An Engineered Protein Hydrogel for Promoting Neurite Growth

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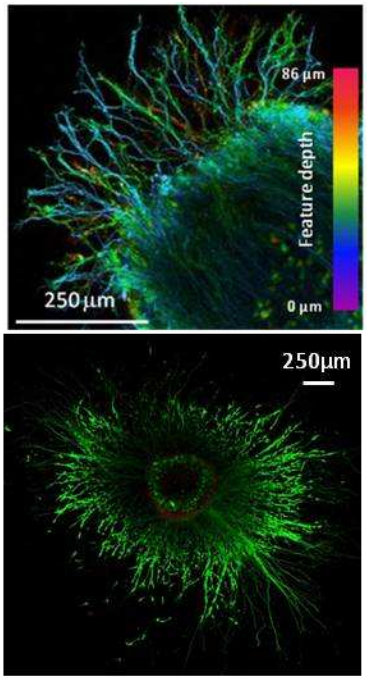
Statement of Purpose: Neural regeneration within the central nervous system (CNS) is a critical unmet challenge as brain and CNS disorders continue to be the leading cause of disability nationwide. Here we have developed a 3D cell instructive hydrogel made of engineered protein, an artificial extracellular matrix (ECM). Previously we engineered a family of elastin-like protein (ELP) hydrogels with the goal of investigating individual and synergistic effects of elastic modulus and cell adhesivity on cell behavior in a tunable microenvironment[1]. Unlike natural matrices, our engineered ELP allows independent modulation of such factors using a defined biological protein hydrogel, but only two-dimensional studies had been conducted.

Methods: ELPs containing either adhesive RGD sequences or non-adhesive RDG scrambled sequences were recombinantly expressed in *Escherichia coli* BL21 DE3 and purified[2]. Crosslinked hydrogels were formed by mixing the ELP solution at 1-3.0 wt% with tetrakis (hydroxymethyl) phosphonium chloride (THPC) which reacts with free amines to crosslink the engineered protein. Ratios of THPC reactive sites to free amines were adjusted from 0:5:1 to 2:1. Elastic Young's modulus was determined by unconfined compression to 15% strain. Storage and loss modulus were determined via oscillatory stress/strain measurements. Gels were created with controlled RGD densities from 0 to 1.9×10^6 ligands/μm^3. Whole embryonic chick dorsal root ganglia (DRGs) were encapsulated within the gels and allowed to grow over the course of 7 days in growth medium containing 10% fetal bovine serum, 1% penicillin/ streptomycin, and 50 ng/mL nerve growth factor (NGF) in DMEM under normal incubation conditions. Confocal images were obtained to determine viability (live/dead staining) and cell type (immunocytochemical staining for neurons and glia). A Matlab code was written to quantify outgrowth. Furthermore, single-cell suspensions of PC12 cells were encapsulatd in 3D within the protein gels and viability was determined via the Alamar Blue assay.

Results: Encapsulated DRGs survived well, with minimal death observed (<10% in all cases), a novel result for 3D encapsulation within engineered protein gels (Figure, top). Over seven days, neurites grew into the surrounding ELP matrix in a 3D manner (Figure, bottom). RGD density had a profound effect on length and number of neurites, with 1.9×10^6 ligands/μm^3 more than doubling the rate of neurite extension. Immunostaining demonstrated that the outgrowth was indeed neurite extension, as the majority of the growth was positive for beta-tubulin with very few glial cells migrating into the matrix. By adjusting the crosslinker concentration, we tuned the elastic modulus to 0.5, 1.5, or 2.1 kPa with constant adhesive site densities. The most compliant gels led to the greatest outgrowth from encapsulated DRGs, with neurites extending over 1800 μm by day 7. In contrast, the stiffest gels permitted far fewer extensions and limited outgrowth to a maximum of 600 μm. This work was limited to large clusters of cells that extended neurites into the surrounding matrix. Preliminary results encapsulating single-cell suspensions of PC12 cells suggest that these cells can survive the encapsulation process and proliferate over the course of 7 days. While PC12 viability increases over time, we are working to tune hydrogel factors to maximize cell growth and viability.



DRGs extend neurites three-dimensionally into the surrounding ELP matrix as early as day 1 (top). At day 7, cells survive well (live/dead imaging, bottom) and extend dense networks of neurites hundreds of microns.

Conclusions: This work demonstrates the versatility of our modularly-designed protein hydrogels for neural cell culture and encourages continued development as a biomaterial tissue construct for treating spinal cord injury. We can viably encapsulate neuronal clusters and neuronal-like single cells in 3D engineered protein hydrogels and directly control the material properties (adhesive site density, mechanical properties) to direct the growth of neurons. We believe this is the first report of single cells surviving and growing in this type of covalently crosslinked protein hydrogel matrix. We have learned from this work the parameters important to viably encapsulate single cells and we now hope to extend this work to encapsulation of more physiologically relevant neural cell types and co-cultures.

References:

1 Straley, K. S. & Heilshorn, S. C. Design and adsorption of modular engineered proteins to prepare customized, neuron-compatible coatings. *Front Neuroengineering* **2**, 1-10 (2009).

2 Straley, K. S. & Heilshorn, S. C. Independent tuning of multiple biomaterial properties using protein engineering. *Soft Matter* **5**, 114-124, doi:Doi 10.1039/B808504h (2009).