OPTIMIZATION OF HYDROGEL ENCASEMENT FOR MICRO TISSUE ENGINEERED NEURAL NETWORK FABRICATION

OLIVIA AMADOR[2,3,4] , SAARANG KARANDIKAR[2,3], DIMPLE CHOUHAN[1,2], AND KACY CULLEN[1,2,3,4]

[1]Center for Brain Injury & Repair, Department of Neurosurgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, [2]Center for Neurotrauma, Neurodegeneration & Restoration, Corporal Michael J. Crescenz Veterans Affairs Medical Center, Philadelphia, PA, [3]Department of Bioengineering, School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, PA. [4]Penn Undergraduate Research Mentorship Program.

INTRODUCTION

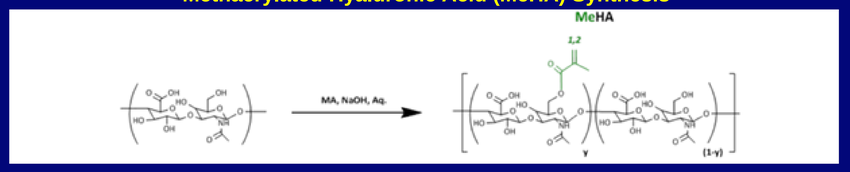
Parkinson's Disease is a neurodegenerative disorder primarily affecting dopaminergic cells in the substantia nigra.[1] The resulting degenerated axons in the nigrostriatal pathway lead to a denervated striatum, which affects circuits controlling patient movement.[2] Nearly one million individuals in the U.S. are currently affected by the disease and approximately 90,000 more are diagnosed each year.[3] While current standards of treatment, including oral Levodopa (L-Dopa) and deep brain stimulation, help to manage symptoms, none can restore circuit level functionality.[4,5] Though ectopic implantation of dopaminergic neurons is an elegant path toward permanent repair, a lack of input modulation can ultimately worsen dyskinesia symptoms for patients [6].

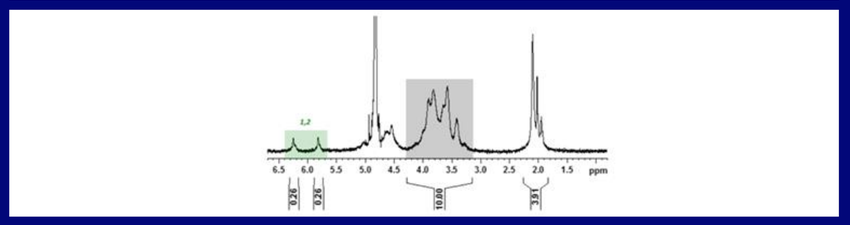
To address these challenges, our group has begun development of implantable micro-tissue engineered neural networks or Micro-TENNs. Micro-TENNs consist of a hollow hydrogel column filled with an extracellular-matrix like solution and seeded with an aggregated mass of immature neurons at one end. In turn, the hydrogel column acts as a guide for proliferating axons, yielding unidirectional outgrowth. When fabricated with dopaminergic neurons, micro-TENNs act as Tissue Engineered Nigrostriatal Pathways (TE-NSPs) by reconnecting the substantia nigra pars compacta and striatum, theoretically restoring proper dopamine signaling required for motor regulation. However, TE-NSP axonal proliferation remains slow, typically tapering to <500µm/day[7]. Given that axonal proliferation is strongly dependent on the local neuronal micro-environment [8], this study aimed to optimize micro-TENN outgrowth through manipulation of the hydrogel encasement. Specifically, micro-TENN casings were fabricated from methacrylated hyaluronic acid (MeHA) with varying degrees of backbone modification (DoM) and solution concentration. As a proof of concept, we then constructed generalized micro-TENNs from embryonic rodent cortical neurons to assess the impact of casing polymeric network density on axonal outgrowth.

(A) Brain pathway, etiology in Parkinson's disease. (B) Our Tissue Engineered Nigrostriatal Pathway implant connecting the substantia nigra and striatum to restore the degenerated pathway

METHODS

Methacrylated Hyaluronic Acid (MeHA) Synthesis

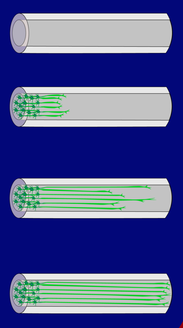




Methacrylated Hyaluronic acid was synthesized by the above esterification reaction. Briefly, 1.0g hyaluronic acid (HA) was dissolved in DI water and then cooled under continuous stirring. pH was adjusted to approximately 8.5 by addition of NaOH, and a calculated volume of methacrylic anhydride (MA) was then added based on desired degree of backbone modification. pH was then monitored and readjusted back to 8.5 as needed for 3 hours before placing the solution in 4°C storage overnight. Thereafter, pH was readjusted to 8.5 and the reaction contents were dialyzed against DI water for 5 days. After dialysis, the solution was frozen at -80°C and lyophilized. Finally, ^{1}H NMR analysis was performed to estimate achieved backbone modification.

Micro-TENN Fabrication & Cortical Neuron Isolation

A schematic of micro-TENN structure is depicted at right, showing progressive axon growth over 14+ days in vitro.



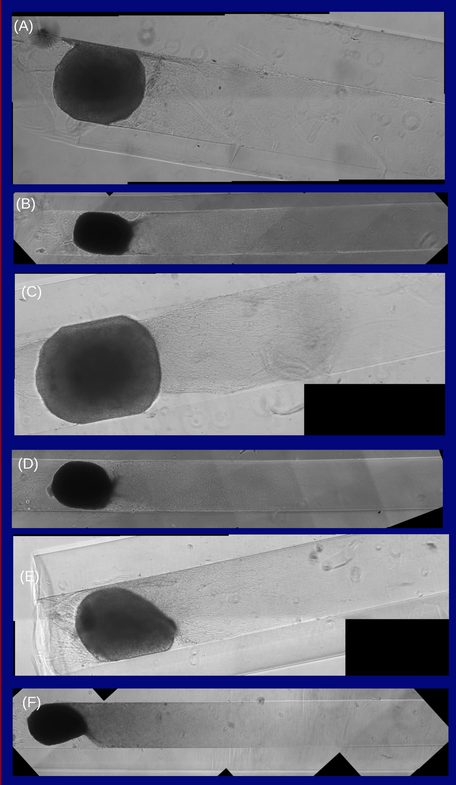
Micro-TENN casings were fabricated by first drawing MeHA solution into a capillary tube coaxially loaded with an acupuncture needle (inner/outer diameter of 500/1049um respectively). MeHA was cured under 10mw/cm^2 UV light, the acupuncture needle was withdrawn, and the resulting casing was then deposited into DPBS for storage. Casings were cut into 12mm lengths and an ECM consisting of rat tail collagen and mouse laminin was injected. After 20 minutes of gelation, an aggregate of rodent cortical neurons (110k neurons per aggregate) was inserted in one end of the column and the construct was placed in neurobasal media for long-term culture.

Cortical Cell Isolation & Aggregate Fabrication

To fabricate neuronal aggregates, cortices were first isolated from E18 Sprague Dawley rat embryos. Cortices were then digested with trypsin and fully dissociated under DNAse. The resultant solution was centrifuged and resuspended in neurobasal medium. Approximately 15uL of cell suspension was then transferred into custom pyramidal PDMS aggregation wells. After final centrifugation, resultant aggregates were allowed to rest for 96h at 37ºC prior to installation within a micro-TENN casing.

RESULTS

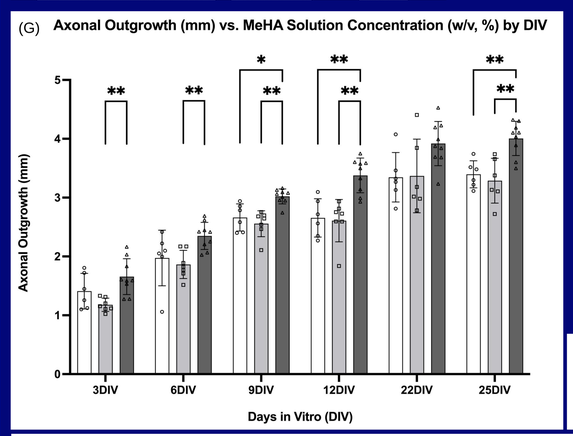
Solution Concentration Comparison Study



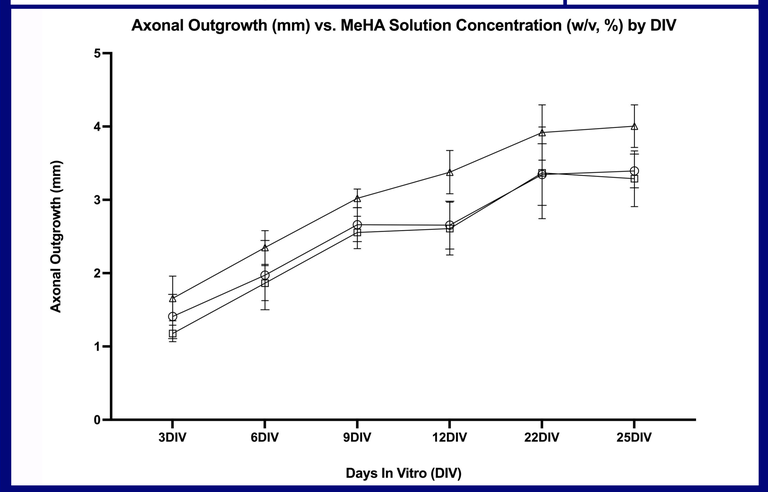
(A) Phase-contrast image of a micro-TENN with a 23% DoM, 3% solution casing at 3 days post plating (days in vitro or DIV).
(B) The same micro-TENN shown in (A) at 28DIV.
(C) Phase-contrast image of a micro-TENN with a 23% DoM, 4% solution casing at 3 DIV.
(D) The same micro-TENN shown in (C) at 28DIV.
(E) Phase-contrast image of a micro-TENN with a 23% DoM, 5% solution casing at 3 DIV.
(F) The same micro-TENN shown in (E) at 28DIV.

*Images are cropped to show the farthest axon outgrowth at their respective time points

This study assessed the impact of MeHA solution concentration on lead axonal outgrowth. DoM was held at 23%, and three groups of micro-TENN casings consisting of 3%, 4%, and 5% solution concentration respectively were fabricated. All groups were plated with a single 110k neuron aggregate.

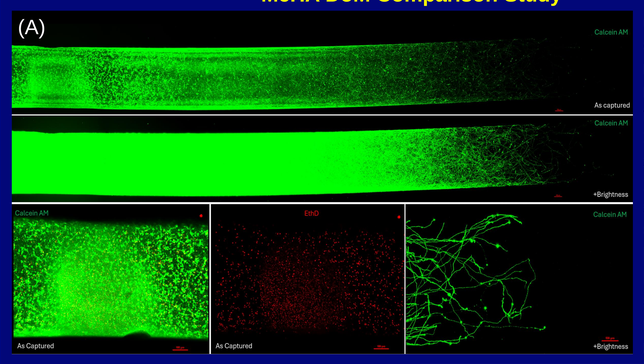


(G) Pairwise graph of Axonal Outgrowth (mm) vs. MeHA Solution Concentration by days in vitro
(H) Linear graph of axonal outgrowth (mm) vs. MeHA Solution Concentration by days in vitro

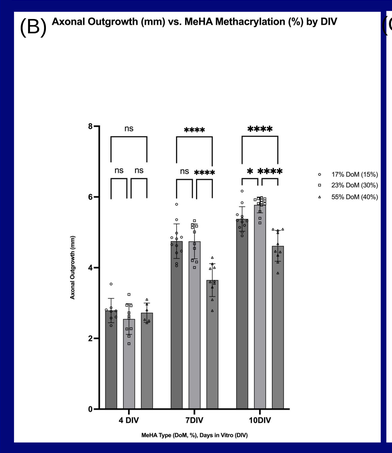


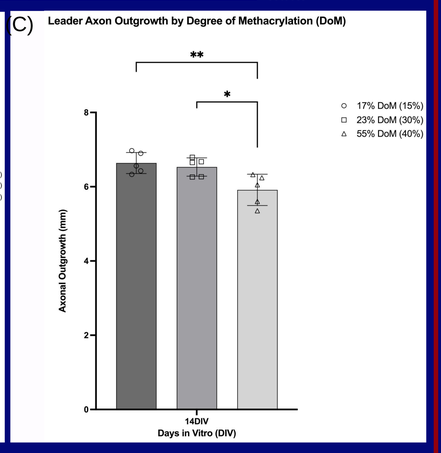
The above results suggest micro-TENN casings fabricated from 23% DoM MeHA at 5% solution enabled significantly faster axonal growth than casings fabricated at 3 and 4% solution. However, it should be noted that the raw difference between groups was typically < 1mm on any given day, and overall Micro-TENN outgrowth was approximately 3 to 4 mm over 25 days.

MeHA DoM Comparison Study



(A) Representative confocal micrograph of a micro-TENN fabricated from 23% DoM, 3% solution MeHA at 14 DIV after staining with Calcein-AM and Ethidium Homodimer. Lead axonal length was measured at 6.53 mm.





(B) Pairwise graph of axonal outgrowth (mm) vs. MeHA DoM by days in vitro. Measurements were taken from live phase images of the micro-TENNs at each time point. (C) Pairwise graph of axonal outgrowth (mm) vs. MeHA DoM by days in vitro. Measurements were taken from confocal micrographs of 5 randomly selected micro-TENNs per group at 14DIV after staining with Calcein AM (green) and ethidium homodimer (red).

This study compared the effects of MeHA DoM on axonal outgrowth. Micro-TENN casings were fabricated from MeHA with 17% DoM, 23% DoM, or 55% DoM, all at 3% solution concentration. Initially, no significant differences in axonal outgrowth were observed among the three groups. However, by day 7, the 17 and 23% DoM groups exhibited significantly faster growth relative to the 55% DoM group. This outgrowth trend continued through remaining time points. By 14 days in vitro, total micro-TENN axonal outgrowths reached approximately 6 to 7 mm.

Conclusions

The optimization of the micro-TENN encasement was investigated through the manipulation of MeHA backbone modification and solution concentration. Our findings suggest that encasements made of higher degrees of methacrylation and lower solution concentrations correlate with less axon outgrowth within the micro-TENN. This may result from higher backbone modification levels driving higher network density within the encasement. In turn, higher polymeric network density could restrict diffusion through the column walls and enforce reliance on diffusion through the open ends of the structure. Ultimately, this change in diffusion balance could reduce the overall amount of available nutrients, restrict removal of wastes, and thus hinder axonal outgrowth through heightened metabolic stress. Future studies will characterize porosity and molecular diffusion through MeHA at varying DoM and solution concentrations. Longitudinal neuronal health will also be assessed via lactate dehydrogenase levels or similar nondestructive outcome measures.

Acknowledgements

We thank the generous funding received from the Penn Undergraduate Research Mentorship Program, and the Department of Veterans Affairs (I01-BX003748, I01-RX001097). D.K.C. is a co-founder of Axonova Medical, LLC and Innervace, Inc., which are University of Pennsylvania spin-out companies focused on translation of advanced regenerative therapies to treat nervous system disorders.

References

[1] Parkinson's Disease. National Institute on Aging. 2022. [2] Sonne J. Neuroanatomy, Substantia Nigra. 2022. [3] Statistics. Parkinson's Foundation. [4] Gandhi KR. Levodopa (L-Dopa). 2023. [5] Lozano A. M. Deep Brain Stimulation. 2019. [6] Greene P. E. Persistent dyskinesias. 2021. [7] Wisberty J. Gordian-Velez, Restoring lost nigrostriatal fibers. 2021. [8] Eissa A. Moving through the crowd. 2023.

Contact information

Presenting author: Olivia Amador, Undergraduate Class of 2026, Department of Bioengineering, School of Engineering and Applied Science, University of Pennsylvania, PA.
Email: oamador@seas.upenn.edu