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Glycosaminoglycan-based hybrid hydrogel encapsulated with polyelectrolyte complex nanoparticles for endogenous stem cell regulation in central nervous system regeneration

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ABSTRACT

The poor regenerative capability of stem cell transplantation in the central nervous system limits their therapeutic efficacy in brain injuries. The sustained inflammatory response, lack of structural support, and trophic factors deficiency restrain the integration and long-term survival of stem cells. Instead of exogenous stem cell therapy, here we described the synthesis of nanohybrid hydrogel containing sulfated glycosaminoglycan-based polyelectrolyte complex nanoparticles (PCN) to mimic the brain extracellular matrix and control the delivery of stromal-derived factor-1α (SDF-1α) and basic fibroblast factor (bFGF) in response to matrix metalloproteinase (MMP) for recruiting endogenous neural stem cells (NSC) and regulating their cellular fate. Bioactive factors are delivered by electrostatic sequestration on PCN to amplify the signaling of SDF-1α and bFGF to regulate NSC *in vitro*. In in vivo ischemic stroke model, the factors promoted neurological behavior recovery by enhancing neurogenesis and angiogenesis. These combined strategies may be applied for other tissue regenerations by regulating endogenous progenitors through the delivery of different kinds of glycosaminoglycan-binding molecules.

1. Introduction

Brain injuries, either traumatic or vascular, typically result in neural tissue damage directly from the initial impact, and trigger subsequent processes that lead to permanent neurologic deficit. The mainstay of treatments today mostly aimed to damage control, which can only be regarded as supportive measures instead of reconstructive interventions. Therefore, there is a huge unmet need to rescue the dysfunctional neural tissues using novel therapeutic strategies [1]. Although many researchers have investigated novel approaches based on stem cell transplantation, currently there is no consensus on the most effective time period and the dosage of delivery when utilizing stem cell-based therapies for human [2]. Additionally, the clinical efficacy of cell transplantation techniques has been limited by scarce donor source, uncontrolled cell maturity, poor cell survival and low engraftment rate. These limitations are mainly attributed to severe inflammation [3], loss of stromal support and trophic factors in the injury site [4]. Therefore, the objective of this research is to overcome the costly and complex *ex vivo* process of cell-transplant and the short therapeutic window of drug-based methodology. To achieve this goal, we aimed to enhance the regenerative capacity of central nervous system (CNS) during acute and chronic stages after brain injury.

The central nervous system can regenerate to certain degree after injury. Evidences revealed that endogenous neural stem cells

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(NSC) may migrate tangentially through blood vessels from the subventricular zone (SVZ) to the neocortex and *in situ* cortical neurogenesis occur [5–8]. Thus, the activation of endogenous NSC, such as migration, proliferation, and differentiation, to modulate the injured area is potentially a promising strategy in CNS regeneration. Stromal cell-derived factor-1α (SDF-1α), a chemoattractant, is important for endogenous NSC to migrate over long distance from the SVZ to the injured site. To be specific, the C-X-C chemokine receptor 4 (CXCR4) on the membrane of NSC can direct migration toward the injured site where SDF-1α are highly expressed via ligand-receptor interaction. The dynamic of this specific interaction appears to be required for NSC moving toward injury [9]. On the other hand, for endogenous NSC proliferation, basic fibroblast growth factor (bFGF) is upregulated after brain injury and persists for more than 2 weeks [10]. The upregulation of bFGF alters tropomyosin receptor kinase B mRNA and brain-derived neurotrophic factor expression, which results in supporting the self-renewing potential of endogenous NSC and promoting neuronal cells survival, synaptic plasticity, and functional recovery in the lesioned CNS system [11]. Therefore, bFGF in injured area appears to be a critical regulator of neural tissue repair. However, these responses are only activated in the restricted pattern spatially and temporally. They provide poor regenerative capacity in the chronic stage of injury [12,13].

The extracellular matrix (ECM) of brain is mainly composed of hyaluronic acid (HA) and proteoglycans [14]. Sulfated glycosaminoglycans (GAGs), such as heparan sulfate and chondroitin sulfate, covalently bound on the side chain of proteoglycan are heterogeneous polysaccharides and serve as a reservoir for binding and stabilization of growth factors, chemokines, and other ECM proteins. Moreover, they also regulate processes associated with growth factor-receptor signaling pathways [15,16]. On the other hand, HA, a kind of non-sulfated GAG, is a unique candidate for neural tissue regeneration. It is known to reduce glial scar formation and minimize local inflammation by attenuating lymphocyte and macrophage motility.

In order to induce chemokines and growth factors, and to modulate cellular responses after CNS injury, we have developed the nanohybrid hydrogel from HA hydrogel matrices incorporated with SDF-1α- and bFGF-loaded polyelectrolyte complex nanoparticles (PCNs) in this work (Fig. 1A). PCN is a proteoglycan-mimetic and GAG-containing carrier, which can sequester cationic proteins through GAGs-protein interaction [17–19]. For endogenous NSC recruitment, SDF-1α was loaded on the heparan sulfate-containing PCN for the optimization of SDF-1α presentation [20]. Furthermore, continuous expression of FGF-2 complexed with chondroitin sulfate-containing PCN within the hydrogel is enabled to regulate injury-induced NSC proliferation [21]. The *in situ* gelation of nanohybrid hydrogel is tunable by the cross-linker, adipic dihydrazide acid (ADH), via Schiff base crosslinking (Fig. 1B) [22]. Since matrix metalloproteinase (MMP) is upregulated after brain injuries and is capable of degrading ECM structural proteins during tissue remodeling, the MMP-cleavable peptides (GCDSGGRMSMPVSDGG) and MMP-inactive peptides (GCRDFGAIGQDGDRGG) modified PCNs were complexed with functionalized HA and offered the controlled release of both growth factors by the selective cleavage of MMP-cleavable rather than MMP-inactive peptides modified on both PCNs in the injured brain with upregulated MMP [23–25] (Fig. 1C). We demonstrated that the locally-injected nanohybrid hydrogel effectively promoted endogenous NSC migration and neurogenesis in the infarcted region. Notably, the activation of NSC was further confirmed by neuroregeneration with significant reduction of stroke cavity and improvement of functional recovery in a rat animal model of photothrombotic ischemic (PTI) stroke. Our study offered the proof of concept for regulating endogenous stem cell by the bioactive factor delivery from GAG-based nanohybrid hydrogel for ischemic stroke.

2. Materials and methods

2.1. Maleimide group conjugation on chondroitin sulfate and heparan sulfate

The CS-maleimide/HS-maleimide were successfully fabricated by modifying from previous study [26]. In brief, chondroitin sulfate (CS) sodium salt (Sigma) or heparan sulfate (HS) sodium salt (Sigma) was dissolved in 6 mL deionized water and the pH value was adjusted to 4.7 by adding 0.1 N hydrochloric acid. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and Nhydroxysuccinimide (NHS) were added to the solution of CS or HS and stirred for 15 min at room temperature to activate carboxylic groups on both CS and HS by EDC crosslinking and form an NHS ester for efficient conjugation to primary amines. *N*-(2-aminoethyl) maleimide trifluoroacetate salt (Santa Cruz Biotechnology) was added and the reaction mixture was incubated for 6 h at room temperature under stirring for direct conjugation of primary amines to the activated carboxylic acids via amide bonds. The resulting solution was dialyzed against deionized water for 24 h (MWCO 6000–8000 Da) under gentle shaking to remove the excess of coupling agents and byproducts of the reaction. The polymer solutions were lyophilized and stored at −20 °C. The percentage of maleimide modification and amide bonding formation of CS and HS were determined by ^{1}H NMR (Bruker) and FT-IR (Thermo Fisher Scientific), respectively.

2.2. Synthesis and characterization of bFGF-loaded mCSPCN and SDF-1α-loaded mHSPCN

To prepare bFGF-loaded mCSPCN, 1.2 mg/mL of CS-maleimide (CS-mal) and 0.6 mg/mL of chitosan (Chi) were dissolved in 0.1 M acetic acid solution and then filtered using 0.22 μm MCE syringe filters. The chitosan solution was then added in one shot to the stirring CS-mal solution in a volume ratio of 1:6 (Chi:CS-mal), and the mixture was vigorously stirred for 3 h. CS-mal/Chi PCN was dialyzed against deionized water for 24 h to remove the uncomplexed polymers. CS-mal/Chi PCN solution was then mixed with customized MMP-inactive peptide (GenScript) solution with maleimide:thiol molar ratio of 1:1 for 4 h at 4 °C to form MMP-inactive peptide modified chondroitin sulfate PCN (mCSPCN) through water-stable thiol-maleimide click reaction. The pH value was adjusted in the range of 6.5–7.5 to prevent from side reactions. bFGF loading was achieved by mixing bFGF and mCSPCN solutions in deionized water with a mass ratio of 100 ng/mg bFGF/mCSPCN. The bFGF-loaded mCSPCN solution was then mixed with sucrose solution (20% w/v) as cryoprotectant, and finally freeze-dried and stored at 4 °C for further use.

For SDF-1α mHSPCN preparation, the 1.2 mg/mL of HS-maleimide (HS-mal) solution was mixed with 0.3 mg/mL of Chi because heparan sulfate contains approximately half as many sulfate groups as chondroitin sulfate. The HS-mal/Chi PCN was conjugated with customized MMP-cleavable peptide (GenScript) with maleimide:thiol molar ratio of 1:1 and form MMP-cleavable peptide modified heparan sulfate PCN (mHSPCN). The pH value was also adjusted in the range of 6.5–7.5 to prevent from side reactions. SDF-1α loading was achieved by mixing SDF-1α and mHSPCN solutions with a mass ratio of 100 ng/mg SDF-1α/mHSPCN. The SDF-1α-loaded mHSPCN was also freeze-dried, followed by mixing with sucrose and storing at 4 °C.

The morphological analysis of PCNs was performed by