oct;248:491-503). These experiments demonstrate that neural stem cells survive well in the injured spinal cord but predominately produce glial cells and astrocytes in particular. The goal of subsequent tasks is to generate an environment that will remove this glial-genic signal.

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

*Subtask 3 Progress: In year 1 we conjugated a model peptide drug, bivalirudin, to sPEG-b-P(MEO<sub>2</sub>MA-co-OEGMA<sub>475</sub>-co-NHSMA) to test conjugation efficiency and to assess the effect of peptide grafting on polymer properties. Successful conjugation of peptide to the polymer was confirmed by an increase in absorbance at 280 nm (Figure 1A). This work resulted in a publication in the Journal of Controlled Release (J Control Release. 2015 Jun 28;208:76-84).*

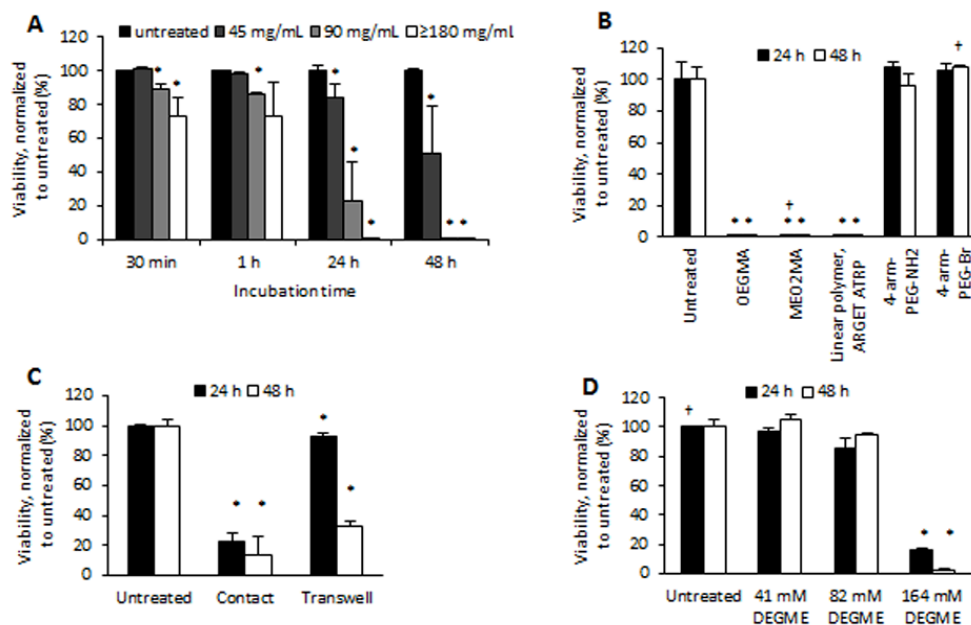
Subtask 4: Evaluate the mechanical properties and lower critical solution temperature of polymers

Subtask 4 Progress: This task is complete. We measured polymer cloudpoints of the oligoethyleneglycol-based star polymers in phosphate buffered saline using an Agilent 8453 UV-Vis Spectrophotometer. The temperature was raised slowly in increments of 1 °C with a 1 min hold at each temperature prior to the absorbance reading. The lower critical solution temperature was calculated as the temperature at which the absorbance reached a midpoint between the baseline and first plateau reading. Conjugation of the peptide to polymer with a 94:6 ratio of MEO<sub>2</sub>MA to OEGMA<sub>475</sub> resulted in polymers with improved solubility, but also an associated increase in the lower critical solution temperature far above physiological temperature. In order to compensate for the hydrophilicity of the peptide, conjugation was repeated with polymers containing a higher molar ratio of MEO<sub>2</sub>MA to OEGMA<sub>475</sub> (98:2 or 100:0). The subsequent peptide-polymer conjugates had lower critical solution temperatures which were at or below physiological temperature (Figure 1B). This makes the material ideal for injection in vivo.

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

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

Subtask 5 & 6 Progress: We have now completed these subtasks. Unfortunately biocompatibility was not optimal with the original material design. In short, in order to determine whether degradation products of the hydrogel may contribute to the observed cytotoxicity, cells were incubated directly on top of hydrogels (rather than encapsulated), or separated from the hydrogel by a Transwell insert. While the Transwell insert seemed to improve cell viability compared to direct gel contact, significant cytotoxicity was still observed at 48 h post-treatment (Figure 1C). In year 2 we made several changes to our design and essentially redesigned the material entirely. We are delighted to report significant progress in the development of a new material (see below). Progress in the respect has prevented the need for any delay in our SOW schedule. This indicates that polymer breakdown products may be cytotoxic over long-term exposure.

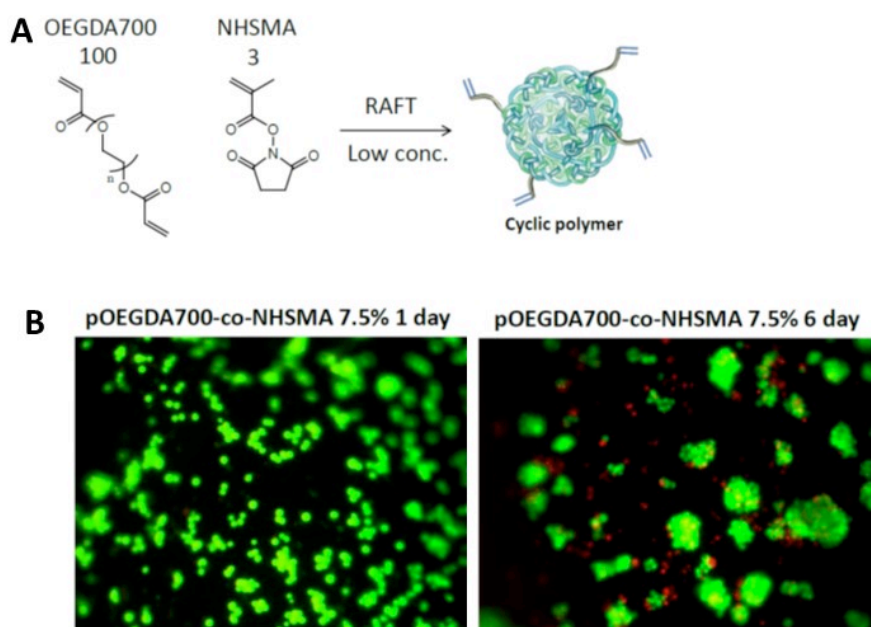


**Figure 1.** **A)** Viability of cells over time, encapsulated in various polymer concentrations. **B)** Viability of cells treated with polymer-equivalent concentrations of monomers, linear polymer, macroinitiator precursor, and macroinitiator. **C)** Viability of cells deposited directly on gel (“Contact”) or separated from gel by a Transwell insert (“Transwell”). **D)** Viability of cells treated with 41 mM, 82 mM, and 164 mM of DEGME, modeling 5%, 10%, and 20% MEO<sub>2</sub>MA hydrolysis, respectively. Treatments performed in triplicate, except when  $n = 2$ . Data are reported as mean  $\pm$  standard deviation. Statistical analysis performed with a two-tailed Student’s t-test, \*p-value < 0.05.

Based on these studies, we have redesigned our hydrogel material with the following design criteria: gelation at lower critical polymer concentration (to reduce polymer exposure), selection of more biocompatible starting monomers, and incorporation of bioactive peptides to improve cell viability. To this end, we have synthesized injectable polymers by RAFT polymerization of bi-functional OEGDA700 and NHSMA (Fig 2A). The resulting material is a cyclic polymer that exhibits thermosensitive behavior, and can be crosslinked to form hydrogels. We have shown that this material is well tolerated by mammalian cells. Cell viability using Live/Dead assay is high (green fluorescent cells) and cell proliferation is observed, as evidenced by spheroid formation within the hydrogel after 6-days of culture (Figure 2B). We have currently synthesized



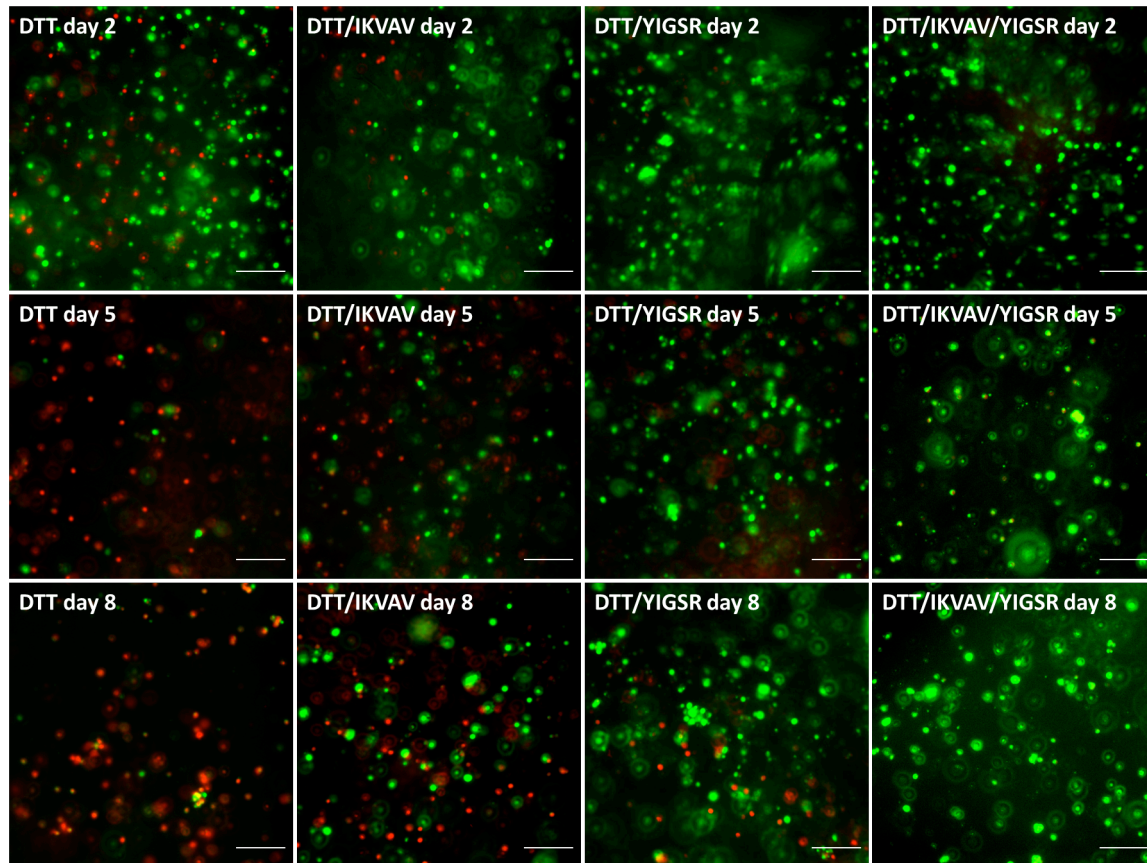
functionalized laminin-derived peptides as crosslinking agents for this hydrogel, as neural progenitor cells growth better in culture on laminin.



**Figure 2.** **A)** Schematic of cyclic polymer synthesis **B)** Epifluorescent images of mammalian cells cultured in cyclic polymer hydrogels for 1 and 6 days. Cell viability is assessed by Live/Dead stain for Green/Red cells, respectively.

We have also tested the final composition of hydrogel using peptide conjugates IKVAV and YIGSR to test for toxicity. In Figure 3 we demonstrate long-term viability with encapsulated NPCS.

**Figure 3.** Neural Progenitor Cells Encapsulation. Hydrogel crosslinked with DTT and DTT/Peptides (IKVAV and YIGSR), DTT:peptide=85:15. These data demonstrate long-term survival with NPCs.



#### 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 Figure 4 we diagram our peptide linkage strategy for conjugating bivalirudin to the scaffold in a material designed to be bio-responsive to MMP3. MMP3 has been shown to be upregulated after neural trauma acutely and represents a ideal target for in vivo release. Also in year 2, we have tested the release of substrate from the material (Figure 5). We have shown that our construction of proteolytic cleavage site produces a selectively responsive drug release that is unique to MMP3 but not other metalloproteinases

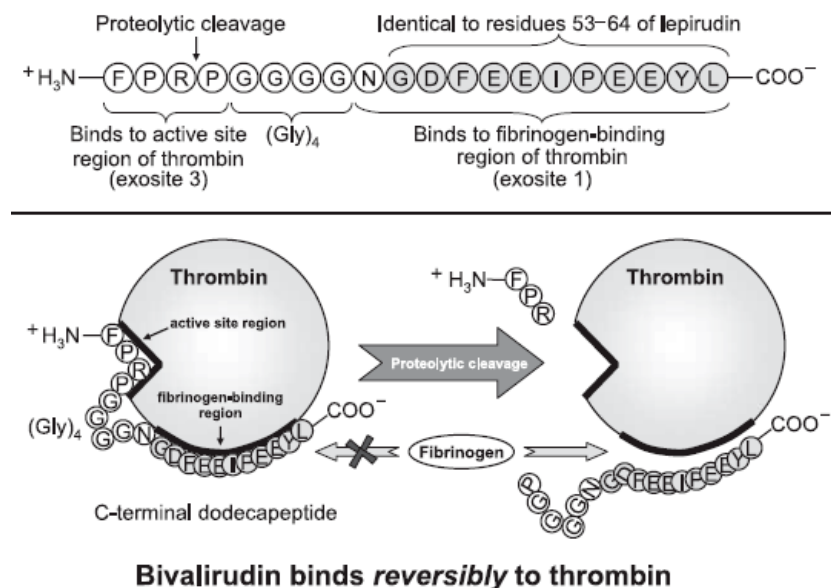
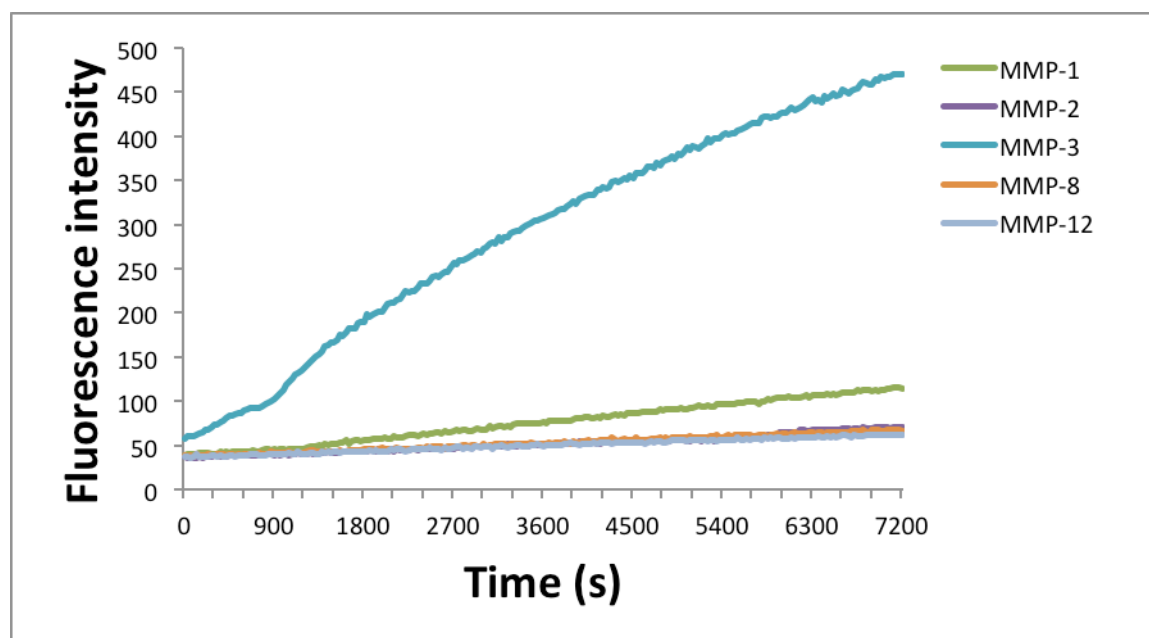


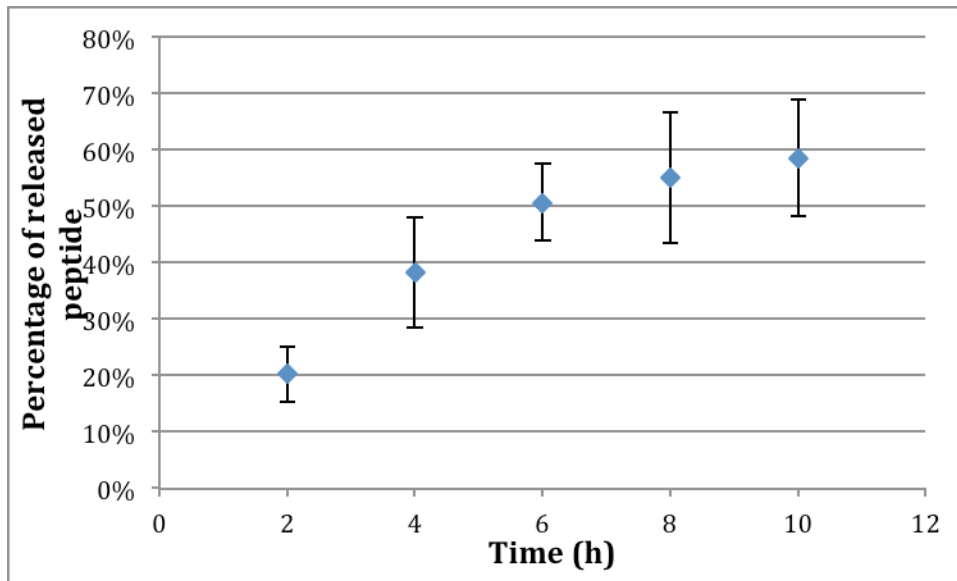
Figure 4 – Material strategy for proteolytic cleavage of bivalirudin from the hydrogel. Polymer-MMP3 conjugates are designed to have specific cleavage by MMP-3 enzyme.



**Figure 5.** In vitro testing of MMP3 select cleavage of MMP-3 peptide cleavage substrate. A fluorescent peptide was designed for testing release connects and substrate was exposed to multiple MMPs. Fluorescence intensity of H-Arg-Pro-Lys(fluorescein-5(6))Pro-Val-Glu-Nva-Trp-Arg-Lys(5(6)-TAMRA)-NH<sub>2</sub> is shown. Note, only MMP3 induces significant cleavage.

Finally, we tested conjugated bivalirudin release from the newly constructed material (Figure 6). These data indicate rapid release of bivalirudin by MMP3 over 12 hours.

***These data show that we have produced a material that 1) is non-toxic and biocompatible with neural progenitor cells, 2) has an incorporated peptide linkage that makes drug release specific to MMP3 and 3) capable of rapid release of our therapeutic drug to directly effect transplanted cells.*** The methods and data for the material formulation testing have been drafted for submission to the journal Chemical Science.



**Figure 6.** This is a test of bivalirudin partial sequence release from hydrogel by MMP3 enzyme cleavage. 0.5nM of Bivalirudin-MMP3 (BM3) peptide (Ac(D)F}PRPGGGGNGDFEEIPEEYLGGRPK(5(6)-TAMRA)PVE-Nva-WRKGGGC-CONH<sub>2</sub>) was conjugated to 50ul of hydrogel which was then immersed in 450ul Tris buffer solution, followed by adding MMP3 enzyme and cultured at 37oC for different time points. The concentration of released peptide sequence (blue) in solution was tested by Nanodrop (mean  $\pm$  SD, n = 3). The release sequence was confirmed by mass spectrum analysis.

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

Subtask 2 Progress: We have made great progress toward this subtask. We have performed extensive in vivo testing of HAMC hydrogel and established its pre-clinical efficacy in an injury model. Due to our observed toxicity to stem cells in vitro of HAMC hydrogel, we will perform our combined stem cell experiments with the newly developed bi-functional OEGDA700 and NHSMA material. However, our in vivo experiments with the HAMC material have shown strong biologic effect in vivo and we have published these findings. In year one we have demonstrated that polymer-conjugated bivalirudin peptides maintained activity while demonstrating enzyme-mediated release upon MMP9 exposure and prolonged release from hyaluronic acid/methylcellulose (HAMC) hydrogels compared to free bivalirudin peptide. Localized administration of bivalirudin copolymers *in vivo* at the site of a rat spinal cord injury decreased cellular proliferation and astrogliosis, suggesting the bivalirudin copolymer and HAMC hydrogel system are a promising therapeutic intervention for reducing immediate inflammatory responses and long term scarring. This work was published in *Biomaterials Science* (Biomater Sci. 2015 Jan;3(1):41-5)

#### **4. Key Research Accomplishments**

- Conjugated and optimized a bio-reactive material for therapeutic delivery of bivalirudin.
- Proven that a metalloproteinase sensitive material can be applied to locally deliver bivalirudin in the injured spinal cord.
- Bivalirudin release in vivo significantly reduces glial scarring after SCI.
- Identified potential material toxicity when stem cells are exposed for long time periods to HAMC hydrogels and engineered a modification of the material to eliminate toxicity.

- Produced a new material based on cyclized vinyl polymers, synthesized by RAFT polymerization and shown this material is not toxic to neural progenitor cells.
- Demonstrated that a cyclized vinyl polymer and dithiol linker system provides a universal template on which biomolecules and their combination can be applied to study the 3D cell-biomaterial interactions and drug release.

## 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 bivalirudin peptide from the newly synthesized material. Combined with our research in year 1 we have now demonstrated the safety and efficacy of delivering a thrombin inhibitor to the injured nervous system. We have also constructed a

versatile material that is biocompatible, over-comes prior toxicity to neural progenitor cells and has a unique and highly versatile bio-responsive element to deliver our therapeutic selectively to injury site.

## **6. Publications, Abstracts, and Presentations**

There are five publications and one meeting abstract 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, Fishedick 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 Zhaoa, Drew L. Sellersa,b, Yilong Chenga, Philip J. Horner, Suzie H. Pun.



Tunable, injectable hydrogels based on peptide-crosslinked, cyclized polymer nanoparticles for neural progenitor cell delivery. *In preparation for submission to Chemical Science.*

### **3. Invited Articles**

Publication 5: James-Kevin Y. Tan, Drew L. Sellers, Binhlan Pham, Suzie H. Pun and Philip J. Horner. Non-Viral Nucleic Acid Delivery Strategies to the Central Nervous System. *Frontiers in Neuroscience*, Rev, *in press*

### **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.

### **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. References**

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. 2015 Mar 4. PubMed PMID: 25747144.

Chu DS, Sellers DL, Bocek MJ, Fishedick 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.

## **11. Appendices - NA**



**INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

1. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).
2. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**
3. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.
4. **CONCLUSION:** Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.
5. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**
  - a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.
    - (1) Lay Press:
    - (2) Peer-Reviewed Scientific Journals:
    - (3) Invited Articles:
    - (4) Abstracts:
  - b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (\*) if presentation produced a manuscript.

6. **INVENTIONS, PATENTS AND LICENSES:** List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.
  7. **REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.
  8. **OTHER ACHIEVEMENTS:** This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.
- For each section, 4 through 9, if there is no reportable outcome, state “Nothing to report.”
9. **REFERENCES:** List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).
  10. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

**NOTE:**

**TRAINING OR FELLOWSHIP AWARDS:** For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

**COLLABORATIVE AWARDS:** For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

**QUAD CHARTS:** If applicable, the Quad Chart (available on this eReceipt System [https://cdmrp.org/Program\\_Announcements\\_and\\_Forms/](https://cdmrp.org/Program_Announcements_and_Forms/) and under “Forms” on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

**MARKING OF PROPRIETARY INFORMATION:** Data that was developed partially or exclusively at private expense shall be marked as “Proprietary Data” and Distribution Statement B included on the cover page of the report. Federal government approval is required before including Distribution Statement B. The recipient/PI shall coordinate with the GOR to obtain approval. **REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE.** It is the responsibility of the Principal Investigator to advise the GOR when restricted limitation assigned to a document can be downgraded to “Approved for Public Release.” **DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS.** See term entitled “Intangible Property – Data and Software Requirements” and [https://mrmc.amedd.army.mil/index.cfm?pageid=researcher\\_resources.technical\\_reporting](https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting) for additional information.