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Design and optimization of PLGA microparticles for controlled and local delivery of Neuregulin-1 in traumatic spinal cord injury

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ABSTRACT

Spinal cord injury (SCI) results in significant tissue damage that underlies functional impairments. Pharmacological interventions to confer neuroprotection and promote cell replacement are essential for SCI repair. We previously reported that Neuregulin-1 (Nrg-1) is acutely and permanently downregulated after SCI. Nrg-1 is a critical growth factor for differentiation of neural precursor cells (NPCs) into myelinating oligodendrocytes. We showed that intrathecal delivery of Nrg-1 enhances oligodendrocyte replacement following SCI. While an effective delivery system, intrathecal and systemic administration of growth factors with diverse biological targets may pose adverse off-target effects. Here, we have developed and optimized an injectable biodegradable poly(lactic-*co*-glycolic acid) (PLGA) microparticles system for sustained and prolonged intraspinal delivery of Nrg-1 in SCI. Recombinant human Nrg-1β1 peptide was encapsulated into PLGA microparticles. Optimal Nrg-1 release rate and duration were achieved by manipulating the porosity and size of PLGA particles. Our *in vitro* analysis showed a direct correlation between particle size and porosity with Nrg-1 release rate, while Nrg-1 loading efficiency in PLGA microparticles was inversely correlated with particle porosity. In SCI, local intraspinal injection of PLGA-Nrg-1 microparticles maintained significantly higher tissue levels of Nrg-1 for a long-term duration compared to Nrg-1 delivered intrathecally by osmotic pumps. Bioactivity of Nrg-1 in PLGA microparticles was verified by promoting oligodendrocyte differentiation of NPCs *in vitro*, and preservation of oligodendrocytes and axons in SCI. PLGA-Nrg-1 also attenuated neuroinflammation and glial scarring following SCI. We show, for the first time, the feasibility, efficacy and safety of PLGA microparticle system for local and controlled administration of Nrg-1 in SCI.

1. Introduction

Spinal cord injury (SCI) is a devastating neurological condition that results in significant cell death, tissue degeneration and progressive functional impairment in patients [1]. To date, repair of SCI has remained an elusive goal and new therapeutic strategies are clearly needed to promote cell replacement in the injured spinal cord [2]. Myelin-forming oligodendrocytes are particularly susceptible to cell death in the acute phase of SCI and their loss leads to subsequent axonal degeneration and functional impairment [1,3]. SCI elicits proliferation and activation of endogenous populations of neural and oligodendrocyte precursor cells (NPCs and OPCs) with the capacity to replace myelinating oligodendrocytes. However, the survival of newly generated oligodendrocytes and their ability for maturation into a fully myelinating phenotype is challenged in the milieu of injured spinal cord. This limitation is largely due to lack of essential supportive growth factors for oligodendrocyte development and maturation [1,3]. Over the past years, treatment strategies have been developed by our group and others to optimize the impermissible microenvironment of SCI for oligodendrocyte replacement [4–6].

We previously reported that SCI results in a rapid and long-lasting decrease in the tissue level of Neuregulin-1 (Nrg-1) [1]. Nrg-1 is a critical growth factor for the development and maintenance of myelin-forming oligodendrocytes and Schwann cells in the central and peripheral nervous system, respectively [7]. Depletion of Nrg-1 following SCI is well-correlated with reports that show insufficient endogenous replacement of oligodendrocytes by NPCs [1]. We have previously shown that Nrg-1 treatment can enhance NPC proliferation and their differentiation into oligodendrocyte lineage *in vitro* and *in vivo* [1]. We administered recombinant human Nrg-1β1 (rhNrg-1β1) peptide intrathecally into the cerebrospinal fluid (CSF) through the subarachnoid space surrounding the spinal cord lesion using an osmotic mini-pump

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attached to an indwelling catheter. We utilized an eight kDa Nrg-1 peptide that contains the functional bioactive epidermal growth factor (EGF)-like domain of all Nrg-1 isoforms [7] and capable of entering CNS through blood-brain-barrier [8]. Intrathecal administration of rhNrg-1 for 14 days following SCI resulted in a significant increase in the tissue content of Nrg-1 that was associated with increased formation of new oligodendrocytes [1]. Interestingly, Nrg-1 treatment also exerts neuroprotective and immunomodulatory effects resulting in tissue preservation and recovery of functions in chronic SCI [1,9]. Evidence from other groups have also shown the promising effects of Nrg-1 treatment for attenuating cell death and improving neuronal survival in ischemic brain conditions [10] suggesting the promise of Nrg-1 therapy for the central nervous system (CNS).

Systemic effects and toxicity are major concerns in pharmacological therapies particularly with the administration of immunomodulators or growth factors such as Nrg-1 with broad biological functions and targets throughout the human body. Intrathecal drug administration through catheter implantation has been an effective method for developing treatment strategies for SCI in preclinical studies. Moreover, intrathecal delivery allows a more effective delivery of reagents to the spinal cord compared to subcutaneous, intravenous or intraperitoneal systemic methods. However, it may pose several risks and limitations in clinical translation. One limitation is the risk of off-target effects in other CNS regions since the treatment is infused into the CSF circulation. This will additionally increase the need for a higher dose to maintain therapeutic effects at the level of SCI compared to local intraspinal delivery [11]. With higher concentration of drug in intrathecal delivery, there is a potential risk for systemic toxicity and side effects as CSF circulation enters the systemic circulation eventually through venous sinuses in the dura matter [12]. Other adverse effects of chronic catheter implantation and intrathecal delivery include the risks of meningeal fibrotic scarring and potential injury to the spinal cord [13]. Moreover, treatment delivery through osmotic minipumps would not allow the simultaneous use of multiple reagents in combinatorial therapies due to the potential undesired cross reactivity of the reagents when stored in one pump. To circumvent these limitations, intraspinal delivery of treatments through biodegradable polymers provides a more reliable method for local administration of therapies directly to the injured spinal cord.

In the present study, we investigated the feasibility and efficacy of local delivery of Nrg-1 in SCI through injectable poly(lactic-*co*-glycolic acid) (PLGA) microparticles as an alternative to osmotic minipump and indwelling intrathecal catheters. PLGA is a copolymer that is widely used in various therapies and devices due to its biodegradability and biocompatibility [14]. Therapeutic agents can be encapsulated into PLGA microparticles by different methods depending on their natural properties [15]. There are various properties of PLGA that can be modified to optimize the release rate and duration of peptide delivery in tissue [16]. This has made PLGA a suitable and versatile vehicle for drug delivery in biological systems.

Here, we encapsulated rhNrg-1β1 peptide into PLGA microparticles using "water-oil-water double emulsion" method. We optimized and evaluated the release rate of Nrg-1 in primary cultures of NPCs and in a preclinical model of compressive SCI in rats by manipulation of particle porosity and size. Our comprehensive *in vitro* and *in vivo* assessments have verified the feasibility and efficacy of Nrg-1 delivery by PLGA microparticles. In SCI, we found a higher level of Nrg-1 in the injured spinal cord tissue of the rats that received intraspinal injections of PLGA-Nrg-1 microparticles at various time-points compared to rats that received Nrg-1 intrathecally using osmotic pumps for the same duration. We demonstrate the feasibility and a higher efficacy for PLGA microparticles in delivery of Nrg-1 into the injured spinal cord compared to intrathecal delivery. PLGA-Nrg-1 was biologically effective and promoted oligodendrocyte differentiation of NPCs, conferred protection to oligodendrocytes and axons, and attenuated neuroinflammation and scar formation following SCI.

2. Materials and methods

2.1. Materials

Poly-lactide-*co*-glycolide (PLGA) copolymers, Pluronic® F-127, glucose, NaHCO3, paraformaldehyde (PFA), HEPES, transferrin, insulin, putrescine, selenium, progesterone, epidermal growth factor (EGF) and heparin was purchased from Sigma Aldrich. Recombinant human Nrg-1β1 and human Nrg-1β1/HRG1 beta1 DuoSet ELISA kit was purchased from R&D systems. Papain enzymatic dissociation kit was purchased from Worthington Biochemical Corporation. Neurobasal-A media. AQueous One Solution Cell Proliferation Assay (MTS) kit and Griess Reagent System kit were purchased from Promega. Basic fibroblast growth factor (bFGF) and Matrigel were purchased from BD Biosciences. B27 supplement, Fetal Bovine Serum (FBS), L-glutamate and penicillin/streptomycin/neomycin (PSN mix) were purchased from Gibco. All other chemicals and reagents used for the experiments were regular laboratory grade.

2.2. Animals and animal care

All experimental protocols were approved by the Animal Committee of the University of Manitoba in accordance with the policies established in the guide to the care and use of experimental animals prepared by the Canadian Council of Animal Care. Pelleted food and drinking water were available *ad libitum*. A total number of 69 adult female Sprague Dawley (SD) rats (250 g) and 16 adult C57BL/6 mice (8 weeks) were used in this study. Animals were provided by the local facility at the University of Manitoba (Winnipeg, Manitoba, Canada).

2.3. Synthesis of Nrg-1 loaded porous and nonporous PLGA microparticles

PLGA- Nrg-1 particles were prepared by the method described by Ilyas and colleagues [15] with few modifications. Briefly, we prepared three solutions. For solution 1, 2–16 μg of rhNrg-1β1 was dissolved in 0.2 ml of deionized (DI) water. Solution 2 contained PLGA in ethyl acetate (45 mg of PLGA was dissolved in 3 ml of ethyl acetate). Solution 3 was composed of 5% Pluronic® F-127 in warm (50 °C) DI water (1.0 g of Pluronic® F-127 in 20 ml of DI water). Solution 1 and 2 were mixed and vortexed for at least 1 min. Then, 1 mg of sodium bicarbonate was dissolved in 1 ml of DI water to form porogen solution. This solution was immediately added to PLGA-Nrg-1 emulsion to introduce pores in the particles. These pores would be formed at a later stage due to salt leaching. Porogen solution step was skipped for the preparation of nonporous microparticles. The emulsion was then sonicated for 2 min at 40 W and then added drop by drop to the stirring beaker of 5.0% Pluronic® F-127 solution. The resultant mixture was emulsified by sonication for 3 min at 40 W for small particle and 30 s for big particle preparations. Smaller particle size was achieved by increasing the duration of sonication. The emulsion was gently stirred overnight allowing ethyl acetate to evaporate. Once the ethyl acetate evaporated completely, microparticles were harvested by centrifugation at 4000–8000*g* for 15 min. The supernatant was collected and frozen for calculating the indirect loading efficiency. The pellet was re-suspended in PBS. To obtain porous microparticles, the microparticles underwent repeated salt leaching. The particles were mixed with 5 ml of PBS and vortexed and then centrifuged at 4000–8000*g* for 15 min. This step was repeated three times to remove the salts from particles completely. The resulting microparticles were centrifuged at 4000–8000*g* for 15 min and re-suspended in a final volume of 10–40 μl of PBS or artificial CSF (aCSF).

2.4. Assessment of Nrg-1 loading efficiency in PLGA microparticles

Nrg-1 loading efficiency was determined directly in the PLGA-Nrg-1 microparticles. PLGA-Nrg-1 particles were dissolved in 0.1 N NaOH for

72 h at 37 °C and pH was normalized by adding equimolar HCl [17,18]. Nrg-1 loading efficiency was also determined indirectly in the supernatant collected during the synthesis stage. Nrg-1 content was assessed by the enzyme-linked immunosorbent assay (ELISA). The loading efficiency was calculated as follows:

Direct method:

$$\text{Nrg}-1 \text{ loading efficiency } (\%) = \frac{(\text{Nrg}-1 \text{ present in PLGA}-\text{Nrg}-1 \text{ particles})}{(\text{Total Nrg}-1 \text{ added})} \times 100$$

Indirect method:

$$\text{Nrg}-1 \text{ loading efficiency}(\%) = \frac{(\text{Total Nrg}-1 \text{ added})-(\text{Nrg}-1 \text{ present in supernatant})}{(\text{Total Nrg}-1 \text{ added})} \times 100$$

2.5. Analysis of Nrg-1 release from PLGA-Nrg1 particles

PLGA-Nrg1 particles from different preparation were suspended in 1.0 ml of PBS or aCSF in a low adhesion 1.5 ml Eppendorf tube and maintained at 37 °C. Sampling was done at various time-points (0–42 day depending on preparations). The particle suspension was centrifuged at 4000–8000*g* for 10 min prior to each sampling to collect 10ul of particle-free solution. Remaining solution was maintained at 37 °C until next sampling. ELISA was performed as per manufacturer's protocol to measure Nrg-1 content in the samples. A standard range of 62.5–4000 pg/ml of Nrg-1 peptide was used for the assay. Values were corrected to 1.0 ml volume to rectify the differences due to a decrease in stock volume after each sampling.

2.6. Visualization of PLGA microparticles using scanning and transmission electron microscopy in vitro and in vivo

We used scanning electron microscopy to visualize PLGA microparticles *in vitro*. PLGA or PLGA- Nrg-1 particle samples were collected at 1-day, 3-day and 7-day time-points. Then, the particles were coated directly onto steel stubs and allowed to air dry for 20 min. Air dried particles on holders were then coated with Gold-Palladium using Edwards Sputter Coater (Model S150zcvbnm,/B) and imaged using Cambridge Stereoscan 120 with EDAX-Genesis software. The presence of PLGA-Nrg-1 particles in the injured spinal cord tissue was examined using transmission electron microscopy (TEM). Rats were perfused transcardially with phosphate buffer followed by 3% glutaraldehyde in 0.1 M phosphate buffer. The injured spinal cord was excised out and cut into 1–2 mm pieces and immersed in 3% glutaraldehyde in 0.1 M phosphate buffer for 3 h. The tissues were then immersed overnight in 0.1 M phosphate buffer containing 5% sucrose (pH 7.4) and post fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 2 to 4 h. Finally, the tissues were dehydrated and embedded in Epon 812 as we did previously [4]. Thin sections (100 nm) were prepared from the fixed tissue and stained with uranyl acetate and Lead citrate. The resulting sections were viewed and photographed in a Philips CM 10 electron microscope (Philips Research Eindhoven, Netherlands).

2.7. Isolation and culture of adult neuronal progenitor cells from the spinal cord

Adult neural precursor cells (NPCs) were isolated from adult C57BL/6 mice (8 weeks of age, both sexes). Mice were euthanized by cervical dislocation under isoflurane anesthesia. Spinal cords were excised and transferred to aCSF solution. Finely chopped spinal cord tissues were digested with 5.0 ml of papain enzymatic solution for 40–50 min at 37 °C. Papain inhibitor mixture was added to stop the enzymatic reaction. The resultant solution was passed through 70 μm cell strainer to remove non-dissociated tissue. Cellular components were isolated using 7.5% BSA gradient and centrifugation at 300*g* for 7 min. The pellet was resuspended and plated in Neurobasal-A growth media. Each 100 ml of media contained 84 ml of Neurobasal-A media, 2 ml of 30% glucose, 1.5 ml of 7.5% NaHCO3, 0.5 ml of 1 M HEPES, 10 mg of transferrin, 2.5 mg of insulin, 0.96 mg of putrescine, 1 μg of selenium, 1 μg of progesterone, 1% L-glutamine, 1% penicillin/streptomycin/neomycin, 1 μg of basic fibroblast growth factor (bFGF), 2 μg of epidermal growth factor (EGF), 200 μg of heparin and 2% B27 supplement. Cells were plated on uncoated tissue culture flasks (Biolite; Fisher Scientific). NPCs formed neurospheres that were passaged weekly by mechanical dissociation in growth media for expansion. NPCs from passage 4–7 were used in this study.

2.8. Cell viability assay

NPCs were plated on Matrigel-coated 96-well plates at a gradient density of 5000, 10,000, 20,000, 30,000, 50,000, and 100,000 in growth media containing DMEM/F12, bFGF, EGF and heparin. MTS assay was performed at 2-day and 6-day post treatment as per manufacturer's protocol and absorbance was read at 490 nm using Synergy H1 hybrid plate reader. Calibration curve was made by plotting absorbance against initial cell density and a density of 20,000 cells per well was selected as an optimal number within the linear range of the curve for further experiments. PLGA-Nrg1 small-particle preparation with a release rate of 255.1 ± 0.9 ng/ml/day was used for the study. PLGA-Nrg-1 particles suspension was diluted further at a ratio of 1:1.7 with serum free media (SFM) to obtain microparticles suspension capable of releasing 150 ng of Nrg-1/ml/day. Similarly, PLGA only particles were also suspended and diluted in SFM. Cells were either non-treated or treated with Nrg-1 (150 ng/ml), PLGA only microparticle suspension or PLGA-Nrg-1 microparticle suspension (diluted suspension corresponding to 150 ng/ml/day). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay was performed as per the manufacturer's protocol at 2-day and 6-day time-points. Cell survival under various treatments was compared with untreated cells.

2.9. Immunocytochemistry for analysis of NPC proliferation and differentiation

For proliferation assay, NPCs were plated on Matrigel-coated 8-well chambers at a density of 20,000 cells per well in growth media. After overnight incubation, cells were switched into SFM or treatment media. PLGA-Nrg1 small-particle preparation with a release rate of 255.1 ± 0.9 ng/ml/day was used for the proliferation and differentiation study. PLGA-Nrg-1 particle suspension was diluted further at a ratio of 1:1.7 with SFM to obtain microparticle suspension capable of releasing 150 ng of Nrg-1/ml/day. Similarly, PLGA only particles were also suspended and diluted in SFM. Cells were either non-treated or treated with Nrg-1 (150 ng/ml), PLGA only microparticle suspension or PLGA-Nrg-1 microparticle suspension (corresponding to 150 ng/ml/day). To label proliferating cells, cultures were treated with 5-Bromo-2′-deoxyuridine (BrdU, 20 μM) for 4 h priors to fixation of NPCs. Two days post treatment, cells were fixed with 3% paraformaldehyde (PFA) and washed three times with PBS. Cells were then incubated in 2 M HCl and 0.5% Triton X-100 for 30 min at 37 °C, and washed with 0.1 M sodium borate in PBS for 10 min. Then immunocytochemistry (ICC) was performed with mouse anti-BrdU to detect BrdU positive cells. The nuclei was counter stained by 4, 6-diamidino-2-phenylindole (DAPI). Immunostained cultures were imaged and percentage of BrdU positive cells among total DAPI positive cells was calculated for various treatments in comparison to the untreated control group. For differentiation assay, NPCs were plated in serum media containing DMEM/F12 and 2% fetal bovine serum (FBS) for 7 days. Experimental conditions included

NPCs grown in: 1) control serum media, 2) rhNrg-1β1 (150 ng/ml), 3) PLGA microparticles only 4) PLGA-Nrg-1 suspension (diluted suspension corresponding to 150 ng/ml/day as mentioned above). Seven days after treatments, cells were fixed with 3% PFA and underwent immunocytochemistry to assess the differentiation pattern of NPCs. Astrocytes and oligodendrocytes were detected by antibodies against GFAP (1:800, Sigma G3893,) and Olig2 (1:2000, Chemicon, AB9610), respectively. Cultures were counterstained with DAPI to label nuclei. Images were taken using a Zeiss AxioImager M2 fluorescent microscope. Astrocytes and oligodendrocytes were quantified as percentage of GFAP + /DAPI + and Olig2 + /DAPI + cells, respectively, among the total number of DAPI + cells.

2.10. Isolation, culture and activation of primary astrocytes/microglia

Primary mixed astrocytes and microglia (astro-microglia) cultures were prepared from the cortex of postnatal rats (P1–P3) and maintained in DMEM containing 10% horse serum, glucose and glutamate as we described before [9]. Upon reaching 80% confluency, cultures were serum deprived for 24 h and then divided into three groups: 1) normal control, received the serum free astrocyte media, 2) lipopolysaccharide (LPS) activated, received (10 μg/ml) LPS and 3) PLGA treated, which received 5 μl (corresponds to the same volume and quantity of microparticles suspension used for other *in vitro* studies) of PLGA microparticle suspension. 4) PLGA-Nrg-1 (diluted suspension corresponding to 150 ng/ml/day). Conditioned media (CM) was then collected at 24- and 72-h after activation and assessed for markers of astrocyte reactivity.

2.11. Griess assay

Griess Reagent System kit (Promega Corp. USA) was used to quantify the nitrite content of conditioned media (CM) at 24-h after activation according to the manufacturer instructions. In order to eliminate any possible interference in Griess assay readings, phenol red free media was used for these cultures.

2.12. Slot blot analysis of CSPGs expression in glial conditioned media and tissue samples

Analysis of chondroitin sulfate proteoglycans (CSPGs) was conducted by slot blotting 8 μg of protein in conditioned media (CM) or 3 μg of tissue protein on a nitrocellulose membrane using Bio-Dot® microfiltration apparatus (Bio-Rad laboratories Inc.). The membrane was then blocked and incubated with CS56 Mouse antibody (1:500; C8056 Sigma Aldrich) for 2 h at room temperature. Blocking solution contained 5% skim milk in 20 mM Tris buffered saline + 0.1% Tween-20 (TBST). The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:4000; BioRad) for 1 h. Membranes were then incubated with enhanced chemiluminescence reagent according to the manufacturer instructions (ECL, Thermo #34080). Densitometry analysis of the immunoreactive bands was performed using NIH ImageJ software. To control for variations in protein loading, values were normalized to total protein in CM detected by Ponceau S stain. Values of treatment groups were normalized with untreated controls.

2.13. Rat compressive spinal cord injury and surgical method

A total of 69 adult female Sprague Dawley (SD) rats (250 g) were used in our SCI experiments (Local Facility at the University of Manitoba, Canada). We utilized a well-established and clinically-relevant model of severe clip-compression SCI at mid thoracic level. Laminectomy was performed under deep anesthesia (4% Isoflurane + 2% O2), at thoracic levels T6–T8. Compressive SCI was induced using a 35 g aneurysm clip (University Health Network, Toronto, Ontario, Canada) for 1 min on spinal cord at T7 level. The surgical wounds were sutured, and the rats were immediately given analgesic treatment that included meloxicam (2 mg/kg, Metacam; Boehringer Ingelheim), buprenorphine (0.03 mg/kg, Temgesic; Schering-Plough) and saline (0.9%; 5 ml) to prevent pain and dehydration. Buprenorphine administration was repeated three more times to manage pain and discomfort. To prevent trauma induced bladder complications and hematuria, rats were given Clavamox (amoxicillin plus clavulanic acid; Pfizer Animal Health) for 5 days, beginning 48 h before the SCI procedure. The clip compression model of rat SCI accurately generates the key pathological features of human SCI and it results in severe incomplete SCI and behavioral evidence of a spastic paresis. After SCI, rats were housed in cages with soft paper bedding to prevent skin erosions and urine scalding. Pelleted food and drinking water were available *ad libitum* and animals were placed in a room with 12:12 h light/dark cycle. Their bladders were expressed manually three times a day until the return of reflexive bladder control.

2.14. In vivo experimental groups and treatments

Before SCI, rats were randomly assigned to 6 experimental groups: 1) Uninjured, no injury was performed; 2) SCI/vehicle control, receiving vehicle solution used for preparation of Nrg-1 (0.1% bovine serum albumin, BSA, in aCSF); 3) SCI/Nrg-1, receiving recombinant human Nrg-1β1 (rhNrg-1 β1) intrathecally at a dose of 500 ng/day; 4) SCI/PLGA, receiving intraspinal PLGA injection (10 μl suspension); 5) SCI/PLGA-Nrg-1, receiving intraspinal PLGA loaded with Nrg-1 (10 μl corresponding to 340 ng/day Nrg-1); 6) SCI/Nrg-1 bolus injection at a dose of 500 ng/rat. Each experimental group included three time-points: 3-day, 7-day and 14-day post-SCI. We used rhNrg-1β1 peptide (Cat# 100-46-50 Shenandoah Biotechnology Inc.) containing the bioactive epidermal growth factor (EGF)-like domain of all endogenous Nrg-1 isoforms. All treatments started at the time of injury. For the first two groups, Nrg-1 or vehicle treatment was delivered using an osmotic mini-pump and an indwelling intrathecal catheter (Alzet –model #1003D for 3-day, model #2001 for 7-day, model #2002 for 14-day, model # 2004 for 28-day).

2.15. Western blot analysis on SCI tissue

We conducted Western blot analysis to determine the expression of GFAP, Iba-1, MBP and CNPase in the injured spinal cord tissue across different treatment groups. Five millimeters (5 mm) of the spinal cord tissue centered at the injury epicenter was homogenized in NP-40 buffer containing Sigma-Fast Protease Inhibitor (S8820-Sigma). We loaded 10 μg (GFAP, Iba-1), 20 μg (MBP) and 50 μg (CNPase) protein per each sample into SDS-PAGE gels and transferred onto nitrocellulose membranes (30 V for 12 h). The membranes were then blocked for 1 h at room temperature and incubated with GFAP (1:6000, Mouse, Sigma, G3893), Iba-1 (1:500, Rabbit, Wako, 016-20001), MBP (1:1000, Rabbit, Millipore, AB980) and CNPase (1:200, Mouse, Chemicon, MAB326R) antibodies overnight at 4 °C. Blocking solution contained 5% non-fat milk in 20 mM Tris buffered saline + 0.1% Tween-20 (TBST). The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:4000; BioRad) for 1 h. Membranes were then incubated with enhanced chemiluminescence reagent according to the manufacturer instructions (ECL, Thermo #34080). Densitometry analysis of the immunoreactive bands was performed using NIH ImageJ software. Protein loading was determined by re-probing immunoblots with Anti-rabbit GAPDH antibody (Rabbit, Santa Cruz, sc-25778). To control for variations in protein loading, GFAP and Iba-1 densitometry values were normalized to their respective GAPDH bands. In our procedures, all samples were processed in parallel on the same gel for relative comparison. Data were presented by normalization to the control condition from the same membrane.

2.16. Immunohistochemical assessment of NF-200 expression following SCI

Frozen spinal cord longitudinal-sections (35 μm) containing the center of injury (epicenter) underwent immunostaining of axons using NF200 antibody (Sigma N0142). Tissue sections were first blocked in a solution contained 5% non-fat milk, 1% BSA, and 0.3% Triton X-100 in 0.1 M PBS for 1 h followed by an overnight incubation with anti-NF200 antibody at 4 °C. Then sections were incubated with goat anti-mouse Alexa Fluor 568 secondary antibodies. The slides were washed three times in PBS, stained with DAPI and cover-slipped using Mowiol mounting media. All samples were processed in parallel under the same condition and imaged using Zeiss AxioImager M2 fluorescence microscope (Zeiss) under consistent exposure time (5000 ms) as we described previously [1]. Using NIH ImageJ analysis software (imagej.nih.gov), an area encompassing 2 mm around the epicenter was traced in each section. After setting the threshold automatically, the immunodensity above the threshold was calculated. The following formula was used to calculate the percentage of NF200 immunointensity in the examined area: normalized immunointensity of tissue section X = total immunointensity of tissue section X/total area of the spinal cord section X.

2.17. Detection of Nrg-1β1 with enzyme-linked immunosorbent assay (ELISA) in SCI tissue and in vitro samples

Five millimeters (5 mm) of the spinal cord tissue centered at the injury center was homogenized in RIPA buffer containing Sigma-Fast Protease Inhibitor (S8820-Sigma). We used a commercial ELISA kit (DuoSet ELISA Development System; R&D Systems; DY377) to specifically detect Nrg-1β1 in spinal cord tissue samples. The assay was performed according to the manufacturer's instructions, with standards (125–4000 pg/ml) and loading of 50 μg protein per sample.

2.18. Statistical analysis

Statistical analyses were performed using SigmaStat software. Student's *t*-test was employed where only two groups were compared. Multiple groups were compared using one-way ANOVA. Multiple comparisons over time among groups were performed using a two-way ANOVA. The Holm–Sidak post-hoc correction was used for all multiple comparisons. Data are reported as means ± standard error of the mean and $p < 0.05$ was considered as significant.

3. Results

3.1. Optimization of Nrg-1 controlled release by PLGA microparticles

We assessed the efficacy of encapsulating Nrg-1 in PLGA microparticles for the sustained sequential release of Nrg-1. We encapsulated 2-16 μg of Nrg-1 in PLGA in different microparticle preparations. Using longitudinal ELISA, our initial assessment showed a daily release of 114.5 ± 0.5 ng of Nrg-1 from PLGA-Nrg-1 nonporous microparticles preparation for 6 days (Fig. 1A, Table 1).

This release rate was lower than our optimized therapeutic dose (150 ng/ml) for Nrg-1 treatment identified in our previous *in vitro* studies where we showed a positive effect for Nrg-1 in promoting oligodendrocyte differentiation of NPCs [1]. To achieve a higher release rate closer to the optimized Nrg-1 dose *in vitro*, we modified properties of PLGA-Nrg-1 particles by introducing porosity that has been shown to increase drug release rate from PLGA particles [19]. We introduced porosity by salt leaching method using sodium bicarbonate (NaHCO3) as the porogen. Porosity, pore size and distribution depend on the concentration of the porogen [20]. Therefore, we examined various concentrations of NaHCO3 ranging from 0.5 mg to 2.0 mg (Fig. 1A, Table 1). When both porous and nonporous forms of PLGA microparticles were loaded with the same amount of Nrg-1, higher Nrg-1 release rate was achieved from porous PLGA-Nrg-1 compared to nonporous particles. Our NaHCO3 gradient concentration studies showed a significant increase in the release rate of Nrg-1 from particles prepared with 1.0 mg and 2.0 mg concentration of NaHCO3 compared to nonporous or 0.5 mg NaHCO3 preparation at all time-points after 1 day (*$p < 0.05$, **$P < 0.01$, two-way ANOVA; $N = 3$) (Fig. 1A). The average daily release of Nrg-1 from PLGA small-microparticles prepared with 1.0 mg of NaHCO3 was 255.1 ± 0.9 ng/ml when particles were suspended in PBS. To achieve an Nrg-1 release rate of 150 ng/ml/day, an effective concentration in our previous studies in cultures of adult NPCs, we further diluted these particles at a ratio of 1:1.7 with media [1] (Fig. 1A; Table 1). We observed a decrease in Nrg-1 peptide loading efficiency with an increase in porosity. This inverse relation of porosity and loading efficiency has been previously reported [15]. Altogether, our results show that particle porosity has a positive correlation with release rate and a negative correlation with loading efficiency of PLGA microparticles. Table 1 provides values of loading efficiency and daily release rate for different preparations of PLGA small-microparticles including nonporous and porous preparations with 0.5 mg, 1.0 mg and 2.0 mg of NaHCO3. The amount of Nrg-1 loaded into the particles was calculated indirectly by subtracting the amount of Nrg-1 in the washing supernatants. Additionally, we employed a direct method to quantify the amount of Nrg-1 loaded into PLGA microparticles prepared with 1.0 mg NaHCO3 (described in the Method section). Our direct analysis showed a comparable loading efficiency for Nrg-1 (78.13 ± 1.69%) which was slightly lower than the indirect method (81.1 ± 0.485) ($N = 3$ independent *in vitro* preparations). Percentage of loaded Nrg-1 was calculated based on the total amount of Nrg-1 used for encapsulation. Daily release rate was calculated from the cumulative release of Nrg-1 up to day 6.

3.2. Modification of particle size modulates the duration of Nrg-1 release from porous PLGA microparticles

After optimizing the release rate, we focused on calibrating the duration of Nrg-1 release from PLGA-Nrg-1 microparticles. We conducted a 21-day longitudinal analysis of Nrg-1 release by PLGA-Nrg-1 microparticle prepared with 12.0 μg of Nrg-1, 1.0 mg NaHCO3 as porogen and 3-min sonication for dispersion. Our data showed that PLGA-Nrg-1 microparticles maintained a steady release rate up to 19 days and reached a plateau at 21 days (Fig. 1B). To increase the duration of Nrg-1 release, we postulated that porous microparticles with bigger particle size may maintain similar release rate for a longer duration. Several studies have demonstrated that nonporous bigger particle size causes faster degradation rate and consequently faster drug release [21–24]. However, porous particles follow a different degradation rate due to difference in mechanism as discussed in the literature [19]. Thereby, we prepared bigger porous-microparticles by encapsulating 16 μg of Nrg-1 in PLGA microparticles prepared with 1.0 mg NaHCO3. We also reduced the sonication time from 3 min, which was used in the preparation of smaller particles, to 30 s that resulted in reduced dispersion time. Shear force or energy applied per unit volume has a direct effect on droplet size of the emulsion produced [25]. Since the degree of shear stress has an inverse correlation with the droplet size in the emulsion, we used this paradigm to produce two types of PLGA-Nrg-1 particles, termed as small and big particles in the present work (Fig. 1B–C). One day after the preparation of PLGA-Nrg-1 microparticles, smaller particles loaded with 12 μg of Nrg-1, released 0.7 μg of Nrg-1 while bigger particles loaded with 16 μg released 2.69 μg of Nrg-1 (Fig. 1B–C). This may be attributed to an initial burst of drug release from microparticles as previously described in the literature [26].

After the initial burst release, average release rate progressively decreased over time. To confirm that the progressive decrease in the Nrg-1 release rate did not reflect the degradation of secreted Nrg-1 peptide, we performed thermal and functional stability assay for Nrg-1. We tested the bioactivity of Nrg-1 at various time-points at 37 °C

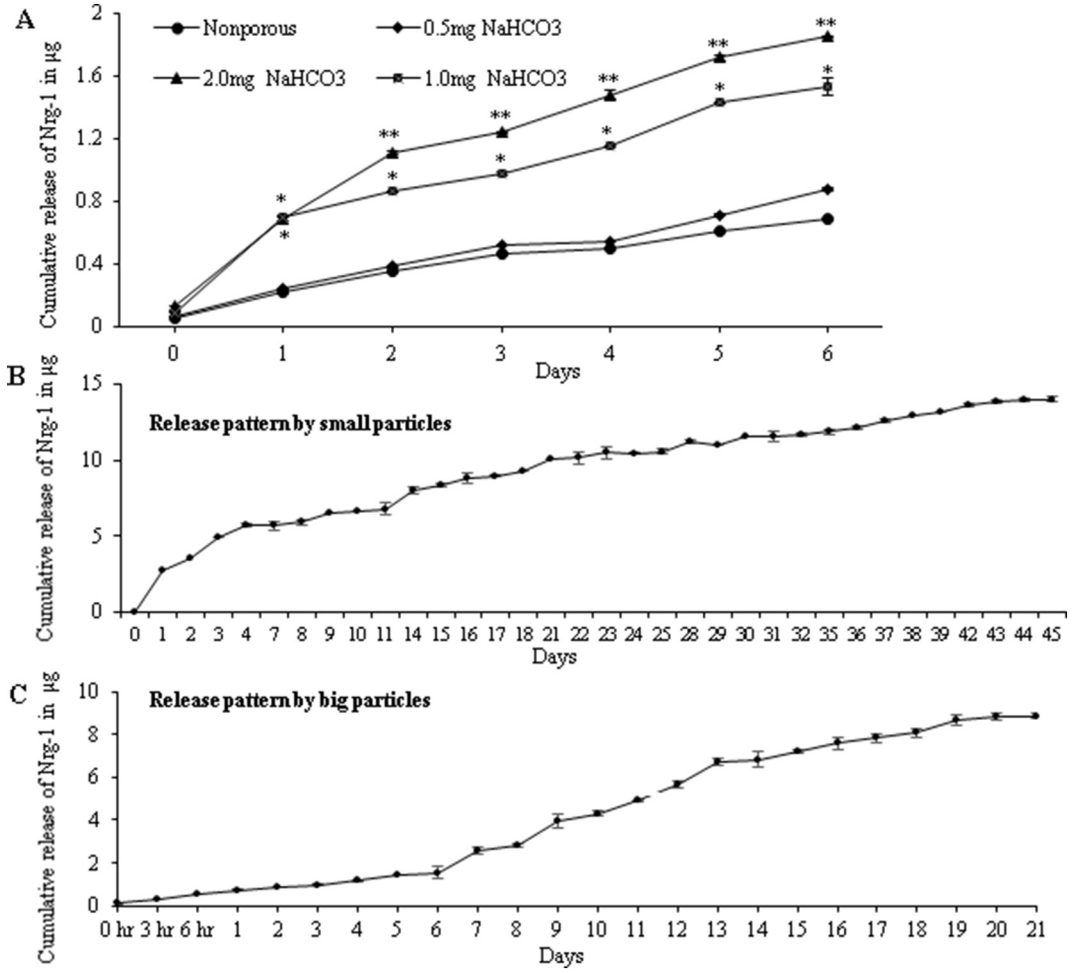

Fig. 1. Cumulative release profile of Nrg-1 from PLGA-Nrg-1 microparticles. (A) Comparison of cumulative Nrg-1 release from different preparations of PLGA microparticles including nonporous and porous preparations with 0.5, 1.0 and 2.0 mg NaHCO3. Our quantification of Nrg-1 using ELISA showed a positive correlation between Nrg-1 release rate and an increase in particle porosity. Our quantification showed a daily release rate of Nrg-1 at 114.5 ± 0.5 ng, 145.9 ± 1.9 ng, 255.1 ± 0.9 ng and 308.1 ± 1.0 ng from nonporous, 0.5, 1.0 and 2.0 mg NaHCO3 preparations, respectively. The release rate was significantly higher in microparticles prepared with 1.0 and 2.0 mg NaHCO3 from day 1 and onwards compared to nonporous microparticles or microparticles prepared with 0.5 mg NaHCO3. Increase in porosity resulted in higher release rate, but it negatively affected loading efficiency. Overall, PLGA-Nrg-1 microparticles prepared with 1.0 mg of NaHCO3 showed the most optimal release and loading efficiency parameters. The data represents the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, Two-Way ANOVA, followed by Holm Sidak post-hoc test, $N = 3$/each type of preparation. (B–C) We assessed Nrg-1 release profile for 21 days in small particle preparation (B) and for 45 days for big particle preparation (C) in PLGA-Nrg-1 prepared with 1.0 mg of NaHCO3 porosity. Release reached a plateau at 21 days for small particles and at 38 days for big particles. Daily release rate was calculated as 255.1 ng and 251.44 ng of Nrg-1 for smaller and bigger particles, respectively. Values represent mean ± SEM; $N = 3$ per preparation.

including 0, 21 and 42 days (Supplementary Fig. 1A–C). As we demonstrated previously [1], Nrg-1 induces a shift in NPC differentiation from astrocyte formation towards oligodendrocyte differentiation *in vitro*. Using this NPC differentiation assay, our immunocytochemical analysis showed the efficacy of Nrg-1 in promoting oligodendrogenesis in NPCs with a highly comparable potency among different Nrg-1 preparation. These data confirmed that Nrg-1 maintains its bioactivity at physiological temperature (37 °C) even when stored for a prolonged duration ($N = 3$ independent experiments) (Supplementary Fig. 1 A–C). Given this evidence, our data suggest that the progressive decrease in Nrg-1 release is a property of the PLGA-Nrg-1 particles and not due to Nrg-1 degradation.

We additionally performed scanning electron microscopy [27] to study the size distribution of PLGA-Nrg-1 microparticles in our small and big particle preparation *in vitro* (Fig. 2A–C). Our examination showed a mixture of particles of different size in both preparations. We found a negative correlation between sonication time and particle size. Decrease in sonication time to 30 s resulted in particles with an average diameter of 2.37 µm compared to an average particle diameter of 1.43 µm prepared with 3-min sonication. Diameter distribution in smaller particle preparation was: 1–1.49 µm (50.40 ± 1.91%), 1.5–1.99 µm (34.55 ± 0.36%), 2–2.99 µm (13.52 ± 2.65%), 3–3.99 µm (< 1%), 4–4.99 µm (< 1%) and > 5 µm (< 1%) (Fig. 2A–B). This mixture of particles released Nrg-1 up to 21 days

Table 1
Nrg-1 loading efficiency and release rate for PLGA-microparticles with different porosities.

Particle type	Nonporous (no NaHCO3)	Porous NaHCO3 concentration		
		0.5 mg	1.0 mg	2.0 mg
Loading efficiency (%)	85 ± 0.192	83.5 ± 0.254	81.1 ± 0.485	72.24511 ± 0.658
Release rate (ng/day)	114.5 ± 0.5	145.9 ± 1.9	255.1 ± 0.9	308.1 ± 1.0

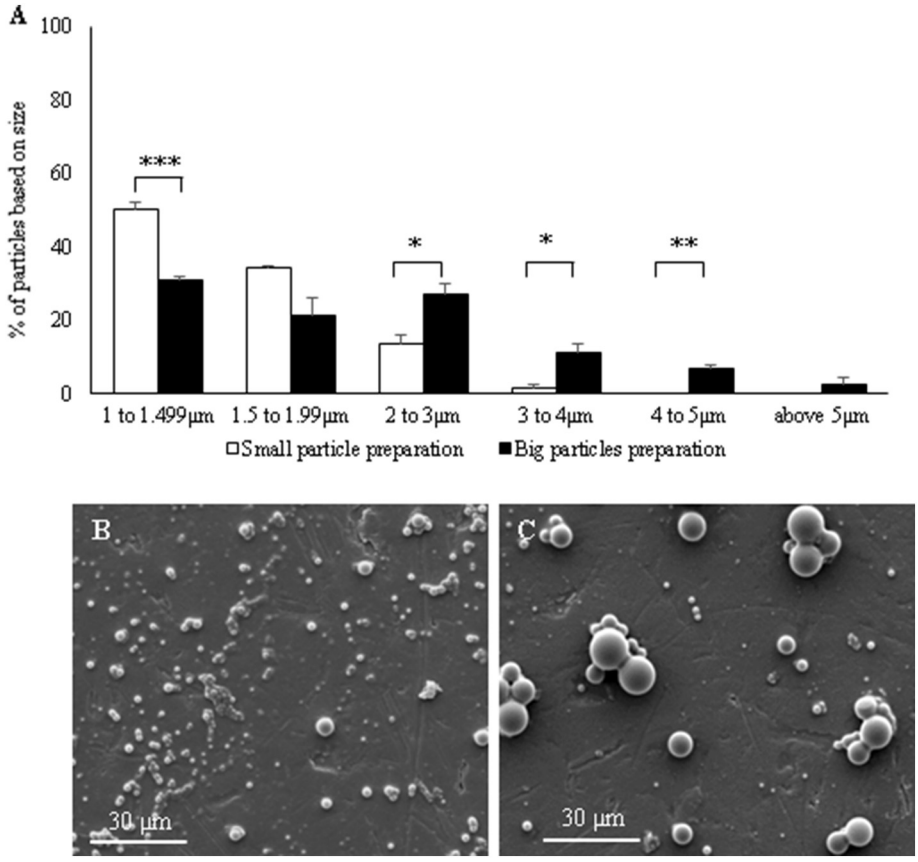

Fig. 2. Morphometric analysis and biodegradation of PLGA and PLGA-Nrg-1 particles *in vitro* and *in vivo*. (A–D) Morphometric analysis and size distribution of porous PLGA-Nrg1 microparticles (100–200 particles/preparation) was performed on smaller (B) and bigger (C) particles using scanning electron microscopy [27]. Both preparations produced a mixture of particles with a different size and lower sonication time produced a higher percentage of bigger particles. $N = 3$, student *t*-test.

(Fig. 1B).

Our morphometric analysis of bigger microparticle preparation revealed a particle diameter distribution of: 1–1.49 μm (30.84 ± 1.26%), 1.5–1.99 μm (21.22 ± 4.94%), 2–2.99 μm (27.12 ± 3.12%), 3–3.99 μm (11.32 ± 2.55%), 4–4.99 μm (6.89 ± 1.22%) and > 5.0 μm (2.60 ± 2.06%) (Fig. 1C). Percentage of particles in 1–1.49 μm diameter range decreased significantly while 2–2.99 μm, 3–3.99 μm and 4–4.99 μm diameter range increased significantly (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Student's *t*-tests, $N = 3$ independent preparations). This particles composition released Nrg-1 up to 42 days before plateauing with an average release rate of 251.44 ng/day (Fig. 1C). These results indicate that release duration of Nrg-1 is positively correlated with porous PLGA particles size.

3.3. Degradation of PLGA-Nrg-1 particles in vitro

Previous studies have shown that polymer composition influences the degradation of PLGA. These studies identified that an increase in glycolic acid percentage in the oligomers accelerates the degradation of the polymer [24,28]. The polymer composition of PLGA used in our study was 50:50 ratio. The size distribution of porous PLGA microparticles was in a range of 0.1 to 5 μm. Using SEM, we found no significant change in the size distribution of particles in porous PLGA and porous PLGA-Nrg-1 microparticles after 7 days of incubation in PBS at 37 °C *in vitro* (Supplementary Fig. 2 A–F). Our results are in agreement with previous reports that showed no change in the weight and water absorption properties of porous PLGA particles with 50:50 polymer compositions up to 6 weeks, and on particle thickness up to 2 weeks [24]. These results suggest that during the first 7 days, the amount of Nrg-1 released is mainly by mechanisms such as dissolving and diffusion through the porous structure of the microparticles. Although autocatalytic hydrolysis of the particles occurs, it is not up to a measurable degree in terms of size.

3.4. Efficacy and safety of Nrg-1 delivered by encapsulation in PLGA microparticles

We next evaluated the functional efficacy of Nrg-1 released by PLGA microparticles in primary cultures of spinal cord derived NPCs. Our previous studies showed that adding Nrg-1 peptide to NPCs at 150 ng/ml concentration positively enhance their proliferation and oligodendrocyte differentiation [1]. Here, we used the same NPC parameters to evaluate the efficacy of PLGA-Nrg-1 microparticles in achieving a sustained delivery of Nrg-1 at this concentration (Figs. 3–4). We evaluated several aspects of NPCs properties including their survival, proliferation and differentiations. Metabolic activity and survival of NPCs was assessed by MTS assay at 2 and 6 days after addition of PLGA and PLGA-Nrg-1 treatment to NPC cultures. Our analysis confirmed a significant 2.5 to 3.9 fold increase in the survival and metabolic activity of NPCs treated with Nrg-1 and PLGA-Nrg-1 compared to control and PLGA alone conditions at both 2 and 6 days ($p < 0.001$, One-way ANOVA, $N = 3$) (Fig. 3A–B). Importantly the positive effect of PLGA-Nrg-1 was comparable to direct addition of Nrg-1 treatment (150 ng/ml) to culture (Fig. 3A–B). We next examined the effects of PLGA-Nrg-1 on NPC proliferation using BrdU pulsing approach that labels mitotic cells and their offspring. Our BrdU immunolabeling and quantification studies at 2 days post treatment revealed a significant increase in NPC proliferation in both Nrg-1 and PLGA-Nrg-1 conditions compared to the control and PLGA alone conditions ($p < 0.001$, one-way ANOVA. $N = 3$/condition) (Fig. 3C–G). We found no significant change in NPC survival and proliferation between PLGA alone and control conditions confirming that PLGA itself does not alter NPCs properties.

We next performed NPC differentiation assays to further confirm bioactivity and efficacy of Nrg-1 release from PLGA-Nrg-1 microparticles. Our previous studies showed that Nrg-1 treatment enhances differentiation of spinal cord derived NPCs into oligodendrocytes while reducing their astrocyte differentiation [1]. We used

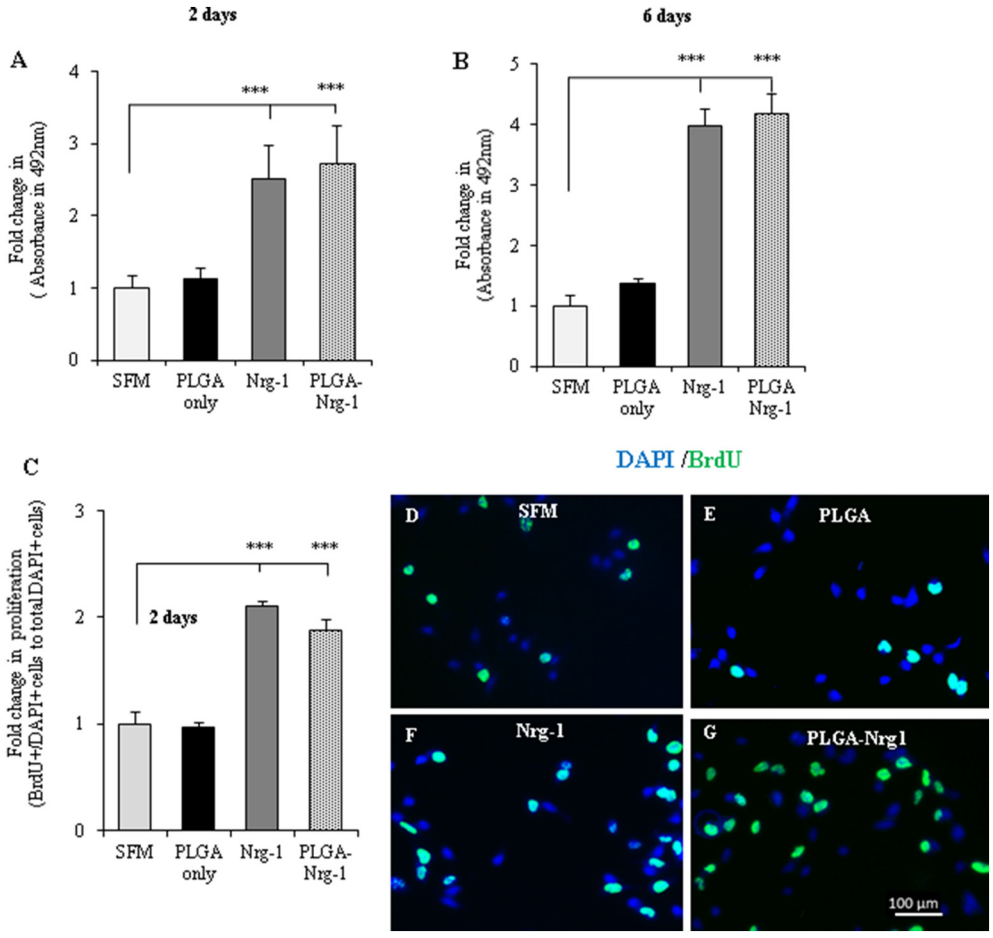

Fig. 3. PLGA microparticles had no adverse effects on NPCs properties and bioactivity of Nrg-1 was not altered by encapsulating into PLGA microparticles. (A–B) Survival of NPCs was measured in culture by MTS metabolic assay at 2 days (A) and 6 days (B) after treatments. PLGA itself had no effects on metabolic activity of NPCs compared to the control condition (serum free media, SFM). Nrg-1 and PLGA-Nrg-1 treatments resulted in significant increase in NPCs survival in both time-points, compared to the control condition. (C, D–G) Our quantitative BrdU proliferation assay revealed that PLGA itself did not affect NPCs proliferation compared to control SFM condition at 2 days after treatment, while there was a significantly higher percentage of BrdU + /DAPI + proliferating NPCs under Nrg-1 and PLGA-Nrg-1 treatments. DAPI marks nucleus. Data shows mean ± SEM; $^{*}P < 0.001$, One-Way ANOVA, followed by Holm Sidak post-hoc test; $N = 3$ independent experiments.

immunohistochemical analysis for Olig2 and GFAP to mark oligodendrocytes and astrocytes, respectively (Fig. 4A–H). Under low concentration of fetal bovine serum (FBS, 0.2%) control condition, about 41% of NPCs differentiated into astrocytes while 23% gave rise to oligodendrocytes (Fig. 4I–J). PLGA alone had no effects on the differentiation pattern of NPCs compared to the control serum condition (Fig. 4A–B) while Nrg-1 and PLGA-Nrg-1 treatments significantly increased oligodendrocyte differentiation at the expense of astrocyte differentiation. ($p < 0.001$ for Olig2 data, $p < 0.01$ for GFAP data, one-way ANOVA, $N = 3$). We found no significant difference in the percentage of Olig2 and GFAP positive cells between Nrg-1 and PLGA-Nrg-1 treated groups indicating efficacy of Nrg-1 release after encapsulation into PLGA microparticles (Fig. 4A–H). Altogether, our analysis shows that PLGA microparticles do not alter NPC activity on its own while release Nrg1 effectively and in a sustained manner. Importantly, we found that Nrg-1 maintains its bioactivity during PLGA encapsulation.

3.5. PLGA has no adverse effect on the activity of astrocytes and microglia in vitro

We next evaluated the effects of PLGA on activity of astrocytes and microglia as a safety measure. Resident astrocytes and microglia rapidly respond to injury and insult, and initiates neuroinflammation and glial scar formation in brain and spinal cord injuries [29,30]. Activation of microglia and astrocytes (microgliosis and astrogliosis) has both negative and positive consequences on repair process following SCI [29]. To assess whether PLGA *per se* may have undesired effects on activation of glia, we employed a mixed culture of astrocytes (85%) and microglia (15%) to recapitulate the composition of these cells in the microenvironment of SCI. We focused on production of nitrite and chondroitin sulfate proteoglycans (CSPGs), two key markers of microgliosis and astrogliosis with detrimental effects [30,31]. Mixed astro-microglia cultures were treated with lipopolysaccharide (LPS), which is known to potently induce activation in cultures of astrocytes and microglia [9,32]. Analysis of conditioned media collected from LPS-treated cultures showed a 25-fold increase in nitrite levels (Fig. 5A, $p < 0.001$, one-way ANOVA, $N = 3$) and a 3-fold increase in CSPGs release (Fig. 5B, $p < 0.05$, one-way ANOVA, $N = 3$) compared to the control condition confirming glial activation. Treatment with PLGA or PLGA-Nrg-1 did not elicit glial activation, as there were no significant changes in nitrite or CSPGs release compared to control condition. These results confirmed that PLGA has no detrimental effect on glial cells and can be employed safely in therapeutic applications in SCI.

3.6. Intraspinal administration of PLGA-Nrg-1 microparticles effectively increases the tissue availability of Nrg-1 in the injured spinal cord

Following our *in vitro* optimization and validation studies, we evaluated the efficacy of PLGA microparticles for delivery of Nrg-1 in a rat model of compressive SCI. We compared intraspinal delivery of Nrg-1 through PLGA-Nrg-1 microparticles to intrathecal delivery *via* indwelling catheter and osmotic minipumps. PLGA-Nrg-1 intraspinal or Nrg-1 intrathecal delivery was performed at the time of SCI for 3, 7, 14 and 28 days representing acute, subacute and early chronic stages of SCI in our rat model. Daily dose of Nrg-1 was 500 ng for intrathecal pump delivery (as we used previously) [1,9] and 340 ng for PLGA-Nrg-1 microparticles delivery. We also allocated two SCI groups as a control for each delivery method in which the rats received vehicle (0.1% bovine serum albumin, a control for protein delivery, in aCSF) either intrathecally or through PLGA microparticles. Additionally, we included one SCI group that received one intraspinal bolus injection of Nrg-1

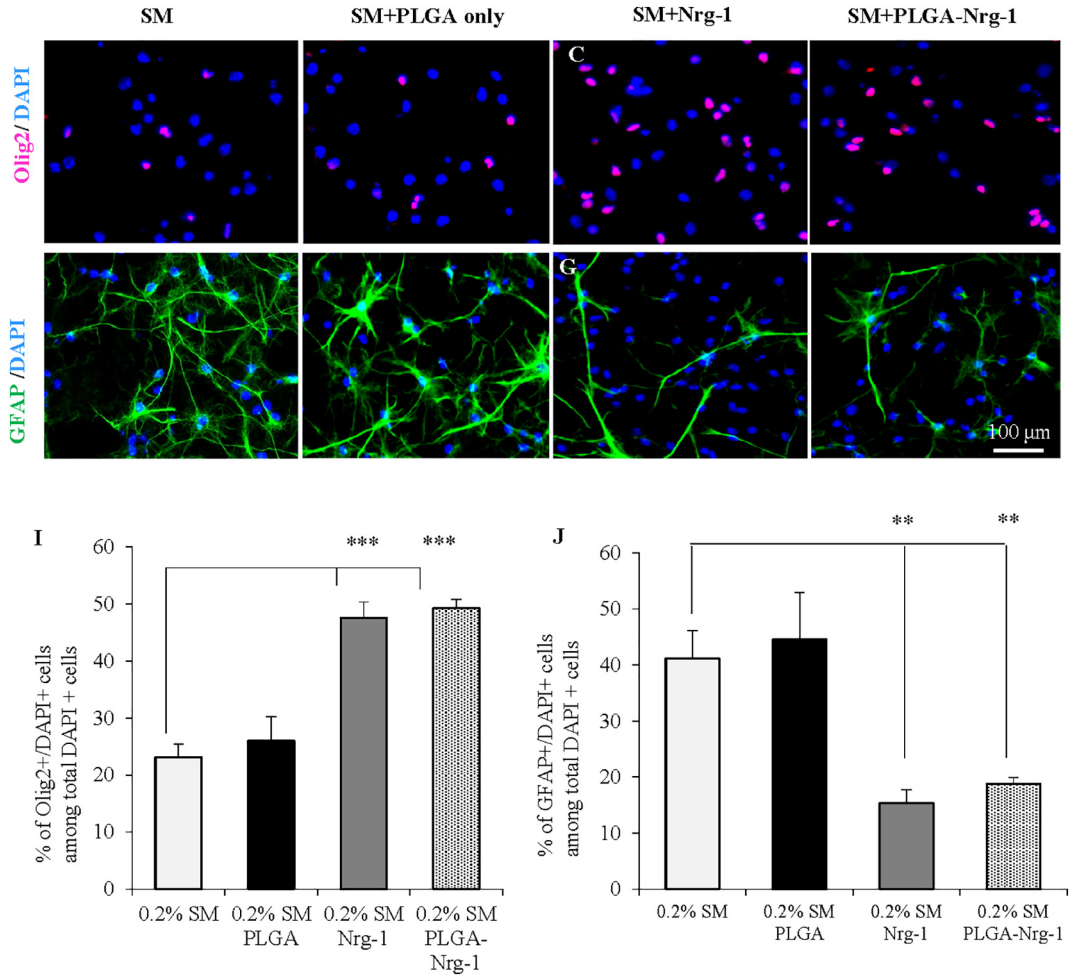

Fig. 4. Effects of PLGA and PLGA-Nrg-1 microparticles on differentiation pattern of NPCs. (A–H) Undifferentiated spinal cord derived NPCs were plated on matrigel and exposed to 0.2% serum for 7 days to allow differentiation. Quantitative immunocytochemistry for oligodendrocytes (Olig2 + /DAPI +) and astrocytes (GFAP + /DAPI +) showed that PLGA itself did not alter NPC differentiation pattern compared to control condition. Nrg-1 and PLGA-Nrg-1 significantly increased the number of Olig2 + cells (I) while decreasing differentiation to GFAP + astrocytes (J) compared to control and PLGA treated conditions. No significant change in NPC differentiation was observed between Nrg-1 and PLGA-Nrg-1 conditions. Data shows mean ± SEM; ***p < 0.001, **p < 0.01, One-Way ANOVA, followed by Holm Sidak post-hoc test; $N = 3$ independent experiments.

comparable to the dose of Nrg-1 encapsulated in PLGA. At 3, 7, 14 and 28 days post-SCI and treatment, we assessed the tissue levels of Nrg-1 in the injured spinal cord tissue using ELISA (Fig. 6A–D). At all time-points, our analysis of Nrg-1 content in the SCI/vehicle group (our baseline SCI control level) confirmed our previous data that SCI causes a significant reduction in tissue levels of Nrg-1 with no subsequent recovery [1]. Nrg-1 content in SCI/vehicle group showed a significant 65–70% decrease after SCI compared to the uninjured tissue levels at all examined time-points post-SCI (Uninjured: 14.49 ± 0.73 pg/µg, 3 day SCI/vehicle: 4.61 ± 0.21 pg/µg; 7 day SCI/vehicle: 4.51 ± 0.42 pg/µg; 14 day SCI/vehicle: 4.06 ± 0.14 pg/µg; 28 days: 6.19 ± 0.42 pg/µg) (One-way ANOVA, $p < 0.001$) (Fig. 6A–D). Similar to our previous

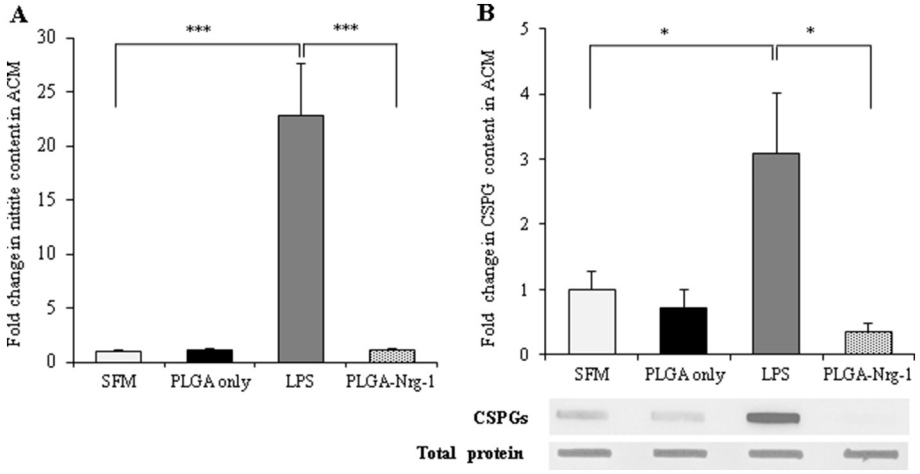

Fig. 5. PLGA and PLGA-Nrg-1 particles had no adverse effects on astrocytes and microglial activity *in vitro*. We assessed production of nitrite and CSPGs in condition media (CM) of mixed cultures of astro-microglia under normal and LPS (lipopolysaccharide) induced-activation conditions. (A) Greiss assay on astro-microglia CM showed that LPS activation resulted in a significant 25-fold increase in nitrite release compared to the control normal condition after 48 h. PLGA and PLGA-Nrg-1 particles had no effects on nitrite production in comparison to control SFM condition. (B) Slot blot analysis on astro-microglia CM showed 3-fold increase in CSPG levels at 72 h after LPS activation, while PLGA and PLGA-Nrg-1 did not induce CSPG production when added to normal astro-microglia cultures. Values are expressed as fold change compared to the control SFM condition. Data shows mean ± SEM; *$p < 0.05$, ***$p < 0.001$ One-Way ANOVA, followed by Holm Sidak post-hoc test; $N = 3$ independent experiments.

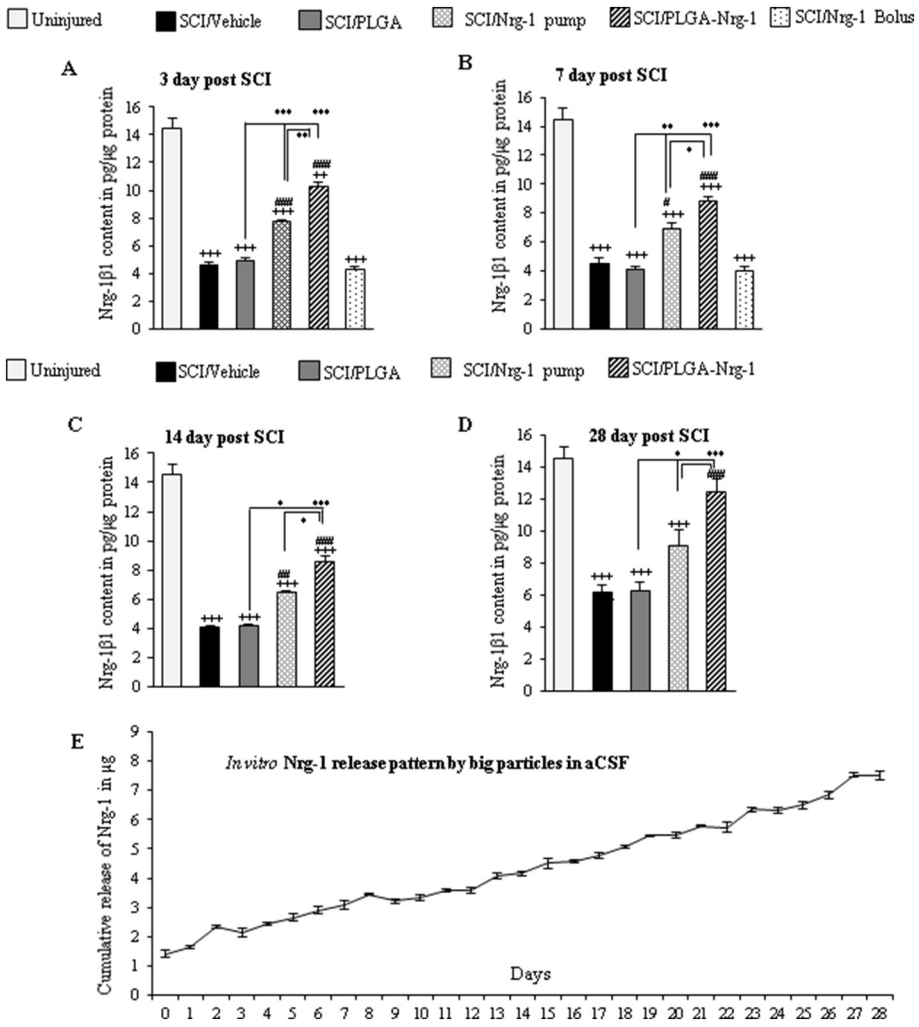

Fig. 6. Intraspinal release of Nrg-1 by PLGA microparticles and comparison of its efficacy to intrathecal delivery following SCI. (A–D) Quantitative ELISA was conducted on spinal cord tissue samples around the injury/injection site to determine the tissue level of Nrg-1β1 at various time-points (3, 7, 14 and 28 days) following SCI and injection of PLGA-Nrg-1 microparticle. ELISA results confirmed a significant reduction in Nrg-1β1 tissue content in SCI/vehicle rats (representing injury baseline) at all examined time-points. SCI/PLGA and SCI/bolus Nrg-1 treated groups also showed significantly decreased tissue Nrg-1 content similar to the SCI/vehicle group. SCI/Nrg-1 pump and SCI/PLGA-Nrg-1 microparticles groups showed significantly increased Nrg-1 content in the spinal cord compared to all other SCI group at all examined time-points. Nrg-1 delivery by PLGA-Nrg-1 microparticles more effectively and significantly increased Nrg-1 content compared to Nrg-1 delivery by osmotic pump at all time-points. SCI group that received Nrg-1 by bolus injection at the time of SCI did not show a restoration in Nrg-1 levels. (E) *In vitro* release profile of Nrg-1 from PLGA-Nrg-1 microparticles was also studied in artificial cerebrospinal fluid (aCSF), a more biologically compatible media for SCI milieu. Sampling of media (excluding the particles) was performed at every 24 h, up to 28 days, recapitulating our early chronic SCI time-point. ELISA showed a daily release rate of 267 ± 4 ng using aCSF, which was comparable to the release rate that we found for PLGA-Nrg-1 microparticle suspended in PBS in our earlier *in vitro* release studies. Data shows mean ± SEM; +++$p < 0.001$, ++$p < 0.01$, compared to uninjured; ###$p < 0.001$, ##$p < 0.01$, #$p < 0.05$, compared to injured; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, compared to respective treatments, One-Way ANOVA, followed by Holm Sidak post-hoc test. N = 3–4 independent experiments.

work, intrathecal infusion of Nrg-1 using an osmotic pump significantly augmented the tissue level of Nrg-1 following SCI in a sustainable fashion compared to SCI/vehicle group (SCI/Nrg1 pump group: 3 day- 7.78 ± 0.34 pg/μg; 7 day- 6.90 ± 0.45 pg/μg; 14 day- 6.42 ± 0.16 pg/μg; 28 day- 9.10 ± 0.94 pg/μg). However, SCI group that received PLGA-Nrg-1 microparticles showed a significantly higher level of Nrg-1 compared to SCI/Nrg-1 pump at all time-points (3 days: 10.25 ± 0.34 pg/μg; 7 days: 8.84 ± 0.27 pg/μg; 14 days: 8.57 ± 0.35 pg/μg; 28 days:12.41 ± 0.80 pg/μg) (Fig. 6A–D, One-way ANOVA, $p < 0.001$, N = 3–4 rats/group/time-point). Importantly, SCI group that received Nrg-1 as bolus intraspinal injection at the time of injury showed no increase in Nrg-1 levels compared to SCI/Vehicle rats. This evidence suggests that PLGA-Nrg-1 microparticles maintain an elevated level of Nrg-1 in the injured spinal cord tissue through sustained PLGA biodegradation and Nrg-1 release, and that deficient level of Nrg-1 cannot be restored in a sustained manner by one bolus treatment. Altogether, we show that intraspinal PLGA-Nrg-1 delivery system has a higher efficacy in restoration of Nrg-1 levels in comparison to intrathecal pump delivery.

To confirm our *in vivo* release data in a more physiologically relevant condition, PLGA-Nrg-1 microparticles were suspended in artificial cerebrospinal fluid (aCSF), which is utilized in our SCI drug delivery approaches. We detected a baseline level of Nrg-1β1 in the release media even at 0 time-point. This basal level of Nrg-1β1 may be due to an initial burst release of peptide that is also reported in the literature [33]. Our longitudinal 28-day *in vitro* release assay showed a daily release rate of 267 ± 4 ng using aCSF that was comparable to the release rate that we found for PLGA-Nrg-1 microparticle suspended in PBS (255.1 ± 0.9 ng/day in our other *in vitro* release studies (Fig. 6E). We also performed time-point transmission electron microscopy (TEM) on injured spinal cord and confirmed the presence of PLGA and PLGA-Nrg-1 microparticles in the spinal cord tissue (Supplementary Fig. 3B–E). A positive control with PLGA microparticles embedded into agar was used as a reference (Supplementary Fig. 3A).

3.7. PLGA-Nrg-1 delivery system attenuates chronic glial scar formation and neuroinflammation following SCI

We next evaluated the safety and biological efficacy of PLGA-Nrg-1 delivery system in the milieu of SCI. We first focused on astrogliosis and neuroinflammation; two hallmarks of SCI pathophysiology. After SCI, astrocytes increase their activity and form a glial scar around the lesion that poses a challenge to axon regeneration and cell replacement in chronic stage of SCI [29]. One of the characteristics of astrogliosis is an increase in the expression of GFAP, an intermediate cytoskeletal filament that is found abundantly in the glial scar [1,34]. We conducted Western blot analysis on the injured spinal cord tissue to study the protein expression of GFAP under various treatments at 3, 7, 14 and 28 days post-SCI (N = 4–6, Fig. 7A–D). As expected, our analysis confirmed that SCI resulted in 2 to 4 fold increase in GFAP expression in SCI/vehicle group (our SCI baseline) at 3, 7 and 14 days post-SCI compared to the uninjured group. However, at the 28-day timepoint of SCI, GFAP increase was more prominent and significantly higher (approximately 7 folds) in the SCI/vehicle group compared to the uninjured tissue (one-way ANOVA, $p < 0.05$, N = 4–5 rats/group/time-point). This timepoint represent an early chronic stage in our model

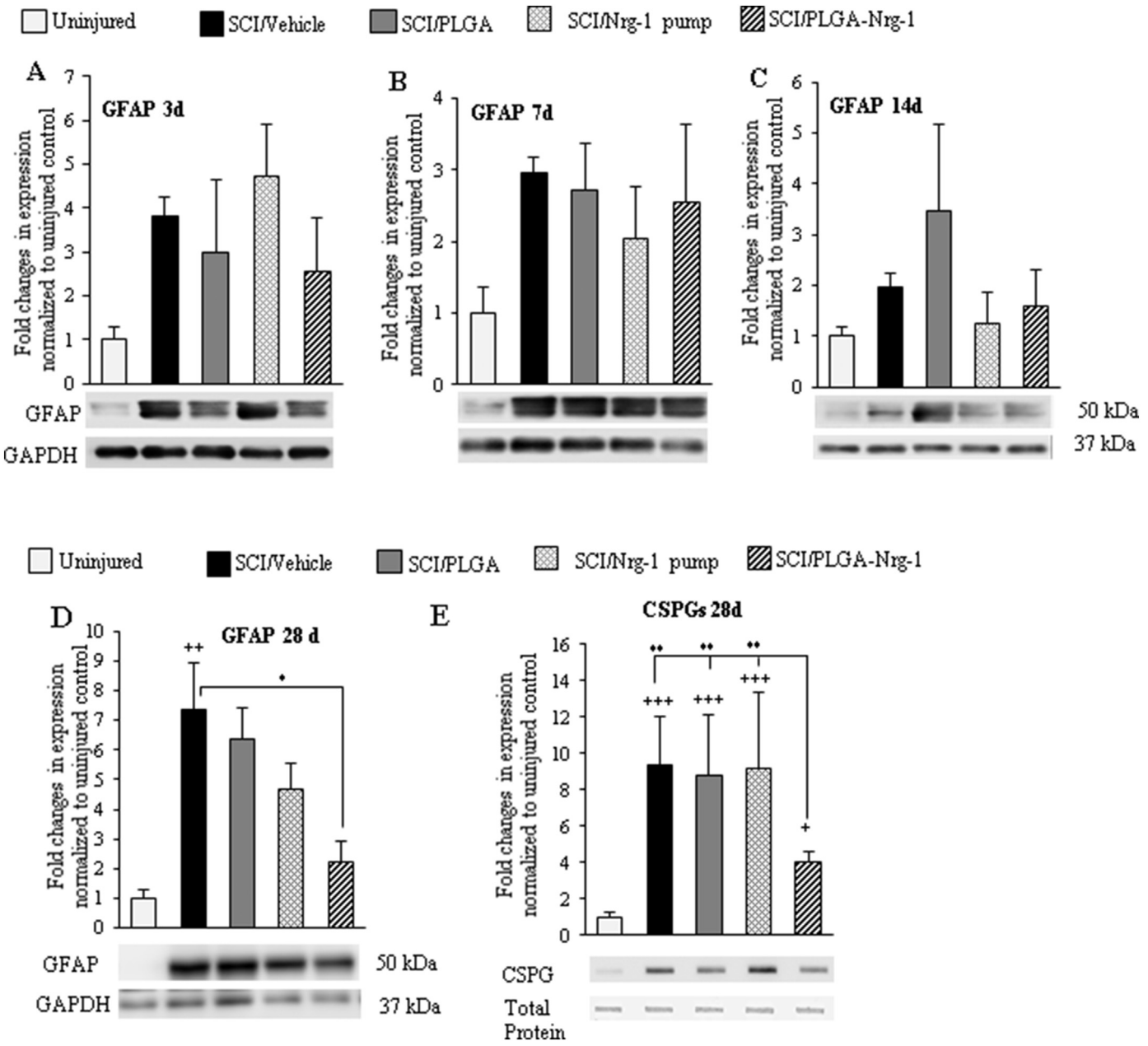

Fig. 7. PLGA-Nrg-1 positively regulates scar formation in chronic SCI. (A–D) Western blot analysis of GFAP protein expression, an indicator of astrogliosis, was conducted in the injured spinal cord under various treatment conditions. There was an increase in GFAP expression in SCI/vehicle group at 3, 7, 14 and 28 days following SCI compared to the low normal level in the uninjured group, which was significant at the 28 days chronic time-point. PLGA alone caused no significant change in GFAP expression pattern at any-timepoint. PLGA-Nrg1 led to a significant decrease in GFAP expression compared to SCI/vehicle at the 28 day time-point. (E) Slot blot analysis at 28 days post-SCI also showed that CSPGs were upregulated after SCI in all groups. PLGA-Nrg-1 treatment significantly reduced CSPGs in the injured spinal cord tissue compared to all other treatment groups at 28 day post-SCI. All the data represents mean ± SEM; $++p < 0.01$ compared to uninjured control, $*p < 0.05$, compared to respective treatments, One-Way ANOVA, followed by Holm Sidak post-hoc test. $N = 4–5$/time-point.

when the astrocytic glial scar is developed around the lesion. All other SCI experimental groups also showed GFAP upregulation compared to the uninjured animals at all examined time-points. Nrg-1 delivery by PLGA or pump had no significant effects on GFAP expression at acute (3 days) or subacute (7 and 14 days) time-points, which is in agreement with our recent studies [9]. Interestingly, at the chronic 28-day time-point of SCI, we found a significant 37% reduction in GFAP expression in the group that received Nrg-1 by PLGA delivery compared to the vehicle control group ($p < 0.05$, one-way ANOVA, $N = 4–5$per group). While there was a trend towards reduced GFAP expression in the rats that received Nrg-1 by osmotic pump, the difference was not significantly different from the vehicle or PLGA alone control SCI groups. PLGA had no effects on scar formation *per se* indicating the specificity of Nrg-1 effects. This evidence also suggests that PLGA is safe and does not negatively alter the microenvironment of the injured spinal cord. We also assessed the effects of PLGA-Nrg-1 treatment on the expression of CSPGs in the chronic glial scar. CSPGs are extracellular matrix molecules that are upregulated in the glial scar by activated astrocytes and are well-known for their inhibitory role in impeding axonal regeneration and oligodendrocyte replacement after SCI [35,36]. SCI caused a significant 9.4 fold increase in CSPGs in SCI/vehicle group compared to uninjured rats (Fig. 7E, $p < 0.05$, one-way ANOVA, $N = 4–5$per group). Similar to GFAP, PLGA-Nrg-1 administration also significantly diminished CSPGs expression (59%) in the glial scar compared to vehicle treatment at 28 days post-SCI (Fig. 7E). Interestingly, Nrg-1 administration by osmotic minipumps showed no

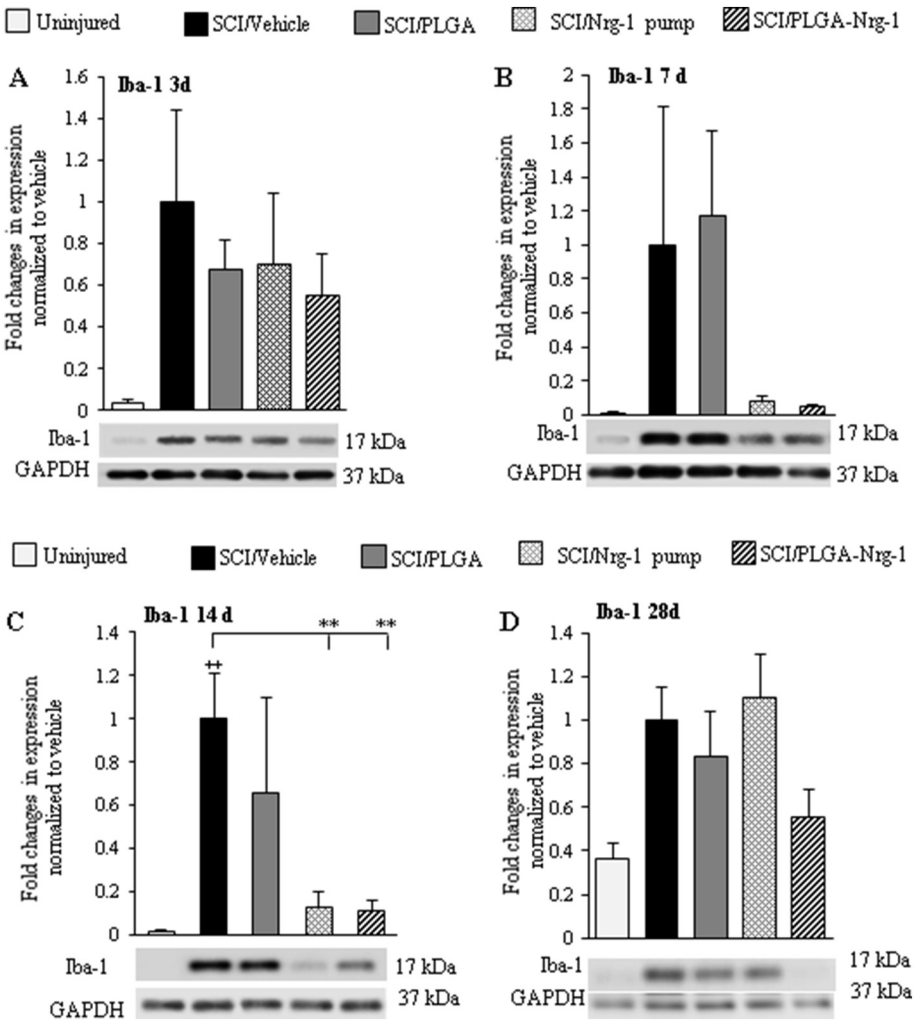

Fig. 8. PLGA-Nrg-1 attenuates inflammation in subacute SCI. (A–D) Western blot analysis of Iba-1, a marker of macrophages/microglia, showed an increase in recruitment of microglia/macrophages in all SCI rats compared to the uninjured group at 3, 7, 14 and 28 days following SCI. PLGA treatment on its own showed no significant effect on macrophages/microglial infiltration to the lesion. Nrg-1 administration by intrathecal catheter-pump or intraspinal PLGA microparticles resulted in a reduction in the Iba-1 level at 7 and 14 days post-SCI, which was significantly different at the 14 day time-point. There was no significant difference between SCI/Nrg-1 pump and SCI/PLGA-Nrg-1 groups at any given time points. The data represents mean ± SEM; $++p < 0.01$, compare to uninjured; $**p < 0.01$, compared to respective treatments, One-Way ANOVA, followed by Holm Sidak post-hoc test. $N = 4–5$/time-point.

difference in the level of CSPGs compared to the baseline levels in SCI/vehicle group at 28 days. Collectively, our data suggest a positive role for Nrg-1 in modulating scar formation in chronic SCI when it is administered intraspinally in a sustained manner by PLGA microparticles. We additionally assessed whether PLGA or PLGA-Nrg-1 delivery has any effect on neuroinflammation. We focused on the recruitment of microglia/macrophages to the injured spinal cord. Resident microglia along with infiltrating macrophages from the bloodstream play a pivotal role in the onset of neuroinflammation and secondary injury mechanisms following SCI [37]. We assessed microglia/macrophages using Western blotting for Iba-1 (a marker of microglia and macrophages) at 3, 7, 14, and 28 days post-SCI. As expected, Iba-1 expression was increased at all time-points after SCI that represent an increase in the number of microglia/macrophages (Fig. 8). We found that intraspinal delivery of PLGA alone had no significant effect on the recruitment of macrophage/microglial into the SCI lesion indicating its safety with regards to neuroinflammation (Fig. 8A–D). Our data is in agreement with previous *in vivo* studies that indicated the safety of PLGA with regards to glial activation and neurotoxicity [38]. Interestingly, administration of Nrg-1 through either PLGA microparticles or osmotic pump attenuated the recruitment of macrophages/microglial particularly at 7 days and 14 days post-SCI compared to vehicle and PLGA treated groups. However, the 14 day time-point was statistically significant compared control SCI/vehicle group (Fig. 8, $P < 0.05$, one-way ANOVA, $N = 4–5$/group).

Taken together, our complementary *in vitro* and *in vivo* findings indicate that PLGA particles on their own do not pose any adverse effects on the response of astrocytes and microglia/macrophages while they successfully deliver bioactive Nrg-1 with positive effects on scar formation and neuroinflammation following SCI.

3.8. Intraspinal delivery of PLGA-Nrg-1 promotes oligodendrocyte and axon preservation following SCI

Nrg-1 is known for its critical role in development and survival of oligodendrocytes in the spinal cord [39,40]. We previously showed that intrathecal delivery of Nrg-1 was sufficient to promote preservation of mature oligodendrocytes and the replacement of damaged oligodendrocytes after SCI [1]. We also found that bioavailability of Nrg-1 through osmotic pump delivery can promote axonal preservation following injury [1]. Here, we tested the bioactivity and efficacy of Nrg-1 delivered through intraspinal administration of PLGA-Nrg-1 microparticles on oligodendrocytes and axons at the 28 day chronic stage of SCI. We assessed oligodendrocyte preservation by Western blot analysis for CNPase (a specific marker for mature oligodendrocytes). As expected, there was a robust 80% reduction in CNPase expression in SCI/vehicle group compared to uninjured normal group indicating a significant loss of mature oligodendrocytes as a result of injury (Fig. 9A, $p < 0.05$, one-way ANOVA, $N = 4–5$per group). Among our experimental groups, only PLGA-Nrg-1 treatment promoted preservation of oligodendrocytes after injury, as there was a significant 24% increase in CNPase expression in the PLGA-Nrg-1 group in comparison to the SCI/vehicle group.

We also studied myelin preservation by assessing the expression of myelin basic protein [32], a signature marker for myelin in the central nervous system. Our Western blot analysis confirmed a 55% significant

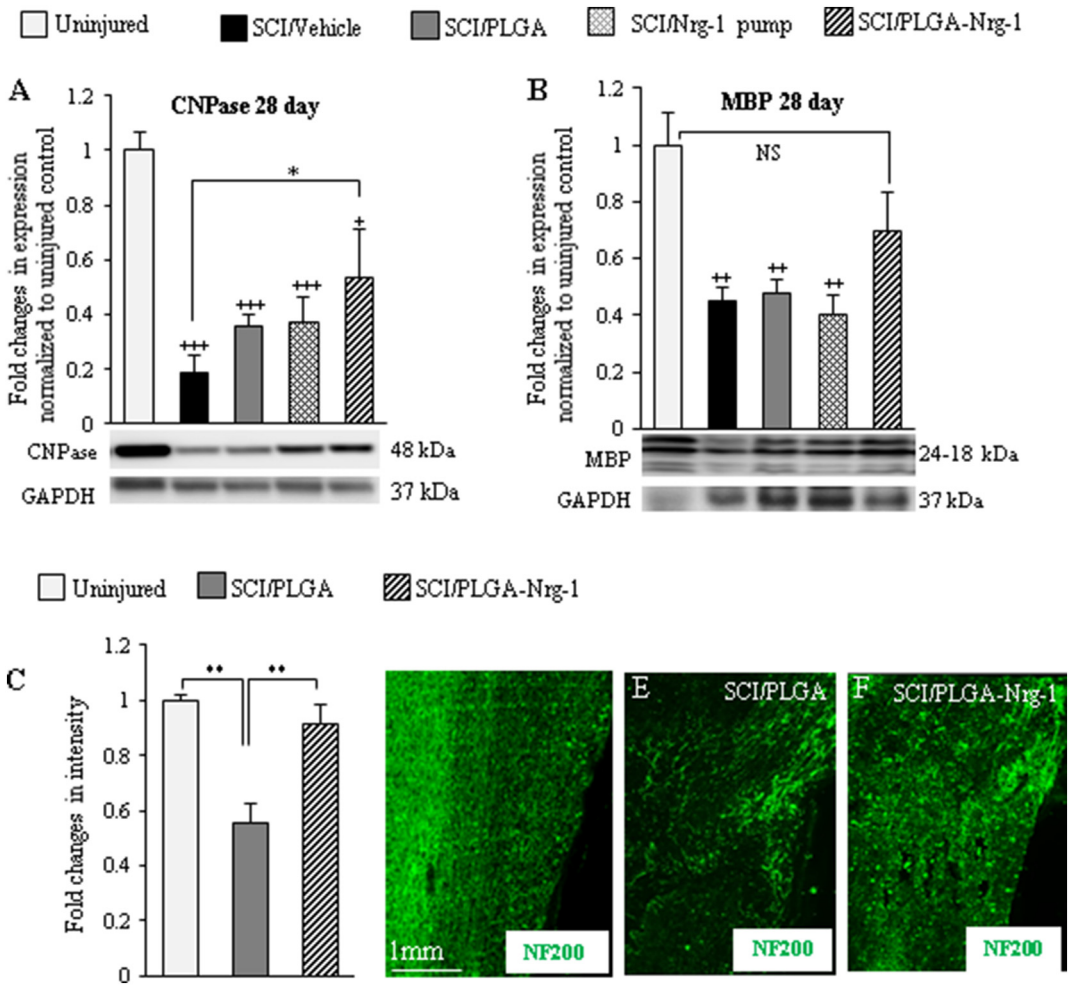

Fig. 9. PLGA-Nrg-1 promotes oligodendrocyte and axonal preservation in chronic SCI. (A) Western blot analysis of CNPase (a mature marker for oligodendrocytes) showed a 5-fold decrease after SCI. PLGA-Nrg1 treatment group showed a significant increase in CNPase expression compared to SCI/vehicle group. (B) SCI also resulted in a 2-fold reduction in MBP expression in SCI/vehicle rats compared to the uninjured group. PLGA-Nrg-1 treatment promoted MBP expression to a level that was non-significantly different than uninjured spinal cord tissue. (C–F) We also studied axonal preservation by immunohistochemical analysis of axonal neurofilament 200 (NF200) in the spinal cord at 28 days chronic SCI. As expected, SCI caused axon degeneration and thereby a reduction in NF200 expression. SCI/PLGA group showed a significant 1.8-fold decrease in NF200 immunointensity. PLGA-Nrg-1 treatment resulted in a significantly higher axonal preservation compared to PLGA only group, which was close to normal level. The data represents mean ± SEM; + + + p < 0.001, + + p < 0.01, + p < 0.05 compare to uninjured; *$p < 0.05$, compared to respective treatments, One-Way ANOVA, followed by Holm Sidak post-hoc test. N = 4–5/treatment.

reduction in MBP expression in SCI/vehicle group compared to uninjured normal group suggestive of demyelination (Fig. 9B, $p < 0.05$, one-way ANOVA, N = 4–5per group). Importantly, PLGA-Nrg-1 treatment increased MBP expression (25%) to a level that was non-significantly different from normal spinal cord tissue (Fig. 9B). Furthermore, we conducted analysis of axonal preservation in the white matter of injured spinal cord using neurofilament 200 (NF200) that specifically marks axons at 28 days post-SCI. SCI resulted in a significant 45% reduction in NF200 immunointensity compared to the uninjured spinal cord (Fig. 9C). Availability of Nrg-1 through PLGA microparticle resulted in a significant 60% increase in axonal preservation following SCI compared to PLGA alone SCI control group ($p < 0.05$, one-way ANOVA, N = 4–5per group). Collectively, our designed PLGA microparticle system presents an effective strategy for local delivery of bioactive Nrg-1 with the potential to promote oligodendrocytes and axonal preservation following SCI.

4. Discussion

SCI results in profound changes in the expression of key growth factors with critical roles in function and maintenance of neural cells. Therefore, pharmacological therapies, to recondition the injury milieu, are an integral part of repair strategies for SCI [41]. Sustained and controlled delivery of therapeutic reagents to the spinal cord is a key in achieving the optimal biological effects of growth factors; and single bolus intraspinal or systemic injection would not provide the same efficacy [42,43]. Our original work showed that Nrg-1 bioavailability is robustly and permanently reduced in SCI lesions that negatively affect the survival of axons and oligodendrocytes, and cell replacement activities of endogenous precursor cells and inflammatory cells [1,9]. We have shown the promise of sustained intrathecal infusion of recombinant Nrg-1 in improving cell response and recovery of function after SCI. Although intrathecal delivery with an osmotic mini pump provides continuous delivery of therapeutic agents such as Nrg-1, implantation of indwelling catheters may cause chronic inflammation, dural fibrosis, spinal cord compression and infection [13,44]. Importantly, entry of the drug into the CSF and eventually draining to the systemic circulation during intrathecal delivery may cause adverse effects for agents with multimodal activities and diverse cellular targets. Nrg-1 is a potent growth factor with important roles in both cardiovascular and nervous systems [45] and continuous systemic administration of Nrg-1 may cause undesired off-target effects in other systems. The need for a second surgical intervention to remove the indwelling catheter and pump may also add to the invasiveness of intrathecal delivery for clinical practice [46,47]. Hence, intraspinal delivery can circumvent the limitations associated with intrathecal and systemic

drug administrations for SCI in particular in combinatorial cellular therapies. Here, we demonstrate that biodegradable PLGA microparticles can be utilized as an optimal system for controlled and sustained delivery of Nrg-1 in SCI.

Use of PLGA-microparticles for delivery of Nrg-1 alone or in combination with other growth factors has been demonstrated previously in cardiac tissue [48]. However, to our knowledge, PLGA-based Nrg-1 delivery has not been developed for SCI therapeutics. We have manipulated several properties of PLGA to optimize rate and duration of Nrg-1 delivery for therapeutic purposes. In our PLGA preparations, we complied with the basic prerequisites for designing drug delivery microparticles, which are (i) drug incorporation and release, (ii) stability [49] biocompatibility, and (iv) functionality [50]. We have evaluated and verified the efficacy of the encapsulated PLGA-Nrg-1 microparticles in exerting positive biological effects of Nrg-1 both *in vitro* and in a clinically relevant model of SCI. We utilized an established *in vitro* NPC differentiation system to determine the desired therapeutic dosage and duration of Nrg-1 treatment and verify its functional efficacy. Our choice of primary NPC culture system was based on the known positive effects of Nrg-1 in promoting oligodendrocyte differentiation identified by our group and others [1,51]. Our previous studies showed that a dose of 150 ng/ml of Nrg-1 enhances NPCs proliferation and their differentiation along an oligodendrocytes lineage [1]. Using this model, we focused on optimization of the Nrg-1 release rate from PLGA microparticles to achieve a daily dose closer to the optimal dose of 150 ng. PLGA-Nrg-1 particles formed by the "water-oil-water emulsion" method were capable of releasing Nrg-1, but release rate was suboptimal. Several factors can challenge optimal release rate in protein-encapsulated microparticle systems that include instability of encapsulated proteins, their incomplete release and initial burst release [33]. Major release mechanisms of PLGA particles are described as "solvent penetration or device swelling, diffusion, degradation and erosion of the polymer matrix, or a combination of the mentioned mechanisms occurring on different time scale" [52]. Degradation and release rate are generally faster for PLGA polymers with lower molecular weight than more hydrophilic or amorphous particles with higher glycolide content [53]. The most widely used PLGA copolymer consists of a 50:50 proportion of D-L-lactide and glycolide and has the fastest release and degradation rate with approximately 50 to 60 day half-life *in vivo*. Introducing porosity has been an effective approach in increasing release rate of water soluble protein/peptide encapsulated PLGA particles [15]. Based on these criteria, we developed a PLGA copolymer consisting of a 50:50 proportion of D-L-lactide and glycolide. We also used various concentrations of NaHCO3 as porogen and subsequent salt leaching step to produce microparticles with porosity, and thereby higher surface and diffusion areas. This strategy resulted in an increase in release rate at the expense of loading efficiency. Among our four different porosity preparations, the 1 mg NaHCO3 preparation showed the closest and most effective daily release rate to the optimal concentration of rhNrg-1β that we identified in our original *in vitro* studies [1].

Duration of Nrg-1 delivery is also an important factor in the development of PLGA-Nrg-1 microparticles for pharmacological therapies in SCI. Sustained and prolonged drug delivery is necessary to achieve neuroanatomical effects and functional recovery [4,35]. Duration of drug delivery depends on the release rate and biodegradation of PLGA microparticles. Evidence shows that increasing the D-L-lactide ratio in the PLGA composition would progressively prolong the half-life of PLGA biodegradation up to 200 days *in vivo* [26,54]. In our study, we achieved a continuous release rate for a longer duration of Nrg-1 delivery by manipulating the size of PLGA microparticles instead of altering the polymer composition. Bigger particle size was obtained by reducing the shear force during "water-oil-water emulsion" formation. This caused a decrease in dispersion resulting in the formation of bigger PLGA-Nrg-1 particles [25]. While this procedure produced mixed particles, size distribution was shifted towards the bigger particle size and therefore a change in the rate, duration and efficiency of release. Variations in particle size in water-oil-water double emulsion preparations are also well documented in previous studies [15,55].

We found an initial burst in Nrg-1 release in PLGA microparticles that likely reflects particle porosity, property of release media and properties of peptide/protein/drug loaded into the microparticles. It has been shown that nonporous and porous PLGA particles exhibit the same hydrolytic biodegradation mechanism but with different degradation rate [19]. The degradation rate is also influenced by the size of particle where bigger nonporous particles degrade faster compared to smaller nonporous [19]. Mechanistically, an increase in the size of microparticles leads to an increase in the length of diffusion path and conversely a decrease in concentration gradients and mass transport rates. Moreover, pH inside the particles will be neutralized virtually in real time that can decelerate the autocatalytic cleavage of bigger porous PLGA particles and allow the release of Nrg-1 for a longer duration or as long as the particles exist [19]. While these explanations represent proposed mechanisms, it is yet to be affirmed whether the control of drug release in a porous microparticle delivery system is regulated by autocatalytic effects [21]. The initial drug release is also determined by drug type and concentration as well as polymer hydrophobicity. Drugs loaded on the surface of microparticles are in direct contact with the media and their release is mediated by solubility [26]. Nrg-1 is a hydrophilic peptide containing 66 amino acids, with a molecular weight of 7.6 kDa and readily soluble in water up to 100 μg/ml as per the information provided by the manufacturer. Unlike hydrophobic drugs, Nrg-1 has a substantially high loading efficiency when using double emulsion method, and follows the release mechanism of any other hydrophilic drug. Therefore, the initial increase that we have observed in Nrg-1 levels on day 1 may reflect the release of Nrg-1 loaded on the surface of PLGA microparticles.

From a biological and safety point of view, *in vitro* [56] and *in vivo* [27] Studies have identified PLGA as a safe biodegradable polymer since by-products of its hydrolysis, lactic and glycolic acid can be easily metabolized in the body [57]. Owing to its properties and safety, the United States Food and Drug Administration (FDA) and European Medicine Agency (EMA) have approved the use of PLGA in development of medical materials in humans [58]. In our studies, we extensively evaluated the safety of PLGA particles for SCI and NPC therapeutic purposes. Using multiple *in vitro* assays, we confirmed that PLGA itself has no detrimental effect on the cardinal properties of primary spinal cord derived NPCs including their survival, proliferation and multipotential capacity. Our complementary *in vitro* assays and *in vivo* SCI studies revealed that PLGA had no adverse effect on astrocytes and microglia; two major cell populations in the injured spinal cord involved in glial scarring and neuroinflammation indicating its safety for use in SCI [29,30]. Importantly, we confirmed that Nrg-1 peptide preserved its bioactivity for weeks after encapsulation in PLGA microparticles and exert positive effects *in vitro* and in SCI. Nrg-1 significantly attenuate SCI-induced upregulation of GFAP and CSPGs in chronic glial scar. Of note, CSPGs are the main inhibitory molecules associated with the matrix of SCI [59,60]. We have previously shown that CSPGs negatively influence endogenous and transplanted NPCs in the injured spinal cord and modulating their deposits after injury have significant impact on repair process [5,31,35].

5. Conclusion

In the current study, we have developed a PLGA-based biodegradable microparticle system for localized, controlled and sustained delivery of Nrg-1 to the injured spinal cord. We demonstrate that the rate of Nrg-1 release by the PLGA microparticles can be controlled by adjusting particle porosity. Additionally, the duration of Nrg-1 release can be regulated by alterations in particle size resulting in prolonged continuous Nrg-1 delivery for weeks while maintaining a therapeutic dosage in the injured spinal cord tissue. In comparison to intrathecal

delivery using osmotic mini pumps, intraspinal PLGA drug delivery led to a higher level of Nrg-1 in spinal cord tissue with reduced dosing and minimized systemic effects. Importantly, Nrg-1 delivery by PLGA microparticle resulted in several neuroprotective effects after SCI including reduced glial scar formation and neuroinflammation, and increased oligodendrocyte and axonal preservation. This Nrg-1 delivery strategy would have broader applicability and can be exploited in combinatorial pharmacological and stem cell-based therapies in SCI or other CNS conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2017.06.030.

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