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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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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.

We have made significant progress toward completing our aims. One significant change to our structure has been the departure of Dr. Horner and his lab to the Houston Methodist Research Institute. Through discussions with Sandra Rosario we have changed the PI designation to Dr. Pun. We expect limited disruption of our research goals and timeline. Dr. Horner will retain responsibility for the in vivo tasks in Aims 2 and 3. Specifically, materials being developed in the Pun lab will be transferred to Houston where they will be combined with cells and injected in to the spinal cords of rats. Dr. Horner and Dr. Pun have maintained a 15-year collaborative relationship and we do not anticipate any changes in the research plan, personnel effort or timeline. We have modified our SOW and are preparing a sub-budget for Dr. Horner. Dr. Horner has obtained IACUC approval for in vivo experiments from the Houston Methodist Research Institute and an separate ACURO will be filed for the future sections of Aims 2 and 3 described below, when experiments are performed in Houston. Dr. Horner's new contact information is:

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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: We have successfully synthesized and characterized an injectable hydrogel biomaterial with tunable thermosensitivity and the capability for covalent attachment of therapeutic peptides Figure 1. We have tuned and tested the thermosensitive monomers that can be varied to compensate for the incorporation of a relatively hydrophilic drug such as hyrudin. We have developed parameters for maintaining an appropriate gelation temperature for physiological applications such as co-culture with neural stem cells and injection in vivo. Although the required polymer concentration of the present formulation is not suitable for cell

encapsulation, the hydrogel has potential utility for combinatorial, controlled drug delivery in soft tissue applications. This work resulted in a publication in the Journal of Controlled Release (J Control Release. 2015 Jun 28;208:76-84).

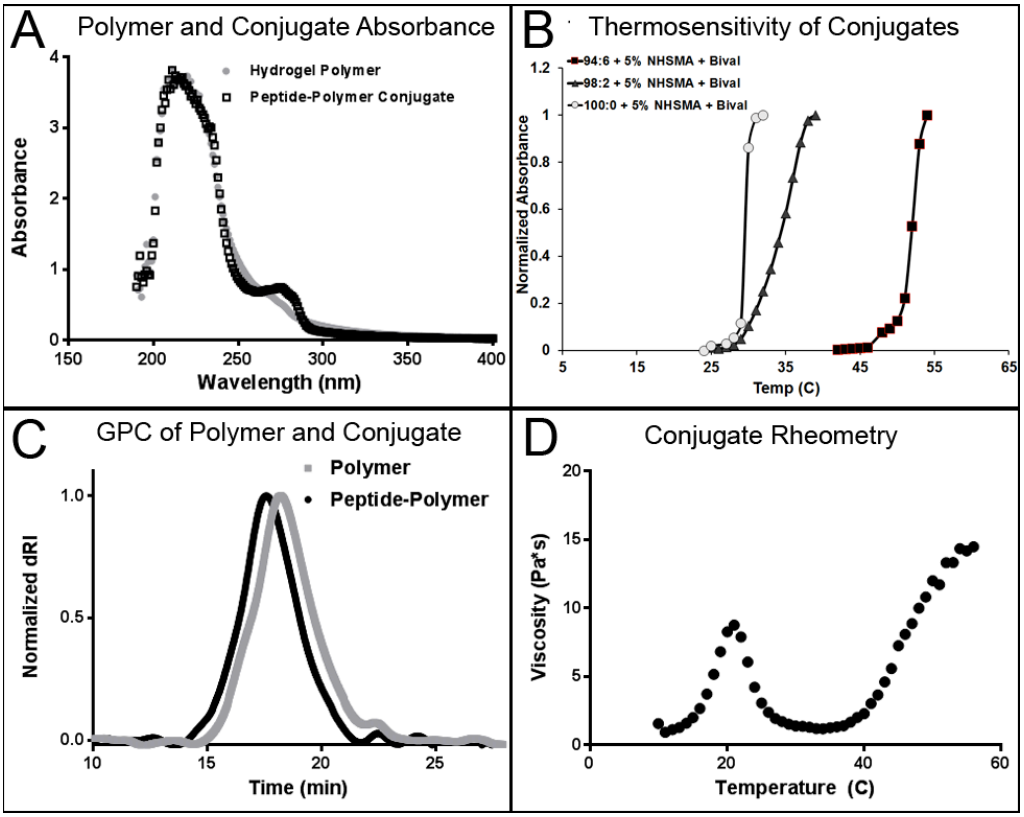

Figure 1. A) Absorbance spectrum of the peptide-polymer conjugate, showing an increase at 280 nm relative to polymer without peptide. **B)** Absorbance (670 nm) measurements for peptide-polymer conjugates with varying proportions of MEO_2MA and $OEGMA_{475}$. The LCST can be tuned using the ratio of thermosensitive monomers to compensate for the hydrophilic peptide. **C)** GPC traces of sPEG-*b*-P(MEO_2MA-*co*-$OEGMA_{475}$-*co*-NHSMA) polymer before and after peptide conjugation. The conjugate shows a leftward shift, consistent with an increase in molecular weight from the peptide. **D)** Rheometry of peptide-polymer conjugate, showing distinct phases in which the viscosity rises, falls, and rises again with increasing temperature. A tube inversion test at 37 °C demonstrated a free standing gel.

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

Subtask 2 Progress: We have completed this subtask. 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_2MA-*co*-$OEGMA_{475}$-*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 almost 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_2MA to $OEGMA_{475}$ 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_2MA to $OEGMA_{475}$ (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: Unfortunately biocompatibility was not optimal with the material produced. We observed cell toxicity of the hydrogel in initial testing of the material with cell co-culture. We spent significant time to determine the source of toxicity but in the end determined to work on the development of a new material that is not-toxic and we

are happy 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. 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 2C). This indicates that polymer breakdown products may be cytotoxic over long-term exposure.

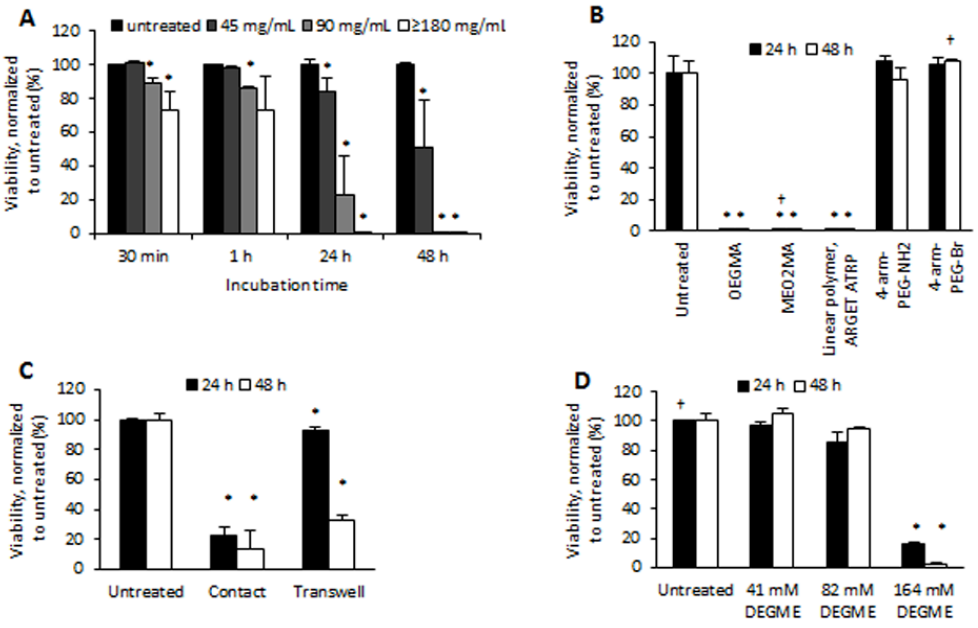

Figure 2. **A)** Viability of cells over time, encapsulated in various polymer concentrations. **B)** Viability of cells treated with polymer-equivalent concentrations of monomers, linear polymer, macroinitiator precursor, and macroinitiator. **C)** Viability of cells deposited directly on gel ("Contact") 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_2MA hydrolysis, respectively. Treatments performed in triplicate, except when †n = 2. Data are reported as mean ± 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 3A). 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 3B). We have currently synthesized functionalized laminin-derived peptides as crosslinking agents for this hydrogel, as neural progenitor cells growth better in culture on laminin.

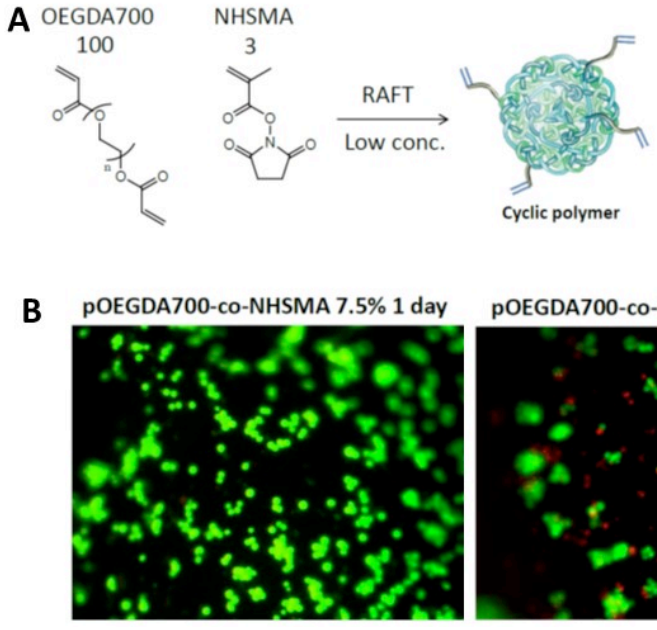

Figure 3. **A)** Schematic of cyclic polymer synthesis **B)** Epifluorescent images of mammalian cells cultured in cyclic polymer hydrogels for 1 and 6 days. Cell viability is assessed by Live/Dead stain for Green/Red cells, respectively.

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.

Subtask 2: Synthesize dual-labeled polymers and evaluate bivalirudin release and hydrogel resorption in rat spinal cord after contusion injury by fluorescence imaging.
Substask 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 delver bivalirudin in the injured spinal cord.
- Bivalirudin release in vivo significantly reduces glial scaring 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.

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. To create this material we spent the first year optimizing materials to be bio-responsive but also retain their thermo-sensitivity and softness to be injectable and compatible with the brain. We made one major accomplishment but also encountered one setback. Our accomplishment was to show that the material containing bivalirudin could be safely injected in to an injured spinal cord without causing damage or bleeding. Bivalirudin release led to a decrease in scar formation. Our setback was the observation that long-term exposure of the material with stem cells can result in modest toxicity. We were not able to isolate the toxic component but did troubleshoot a solution in parallel by

creating a modified formulation that does not seem to have a toxic effect. In the next phase of this proposal, we will be combining human neural stem cells with the material. Our thesis is that the release of bivalirudin will create a protective environment surrounding the neural stem cells and prevent the gliogenic signals that decrease stem cell therapeutic potential. With the development of a multistage, brain-compatible hydrogel and a safe, non-toxic platform for cell delivery, we are on target to accomplish our goals on schedule. Overall, our initial findings indicate application of a hydrogel-based inhibitor of scar formation has therapeutic value for the sub-acute treatment of head and spinal cord injury.

6. Publications, Abstracts, and Presentations

There are three 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, 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.

3. Invited Articles

We are currently writing a review based in part on the material developed here to be published in Frontiers.

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. Epub 2015 Mar 4. PubMed PMID: 25747144.

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11. Appendices

N/A