Assembly of Protein-Based Hollow Spheres Encapsulating a Therapeutic Factor

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ABSTRACT

Neurotrophins, as important regulators of neural development, function and survival, have a therapeutic potential to repair damaged neurons. However, a controlled delivery of growth factors into injured tissue remains one of the greatest challenges facing the drug discovery field. This study presents the development of an innovative protein-protein delivery technology of nerve growth factor (NGF) by collagen microspheres that can be directly injected into the injury site and provide long term release of the therapeutic. A biomimetic hollow reservoir system was fabricated using a novel template. The capability of neurotrophins to localise in these reservoir systems was confirmed by confocal images of fluorescently labelled collagen and neurotrophins. In addition, high loading efficiency of the reservoir system was proven using ELISA. By comparison of release profile from several types of microspheres, highly cross-linked collagen spheres were chosen as they give the biological effect most desired. Finally, biological activity of released NGF was assessed using PC12 and primary rat dorsal root ganglion (DRG) cell bioassay where cell treatment with NGF-loaded reservoirs induced significant neuronal outgrowth, similar to that seen in NGF treated controls. Data presented here highlight the potential of a high capacity reservoir-growth factor technology as a promising therapeutic treatment for spinal cord injury and other neurodegenerative diseases.

KEYWORDS: Neurotrophins, Nerve Growth Factor, Microspheres, Drug Delivery, Dorsal Root Ganglia

Introduction

The ability of neurotrophins to promote neuronal survival, development, synaptic plasticity and neurotransmission have inspired researchers to use these molecules as possible therapeutic agents for damaged neurons in a variety of neurodegenerative diseases [1] or acute injuries [2]. However, despite their undisputed therapeutic potential, neurotrophins have a short half-life and limited *in vivo* availability. Therefore, a successful therapy requires a sustained and controlled delivery of growth factors into injury site. Several promising therapies have been identified to achieve this constant delivery; these include transplantation of stem cells over-expressing various neurotrophic factors [3-5] or viral vector delivery[6, 7]. Despite promising results obtained from *in vitro* and *in vivo* studies, research in these fields still fall short of clinical acceptance mainly due to poor survival of grafted cells and continuing safety issues related to viral transduction. More recently, another interesting approach engaging biomaterial particles as a neurotrophin reservoir and delivery system has been also studied [8-12]. Biomaterial particles are promising tools for controlled drug delivery system as they can overcome some limitations of cell- or gene-based therapy; however other restrictions such as immunogenity, biodegrability or cytotoxicity may arise. Therefore, to address these issues related to synthetic polymers usage, in this study we present the development of an innovative protein-protein delivery technology of nerve growth factor (NGFsecond abbreviation of NGF) by collagen microspheres that can be directly injected into the injury site and provide long term release of therapeutic. Collagen type I, as a naturally occurring protein in mammalian tissue (approximately 30% of the whole-body protein content) [13] would be recognised

in vivo as an extracellular matrix (ECM) and at the same minimises the immune response. Because of its suitability as a biomaterial, it is widely employed in a variety of drug delivery systems [14]. It was also shown that collagen mediates neuronal adhesion to the substratum and guidance growth of neurites *in vitro* [15, 16]. Moreover, collagen has a potential to improve the survival and repair of damaged nerves [13], and is therefore readily use as a material for 3D scaffolds for nerve tissue repair [17, 18]. Finally, collagen is one of the three biomaterials that have progressed to clinical use.

Recently, a novel template method was used to generate a protective ECM-based gene delivery system [19]. Here we hypothesised that biomimetic hollow microsphere technology will provide a sustained growth factor delivery platform for multiple neurotherapeutic applications where the release profile could be tailored due to the amount of cross-linker. The aim of this study was to compare and to select from several types of collagen and gelatin microspheres with various cross-linker ratios the most appropriate growth factor reservoir system as a potential treatment for spinal cord injury and other neurodegenerative diseases.

Materials and Methods

All materials and reagents used in this study were purchased from Sigma Aldrich Ireland Ltd. (Dublin, Ireland) unless otherwise stated. Collagen type I and pentaerythritol poly(ethylene glycol) ether octasuccinimidyl glutarate (8a15kSG PEG) were supplied by Covidien (North Haven, US).

Fabrication of collagen/gelatin hollow-spheres

Collagen microspheres were fabricated according to the method previously described (Browne, 2012). Briefly, commercially available 4.5 μm polystyrene beads were

negatively charged by 18 hours incubation with sulphuric acid at 40ºC. Following sulfonation, beads were resuspended in 0.1M acetic acid and mixed with 5 mg/ml of collagen or gelatin solution at a weight ratio of 1:7 (collagen/gelatin: beads). The mixture was stirred for 2 hours at RT to allow coating. Then, the polystyrene template was removed by three washes with 20% (v/v) tetrahydrofuran (THF) to produce non cross-linked spheres (0x). To generate cross-linked spheres free amino groups of collagen were first cross-linked using 8a15kSG PEG at a weight ratio 1:1 (1x) or 1:2 (2x) (collagen : cross-linker). Hollow spheres were washed three times with 20% ethanol to ensure removal of any remaining THF and then stored in 70% ethanol to prevent potential contamination.

Morphological characterisation of microspheres

Following preparation, morphology of collagen spheres as well as of polystyrene beads was evaluated using scanning electron microscopy (SEM) (Hitachi S-4700 field emission microscope operating with a beam voltage of 15 kV). A drop of sample was placed on carbon tabs mounted on the SEM specimen stubs and then dried and gold coated using an Emitech K550 coating system.

Primary DRGs preparation

Female Sprague-Dawley rats were mated with males of the same strain and females and allowed to gestate to embryonic (E) day 16. At E16 the pregnant mother was euthanized and the embryos were removed by hysterectomy. Dorsal Root Ganglia (DRG) were dissected and stored in cold Hanks Buffered Salt Solution (HBSS). DRGs were washed once in fresh HBSS prior to purification. Whole DRGs were incubated in 25 ng/ml Trypsin at 37°C for 15 minutes. Embryonic DRGs were centrifuged at 1000 *g* for 10 minutes to pellet the cells. The supernatant was removed

and the pellet of cells was washed three times in DMEM supplemented with 15% FBS, 0.7% L glutamine, 1% Pen/Strep, 10 ng/ml nerve growth factor, 0.02M FrdU and 0.8M uridine. The DRG pellet was resuspended in culture medium and counted using a haemocytometer. Embryonic DRGs were seeded in 96 well plates at 4000 cells/well or in 24-well plates at 100,000 cells/well in a volume of 500 µl/well.

Cytotoxicity of microspheres

The toxicity of three types of collagen spheres (0x, 1x and 2x) was evaluated on PC12s as well as on primary DRGs. Cells were seeded in 24-well plates for Live/Dead assay (Molecular Probes) or in 96-well plates in order to evaluate metabolic activity by AlamarBlue assay (Invitrogen, Dublin, Ireland). Two doses of each type of sphere (10 and 20 mg per cm^2) were applied on cells and after 24 hours cells were stained with calcein (live) and ethidium homodimer (dead). Metabolic activity was measured following 24 or 48 hours. Non-treated cells and 10% DMSO-treated cells were used as controls.

Fluorescent labelling of NGF and collagen

NGF (Alomone Labs, Jerusalem, Israel) which is commonly available and relatively inexpensive was chosen as a model protein to demonstrate loading and release rates from the spheres. Fluorescently labelled NGF was used to facilitate detection of loading process. DyLight594 Microscale Antibody Labelling Kit (Thermos Scientific, Rockford, US) was used to fluorescently tag NGF. 1 mg of collagen spheres where incubated with 1 mg/ml FITC (Invitrogen) for 4 hours on shaker at RT and then washed twice with 80% ethanol and distilled water, respectively.

Loading of microsphere reservoirs with growth factors

Spheres were mixed with various concentrations of NGF in PBS solutions (loading buffer) and incubated on shaker at RT overnight to allow proteins to translocate into spheres. Samples were then spun down to separate loaded spheres from the loading buffer, and supernatant was collected for Elisa (R&D Systems, Abingdon, UK) analysis. Based on concentration of NGF in the supernatant, the loading efficiency was calculated. Fluorescently labelled NGF was also loaded into FITC-labelled collagen microspheres before being mounted and examined under an inverted confocal microscope.

Release study

The release profile of NGF was characterized in PBS-0.05%Tween 20 (release buffer) at 37^{o}C (non cell release system). 25 μg of loaded microspheres was incubated with 500 μl of the release buffer and at various time points microspheres were spun down and supernatant was collected and replaced. The collected supernatant was frozen at -20 ^{o}C until Elisa analysis.

Bioactivity of released NGF

In parallel to non cell system, loaded microspheres were added to PC12 cells seeded on coverslips in 24-well plate (25 μg per 1 well) and cells were incubated at 37^{o}C. Every second day, cell morphology was evaluated under inverted light microscopy and supernatants were collected for Elisa analysis (cell release system). At the final time point, supernatants were collected and cells were fixed with ice-cold methanol and stored at -20^{o}C before immunocytochemical staining for neurofilaments. The

bioactivity of released NGF was also confirmed on primary DRGs in a similar manner, except that cell morphology was evaluated every 12 hours.

Immunocytochemistry

Cells fixed on coverslips were washed twice in PBS for 5 minutes at RTRT abbreviation. The samples were then incubated in blocking buffer (2% BSA in PBS) for 1 hour. To determine neuronal morphology, a neuronal specific bIII tubulin monoclonal antibody (Milipore) was used at 1:200 dilution in blocking buffer. Following overnight incubation with primary antibody at 4^{o}C, coverslips were washed twice with PBS and secondary fluorescent antibody Alexa Fluor 488 donkey anti mouse (Invitrogen) was used for 1 hour at RT. Coverslips were washed two twice in PBS and incubated with DAPI-PBS solution (1 µg/ml) for 5 min. After washing in PBS, coverslips were mounted on slides with Vectashield (Labkem, Ireland), and stored at 4^{o}C be for image analysis. Imaging was done using an inverted epifluorescent microscope (Olympus IX81, Mason Technologies, Dublin, Ireland).

Analysis of neurite length and area

Neurite outgrowth from differentiated PC12 and DRG cells was determined from images stained with βIII tubulin using ImageJ (MosaicJ, Philippe Thevenaz) where the area of positively stained DRGs was measured, or individual PC12s neurites were traced manually (Neuron J plugin).

Statistical analysis

All statistical analyses were performed using GraphPad Prism Version 5 (GraphPad Software, Inc.). Data were compared using one-way analysis of variance (ANOVA)

followed by multiple comparison procedures (Tukey's test). Values were considered as significantly different with a $p<0.05$.

Results

Fabrication and morphological characterisation of collagen spheres

Hollow microspheres were fabricated according to the method developed in our laboratory for other natural polymers such as chitosan [20], elastin [21] and collagen [19]. Hollow microsphere fabrication, graphically illustrated in Schematic 1, involves a negatively charged polystyrene template which is subsequently coated by positively charged collagen or solution. Following coating, the collagen is cross-linked to stabilize the matrix. This process can be controlled by the amount of the cross-linker added. Here, we have fabricated three types of spheres; highly (2x) and moderately (1x) cross-linked particles, where a weight ratio of collagen/gelatin to PEG is 2:1 and 1:1, respectively. The third type of sphere has not been cross-linked (0x). Finally, the polystyrene template is dissolved by washing with THF which generates a hollow sphere with the same shape and size as the polystyrene bead. SEM images of resulting hollow spheres, as well as steps of sphere fabrications are shown in Fig. 1. The majority of spheres have a similar size, of between 4-5 μm, and evident hollow morphology obtained following beads removal. The presence of hollow core is manifested by the stamp shape of the spheres due to the high vacuum in the SEM chamber.

Sphere interactions with neuronal cells

The overall goal is to develop a platform delivery technology for multiple neurotherapeutic application, where microspheres will serve as a reservoir system for growth factor delivery. With this in mind, we first checked for cytotoxicity of spheres to a PC12 cell line as well as to primary DRGs. Two doses of each type of sphere (10 and 20 mg per cm^2) were applied on cells and after 24 hours cells were stained with calcein (live) and ethidium homodimer (dead). Spheres of all levels of cross-linker did not exhibit any toxicity to PC12s nor to primary DRGs when compared to PBS and DMSO treated controls (an example of 2x spheres is presented in Fig. 2A). Similarly, when analysed with AlamarBlue assay (Fig. 2B), no significant effect was seen on metabolic activity of cells treated with all types of spheres in comparison to PBS-treated controls. In contrast, following 10% DMSO treatment metabolic activity was significantly ($p < 0.01$) decreased to approximately 20-30%. In addition, following treatment with microspheres PC12 cells showed no changes in morphology within the period of 6 days when compared with a non-treated control (Fig. 5A, cells alone vs. spheres).

Loading and release of NGF from hollow spheres

NGF was chosen as a model protein to demonstrate loading and release rates from the spheres. We began by fluorescent labelling of NGF to facilitate detection of loading process. Then, fluorescently-tagged NGF was loaded into FITC stained collagen spheres and samples were viewed under a confocal microscope. Figure 3A shows FITC stained collagen spheres, NGF-loaded spheres and a merged image of collagen spheres loaded with NGF that confirmed growth factor interaction with microspheres. Furthermore, using an Elisa assays, high loading efficiency of about 99% with high

loading capacity up to 10 μg of protein per mg of collagen was observed regardless of the sphere type (0x, 1x and 2x) (Fig. 3B).

Each of the three types of collagen hollow sphere showed an ability to slowly release NGF in both the cells and no cells system. No significant difference was observed between 0x and 1x spheres; however, a significantly slower release rate was seen in the PC12s system from highly cross linked spheres (2x) (Fig.4). In the non-cell system, release of therapeutic doses of growth factor was observed from all types of spheres up to 13 days with a burst release in the first 4 days.

Bioactivity of released NGF

First, bioactivity of released NGF was evaluated using a neuronal outgrowth assay in PC12 cells, which have ability to differentiate into neuronal cells in the presence of NGF. 2x collagen spheres were loaded with NGF and applied to non-differentiated PC12 which were than cultured for 6 days. Cells treated with NGF-loaded spheres exhibited similar morphology to NGF-treated positive controls. In contrast, PC12s treated with unloaded spheres did not differentiate and maintained round cell body shapes similar to untreated cells (Fig. 5A). This experiment gave similar results when repeated using non cross-linked spheres (See Supplementary Information). For quantitative assessment of this phenomenon, βIII tubulin stained cells treated for 6 days with three types microsphere (0x, 1x and 2x) were analyzed. Significant neuronal outgrowth ($p < 0.05$) was observed in all groups treated with NGF-loaded spheres and in constant NGF treatment in comparison to single NGF-treated control (Fig. 5B). Cells treated with empty spheres and untreated control did not exhibit any neurites.

Bioactivity of released NGF was also assessed on embryonic rat DRGs which were seeded in medium with or without NGF and then treated with NGF-loaded or unloaded 2x collagen spheres. Measurements of βIII tubulin staining area following 36 hours of treatment reveal a significant difference ($p < 0.05$) between cells treated with NGF-loaded and unloaded spheres cultured in medium with or without NGF (Fig. 6).

Discussion

With an emphasis on SCI, for which no therapy currently exists, but also in respect to other neurodegenerative diseases, our study investigated the use of newly developed biocompatible, hollow sphere technology as a growth factor delivery platform for neuronal tissue repair. Since the use of collagen offers one of the most promising opportunities for safe and effective controlled drug delivery, the aim was to develop and characterise natural polymer-made particles that would be highly effective in terms of loading and release rate as well as having minimum toxicity.

Schematic 1 illustrates the main objectives of this work that was first, to generate appropriate collagen hollow microspheres, then to load them with a model growth factor (NGF), and finally to investigate the bioactivity of released NGF and any cytotoxic effect of spheres on neuronal cells.

The template method allows the fabrication of hollow spheres which are uniform in size and shape, as shown in Fig. 1. To avoid sphere phagocytosis *in vivo*, we produced particles which were relatively larger, having a diameter varying between 4 – 4.5 μm. By addition of different amounts of PEG, three types of spheres with different protein release properties were created. However, the degree of cross-linking did not influence loading properties and very high loading efficiency with larger amount of

protein was observed over the three types of spheres. In contrast to previously published reports, the amounts of growth factor loaded per mg of delivery vehicles were considerably lower and oscillated between ng rather than μg [22, 23] or was not specified at all [24]. Another advantage of our delivery platform is 90- 100% loading efficiency that has not been observed in any other neurotrophin delivery system. The hollow structure of spheres allows for diffusion of growth factor inside of the sphere, where it can be protected from unfavourable external environment. Post cross-linking loading prevents protein structural damage that may occur during for example emulsion processing, lyophilisation, freeze/thaw cycles and other processes involved in preparation of biodegradable nano- and microparticles [25, 26]. Nevertheless, confocal images of fluorescently tagged NGF and spheres showed that some portion of protein localises also on the outer surface of the spheres (Fig.3A).

NGF *in vitro* release profile was investigated in a PC12 system as well as in an acellular system where 0.05% Tween 20 – PBS, which improves NGF stability [27], was used as a release buffer. Although growth factor release profile form variety of biomaterial devices has been monitored from hours [28], days [27] to weeks [9], our 13 days duration limit of testing was selected based on the instability of NGF upon extended incubation times [29]. Characterisation of the release profile in the presence of cells showed that NGF release from highly cross-linked (2x) spheres was significantly slower in comparison to other types, and for this reason 2x spheres were selected for further analysis (Fig.4). It is worth emphasizing that the amounts of NGF detected in the supernatants of PC12s were lower than those detected in no cell systems. This difference is probably due to consumption of NGF by PC12 cells.

Although not investigated in this work, embedding of NGF-loaded spheres within collagen hydrogel could further slow down protein release [30]. Therefore, growth

factor-loaded spheres may be directly injected into injury site or, as illustrated in Schematic 1, spheres can be embedded within hydrogel and then placed directly into a lesion area. This would overcome blood-brain barrier limits.

Another advantage of the application of collagen hollow spheres is its great biocompatibility with both neuronal cell line and primary sensory neurons. Cell viability assays showed no difference between cells treated with high dose (20 mg per cm^2) of three types of collagen spheres and PBS-treated cells while metabolic activity of cells treated with 10% DMSO decreased significantly to 15-25% (Fig.2). Surprisingly, many studies describing neurotrophin delivery through the biodegradable spheres or other scaffolds did not include any cytotoxicity experiments to prove biocompatibility of the developed devices [22, 27, 28], or cell viability was checked only following treatment with growth factor-loaded biomaterial [11, 24]. Thus, any potential cytotoxic effect of neurotrophin carrier/hydrogel could be masked by the presence of beneficial growth factor.

Finally, we proved that released NGF promotes PC12s and DRGs differentiation. A significant increase in PC12s neurite length was seen in all groups treated with NGF-loaded spheres when compared to single treated cells (Fig. 5). Similarly, there was significant increase in DRGs staining area when treated with NGF-loaded spheres (Fig. 6). This shows that NGF loading into collagen hollow spheres and subsequent release does not influence growth factor activity.

In conclusion, we have demonstrated that collagen hollow microspheres generated using a template method can be loaded with a large amount of NGF with very high loading efficiency. We have also shown that release of bioactive NGF can be tailored by the amount of cross linker, and that release of therapeutic dose can be achieved

over a period of 13 days. In addition, cytotoxicity testing did not show any negative effects on viability or metabolic activity of both neuronal cell line and primary rat neurons. Hence, we believe that collagen hollow microspheres have a potential for use as a reservoir for neurotrophic factors for multiple neurotherapeutic applications. Currently, the effect of neurotrophin-3 loaded microspheres on rat spinal cord injury is being evaluated.

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Figure Caption

Schematic 1: Graphical representation of the fabrication of hollow collagen microspheres and protein loading/release. The process involves the sulfonation of a commercially available polystyrene bead of defined size, coating of these beads in a collagen solution, crosslinking of the coating and removal of the polystyrene bead core. Spheres are incubated with loading buffer overnight to allow NGF loading into the spheres. Growth factor–loaded spheres are applied to neuronal cells and promote their survival and growth by controlled delivery of neurotrophins.

Figure 1: Steps of sphere fabrication: SEM images of the coating process showing (A) 4 μm polystyrene beads, (B) collagen-coated beads, (C) 2x collagen spheres following treatment with THF to dissolve the template.

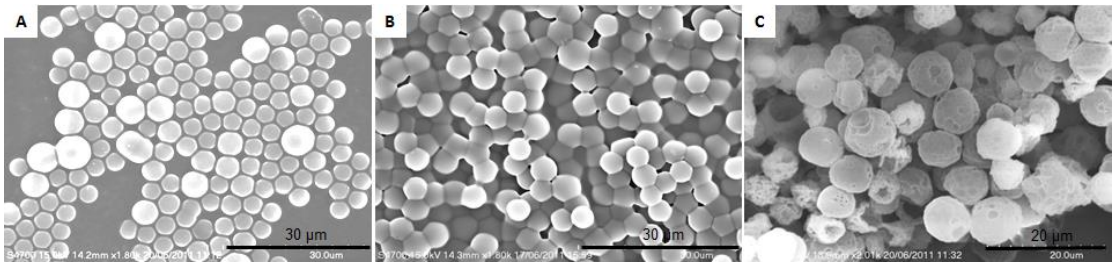

Figure 2: Cells-microsphere interaction. (A) Fluorescent images of PC12s (Top Panel) and DRGs (Bottom Panel) following 24 h treatment with PBS, 10% DMSO and 20 mg/cm^2 of 2x collagen spheres (green-live, red-dead). (B) Metabolic activity of PC12s and DRGs compared with PBS-treated cell control as measured by the AlamarBlue assay. ($p<0.01$).

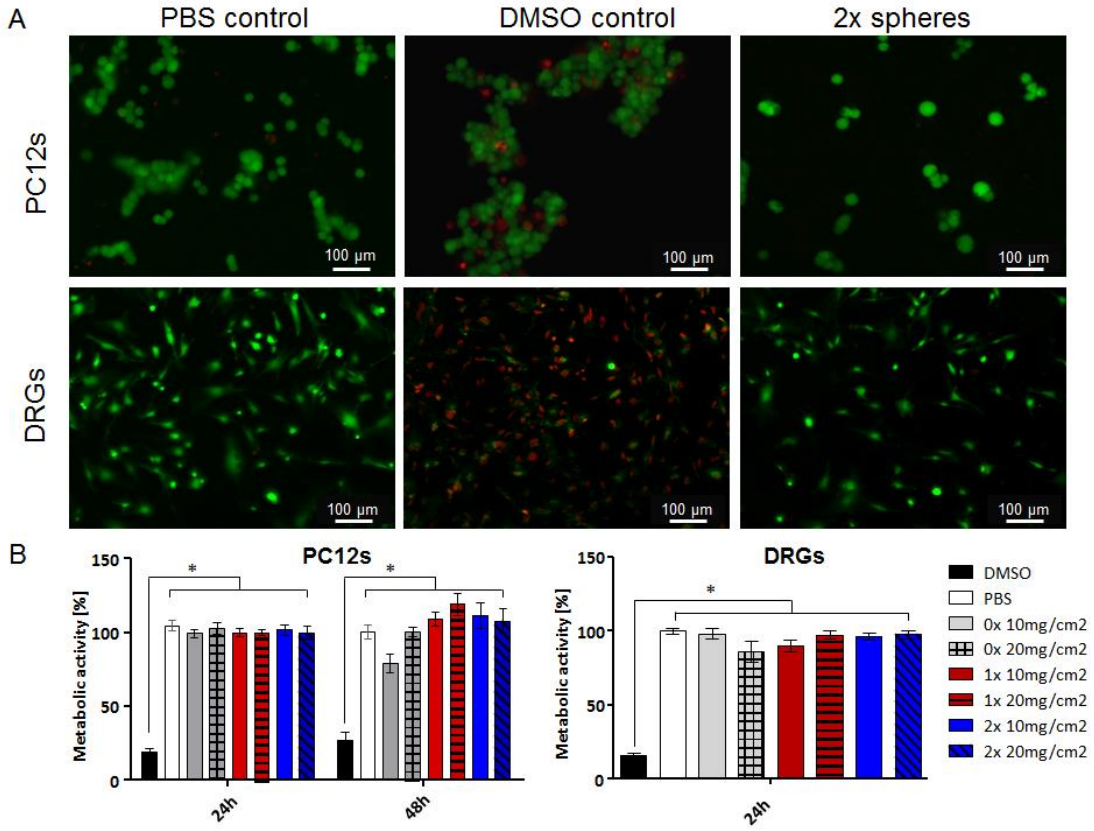

Figure 3: Loading of NGF into collagen microspheres. (A) Confocal images of loaded spheres (green-collagen spheres, red-NGF). (B) Loading efficiency with different doses of NGF (LH panel) and into three types of spheres (0x non-cross-linked, 1x cross-linked and 2x highly cross-linked (RH panel). Data represents mean ± SD, n=3.

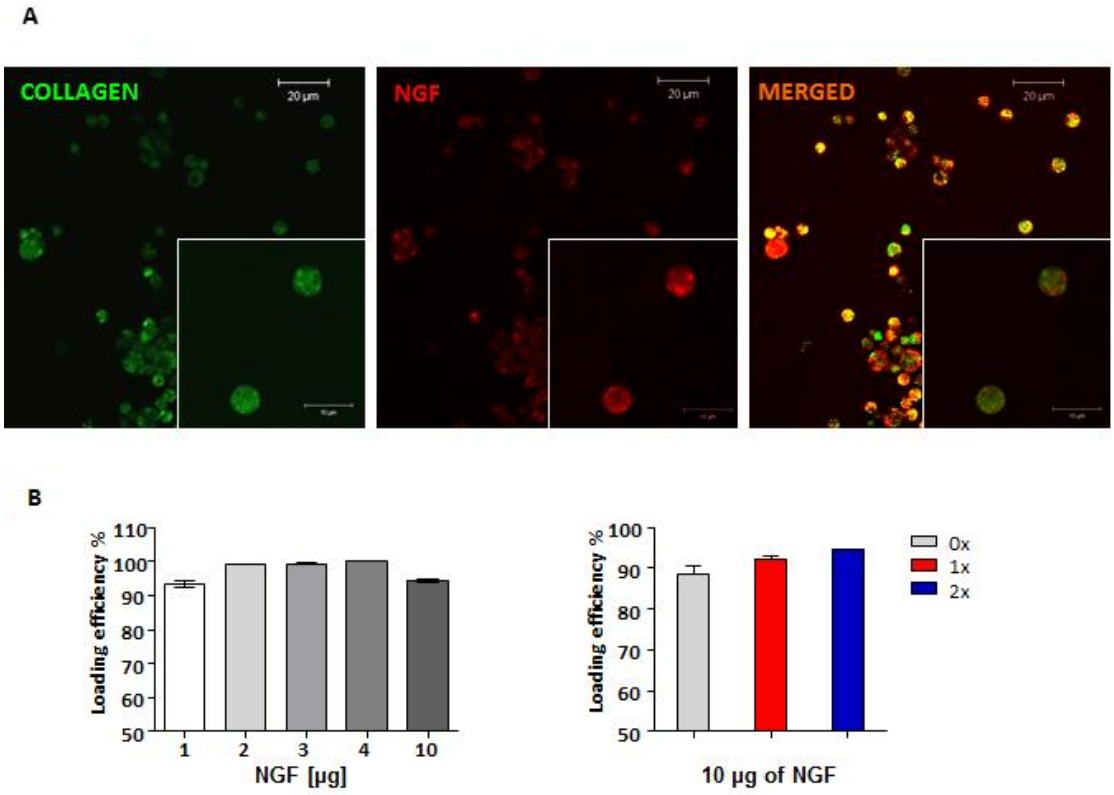

Figure 4: Release profile of NGF from collagen spheres measured by ELISA. In the presence of PC12s a slower release was observed from highly cross-linked spheres (2x) (LH panel). A therapeutic dose of NGF was released from spheres up to 13 days post loading (RH panel). Data represents mean ± SD, n=3.

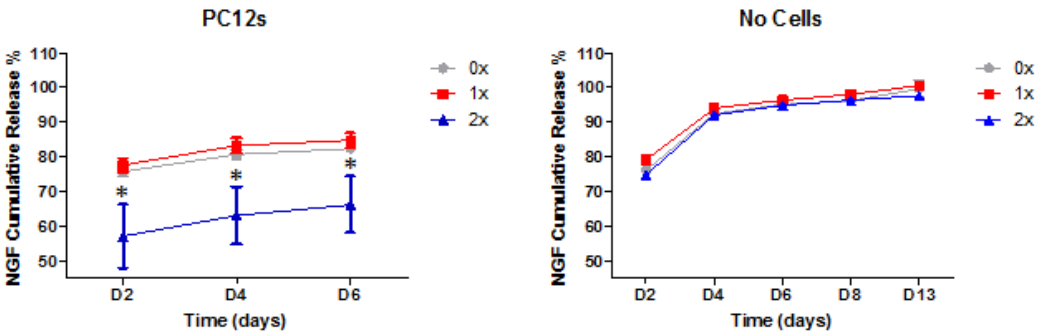

Figure 5: The bioactivity of released NGF assessed on PC12s. (A) PC12s differentiates following treatment with NGF-loaded 2x collagen spheres. (B) Neuronal outgrowth in PC12s following 6 days culture with NGF-loaded spheres (β-III tub staining (green) of differentiated cells). (I) Representative image of non treated cells/ unloaded spheres treatment, (II) single treatment with 50 ng/ml NGF, (III) Constant treatment with 50 ng/ml NGF, (IV, V, VI) 0x, 1x and 2x collagen spheres treatment loaded with 250 ng of NGF. (VII) Significant ($p < 0.05$) neuronal outgrowth was observed in constant NGF treatment and in all groups treated with NGF-loaded spheres. Data represents mean ± SD, n=50 (neurites per group).

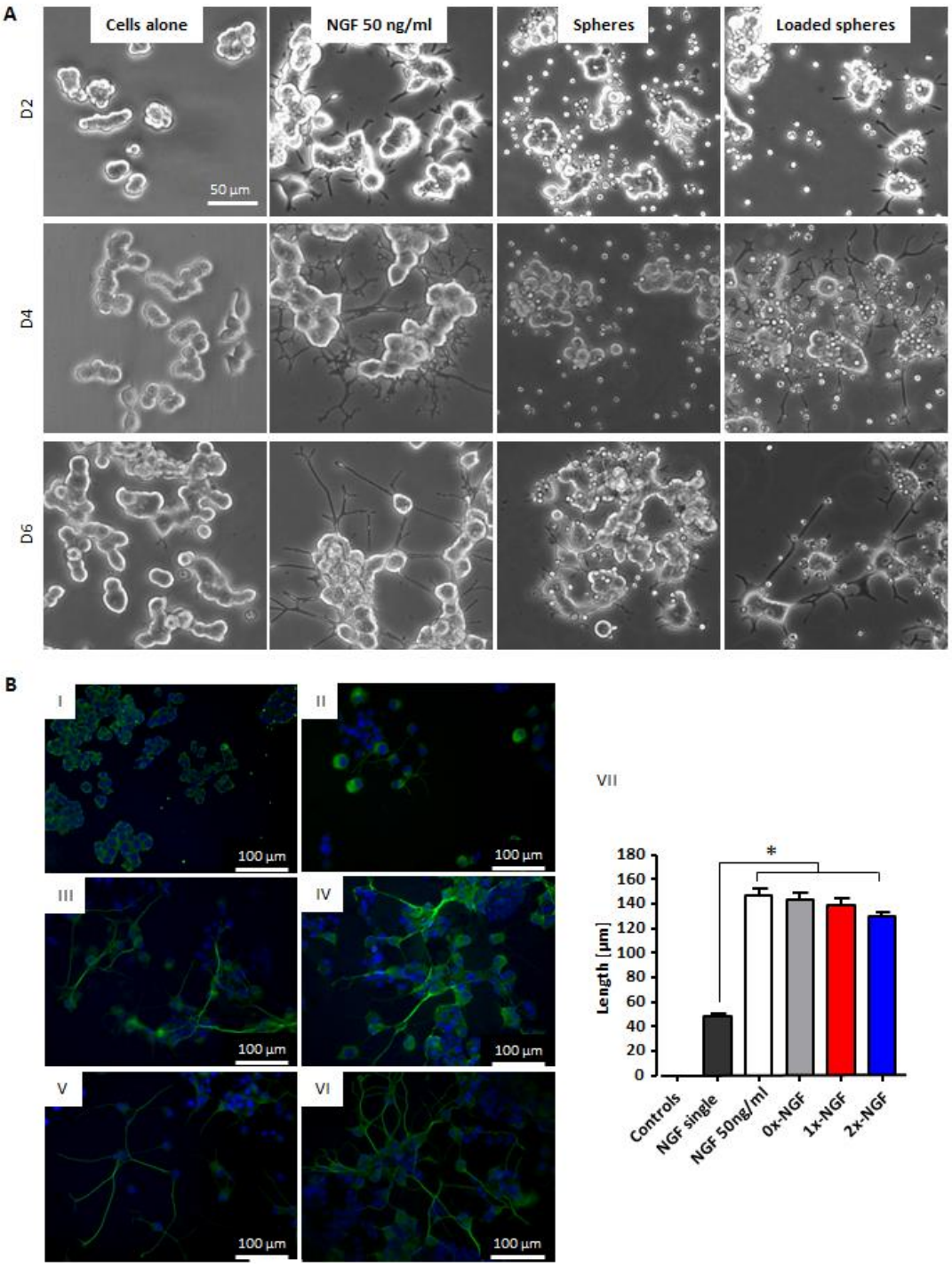
A
Cells alone
NGF 50 ng/ml
Spheres
Loaded spheres
D2
D4
D6
50 µm
B
I
II
III
IV
V
VI
100 µm
VII
Length [µm]
180
160
140
120
100
80
60
40
20
0
*
Controls
NGF single
NGF 50ng/ml
0x-NGF
1x-NGF
2x-NGF

Figure 6: NGF released from highly cross-linked collagen spheres promotes neurite growth in embryonic DRGs. Neurite outgrowth was visualised by β-III tub staining (green). (I, III, V) DRGs cultured in medium with 10 ng/ml of NGF and treated for 36 h with PBS, unloaded and 250 ng NGF-loaded spheres, respectively. (II, IV, VI) DRGs cultured in medium without NGF and treated for 36 h with PBS, unloaded and 250 ng NGF-loaded spheres, respectively. (VII) Measurements of βIII tubulin staining area show a significant difference ($p < 0.05$) between cells treated with NGF-loaded and unloaded spheres cultured in medium with or without NGF. Data represents mean ± SD, n=10.

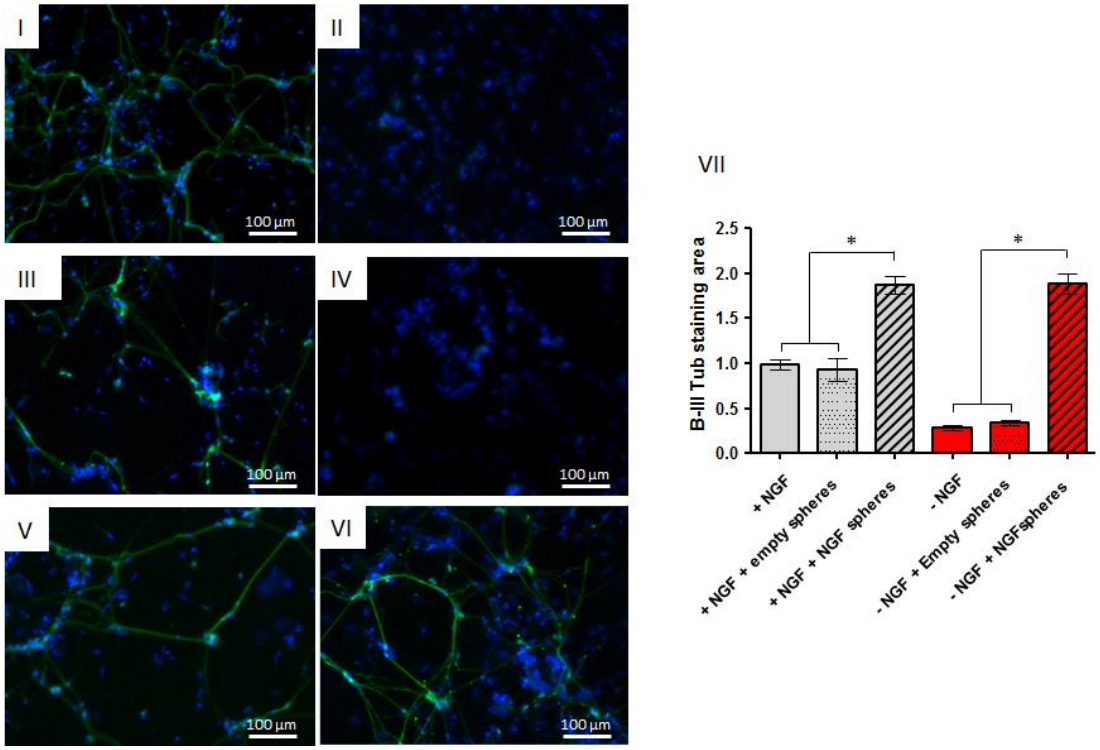

Figure S1: The bioactivity of released NGF assessed on PC12s following treatment with NGF-loaded non cross-linked collagen spheres.