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A modular, plasmin-sensitive, clickable poly(ethylene glycol)-heparin-laminin microsphere system for establishing growth factor gradients in nerve guidance conduits

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ABSTRACT

Peripheral nerve regeneration is a complex problem that, despite many advancements and innovations, still has sub-optimal outcomes. Compared to biologically derived acellular nerve grafts and autografts, completely synthetic nerve guidance conduits (NGC), which allow for precise engineering of their properties, are promising but still far from optimal. We have developed an almost entirely synthetic NGC that allows control of soluble growth factor delivery kinetics, cell-initiated degradability and cell attachment. We have focused on the spatial patterning of glial-cell derived human neurotrophic factor (GDNF), which promotes motor axon extension. The base scaffolds consisted of heparin-containing poly(ethylene glycol) (PEG) microspheres. The modular microsphere format greatly simplifies the formation of concentration gradients of reversibly bound GDNF. To facilitate axon extension, we engineered the microspheres with tunable plasmin degradability. 'Click' cross-linking chemistries were also added to allow scaffold formation without risk of covalently coupling the growth factor to the scaffold. Cell adhesion was promoted by covalently bound laminin. GDNF that was released from these microspheres was confirmed to retain its activity. Graded scaffolds were formed inside silicone conduits using 3D-printed holders. The fully formed NGC's contained plasmin-degradable PEG/heparin scaffolds that developed linear gradients in reversibly bound GDNF. The NGC's were implanted into rats with severed sciatic nerves to confirm *in vivo* degradability and lack of a major foreign body response. The NGC's also promoted robust axonal regeneration into the conduit.

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1. Introduction

The treatment of peripheral nerve injury has advanced greatly in recent years. However, complete functional recovery continues to be difficult to achieve, suggesting it is critical that alternatives to the current standard of care (nerve autografts) be developed [1–3]. A promising strategy involves the use of nerve guidance conduits (NGCs), which can be filled with synthetic and/or biological matrices along with growth factors, to span nerve gaps and enhance axonal regeneration [4]. Glial-derived neurotrophic factor (GDNF) has been reported by several studies to be a potent motor neuron trophic and survival factor, showing great promise in the treatment of peripheral nerve injuries [5–10]. NGC's delivering growth factors such as GDNF have been shown to shown to promote axonal regeneration equivalent to isograft controls [3].

Gradients of biological molecules are known to significantly affect nerve regeneration, as well as other biological processes such as, wound healing, embryogenesis, angiogenesis, and immunity [11–17]. Our laboratory has created nearly linear gradients in reversibly-bound GDNF within heparinated poly(ethylene glycol) (PEG) microsphere scaffolds [18,19]. These GDNF gradients persist for more than a week and potentially might enhance nerve regeneration within an NGC. The microsphere-based 'modular' scaffolds have also been used for the culture for culture of various cell types [20–23]. However, before these microsphere scaffolds

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could be useful for *in vivo* nerve regeneration, several functionalities, including cell-initiated degradability, inter-microsphere cross-linking, and cell adhesion, had to be incorporated into the microspheres.

Recent biomaterials approaches to tissue regeneration have sought to replicate the native degradability of natural biomaterials, such as fibrin, thereby stimulating the regeneration process [24,25]. Peptide sequences sensitive to enzymatic cleavage have been integrated into hydrogels to this end. Matrix metalloproteinase sensitive sequences have been used in a number of biomaterial systems [26–33]. Plasmin is another enzyme that plays a key role in cell migration, especially during wound healing [33]. Plasmin sensitive sequences have also been used extensively [28,34–38]. For the current system, the sequence must not contain any internal lysines or cysteines in order to prevent unwanted crosslinking. The sequence GGVRNGGK is one previously used plasmin-degradable sequence that fits these constraints [37]. This sequence, modified by adding a GC to the N-terminus to make it reactive to vinylsulfone groups, could impart plasmin degradability to these PEG microspheres.

To promote scaffold stability, it was necessary for the microspheres to cross-link to one another. To accomplish this under physiological conditions without using agents that might react with the GDNF, other ambient proteins, or the extending nerves themselves, we sought to utilize a Click reaction [39]. Click reactions are bioorthogonal reactions such as the Huisgen 1,3-dipolar cycloaddition between azides and alkynes, thiol-ene/yne photoadditions, and Staudinger ligation [40–43]. Our lab has already utilized click reactions for both microsphere formation and inter-microsphere cross-linking for scaffold stability [44]. Because copper, a common catalyst for these reactions, can be toxic to cells, we have focused on copper-free strain-promoted azide–alkyne cycloadditions, which have high conversions, fast kinetics, insensitivity to oxygen and water, stereospecificity, regiospecificity, and mild reaction conditions [45–48].

To allow extending nerves to attach to and subsequently grow through the scaffold, it was necessary to affix a cell adhesion protein, such as laminin 111, to the microspheres. Laminin 111, a basement membrane protein, has been shown to be important to neural system development [49]. Laminin 111 not only influences cell adhesion, but also neurite outgrowth and growth cone movement, and acts as a neuronal cue [49–51]. Many studies have already utilized laminin in their biomaterial systems to enhance neurite outgrowth [49,52–54]. The cell adhesion molecules fibronectin and an RGD peptide have previously been attached to the PEG microspheres via reaction of lysines or cysteines in the molecule with vinyl-sulfone groups on the PEG, and the same chemistry was used for conjugating laminin to the microspheres herein [21,55].

Although these various functionalities have been extensively studied individually, combining all of the functionalities to produce a modular NGC requires a precise ordering of the various orthogonal chemistries. Herein, we describe a complex methodology to produce modular NGC's with the described functionalities that make the leap from *in vitro* testing of each property in isolation to *in vivo* implantation in a rat sciatic nerve injury model.

2. Materials and methods

Unless otherwise noted, all reagents were purchased from Sigma–Aldrich.

2.1. PEG synthesis

PEG_8-vinyl sulfone (PEG_8-VS) and PEG_8-amine were synthesized from eight-arm PEG-OH (PEG_8-OH; mol. Wt. 10,000; Shearwater Polymers, Huntsville, AL) as previously described [56]. PEG macromonomers were dissolved separately at 200 mg/mL in Dulbecco's phosphate buffered saline (PBS; 8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM sodium chloride, 10 mM potassium chloride, pH 7.4) and sterile filtered with 0.22 μm syringe filters (Millipore).

2.2. Heparin attachment pre-microsphere formation (for high heparin microspheres)

A solution of 244 mg/mL Heparin sodium salt (mol. wt. ~18,000, ~2.78 mM), 0.081 mM N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), and 0.203 mM N-Hydroxysuccinimide (NHS) in MES buffer (10 mM, pH 6.0) was incubated at room temperature for 30 min. L-Cysteine (free base) was added to the activated heparin solution to make a 6:1 cysteine:heparin molar ratio and allowed to react overnight. The solution was dialyzed in 10× PBS (pH 7.4) to remove unreacted cysteine. Ellman's assays were performed to determine substitution of cysteine on heparin (44% of heparin molecules determined to have cysteine). PEG_8-VS was added at a 10:3 PEG_8-VS:cysteinated-heparin molar ratio and incubated at room temperature overnight. For microsphere formation, heparin-conjugated PEG_8-VS was mixed with PEG_8-amine in a 1:1 ratio of the two PEG types.

2.3. Ellman's assay

Ellman's reagent was dissolved in 0.1 M phosphate buffer (pH 8.0) at 40 mg/mL 0.05–0.15 μmol of cysteinated heparin was added to 3 mL of 0.1 M phosphate buffer (pH 8.0) along with 100 μL Ellman's solution. The solution was mixed and incubated at room temperature for 15 min. Absorbance at 412 nm was measured and compared to standard to determine cysteine content.

2.4. High heparin microsphere formation

Heparinated PEG_8-VS solutions were combined with PEG_8-amine solutions at a 1:1 ratio. The PEG solutions were diluted to 20 mg/mL PEG with PBS and 1.5 M sodium sulfate (in PBS) to a final sodium sulfate concentration of 0.6 M. The PEG_8-VS/PEG_8-amine solutions were then incubated above the cloud point at 70 °C for 11 min. Suspensions of microspheres were subsequently buffer exchanged into 8 mM sodium phosphate twice to remove the sodium sulfate by: (1) diluting the microsphere solution 3:1 with PBS and titurating, (2) centrifuging at 14,100 *g* for 2 min, and (3) removing the supernatant. Fluorescent and phase contrast images were captured using a MICROfire (Olympus, Center Valley, PA) camera attached to an Olympus IX70 inverted microscope.

2.5. Heparin attachment post-microsphere formation

A solution of 515 mg/mL Heparin sodium salt (mol. wt. ~18,000, ~2.78 mM), 0.101 mM N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), and 0.042 mM N-Hydroxysuccinimide (NHS) in MES buffer (10 mM, pH 6.0) was incubated at room temperature for 30 min. L-Cysteine (free base) was added to the activated heparin solution to make a 8.82:1 cysteine:heparin molar ratio and allowed to react overnight (see Fig. 1A). The solution was dialyzed in 10× PBS (pH 7.4) to remove unreacted cysteine. Ellman's assays were performed to determine substitution of cysteine on heparin (109% of heparin molecules determined to have cysteine). The solution was diluted to 130 mg/ml heparin and stored at −20 °C. For heparination of microspheres, cysteine-conjugated heparin was added to PEG microspheres at 2.6 mg/mL and incubated overnight.

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