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Photoreactive interpenetrating network of hyaluronic acid and Puramatrix as a selectively tunable scaffold for neurite growth

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ABSTRACT

The reconstruction of soft tissue, such as that which is found in the nervous system, is governed by the mechanical cues of the growth microenvironment. The complexity of the nervous system, particularly in cases of nerve repair and reconstruction, necessitates the development of facile high-throughput investigational tools. This study assesses the hypothesis that a mechanically tunable photoreactive interpenetrating network (IPN) of hyaluronic acid and Puramatrix can be manipulated in order to demonstrate that 3-D environmental stiffness influences neurite growth and proliferation. For these studies we employed photocrosslinkable glycidyl methacrylate hyaluronic acid (GMHA) and Puramatrix, a self-assembling peptide scaffold, leading to a structurally adjustable IPN system. Our *in vitro* model provides us with a simple, reproducible environment to generate different properties in a single specimen. Mechanically manipulated IPN systems with different degrees of methacrylation were fabricated using a dynamic mask projection photolithography apparatus and characterized. To gauge the impact of IPN stiffness on neurite outgrowth, dorsal root ganglia (DRG) explants were cultured in the hydrogels. We found that neurite outgrowth in 3-D was more likely to happen in an environment with a lesser degree of methacrylation, which corresponded to structures that were more compliant and more porous. Overall, tuning the mechanical behavior of our IPN systems led to statistically significant ($p < 0.05$) differences in cellular growth and extension that warrants further investigations.

1. Introduction

The regional architectural morphology and mechanical properties of the nervous system microenvironment significantly influence nerve regeneration. Hydrogels with inherently tunable mechanical properties, provide a useful, biocompatible platform to investigate the structural–functional interdependence. The microstructure of a hydrogel can be tweaked through chemical manipulation to establish a micromechanical environment that most closely mimics that of the nervous system. The present study examines the effects of altering the regional composition of a novel hydrogel on neurite growth and development in an effort to improve future nervous system regeneration therapies. To this end we have examined a novel dual hydrogel system that incorporates hyaluronic acid (HA) and Puramatrix (PM).

HA is a glycosaminoglycan polymer composed of disaccharides D-glucuronic acid and D-N-acetylglucosamine that are linked by alternating β-1,4 and β-1,3 glycosidic bonds [1,2]. Abundant throughout the extracellular matrix (ECM), HA is an important structure within the nervous system and throughout the body [3–5]. HA has been shown to improve peripheral nerve regeneration *in vitro* by organizing the ECM into a more favorable structure for axon migration [6]. The present analysis investigates the chemical modification of HA utilizing polymerizable methacrylate groups to alter the regional structural and mechanical properties of a hydrogel scaffold in an *in vitro* model of nerve regeneration.

While HA has been utilized in biomaterial applications to create tissue-like scaffolds, there is no single ideal design that can closely mimic the body's properties. As such, considerable research has been devoted towards its application as a biomimetic scaffold [7–12]. Incorporating HA with other hydrogels in order to achieve a more desirable cell support and protein adhesion has been repeatedly reported for tissue engineering applications [11,12]. There are a variety of methods for utilizing the functional properties of HA in order to employ it for nerve tissue studies. One of these approaches is through the use of an interpenetrating network of polymers (IPN).

IPNs are hybrid structures composed of two or more polymers that are physically or chemically crosslinked into a network that is then re-entangled. These highly entangled networks retain the

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structural and mechanical properties of component polymers while strengthening and reinforcing the overall scaffold. Incorporation of PM into the IPN system designed in this article adds cell-adhesive properties to mechanically tunable HA, which is not typically cell-adhesive on its own [11–15].

In this study, we created an *in vitro* model that incorporated HA with PM. PM is a self-assembling peptide scaffold structurally composed of 99% water and synthetic peptide (1% w/v) that is composed of 16 amino acids which have alternating hydrophilic and hydrophobic side chains [16–19]. It is widely used to recreate the 3-D microenvironments that are favorable for cell growth in the absence of animal-derived materials and pathogens [16].

We utilized an integrated system with a digital micromirror device (DMD) capable of structural and molecular 3-D micropatterning to create the dual hydrogel tissue culture constructs used in our investigations. Our model consisted of a photocrosslinkable polyethylene glycol (PEG) that surrounded an IPN hydrogel of methacrylated-HA and PM. By projecting UV light in defined geometries onto the photocrosslinkable substrates, the DMD enabled us to create a dynamic mask that could irradiate PEG and the IPN throughout the depth of the gel [17,20–23]. We took advantage of this method in order to create a dual hydrogel system with a 3-D microenvironment to create an ideal *in vitro* environment for investigating neurite growth in the presence of varying mechanical properties.

In summary, this work describes a novel hydrogel that utilizes photocrosslinkable HA and PM components to promote cellular growth and allow adjustment of mechanical properties by varying the degree of methacrylation. This analysis measures the effects of the mechanical and microstructural changes on neuronal growth and guidance.

2. Materials and methods

2.1. Synthesis of glycidyl methacrylate modified hyaluronic acid

All materials were obtained from Sigma–Aldrich, St. Louis, MO, unless otherwise specified. Photocrosslinkable glycidyl methacrylate-modified HA (GMHA) was synthesized as previously described by adding methacryloyl groups to HA (Molecular Weight: 1.5–1.8 $\times$ 10^6 Da) to obtain 32% and 90% degrees of methacrylation (%Me) [24]. Briefly, 1.0 g of HA was combined with 200 mL PBS (pH 7.4), 67 mL of dimethyl sulfoxide (DMSO), 13.3 g glycidyl methacrylate (GM), and 6.7 g of triethylamine (TEA) to prepare 32% GMHA (%Me); similarly 132 mL PBS, 132 mL DMSO, 40.8 g GM, and 13.3 g TEA to prepare 90% GMHA (%Me). After 10 days of reaction at room temperature, the solutions were dialyzed against d-H_2O for 3 days and then lyophilized for 3 days. %Me was confirmed with ^{1}H NMR.

2.2. Preparation of PM–GMHA interpenetrating network gels

For the preparation of interpenetrating network (IPN) and semi-interpenetrating network (S-IPN) hydrogels, both 32%Me and 90%Me GMHA were dissolved overnight to 4% (w/v) in PBS, pH 7.4, containing 1% (w/v) Irgacure 2959 (Ciba Specialty Chemicals, Basel, Switzerland) and 0.03% (v/v) N-vinyl-pyrrolidinone. 0.001% (v/v) laminin (Invitrogen, Carlsbad, CA) was added to 1% (v/v) PM solution (BD Biosciences, Bedford, MA). While PM alone will support axon outgrowth, laminin was added according to the manufacturer's protocol to obtain optimal DRG neurite outgrowth in PM. To choose the best composition for each IPN system, either with 32%Me (IPN_{32}) or 90%Me (IPN_{90}), three different proportions of PM/GMHA were prepared: 1:2, 1:1 and 2:1. To form S-IPNs the solutions were placed in an incubator at 37 °C and 5% CO_2 for at least 30 min to allow PM to complete its gelation in the presence of PBS or neuronal growth media and GMHA. The S-IPNs then were changed into complete IPN systems by a 14 min constant irradiation with UV light. Photopolymerization of GMHA chains occurred by free radical polymerization, and so gelation of IPNs was assumed to be complete after 30 min of incubation followed by 14 min of UV irradiation.

2.3. Fabrication of dual hydrogel culture systems

Dual hydrogel culture systems were fabricated via digital projection photolithography, as previously described and seen in Fig. 1 [17]. Briefly, photoreactive hydrogel solutions contained in permeable cell culture inserts with 0.4 μm pore size (Corning Inc., Corning, NY) were irradiated using an apparatus consisting of a collimated UV light source (OmniCure 1000 with 320–500 nm filter, EXFO, Quebec, Canada), a digital micromirror device (DMD) (Discovery™ 3000, Texas Instruments, Dallas, TX) as a dynamic photomask, and a 2$\times$ Plan Fluor objective lens (Nikon Instruments, Tokyo, Japan) [17,20,25,26]. A solution of 10% (w/v) PEG-diacrylate (M_n 1000; Polysciences Inc., Warrington, PA) and 0.5% (w/v) Irgacure 2959 in PBS was irradiated with 181 mW/cm^2 UV light, as measured by a radiometer (306 UV Powermeter,

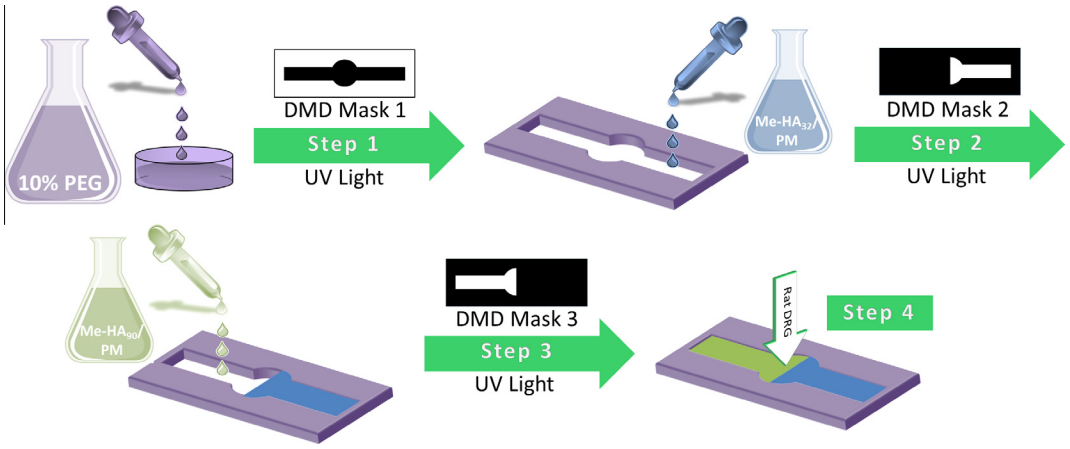

Fig. 1. *In vitro* model was made by fabricating the PEG mold utilizing DMD and related photomask (Mask 1). Each side of the mold was filled with either S-IPN_{90} or S-IPN_{32}. For each side, a different photomask (Mask 2 and 3) was used to form a complete IPN system. Dorsal root ganglion (DRG) E15 was cultured in the center of the model. Step 1: PEG mold fabricated utilizing DMD. Step 2: Mold filled with semi-IPN_{90} and irradiated for 14 min to form IPN_{90}. Step 3: Other mold half filled with S-IPN_{32} and irradiated to make IPN_{32}. Step 4: DRG explants cultured in the center of the model.