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Systematic optimization of an engineered hydrogel allows for selective control of human neural stem cell survival and differentiation after transplantation in the stroke brain

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ABSTRACT

Stem cell therapies have shown promise in promoting recovery in stroke but have been limited by poor cell survival and differentiation. We have developed a hyaluronic acid (HA)-based self-polymerizing hydrogel that serves as a platform for adhesion of structural motifs and a depot release for growth factors to promote transplant stem cell survival and differentiation. We took an iterative approach in optimizing the complex combination of mechanical, biochemical and biological properties of an HA cell scaffold. First, we optimized stiffness for a minimal reaction of adjacent brain to the transplant. Next hydrogel crosslinkers sensitive to matrix metalloproteinases (MMP) were incorporated as they promoted vascularization. Finally, candidate adhesion motifs and growth factors were systemically changed *in vitro* using a design of experiment approach to optimize stem cell survival or proliferation. The optimized HA hydrogel, tested *in vivo*, promoted survival of encapsulated human neural progenitor cells (iPS-NPCs) after transplantation into the stroke core and differentially tuned transplanted cell fate through the promotion of glial, neuronal or immature/progenitor states. This HA hydrogel can be tracked *in vivo* with MRI. A hydrogel can serve as a therapeutic adjunct in a stem cell therapy through selective control of stem cell survival and differentiation *in vivo*.

1. Introduction

Stroke is the leading cause of long-term disability [1]. There are no therapies that promote recovery in this disease. New strategies aimed at enhancing post-stroke brain plasticity have utilized trophic factors, stem cell therapies or a combination of the two. However, their clinical translation has been limited because of the short half-life and undesirable systemic effects of injected growth factors [2,3] and poor survival of transplanted cells [4], partly due to the abrupt withdrawal of adhesive support and the inflammatory environment of the damaged brain [5]. Although there are many protocols to differentiate *in vitro* stem/progenitor cells to desired fates, *in vivo* these protocols are either not possible or fail to differentiate the cells to the same extent. The lack of a successful medical therapy that promotes long-term recovery in stroke imposes a substantial clinical and economic burden, indicating the need for a novel therapeutic solution.

Recent advances in tissue engineering have developed injectable hydrogels that can serve both as a protective vehicle for cells and a depot release platform for trophic factors, with distinct synergistic advantages of both strategies. Cell-loaded hydrogels provide structural support that not only directly promote the survival of encapsulated cells [6], but also promote cell infiltration from the surrounding parenchyma [7] and reduce the glial scar and inflammation at the ischemic border [8,9]. Similarly, local drug delivery from injectable hydrogels can achieve sustained [3,10,11] or sequential delivery [12] in a time- and space-controlled manner, while enhancing protein stability, diffusion distance and *in vivo* bioactivity [11]. Polymer-based hydrogels are highly

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customizable as their composition can be modified to adapt to the host tissue. For example, a simple alteration of the polymer length, the crosslinking points and/or the scaffold mesh size can modulate gel stiffness, nutrient diffusion, and even cell motility [13]. Recent studies have shown that a wide variety of chemical, mechanical and spatial cues can be incorporated into cell–material interactions to ultimately allow for greater control over cell behavior [14].

Hyaluronic acid (HA) gels are appealing choices for cell encapsulation in a transplant approach. HA is abundantly found in the brain, particularly in the endogenous environment for neural progenitor cells (NPCs) [15] and it is both a biocompatible and bioresorbable material that allows cells to degrade it as they spread within the gel [16]. We have previously reported a hyaluronic acid hydrogel crosslinked *in situ* via thiol/acrylate Michael type addition for human induced pluripotent neural precursor (iPS-NPC) culture *in vitro*, which demonstrated biocompatibility after transplantation *in vivo* [13].

Stroke offers a unique opportunity for a tissue engineering neural repair therapy. After initial cell death in stroke, the clearance of debris in the lesion leaves a compartmentalized cavity that can accept a large volume transplant without further damaging the surrounding healthy parenchyma [17]. This stroke cavity is situated directly adjacent to the peri-infarct tissue, the region of the brain that undergoes the most substantial repair and recovery, meaning that any therapeutic delivered to the cavity will have direct access to the tissue target for repair [2]. Although utilizing a hydrogel material to promote differentiation of transplanted stem cells is a logical next step, no published study has shown substantial differentiation or differential control over post-transplantation cell fate. We developed a hydrogel material that can control transplanted human neural progenitor cell fate *in vivo* after the injection through the modulation of adhesive and trophic signals delivered from the scaffold. We show that the material can be optimized *in vitro* to result in either maintenance of the progenitor state or differentiation towards different central nervous system fates *in vivo*. Our findings mark the first time a hydrogel has been engineered to control transplanted human stem/progenitor cell fate *in vivo* utilizing *in vitro* findings.

2. Materials and methods

2.1. Cell culture

Induced pluripotent stem cells (iPS) were generated from human fibroblasts and characterized [18] under approved protocols from the UCLA ESCRO. NPCs were differentiated from iPS through formation of neural rosettes and maintained in culture [19]. On the day of transplantation human iPS-NPCs were harvested by TrypLE treatment (3–5 min; Life Technologies), centrifuged at 300 g for 5 min, re-suspended in maintenance culture medium and kept on ice. Cell viability throughout transplantation process remained above 95%–97% as determined by Trypan blue exclusion method. Animal origin-free products were used for the cell culture.

2.2. Hyaluronic acid modification

Hyaluronic acid (60,000 Da, Genzyme, Cambridge, MA) was functionalized with an acrylate group using a two-step synthesis as previously described [20]. After dissolving the HA (2.0 g, 5.28 mmol) in water, it was reacted with adipic dihydrazide (ADH, 18.0 g, 105.5 mmol) in the presence of 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC, 4.0 g, 20 mmol) overnight at a pH of 4.75. The hydrazide-modified hyaluronic acid (HA-ADH) was purified with decreasing amounts of NaCl (100, 75, 50, 25 mmol) for 4 h each via dialysis (8000 MWCO). The solution was then purified via dialysis (8000 MWCO) in deionized water for 2 days and lyophilized. The HA-ADH was re-suspended in 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid (HEPES) buffer (10 mM HEPES, 150 mM NaCl, 10 mM EDTA, pH 7.4) and reacted with N-acryloxysuccinimide (NHS-AC), 1.33 g, 4.4 mmol overnight. After purification via dialysis as described earlier, the acrylated HA (HA-AC) was lyophilized. The product was analyzed with 1H NMR (D_2O) and the degree of acrylation (14.9%) determined by dividing the multiplet peak at $\delta = 6.2$ (cis and trans acrylate hydrogens) by the singlet peak at $\delta = 1.6$ (singlet peak of acetyl methyl protons in HA). This gel was chosen because of its biocompatibility with human tissue, as it is constituted of naturally occurring brain extracellular matrix constituents. The mechanical properties of this gel are similar to those of normal brain. HA has been shown to promote angiognesis in a mouse model of skin wound healing [21,22]. Biotin was then introduced to HA-AC using sulfo-NHS–LC–biotin (NHS–biotin). NHS–biotin (100 mg) was added as a solid to a solution of HA–AC (300 mg; 3 mg/mL in PBS; pH 7.4) as previously described [22]. The reaction proceeded for 16 h at room temperature with stirring. The reaction product was then purified by dialysis (MWCO 14,000) against distilled water for 24 h. The final purified product was lyophilized and stored at 4 °C until used.

2.3. Heparin modification

Heparin Carboxylic acid groups (Alfa Caesar, Ward Hill, MA) were functionalized with thiol groups by dissolving 2 mg/mL of heparin in PBS. To this solution, EDC, NHS, and an excess amount of cysteamine were added. The pH of the reaction mixture was adjusted to 4.75 and the reaction was allowed to continue overnight with stirring at room temperature. DTT was then added to reduce the oxidized disulfide groups in order to release thiol groups. This solution was allowed to react overnight at pH 7.5 and then adjusted to pH 3.5 by the addition of 1.0 N HCl. Next, the reaction solution was dialyzed and lyophilized. The amount of the thiol group attached to heparin was measured using the molar absorptivity of Ellman's reagent at 412 nm.

2.4. Gelation

Lyophilized acryl hydrazide hyaluronic acid was dissolved in 4-(2-hydroxyethyl)-1- piperazine ethane-sulfonic acid (HEPES, 0.3 M) buffer for 15 min at 37 °C for the *in vitro* experiments, and 20 min at room temperature for the *in vivo* injections. The appropriate concentration of Ac-GCGYGRGDSPG-NH2 adhesion peptide (RGD, Genscript, Piscataway, NJ), Ac-GCGYGYIGSR-NH2 (YIGSR, Genscript), Ac-IKVAVGYGCG-NH2 (IKVAV, Genscript) was dissolved in 0.3 M HEPES and added to the dissolved HA-AC and allowed to react for 20 min. For *in vitro* studies gel culture media, iPS-NPCs (3000 cells/μL final concentration), were then added. Heparin and growth factors were then added to the gel precursor solution, before the addition of a MMP-degradable peptide cross-linker (Ac-GCRDGPQGIWGQDRCG-NH2, Genscript), dissolved in 0.3 M HEPES. For in the *in vitro* experiments, 5 μL of this solution was pipetted onto, and sandwiched between two Sigmacote (Sigma-Aldrich) functionalized glass coverslips and placed in an incubator for 30 min at 37 °C to gel. For injections into stroke brains, the precursor was loaded into the Hamilton syringe directly after mixing in the desired cross-linking peptide. The gel remains liquid for at least 10 min after mixing, allowing it to be injected.

2.5. Rheometry

Gels without cells were made as described above and cut to size using a 8.0 mm biopsy punch. The modulus was measured with a