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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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Competing Financial Interests

The Authors have no competing financial interests.

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Keywords

microsphere; gradient; peripheral nerve regeneration; Click chemistry; degradable; scaffold

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 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 of various cell types [20–23]. However, before these microsphere scaffolds 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 vinyl-sulfone 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, thiolene/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, regioselectivity, 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.

Materials and Methods

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

PEG Synthesis

PEG₈-vinylsulfone (PEG₈-VS) and PEG₈-amine were synthesized from eight-arm PEG-OH (PEG₈-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).

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-Hydroxy-succinimide (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 10X 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). PEG₈-VS was added at a 10:3 PEG₈-VS:cysteinated-heparin molar ratio and incubated at room temperature overnight. For microsphere formation,

heparin-conjugated PEG₈-VS was mixed with PEG₈-amine in a 1:1 ratio of the two PEG types.

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 μ mol of cysteinated heparin was added to 3 mL of 0.1 M phosphate buffer (pH 8.0) along with 100 μ L Ellman's solution. The solution was mixed and incubated at room temperature for 15 minutes. Absorbance at 412 nm was measured and compared to standard to determine cysteine content.

High Heparin Microsphere Formation

Heparinated PEG₈-VS solutions were combined with PEG₈-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 PEG₈-VS/PEG₈-amine solutions were then incubated above the cloud point at 70°C for 11 minutes. 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 titrating, (2) centrifuging at 14,100g 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.

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-Hydroxy-succinimide (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 Figure 1A). The solution was dialyzed in 10X 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.

Heparin Labeling

To confirm post-microsphere formation attachment, cysteinated heparin was labeled with Dylight-488 NHS-ester (Pierce). Cysteinated heparin (130 mg/mL) and Dylight-488 (560 μ g/mL) in PBS was incubated overnight at room temperature. The labeled heparin solution was dialyzed using Slide-A-Lyzer MINI Dialysis Units (Thermo Scientific, Rockford, IL, 3500 MWCO) in PBS (pH 7.4) to remove any unbound Dylight-488. The heparin solution was then used in the heparination post-microsphere formation protocol as described above. To determine heparin content, fluorescence of the suspended microsphere solution was measured using a plate reader in triplicate and compared to a standard curve of fluorescently labeled heparin in solution.

Plasmin-Degradable PEG Synthesis

Peptide sequence Ac-GCGGVRNGGK-NH₂ (N-Terminal Acetylation, C-Terminal Amidation, Purity >95%, GenScript USA Inc., Piscataway, NJ) was dissolved in 0.1 M phosphate buffer at 117.9 mg/mL with PEG₈-VS (200 mg/mL, 78% substitution) and brought to a pH of 7.4. The solution was incubated overnight at room temperature before storage at 4°C.

Plasmin-Degradable Microsphere Formation

PEG₈-VS solutions were combined with plasmin degradable PEG₈-VS (PEG-(VRN)₈) solutions at a 1:1 molar ratio and incubated at 37°C for 1 hour. 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. PEG₈-Azide/Amine or PEG₈-Cyclooctyne/Amine were added to the PEG solution at a 50:1 PEG₈-VS/PEG-(VRN)₈ to Clickable PEG ratio. The PEG solution was then incubated above the cloud point at 70°C for various times. Suspensions of microspheres were subsequently buffer exchanged into 8 mM sodium phosphate buffer twice to remove the sodium sulfate by: (1) diluting the microsphere solution 3:1 with PBS and titrating, (2) centrifuging at 14,100g for 2 min, and (3) removing the supernatant (see Figure 1B).

PEG₈-Azide/Amine Synthesis

Eight arm PEG-mesylate (PEG₈-mesylate; mol wt 10,000) was first synthesized from four arm PEG-OH (PEG₈-OH; mol wt 10,000; Creative PEGWorks) by mesylating the alcohol group on PEG₈-OH with mesyl chloride. This was done by dissolving PEG₈-OH in dichloromethane (DCM), adding four equivalents of triethylamine and four equivalents of methanesulfonyl chloride while on ice, and letting it react overnight under constant stirring and nitrogen flow. After removing the salt byproduct, excess DCM was removed by using a rotovap, and the PEG₈-mesylate was precipitated using cold diethyl ether. The product was dried under vacuum overnight to remove remaining diethyl ether. The next step was the nucleophilic azidation of the mesylate group with sodium azide. Three equivalents of sodium azide were dissolved in dimethylformamide (DMF). PEG₈-mesylate was then dissolved in the DMF mixture and put under nitrogen and constant stirring in a hot water bath at 60°C. The reaction was run overnight. The following day required the filtration of excess salt followed by rotovapping, diethyl ether precipitation, and drying as was done for the PEG₈-mesylate. The product was dissolved in a basic water solution with a pH between 9 and 12, and then extracted with DCM over anhydrous sodium sulfate (Na₂SO₄). A standard extraction procedure was done to extract the product into DCM. After three extractions, the Na₂SO₄ was filtered out and the process of rotovapping, diethyl ether precipitation, and drying was repeated as before. ¹H NMR (300 MHz, CDCl₃, δ): (s, 902.55H, PEG), 3.0 (s, 3H, -SO₂CH₃), 4.3 (t, 2H, -CH₂OSO₂-). NMR of the product confirmed that no mesylate features remained at 3.0 ppm and 4.3 ppm.

PEG₈-azide was dissolved in tetrahydrofuran (THF) and 1.15 equivalents of triphenylphosphine (TPP) and 30 equivalents of ultrapure H₂O were added while on ice, and the reaction was allowed to go overnight under constant stirring and nitrogen flow. A large excess of H₂O to TPP was needed for amine formation. Excess THF and H₂O were removed

by rotovapping, and PEG₈-Azide/Amine and triphenylphosphine oxide (TPPO) were precipitated out using cold diethyl ether. The product and byproduct were dried under vacuum overnight to remove remaining diethyl ether. Once dry, the PEG₈-Azide/Amine and TPPO were added to cold toluene, because TPPO is soluble in cold toluene while PEG is insoluble. The PEG₈-Azide/Amine was then vacuum filtered to remove the TPPO. The product then underwent the same extraction procedure with DCM that was described for PEG₈-Azide synthesis. ¹H NMR (300 MHz, CDCl₃, δ): (s, 902.55H, PEG), 2.9 (t, 2H, -CH₂CH₂NH₂). NMR of the product confirmed the reduction of about 50 percent of azides to amines via the amine feature at 2.9 ppm.

PEG₈-Cyclooctyne/Amine Synthesis

Amines on PEG₈-Amine (prepared as previously described) were partially reacted with a cyclooctyne-containing molecule to form PEG₈-Cyclooctyne/Amine. PEG₈-Amine was dissolved in DCM, and 0.5 equivalents of diisopropylcarbodiimide (DIPCDI) were added to a separate flask with DCM while on ice and under nitrogen flow and constant stirring. Next, 0.5 equivalents of hydroxybenzotriazole (HOBt) and 0.5 equivalents of aza-dibenzocyclooctyne with a pendant carboxylic acid (DBCO-acid; Click Chemistry Tools) were added to the mixture and allowed to stir for 10 minutes. While waiting, one equivalent of N,N-diisopropylethylamine (DIPEA) was added to the dissolved PEG₈-Amine. Finally, this mixture was slowly added to the activated DBCO, and the reaction was allowed to proceed for 24 h on an ice bath under constant stirring and nitrogen gas. Following that process, the urea precipitate was filtered out, and rotovapping, diethyl ether precipitation, and drying were performed. The product was then dissolved in distilled H₂O and underwent the same extraction procedure that was done for the PEG₈-Amine. Further rotovapping, diethyl ether precipitation, and drying were done. ¹H NMR (300 MHz, CDCl₃, δ): (s, 902.55H, PEG), 5.1 (d, 2H, -CH₂-). NMR of the product confirmed the conversion of 50 percent of amines to cyclooctynes (PEG₈-Cyclooctyne/Amine) via the presence of a doublet at 5.1 ppm.

Clickable Microsphere Formation

PEG₈-Azide/Amine and PEG₈-Cyclooctyne/Amine were separately dissolved in 0.1 M phosphate buffer (pH 7.4) at 40 mg/mL. Dylight-633 NHS-ester (Pierce) was dissolved in dimethyl formamide at 10 mg/mL and added to the clickable PEG's such that final concentrations were 33.33 mg/mL clickable PEG and 1.67 mg/mL Dylight. Solutions were incubated overnight at 25°C to allow near complete reaction. The same methods for degradable microsphere formation were followed, except that just prior to dilution in 0.6 M sodium sulfate, PEG₈-Azide/Amine and PEG₈-Cyclooctyne/Amine were added to separate batches of the degradable microsphere precursor solution at a 1:50 molar ratio of clickable PEG to all other PEG. The methods for degradable microsphere formation given above were followed from this point, keeping the batches containing PEG₈-Azide/Amine or PEG₈-Cyclooctyne/Amine separate until just prior to scaffold formation (see Figure 1C).

Laminin Attachment

Laminin Mouse Protein, Natural (Life Technologies, Grand Island, NY) was added to microspheres at 20 µg/mL or 2-D gel at 0.8 µg/mL and incubated at 37°C overnight (Smith et al., 2013).

Cysteine capping of Vinyl-Sulfones

After all other functionalities were added to the microspheres (the last step being incubation with thiolated heparin and laminin), the microspheres were washed 2X and re-suspended in 2.5 mg/mL L-cysteine and incubated for 30 min at room temperature. The microspheres were then washed 3X before use.

GDNF Loading of Microspheres

Recombinant human GDNF (Peprotech, Rocky Hill, NJ) was dissolved in 8 mM sodium phosphate buffer (pH 7.4) and added to washed microspheres such that the GDNF concentration within the supernatant was 250 ng/mL (note higher concentrations used for DRG experiments below). The microsphere/GDNF solution was well mixed by titration and incubated 2 h at 4°C to allow diffusion of GDNF into the microspheres. Immediately before scaffold formation, the microspheres were centrifuged at 14,100 g, supernatant was removed, and microspheres were re-suspended in 8 mM sodium phosphate.

GDNF Labeling

Dylight-488 NHS-ester (Pierce) was dissolved in dimethyl formamide at 10 mg/mL. Recombinant human GDNF (Peprotech, Rocky Hill, NJ) was dissolved in 8 mM sodium phosphate buffer (pH 7.4). Dylight-488 was added to the solution for a final GDNF concentration of 10 µg/mL and a final Dylight-488 concentration of 50 ng/mL and incubated overnight at 4°C. The solution was then dialyzed using Slide-A-Lyzer MINI Dialysis Units (Thermo Scientific, Rockford, IL, 3500 MWCO) in 8 mM sodium phosphate buffer (pH 7.4) to remove unbound Dylight-488.

Confirmation of Gradient Formation

The glass walls of Pasteur pipettes were passivated with PLL(375)-g[7]-PEG(5) [57, 58]. The pipettes were filled with a 20 mg/mL PLL-g-PEG solution, incubated for 30 s, and washed with DI water. After sufficient drying time, the tips of the pipettes were sealed with silicone aquarium sealant (DAP Inc., Baltimore, MD). To form scaffolds, microsphere solutions were sequentially added to the pipettes that were placed in 15 mL conical vials. The microsphere solutions were centrifuged at 1000 g for 5 min before the next layer of microspheres was added (if producing a two-tiered scaffold). The supernatant was then removed once more and replaced with 8 mM sodium phosphate.

Confocal microscopy

Fluorescence microscopy was performed with a Nikon Eclipse C1/80i confocal microscope. Microsphere gradients were imaged while still in the Pasteur pipettes with a 10X objective (NA=0.30, DIC L/N1, WD= 16.0mm). Multiple images were taken along the length of the pipette and processed using EZ-C1 3.70 FreeViewer software (Nikon Instruments Inc.) and

then combined. Fluorescence in the composite photographs was analyzed with ImageJ software.

Analysis of GDNF Activity Retention

PEG₈-VS and PEG₈-Amine solutions were combined at a 1:1 ratio and diluted to 66.66 mg/mL PEG in PBS. The PEG solution (0.6 mL) was added to each well of a 24 well plate (BD Falcon, Franklin Lakes, NJ) and incubated at 37°C for 3 days to ensure maximal crosslinking. Wells were washed 2X with 1 mL PBS before adding 0.6 mL of laminin (0.8 µg/mL) in PBS and incubating at 37°C overnight. GDNF (833 ng/mL in 8 mM sodium phosphate buffer) was loaded into microspheres as described above. After incubation, microspheres were centrifuged and supernatant was removed. Microspheres were re-suspended in modified neurobasal (MNB) media (Invitrogen, Carlsbad, CA) containing 0.1% BSA, 0.5 mM L-glutamine, 2.5 µM L-glutamate, 1% N2 supplement, and 1% antibiotic/antimycotic solution (ABAM) (all from Invitrogen) and quickly centrifuged again to remove free GDNF. Supernatant was removed and the microspheres were re-suspended in MNB media (1 mL of media for about 0.5 mL of loaded microspheres) and incubated 2 h at 4°C. The microspheres were centrifuged once again and the supernatant was transferred to the 24 well plate with PEG gels (1 mL per well). Dorsal root ganglions (DRGs) were dissected from day 10 White Leghorn chicken embryos (Sunrise Farms, Catskill, NY) and placed into wells containing either microsphere MNB media or fresh MNB media (no GDNF). At 24, 48, and 72 h, phase contrast images of the neurite extension from the DRGs were taken with a 4x objective.

Conduit Assembly

Sections of standard silicone tubing (Helix Medical, Carpinteria, CA) (1.47 mm inside diameter × 0.39 mm wall thickness) were stretched over the ends of 1 mL pipette tips (Rainin Instrument LLC, Oakland, CA) until secure with ~2 cm protruding from the ends. After autoclaving, a small amount of hot glue was drawn into the tube to form a plug (filling about ~3 mm at the bottom of the tube). The plugged conduits were stored under UV light in a sterile cabinet to enhance sterility. Fibrinogen solutions were prepared by dissolving human plasminogen-free fibrinogen in deionized water at 8 mg/mL for 1 h and dialyzing against 4 L of Tris-buffered saline (TBS) (33 mM Tris, 8 g/L NaCl, 0.2 g/L KCl) at pH 7.4 overnight to exchange salts present in the protein solution. The resulting solution was sterilized by filtration through 5.0 and 0.22 µm syringe filters, and the final fibrinogen concentration was determined by measuring absorbance at 280 nm. Components were mixed to obtain the following final solution concentrations: 8 mg/mL fibrinogen, 2.5 mM Ca²⁺, and 1 NIH U/mL of thrombin. Using a 30 gauge syringe (Exel International Medical Products, St. Petersburg, FL), this solution was added inside the tube on top of the glue plug such that a 1–2 mm plug of fibrin was formed. The conduits were then incubated for 1 hour at 37°C. The pipette tip and conduit were then placed inside a 3-D printed mold designed to allow for centrifugation of the conduit (Supplementary Figure 3, CAD file in supplementary material). Microspheres were then added to the pipette tip and centrifuged to form a scaffold within the tube as previously described. The conduit was then cut away from the tip. The supernatant was removed from the microspheres, and another small fibrin plug was added

on top of the microspheres. The glue plug was then excised by cutting the silicone tube around the plug 1 mm from the top of the plug and pulling the plug free (Figure 6A and B).

Experimental Animals

Twenty four adult male Lewis rats (Charles River Laboratories, Wilmington, MA), each weighing 250–300 g, were used in this study. All surgical procedures and perioperative care was performed in accordance with the National Institutes of Health guidelines, where NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been observed. Animals were randomly assigned to an experimental group in one of two studies. The first study (n=12) assessed the in vivo degradation of the delivery system and preliminary analysis of nerve regeneration using a 13 mm nerve gap injury model. The second study (n=12) quantitatively assessed axonal regeneration into the conduits using a 7 mm nerve gap injury model. This second study also qualitatively assessed the degradation of the delivery system and the presence of a foreign body response, including neutrophil and macrophage accumulation, within the conduits.

Operative procedure

All surgical procedures were performed using aseptic technique and microsurgical dissection and repairs. Under subcutaneous anesthesia with ketamine (75 mg kg⁻¹) and medetomidine (0.5 mg kg⁻¹), the hind leg of the rat was prepped with betadine and alcohol and the sciatic nerve was exposed through a dorsolateral gluteal muscle splitting incision. An ~5 mm nerve segment was excised proximal to the trifurcation of the sciatic nerve and a nerve guidance conduit was sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end. Two 9-0 nylon interrupted microepineurial sutures were used to secure the conduit at each end, resulting in a tension-free gap between the proximal and distal stumps. Wounds were irrigated with saline, dried and closed with a running 5-0 vicryl suture in muscle fascia, and then interrupted 4-0 nylon skin sutures. Anesthesia in experimental animals was then reversed with a subcutaneous injection of atipamezole HCl (1 mg kg⁻¹) (Pfizer Animal Health, Exton, PA), and the animals recovered in a warm environment. After recovery, the animals were returned to a central housing facility.

In the first study, at 1, 2, 4, 6, and 8 weeks postoperatively, all animals were re-anesthetized and the conduits/nerves were exposed by reopening the prior muscle splitting incision. At this time, light and fluorescence photomicrographs were taken, and the wounds were re-closed as before. At 8 weeks, the nerve conduit and a 5 mm portion of native nerve both proximally and distally were harvested. The specimens were marked with a proximal suture and stored in 4% paraformaldehyde in PBS (pH 7.4) at 4 °C and then changed to 30% sucrose in PBS at 4 °C until cryosectioning and immunohistochemical analysis was performed. Following the tissue harvest, the animals were then euthanized with intraperitoneal injection of Euthasol (150 mg kg⁻¹) (Delmarva Laboratories, Des Moines, IA).

In the second study, all animals were re-anesthetized and the conduits/nerves were exposed and harvested at 4 weeks. The nerve conduit and a 5 mm portion of native nerve both

proximally and distally were harvested and stored in 3% glutaraldehyde (Polysciences Inc., Warrington, PA) in phosphate buffer (pH 7.2). These nerves were assessed for histology and quantification of axonal regeneration using histomorphometry.

Immunohistochemistry

Longitudinal sections of the delivery system and regenerated tissue were cut at 10 μm on a cryostat. Slides were stained for S100 with 1:500 rabbit anti-S100 (Dako; GA504) primary antibody followed by goat anti-rabbit Alexa Fluor 555 secondary antibody (ThermoFisher; A-21428), and stained for neurofilament with monoclonal anti-NF-160 primary antibody (Sigma N-5264) followed by goat anti-mouse Alexa Fluor 488 secondary antibody (ThermoFisher; A-11029) using standard immunohistochemistry techniques. Sections were imaged at 20x using the Nanozoomer HT (Hamamatsu, Bridgewater, NJ) with appropriate optical filters.

Histomorphometry

En bloc specimens of the mid-conduit and distal sciatic nerve with the regenerated nerve underwent histomorphometric analysis as previously described [59]. Briefly, nerve was harvested and stored in 3% glutaraldehyde. The nerves were post-fixed in 1% osmium tetroxide and serially dehydrated in ethanol and toluene. The nerves were then embedded in epoxy (Polysciences), and sectioned on an ultramicrotome into 1 μm cross sections. Slides were counter-stained with 1% toluidine blue dye. The slides were then analyzed at 1000x on a Leitz Laborlux S microscope. The Leco IA32 Image Analysis System (Leco, St. Joseph, MI) was utilized to quantify nerve fiber counts, fiber width, fiber density, and percent neural tissue. The sections were also analyzed qualitatively for a foreign body response including neutrophil and macrophage presence. All analysis was done by an observer blinded to the experimental groups.

Results and Discussion

For this system we devised a new heparin attachment chemistry that would alleviate limitations of our previous methods of incorporating heparin into the microspheres (i.e. better control over the extent of the reaction, minimization of heparin self-crosslinking, higher reproducibility, and increased incorporation of heparin). In this new chemistry, an intermediate step of bonding cysteine to the heparin through the previously described EDC/NHS activation of carboxyl groups was used [18]. Although both the amine and thiol on cysteine may react with NHS-activated carboxyls on heparin, the neutral conditions would favor an S-to-N acyl shift leaving a free thiol. The amount of cysteine conjugated to the heparin could be quantified by an Elman's assay for the thiols on the pendant cysteines (free cysteine would be readily removed in the dialysis step). This "thiolated heparin" could then be reacted with PEG₈-VS in a much more controlled manner than the previous reaction using EDC/NHS activated carboxyl groups reacted with PEG₈-Amine directly (reproducibility of the EDC/NHS activation is challenging due to fast reaction kinetics, age of the EDC, and inability to quantify activation extent during each reaction). The new method allowed for the creation of microspheres with as much as 21 percent by weight heparin content. However, the higher amounts of heparin inhibited, and sometimes even

prevented, the formation of microspheres by changing the solubility characteristics of PEG in 0.6 M sodium sulfate. Microspheres that were successfully formed at the high heparin conditions were much smaller (less than 1 micron in diameter, supplementary Figure 1) than the previously fabricated microspheres (5–20 microns). Upon introduction of further functionalities such as plasmin degradability, microspheres no longer formed at all unless the heparin content was dropped to levels below that which the previous chemistry afforded (less than 3 percent by weight). Thus, we introduced a new method, creating the thiolated heparin as described here, but the thiolated heparin was reacted to PEG₈-VS after the microspheres had been formed (thiolation chemistry shown in Figure 1A). To assess the heparin content of these microspheres, we labeled the heparin with Dylight-488. By comparing the fluorescence of a solution of suspended heparinated microspheres to a standard curve of fluorescent thiolated heparin, the heparin content of the microspheres was determined to be at least 4 percent by weight ($3.94\% \pm 0.08$; $n = 4$). This heparin content was greater than the previous published system, while having the added benefit of not affecting the microsphere formation process.

To allow extending axons to degrade the scaffold within the NGC, a plasmin sensitive peptide sequence, Ac-GCGGVRNGGK-NH₂, was incorporated into the microspheres (Figure 1B). The N-terminus and C-terminus of the peptide were acetylated and amidated, respectively, to prevent any unwanted cross-linking during microsphere formation. The cysteine in the peptide contains a thiol group that reacts rapidly with vinyl sulfone groups on the PEG₈-VS. By combining the peptide with PEG₈-VS in a ratio of one peptide chain to every vinyl sulfone group, we created an eight-arm PEG with arms terminated with plasmin-sensitive peptides. We refer to this as PEG-(VRN)₈. The lysine at the C-terminal end of the peptide contains a primary amine group, effectively producing a type of plasmin-degradable PEG₈-Amine. Addition of the primary amine-containing PEG-(VRN)₈ to additional PEG₈-VS essentially recapitulates our original microsphere formation process that reacted PEG₈-VS and PEG₈-Amine. Because of the stepwise nature of the chemical scheme, any cross-linkages between PEG molecules in the microspheres would be vulnerable to attack by plasmin. To form microspheres, PEG-(VRN)₈ and PEG₈-VS was added at a 1:1 molar ratio and reacted as the previous PEG₈-Amine/PEG₈-VS constituents were. The production of microspheres via sodium sulfate phase separation was hindered somewhat by the introduction of the PEG-(VRN)₈, due possibly to electrostatic interactions of the arginine peptide with water. For microspheres to form, a pre-incubation step had to be added, in which the undiluted PEG-(VRN)₈ and PEG₈-VS (200 mg/mL total PEG) were incubated for > 30 min (1 h in final protocol) at 37°C before dilution in 0.6 M sodium sulfate and subsequent incubation at 70°C. The incubation time at 70°C in the phase separated state is known to determine the extent of crosslinking within the microsphere, affecting its swelling, buoyancy and the rate of diffusion of proteins through the material [19, 60]. Because the time in phase separated state ultimately controls the rate of release of growth factor, this time length was carefully monitored during microsphere formation.

PEG microspheres with click cross-linking functionality have already been developed by our laboratory [44]. PEG₈-Amine was first reacted so that it became about fifty percent substituted with either azide or cyclooctyne groups (PEG₈-Azide/Amine and PEG₈-Cyclooctyne/Amine). Microsphere precursor solutions were split into two batches, and one

batch was reacted with PEG₈-Azide/Amine while the other was reacted with PEG₈-Cyclooctyne/Amine, with the amines on the clickable PEGs reacting with vinyl sulfones on PEG-VS. This led to the production of batches of microspheres decorated with either azide or cyclooctyne groups. Upon mixing these two types of microspheres together, the clickable groups would react to one another, allowing covalent coupling between microspheres to produce a scaffold (see Figure 1C). The clickable PEG content had to be relatively low so as not to hinder plasmin-degradability (50:1 non-clickable PEG:clickable PEG molar ratio). At this level, scaffold formation is relatively slow (over the course of a few days). However, when implanted *in vivo* the scaffolds are in place for weeks, so the click cross-linking functionality will still be advantageous for the system. In future studies, use of linear clickable PEG reagents will allow for much faster scaffold formation.

The final functionality added to the microspheres was cell adhesion, via covalent attachment of laminin to the microspheres. To confirm laminin attached to PEG via vinyl sulfone groups would encourage neuronal growth, thin bulk gels made from PEG₈-Amine/PEG₈-VS (6.66% in PBS) were incubated overnight with laminin (20 µg/mL) at 37°C, allowing the cysteines on laminin to react with free vinylsulfones, covalently coupling the laminin to the gels. DRG's were cultured on the gels with laminin and compared to gels without laminin (Figure 2). DRG's cultured on PEG gels without laminin showed no growth. DRG's cultured on PEG gels with laminin extended neurites. This confirmed that laminin was attached to the gel and retained its ability to encourage neurite growth. To attach laminin to the microspheres, laminin (20 µg/mL) was added to previously formed and washed microspheres and incubated overnight at room temperature. Thin bulk gels were used because visualizing neurites on microsphere-based scaffolds is very challenging.

After the addition of each functionality to microspheres or gels was demonstrated individually (heparin binding, plasmin degradability, click cross-linking, and cell adhesion), the functionalities needed to be combined within one material. The final method for fabricating fully functionalized microspheres is shown in Figure 3. This protocol is the combination of all the processes discussed above, ending with a 30 minute incubation in 2.5 mg/mL cysteine to cap any remaining free vinyl-sulfone groups. This capping step prevented unwanted covalent binding of the microspheres to GDNF (or any other proteins) as demonstrated in our previous work [18].

While the lowered amount of click reagents (50:1 non-clickable PEG to clickable PEG) allowed for the retention of plasmin degradability, this is only with a particular range of microsphere formation incubation times. For less than 8 minutes at 70°C, no microspheres formed. For more than 10 minutes at 70°C, the microspheres cross-linked to a degree that eliminated their ability to be degraded by plasmin. Within this range of 8–10 minutes incubation at 70°C, the rate of degradation was tunable. To test the rate of degradability, microspheres were suspended in 1 unit/mL of plasmin and incubated at 37°C, with samples taken periodically to be viewed by phase-contrast microscopy. Microspheres that had been incubated at 70 °C in the phase separated state for less than 10 minutes degraded in a matter of hours, while microspheres incubated for exactly 10 minutes degraded over the course of days or not at all (Figure 4). *In vivo*, however, axons activating plasmin will degrade the

microsphere scaffolds locally, which should take a considerably greater amount of time. Thus, this should represent an accelerated model compared to degradation *in vivo*.

With the protocol for the fabrication of these microspheres finalized, we needed to confirm that the ability to produce linear concentration gradients described in the previous study was retained [18]. A two-tier initial step gradient in GDNF was created as before, using the fully functionalized microspheres. The initial step gradient in GDNF concentration formed a nearly linear concentration profile after one day (release profile of the GDNF visualized by confocal microscopy can be seen in Supplementary Figure 2). This demonstrated that release of GDNF from plasmin degradable, clickable microspheres was similar to non-degradable, non-clickable microspheres from the previous study.

The next important question to answer was whether or not GDNF loaded into the microspheres and subsequently released retained its biological activity. DRG's cultured on thin bulk PEG₈-Amine/PEG₈-VS with attached laminin extended neurites regardless of inclusion of GDNF (100 ng/mL) in the media. Differences between DRG's given media with and without GDNF were only observed after the concentration of laminin incubated on the gel was drastically decreased. It was only within a range of 0.5–1.0 µg/mL laminin incubated on the PEG gel that a difference was observed. DRG's in both conditions (with and without GDNF) extended neurites on these gels initially. However, after 2–3 days, DRG's without GDNF would lose their extensions or even detach from the gel completely, while the DRG's given GDNF (100 ng/mL) in the media would remain attached and maintain extended neurites. With this knowledge, an experiment was performed comparing the growth of DRG's given media without GDNF to those given media with GDNF released from the fully functionalized microspheres.

Fully functionalized microspheres were fabricated and washed thoroughly. Sufficient numbers of microspheres were made such that there would be approximately 0.5 mL of microspheres for every 1 mL of cell culture media needed. The microspheres were then suspended in low salt buffer (8 mM sodium phosphate, pH 7.4) with a high concentration of GDNF (833 ng/mL for the experiment shown in Figure 5) and incubated for 2 hours at 4°C to allow the GDNF time to diffuse into the microspheres (Roam et al., 2010). The microspheres were then centrifuged and the supernatant was removed. However some GDNF that had not interacted with the microspheres might still be in the media between microspheres. To remove unbound GDNF as well as avoid dilution of the cell culture media, the microspheres were resuspended in MNB media and quickly (less than 10 seconds later) centrifuged again. The supernatant was removed and the microspheres were once again resuspended in fresh MNB media. The suspended microspheres were incubated for 2 hours at 4°C to thoroughly release the loaded GDNF into the MNB media. The microspheres were then centrifuged and the supernatant (MNB media with released GDNF, no microspheres) was transferred to a 24 well plate containing the PEG gel (with 0.8 µg/mL incubated laminin) and DRG's. Phase-contrast photomicrographs were taken at 1, 2, and 3 days. Figure 5 shows typical examples of DRG's cultured with microsphere-released GDNF and without GDNF. At day 3, DRG's without GDNF had lost their extensions and detached from the gel completely while the DRG's given GDNF from microspheres remained attached and maintained extended neurites (Figure 5). This drastic difference in the two

cases demonstrates that the GDNF loaded into and subsequently released from the microspheres retained its biological activity.

Lastly, we needed to form the GDNF-containing, plasmin-degradable, laminin-decorated, clickable microspheres into nerve guidance conduits (NGC's). Microspheres were incubated for 8, 9, 9.5 or 10 minutes at 70°C in the phase separated state during formation to alter their plasmin degradation rates. Note that this elevated temperature is prior to addition of heparin, laminin or growth factor - biological molecules are never exposed to temperatures greater than 37°C. Fully functionalized microspheres containing GDNF were centrifuged into silicone tubes by stretching the silicone tube securely over a 1 mL pipette tip and sealing the other end with hot glue (Supplementary Figure 3A). This was enclosed within a custom made 3D printed mold (Supplementary Figure 3 B and C, CAD file in supplementary material), which could be inserted into a 15 mL conical vial for centrifugation (supplementary Figure 3 B–D). The NGC was then excised from the pipette tip, and the glue plug was removed (Figure 6). Though handling and implantation of the conduits was possible, it was also difficult, and the scaffold within was easily destabilized due to the slow kinetics of the click reaction between microspheres in the current chemical scheme. Fibrin plugs were thus formed at either end of the scaffold to increase stabilization as the click reaction proceeded (fibrin plugs can be seen in Figure 6A and B). Conduits were then ready for *in vivo* testing.

To assess the *in vivo* degradation of the delivery system, conduits containing microsphere scaffolds labeled with Dylight-633 were implanted into rats traversing a severed sciatic nerve (Figure 6C and D). Scaffolds were 10 mm in length, with some variation, and with the fibrin plugs the total length of nerve gap was ~13 mm. Fluorescence images indicating the presence of non-degraded scaffold were compared to normal light images of the conduit to determine the percentage of each scaffold's length that had degraded at each time point (Table 1). Example fluorescence photographs and a graphical representation of the typical amounts of degradation can be seen in Figure 7.

Most scaffolds composed of microspheres were largely degraded after 1 week, except those that were incubated in the phase separated state at 70°C for 10 minutes during microsphere formation. In the conduits that did incur an appreciable amount of degradation over their length, some differences were observed in their rates of degradation. The conduits formed from '8 minute' microspheres, especially, degraded faster than the scaffolds with longer incubation times. There was no appreciable change in the length of the scaffolds composed of microspheres incubated at 70°C for 10 minutes, although very small patches of degradation were observed, suggesting that the scaffolds were degradable. Patchy degradation was also observed for one out of three conduits with microspheres incubated for 9 minutes and 9.5 minutes. What is most interesting about these two cases was that these conduits resulted in substantial tissue regeneration across the nerve gap.

Immunohistochemistry for the three cases of regenerated tissue is shown in Figure 8. In the 9 and 9.5 minutes cases, a porous structure can be seen where the scaffold did not degrade (as determined by the presence of fluorescence of Dylight-633). DAPI staining revealed cell growth throughout the tissue in all cases, while S100 staining indicated the presence of Schwann cells in the scaffold-containing regions. Staining for neurofilaments (NF-160) was

weak and did not indicate the presence of axons. All conduits were also evaluated for any observed infection or necrosis (Table 1). None of the conduits were observed to elicit either of these negative biological reactions.

To quantitatively assess axonal regeneration into the conduits and scaffolds, as well as the presence of a foreign body response, conduits containing fully functionalized '9 minute' microspheres were implanted into the rat sciatic nerve injury gap model (Figure 9). In this instance, the PEG scaffold was 5 mm in length with the fibrin plugs again at the ends to yield a total gap of ~7mm, which was compared to an empty conduit of a similar gap. None of the conduits contained GDNF, so as to focus on material effects rather than material + growth factor effects. After 4 weeks, conduits were assessed by histomorphometry at the mid-conduit level to determine if the scaffold promoted nerve regeneration. Conduits containing scaffolds promoted robust nerve regeneration including axonal regeneration in most animals (5 of 6 had regenerated axons mid-conduit) (Figure 9B). Conduits without the scaffold (empty) did not regenerate any axons (0 of 6 had regenerated axons mid-conduit). In addition, conduits containing the scaffolds did not qualitatively demonstrate the presence of a foreign body response, including neutrophil or macrophage accumulation or a fibrotic response, as assessed by histomorphometry. Further distal to the mid-conduit level, the scaffolds demonstrated increased degradation as noted by larger scaffold voids which corresponded with less cellular migration and repopulation of these areas (Figure 9C). Well-vascularized connective tissue was observed adjacent to the silicone conduit but not within the scaffold (Supplementary Figure 4).

Conclusions

We have developed a multistep fabrication process for creating nerve guidance conduits containing nearly 100% synthetic modular scaffolds. PEG microspheres that form the scaffolds were engineered with multiple functionalities. They contained heparin to control the rate of GDNF release. To allow nerves to extend into the conduits, microspheres were degradable by plasmin, with tunable rates of degradation. Click chemistries were used to allow microspheres to crosslink with each other to form a scaffold in the presence of the proteins without risk of reacting with them. The cell adhesion protein laminin was bound to the microspheres to encourage cell growth. The functionalities were combined and the GDNF concentration gradient-making capability of the fully functionalized microspheres was confirmed. GDNF released from these microspheres was confirmed to be biologically active. Finally, we developed methods for forming these scaffolds in silicone conduits using 3D printed plastic molds, and added fibrin plugs to enhance scaffold stability. Conduits were implanted into rats traversing a severed sciatic nerve to demonstrate that the conduit fabrication system was effective, that the scaffolds degrade and promote robust nerve regeneration *in vivo*, and that the scaffolds did not elicit negative biological responses, such as infection, necrosis, or a foreign body response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chemistries for addition of various biological functionalities

A. Thiolation of heparin. B. Production of plasmin degradable microspheres through the formation of PEG-(VRN)₈. C. Scaffold formation through addition of 'Click' cross-linking agents. PEG₈-Azide/Amine and PEG₈-Cyclooctyne/Amine were added to the microspheres during formation producing batches of microspheres decorated with either azide or cyclooctyne groups. Upon mixing and centrifugation, the click agents will react to one another, covalently coupling the microspheres into a scaffold.

Figure 2. Laminin Promotes Growth of DRG

A. Typical DRG growth on PEG₈-VS/PEG₈-Amine gels decorated with laminin at 20 µg/mL. (2 days after seeding, dashes show border of growth). B. DRG growth on PEG₈-VS/PEG₈-Amine gel without laminin. (2 days after seeding, dashed lines show border of growth). C. Average neurite extension in mm for DRG's cultured on PEG gel with and without laminin (n=5). No growth was observed in DRG's without laminin present. Error bars shown but equal to 0 for the –laminin condition.

Figure 3. Final Functionalized Microsphere Procedure

A. $\text{PEG}-(\text{VRN})_8$ and $\text{PEG}_8\text{-VS}$ (200 mg/mL) were combined at 1:1 molar ratio and incubated at 37°C for 1 hour. B. $\text{PEG}_8\text{-Azide/Amine}$ and $\text{PEG}_8\text{-Cyclooctyne/Amine}$ were added at 1:50 click PEG to non-click PEG ratio. C. PEG was diluted to 20 mg/mL in 0.6 M Na_2SO_4 and incubated 8–10 min at 70°C. D. Microspheres were washed 3X in PBS and thiolated heparin (2.6 mg/mL) and laminin (20 $\mu\text{g/mL}$) were added to suspended $\mu\text{spheres}$ and incubated at 25°C overnight. Microspheres were washed 2X in low salt buffer. E. Cysteine (2.5 mg/mL) was added and incubated 25°C for 30 minutes to cap remaining vinylsulfones. Microspheres were washed 2X in low salt buffer. F. The two microsphere types were combined prior to growth factor loading and/or scaffold formation.

Figure 4. Degradation of Microspheres Suspended in Plasmin

Microspheres formed by incubation at 70°C for 8 and 9 minutes were suspended in 1 unit/mL of plasmin and incubated at 37°C to view the rate of degradation. Graph shows average microsphere diameter over time for 8 (blue), 9 (red), and 10 minute (green) formation times (i.e. length of incubation in the phase separated state during microsphere formation at 70 °C). Black dashed line indicates 9 minute microspheres in control conditions (no plasmin). (n=4)

Figure 5. GDNF Activity Retention - DRG growth

Average neurite extension for DRG's grown on thin, bulk PEG gels with 0.8 $\mu\text{g/mL}$ incubated laminin for one, two, and three days under two media conditions: Microsphere released GDNF (833 ng/mL incubation) in MNB media (Blue line, $n=8$), MNB media with no GDNF (Red line, $n=14$). Typical photomicrographs from the 3 day time point are shown both conditions. Yellow dashes indicate boundary of neurite extension. Note: All DRG's in the control condition (No GDNF) had no extensions at Day 3. Error bars shown, but error was zero due to uniformity of samples at this condition.

Figure 6. Fully Formed Conduits

A. Fully formed conduit: microsphere scaffold (blue) flanked by two fibrin plugs, glue plug still intact. B. Fully formed conduit, glue plug excised. Ready for implantation. C. Implanted conduit traversing the severed sciatic nerve in a rat. D. Fluorescent photograph of implanted conduit seen in C. (microspheres labeled with Dylight-633)

Figure 7. *In vivo* degradation of scaffolds

Conduits containing fully-functionalized PEG microsphere scaffolds with gradients in GDNF were implanted in rats traversing a severed sciatic nerve. Degradation of the scaffolds was evaluated using fluorescence microscopy. Sample photographs for each condition are shown. Graph shows average percentage of the scaffold degraded over time for each condition.

Figure 8. IHC for Regenerated Tissue

Fluorescent photomicrographs of sectioned tissue harvested from NGC's at 8 weeks. S100 (red) layered with DAPI (blue) staining over the whole length of the tissue is shown for the 3 instances of regeneration (occurring in different microsphere incubation time conditions). Sample fluorescent photomicrographs at higher magnification (100X) of tissue stained for neurofilaments (green) shown for the 9.5 minute condition.

Figure 9. Axonal regeneration within scaffolds

Conduits containing fully-functionalized PEG microsphere scaffolds without growth factor were implanted in rats traversing a severed sciatic nerve and compared to empty conduits.

A. Representative photomicrograph of axonal regeneration within the scaffolds, taken at the mid-conduit level. Note the presence of nerve regeneration evidenced by the presence of myelinated axons (clear circular area surrounded by dark ring) as well as scaffold degradation (red asterisks). B. Photomicrograph (mid-conduit level) of scaffold with the most extensive nerve regeneration. C. Scaffolds promoted robust axonal regeneration at the mid-conduit level while an empty conduit did not promote any axonal regeneration. Average with standard error of the mean is shown. D. The distal portion of the conduit demonstrated more scaffold degradation and less tissue regeneration.

Table 1***In vivo* degradation of scaffolds**

Conduits containing fluorescently labeled, fully-functionalized PEG microsphere scaffolds with gradients in GDNF were implanted in rats traversing a severed sciatic nerve. Degradation of the scaffolds was evaluated using fluorescence microscopy periodically in living animals. Implants were evaluated visually for the presence of infection or necrosis. The conduits were also evaluated for tissue regeneration across the gap. “Microsphere incubation time” refers to the length of time that microspheres were cross-linked at 70°C during formation, which was prior to introduction of biologically-derived molecules (heparin, laminin and GDNF). Biological molecules were never exposed to temperatures greater than 37°C.

Microsphere Incubation Time		% of Scaffold Degraded					Observed Infection?	Observed Necrosis?	Regenerated Tissue?
		7 Days <i>in vivo</i>	14 Days <i>in vivo</i>	32 Days <i>in vivo</i>	41 Days <i>in vivo</i>	55 Days <i>in vivo</i>			
8 Minutes	#1	86%	89%	96%	98%	99%	No	No	No
	#2	89%	91%	96%	99%	99%	No	No	No
	#3	99%	100%	100%	100%	100%	No	No	Yes
9 Minutes	#1	0%	76%	81%	83%	83%	No	No	No
	#2	0%	0%	0%	2%	4%	No	No	Yes
	#3	45%	57%	74%	76%	84%	No	No	No
9.5 Minutes	#1	80%	80%	98%	97%	98%	No	No	No
	#2	77%	89%	91%	92%	99%	No	No	No
	#3	0%	0%	0%	0%	0%	No	No	Yes
10 Minutes	#1	0%	0%	0%	0%	0%	No	No	No
	#2	0%	0%	0%	0%	0%	No	No	No
	#3	0%	0%	0%	0%	0%	No	No	No