Focal release of neurotrophic factors by biodegradable microspheres enhance motor and sensory axonal regeneration in vitro and in vivo

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Running title: Microspheres released trophic factors for nerve regeneration

Abstract

Neurotrophic factors (NTFs) promote nerve regeneration and neuronal survival after peripheral nerve injury. However, drawbacks related with administration and bioactivity during long periods limit their therapeutic application. In this study, PLGA microspheres (MPs) were used to locally release different NTFs and evaluate whether they accelerate axonal regeneration in comparison with free NTFs or controls. ELISA, SEM, UV/visible light microscopy, organotypic cultures of DRG explants and spinal cord slices were used to characterize MP properties and the bioactivity of the released NTFs. Results of organotypic cultures showed that encapsulated NTFs maintain longer bioactivity and enhance neurite regeneration of both sensory and motor neurons compared with free NTFs. For in vivo assays, the rat sciatic nerve was transected and repaired with a silicone tube filled with collagen gel or collagen mixed with PBS encapsulated MPs (control groups) and with free or encapsulated NGF, BDNF, GDNF or FGF-2. After 20 days, a retrotracer was applied to the regenerated nerve to quantify motor and sensory axonal regeneration. NTF encapsulation in MPs improved regeneration of both motor and sensory axons, as evidenced by increased numbers of retrolabeled neurons. Hence, our results show that slow release of NTFs with PLGA MP enhance nerve regeneration.

1. Introduction

Peripheral neurons are able to regenerate their axons after nerve injuries and reinnervate end organs, although functional recovery is often poor. After nerve transection, surgical repair is mandatory to rejoin the nerve stumps allowing axons to find an adequate substrate to grow distally. Direct suture of the two stumps or interposition of an autologous graft when the interstump defect is long are considered the standard option for repairing a nerve transection. The use of nerve conduits to bridge short or mid length gaps is a good alternative to autografts (Arslantunali et al., 2014), although regeneration is slowed during the initial phase compared to autograft (Boeckstyns et al., 2013). Several parameters affect the rate of axonal regeneration, including the distance between stumps (Scherman et al., 2001), the age of the subject (Kang et al., 2013; Painter et al., 2014; Verdú et al., 2008), the time after repair and the distance between the lesion site and end organ (Gordon et al., 2003). Indeed, the time from injury to target reinnervation is the most important predictor of the degree of functional recovery (Krarup et al., 2002), emphasizing that factors that control early axonal outgrowth influence the final level of recovery attained even years later. Therefore, strategies to accelerate the rate of axonal growth could be useful to stimulate nerve regeneration through longer distances and improve the recovery outcome. In fact, different strategies have already been developed to solve this problem including the use of different neurotrophic factors (NTFs) (Allodi et al., 2012).

After nerve injury, NTFs are synthesized and secreted not only at the spinal cord (SC) and the dorsal root ganglia (DRG) where the somas of the injured neurons are located, but mainly at the site of the lesion by reactive Schwann cells. NTFs have been long studied for their positive effects on nerve regeneration, including their ability to promote neuronal survival, to increase axonal outgrowth and to improve target reinnervation. Different NTFs have been tested in vitro and in vivo to improve

peripheral nerve regeneration, such as nerve growth factor (NGF) (Kemp et al., 2011; Lee et al., 2003), brain-derived neurotrophic factor (BDNF) (Vögelin et al., 2006), neurotrophin-3 (NT-3) (Sternel et al., 1997), glial cell-derived neurotrophic factor (GDNF) (Moore et al., 2010), fibroblast growth factors (FGF1, FGF2) (Midha et al., 2003; Allodi et al., 2013) or insulin like growth factors (IGF1, IGF2) (Ishi et al., 1993; Kanje et al., 1989).

The induction of an increased production of NTFs at the site of the lesion will stimulate axonal regeneration when the local environment is poor. Thus, the combination of a nerve conduit with NTFs appears as a good option to enhance nerve regeneration. However, NTFs added within a nerve conduit can be rapidly degraded (Ejstrup et al., 2010; Tria et al., 1994), diffuse outside the conduit or get diluted after liquid infiltration resulting in sub-optimal concentrations, and thus poor regeneration outcome (de Boer et al., 2012; Wood et al., 2013). Hence, a better way to apply NTFs and prolong their presence in close contact with regenerating axons and Schwann cells is still needed to optimize the effects of NTFs on regeneration. In attempts to increase the NTF availability at the site of injury over time, different approaches have been utilized by using repeated injections through catheters (Mcdonald et al., 2003), osmotic minipumps (Hontanilla et al., 2007), binding to extracellular matrix molecules (Sternel et al., 1997; Sakiyama-Elbert et al., 2000; Lee et al., 2003) or to the wall of nerve conduits (Madduri et al., 2010; Piquilloud et al., 2007) and gene therapy (Allodi et al., 2014; Eggers et al., 2013; Haastert et al., 2006). However, these strategies still present some difficulties to reach optimal nerve regeneration.

In this context, strategies involving biocompatible and biodegradable microspheres (MPs) associated to selected bioactive molecules may provide a sustained and controlled release of factors, offering valuable approaches for overcoming those limitations. Nanotechnology-based carrier technologies offer indeed a customizable approach for locally controlled delivery, by coupling the drug or

molecule of interest to carrier particles such as MPs (Tam et al., 2014), nanoparticles, liposomes or dendrimers (Mura et al., 2013). Thanks to relatively simple fabrication protocols and fair requests in terms of equipment, such technologies have been extensively explored in the last decade, and applied in different fields, spanning from cancer research to nutritional sciences (Mozafari et al., 2008; Peer et al., 2007). The characteristics of carrier technologies have been developed to overcome hindrances arisen in the field of drug administration, thus to improve the selectivity of targeting, to increase the capability to control drug release via chemistry tailoring, and to obtain an increase of stability of otherwise labile payloads. Despite the myriad different chemistries and payloads, the recurring goal has been definitely to exert an otherwise difficult control of drug release and stability at the local level. Among polymer-based microsphere carrier systems that take advantage of the biocompatible character of bio-derived substances, such alginate, chitosan and silk fibroin, PLGA (Poly-lactic Co-Glycolic acid, a synthetic biodegradable polymer) based MPs (Makadia et al., 2011) are particularly attractive for neural regeneration applications, given their favorable biocompatible behavior in physiological environments, low immunogenicity and toxicity, the possibility of size control and predictable, highly tailorable degradation kinetics, not mentioning the fact that they are FDA approved for drug delivery use (Jain et al., 2000).

Finally, the chaotic reinnervation of distal targets due to misdirection of regenerating axons is the main factor that results in poor functional recovery after nerve transection and tube repair (Valero-Cabré & Navarro, 2002), thus study of strategies to improve motor and/or sensory specific reinnervation are still needed. Therefore, the objective of the present study was the initial evaluation of the potential advantages of sustained release from MPs versus acute supply of several neurotrophic factors and their possible selective effects on the regeneration of motor and sensory axons.

2. Results

2.1. MPs fabrication and characterization

To test the correct encapsulation of molecules during the MP fabrication process, fluorescent FITC-BSA was used as tracking dye and its fluorescence in the MP was checked by fluorescence microscopy (Fig. 3). These fluorescent MPs were also used as negative control to initially test their biocompatibility in the organotypic explant experiments (see 3.2).

SEM imaging of MPs showed a fairly rough surface (Fig. 1C) and was used also as a tool for rapid assessment of particle dispersion, allowing quick modifications of the synthesis protocol to obtain narrowly distributed microsphere size by protocol adjustment. A consistent change was observed using non-hydrolyzed PVA instead of hydrolyzed PVA as dispersant of the second emulsification stage in the fabrication protocol. Non-hydrolyzed PVA has higher viscosity and reasonably hampers coalescing of the microdroplets formed during the first homogenization phase, thus permitting smaller and narrowly distributed MP sizes.

To assess the mean MP size, as Dynamic Light Scattering (DLS) was not feasible due the micrometric size of the particles, an optical microscopy-based method was implemented as described. Figure 1 shows the sequential steps of the software pipeline, from the starting image taken in visible light (Fig. 1A), proceeding through normalization, thresholding binarization, particle recognition and counting (Fig. 1B), with final summarization of statistical distribution of the measured diameters (expressed as Feret's diameter) of the MP (Fig. 1D). The particles produced by this protocol have a narrow size distribution with a mean size of 5.9±2.1 µm (n=6823). This is a desirable size range, as nanometer-sized MPs would likely be endocythosed by

cells, while bigger ones would be difficult to handle due to quick precipitation after resuspension.

The encapsulation efficiency was calculated as the actual fraction of a theoretical 100% efficiency. By ELISA tests the estimated encapsulation efficiency resulted to be 64.1±0.02%, that, while being lower than otherwise reachable high reported efficiencies (Péan et al., 1998), also in view of smaller mean particle size, compares with previous work with similar applications (de Boer et al., 2011). This estimate was used to calculate the mass of MP to use when assessing the release profile of NGF. Quantification of the released NGF was performed by ELISA, starting with an estimated 50 ng NGF-containing MP mass (equivalent to 62.5 µg) and measuring the NGF quantity in the supernatant of a suspension of MPs kept in cell culture-like conditions (37°C, 5% CO_2 atmosphere) over one month. The resulting data (shown in Fig. 2 as percentage quantity of the total initial estimated NGF) showed an initial small burst at day 0 (sampling was performed at the end of each considered day) and increasing release over the first 8-10 days of suspension, followed by sustained release until the end of the observation period.

2.2. In vitro effects of NTF loaded MP on neurite outgrowth

Although PLGA has been widely described to be biocompatible and biodegradable (Makadia et al., 2011), there are studies reporting some cell toxicity in culture (Grabowski et al., 2013). Thus, we first evaluated if PLGA MPs could affect neurite outgrowth by loading FITC-BSA in the MPs and embedding them within the collagen matrix surrounding DRG and SC cultures. In both cases neurites grew from the explants and were present in close proximity to the MPs (Fig. 3A, B), indicating no adverse effects caused by PLGA or PLGA degradation products that could interact with neurite outgrowth.

Organotypic cultures of DRG (Fig. 4A-F) and SC (Fig. 5A-F) were performed to test whether MPs loaded with NGF (MP-NGF) and with BDNF (MP-BDNF) increased neurite outgrowth in comparison with the same NTF placed as free protein in the collagen matrix. Without conditioning the collagen matrix, both free and encapsulated NTF increased the amount of neurites that grew from the explant in comparison with cultures without NTF (Fig. 4G and 5G), indicating that MPs released NTF. The longest neurite measured was significantly longer in DRG cultures with free NGF but not in cultures with MP-NGF than in control cultures (Fig. 4H). In SC cultures both free BDNF and MP-BDNF increased the maximal length of neurites with respect to control cultures (Fig. 5H).

After 1 week of conditioning the collagen matrix with no NTF, free NTF or encapsulated NTF, only the explants cultured with MPs increased the amount of neurites in comparison with the control and also free NTF (Fig. 4I and 5I), indicating that after 1 week there was still NTF within the matrix of the MP conditions being able to promote axon outgrowth but not in the free NTF cultures. Similarly, both MP-NGF (Fig. 4J) and MP-BDNF (Fig. 5J) cultures showed increase in the longest neurite measures with respect to control values, while no differences were seen between the free NTF and control conditions.

2.3. NTFs enhance axon outgrowth of motor and sensory neurons in vivo

All the rats showed evidence of axonal regeneration after section of the sciatic nerve and tube repair, as judged by the retrograde labeling of motor and sensory regenerated neurons with FG (Fig. 6). No differences were found between groups treated with collagen alone and with MP-PBS (Fig. 7A, B) indicating again lack of interaction of PLGA MPs with axon outgroth.

GDNF and BDNF applied as free factor in the nerve conduit were the only NTFs that enhanced motor axon regeneration in comparison with the control group, whereas

NT-3, FGF and NGF did not show differences (Fig. 7A). Similarly, free GDNF, BDNF and also FGF yielded significant differences in comparison with animals treated with MP-PBS. On the other hand, all the groups with NTFs encapsulated in MPs had higher number of regenerated motor neurons than the control group. Particularly, groups treated with MP-GDNF and MP-BDNF showed the highest increase in the number of regenerated motor axons. Concerning sensory neurons regeneration, only free NGF showed differences with the two control groups without NTF (Fig. 7B). In contrast, MP-NGF, MP-GDNF and MP-BDNF enhanced sensory axon growth with respect to control and MP-PBS groups.

With regard to the performance of MPs, a comparison of free NTF (dotted line, taken as 100%) with the same factor encapsulated in MPs is shown in Fig. 7C, D. For motor neurons, all the MP groups had higher numbers of neurons with regenerated axons in comparison with free NTF with the exception of FGF (Fig. 7C). For sensory neurons, all the MP groups had increased axon regeneration in comparison with free NTF, except for NGF and BDNF (Fig. 7D). These results indicate that the slow release of NTFs from MPs promoted nerve regeneration in vivo.

Finally, histologic quantification of MF inside the regenerating tube was performed. The biocompatibility of the PLGA MPs used in this study was confirmed, as MPs were in close proximity with regenerated myelinated and unmyelinated axons (Fig. 8 D, F). The regenerated nerve inside the tube had a thin external layer of fibrous tissue and a central region with axons forming the typical morphology of a regenerated nerve inside a tube (Gómez et al., 1996; Lago et al., 2006) in all the groups (Fig. 8 A-C). With regard to the number of MFs, control and MP-PBS groups showed similar values. On the other hand, only NGF and MP-NGF treated groups showed higher number of MFs than controls, whereas the other treated groups had similar values with no differences compared to the control groups (Fig. 8G).

3. Discussion

The results of this study demonstrate that different NTFs encapsulated in PLGA MPs enhance motor and sensory axons regeneration both in vitro and in vivo in comparison with the same NTFs administered as free molecules without encapsulation. The encapsulation in controlled release systems has the advantage to allow a slow and sustained release of desired NTFs focally at selected areas of the nervous system as a therapeutical approach for neurodegenerative diseases and neural injuries (Benoit et al., 2000; Catrina et al., 2013). Due to their small size, MPs can be easily implanted in well localized areas without affecting the surrounding tissues (Fig. 3 & 8), thus avoiding also secondary effects. The controllable delivery of molecules from the MPs is advantageous as compared to a burst supply by direct injection and allows to overcome the limited therapeutic protein stability. Advantages of MPs (and conduits) as carriers for local, prolonged delivery of NTFs include that they are biocompatible and biodegradable, minimizing the foreign-body reaction, they avoid the implantation of catheters and external devices needed with injection ports and osmotic pumps, or the grafting of engineered cells with immunogenicity issues.

First, a characterization of the PLGA MPs was made before their in vitro and in vivo use. ELISA results demonstrate that 10 days after MP fabrication, 30% of the encapsulated NTF has been already released, and that slow release is maintained for more than a month. This releasing profile seems adequate for enhancing nerve regeneration, since the expression of different NTF receptors remains upregulated during the firsts months after injury (Boyd and Gordon, 2003a). Despite previous studies have used PLGA MPs to deliver NTFs, mechanical and chemical stresses during MP manufacturing may affect the structure and the stability of the encapsulated protein (Yeo et al., 2004; Pfister et al., 2007). To address this issue, we first used organotypic cultures of DRG (Fig. 4) and SC (Fig. 5) to assess the bioactivity of NTFs released from the prepared MPs. The obtained results showed that the NTFs tested,

NGF and BDNF, are released in effective concentration beyond one week, and after this period still keep bioactivity to promote neurite outgrowth in comparison with the application of free NTFs. The neurite growth observed in cultures without preconditioning (i.e. immediate use) was similarly enhanced by free NTFs and by NTFs loaded in MPs added within the collagen matrix, which can be explained by the initial burst release of enough amount of the NTFs (Fig. 2). However, after 1 week of preconditioning the medium, only NTFs encapsulated in MPs produced an increase in neurite outgrowth in comparison with control cultures, indicating that MPs were still releasing NTFs at a concentration high enough to stimulate the growing response. Thus, rather than an increased effect of encapsulated NTFs over free NTFs, it seems that the elution of the free NTFs outside the matrix or the loss of their activity is responsible of the low effect in the conditioned setting, while the NTFs continuously released from the MPs were still able to enhance neurite outgrowth to similar levels than in the non-conditioned matrix.

Second, after testing that the manufactured MPs did not interact with growing neurites and improved regeneration in vitro, the same preparations were applied in vivo. A silicone tube filled with a collagen gel containing either free or MPs encapsulated NTFs was implanted to repair a nerve gap. The NTFs applied were expected to act during the period of axonal regeneration across the tube, i.e. 2-3 weeks (Williams et al., 1983; Mcdonald et al., 2003). As the MP encapsulation increased the therapeutic window of NTFs delivery in vitro, its effects were presumed to be also higher in vivo than those of free NTFs. Thus, both retrotracer and histological analyses were conducted to test whether application of encapsulated NTF improved nerve regeneration in vivo.

The number of regenerated neurons (Fig. 7A-B) and MFs (Fig. 8G) in both control groups was similar, indicating that the presence of PLGA MPs did not interfere in the regeneration process. On the other hand, while retrotracer analysis

demonstrates an enhancement of regeneration in all the groups with NTFs, this improvement is not seen in the histological analysis where only groups treated with NGF showed differences compared to control. Moreover, we found less regenerating neurons in the retrotracer study than MFs in the histology. Although this results could seem contradictory, it has been reported that neurons initially generate several axonal sprouts after axotomy (Mackinnon et al., 1991), and even at long term each neuron may maintain more than a single regenerated axonal branch (Gómez et al., 1996; Jenq & Coggeshall, 1985). Furthermore, more regenerated axons (sprouts) do not necessarily indicate more neurons regenerating (Piquilloud et al., 2007) and may be also considered deleterious in terms of functional recovery (Guntinas-Lichius et al., 2005). Hence, the increase of MF in the NGF groups would be in accordance with other studies that describe an important role of NGF on axonal sprouting (Diamond et al., 1987; Gloster et al., 1992; Ruiz et al., 2004). Thus, histological quantification of MFs may result in an overestimation of the number of regenerated neurons (Navarro 2015) and only the retrotracer study provides reliable information on the number of regenerating neurons. For these reasons, in our study the positive effects of the NTFs loaded MPs were indicated by the effective growth of axons that were able to capture the retrotracer placed distal to the nerve conduit.

The results found in this work indicate that an increased number of motor and sensory neurons regenerate distally to the nerve conduit in the groups with MP-encapsulated NTFs. This can be explained by an acceleration of axonal regeneration, as well as by an indirect effect inducing endogenous Schwann cells to a proregenerative state (Gordon et al., 2003). After nerve lesion reactive Schwann cells highly induce the expression of several NTFs, including NGF, BDNF, GDNF, and their receptors (Boyd and Gordon, 2003a; Allodi et al., 2012). The increased neurotrophic secretion peaks at about 1 week postaxotomy and declines with time. Our results give further support to the view that additional exogenous supply of NTFs may enhance the

regenerative response of peripheral neurons. Nevertheless, the concentration and time window of the NTFs applied at the site of nerve injury, such as within a nerve conduit, need to be carefully investigated. Indeed, a small supply of a NTF at the site of injury does not always have beneficial effects, and on the opposite an excessive concentration may even hinder axonal growth. Thus, exogenous application of NGF to axotomized sensory neurons may interfere with the regenerative response and delay axonal regrowth (Gold et al., 1997; Mohiuddin et al 1999). An increased supply of GDNF at the focal site has been shown to stimulate axonal sprouting, thus multiplying the number of regenerating fibers within a nerve conduit, but did not result in positive outcomes in terms of distal regeneration and return of motor function (Piquilloud et al., 2007). A further problem was reported after the injection in the injured nerve of viral vectors transducing the over-expression of NTFs. The high amount of GDNF secreted created a "candy-store" effect (Tannemaat et al., 2008), in which regenerating axons stay in areas with high GDNF concentration and fail to grow distally towards the target organs. Therefore, our results sustain that prolonged, controlled release of NTFs is beneficial to improve nerve regeneration.

Third, after fluorogold retrolabeling and quantification of regenerated neurons in DRG and SC (Fig. 7), we were able to distinguish if different NTFs exerted a selective effect on the axonal regeneration of motor or sensory neurons in vivo, an information that is not provided by standard microscopy methods. The in vivo results corroborated some results previously reported using the organotypic cultures in vitro. Thus, in cultures with NTFs added directly to the collagen matrix, NGF had a positive effect only on sensory neurons, BDNF mainly enhanced motoneurons growth, GDNF promoted neurite outgrowth in both types of neurons, whereas NT-3 did not have a noticeable effect (Allodi et al., 2011), similarly to our results in vivo with free NTFs. Nevertheless, we found that when applied encapsulated in MPs, the same NTFs had stronger effects

on axonal regeneration of both populations of peripheral neurons, although the differential effects were maintained.

Previous studies demonstrated that an additional supply of GDNF and of BDNF exerts beneficial effects on motoneuron survival and regeneration (Boyd and Gordon, 2003b; Pajenda et al., 2014), but no positive effects were found for sensory neurons (Tannemaat et al., 2008). In agreement with those reports, we observed improved motor but not sensory neuron regeneration when BDNF and GDNF were embedded as free factors in the collagen filling the silicone tube. However, when they were encapsulated in MPs, regeneration was enhanced in both motor and sensory neurons. Although sensory neurons and Schwann cells express both BDNF and GDNF receptors (Boyd and Gordon, 2003a), it is likely that a single administration immediately after nerve injury is not enough to exert a significant effect on sensory regeneration.

FGF-2 is expressed in three isoforms that are upregulated after nerve injury (Klimaschewski et al., 2013). FGF-2 18 KDa isoform mainly enhances motor functional recovery with some effects on sensory functional recovery (Allodi et al., 2014; Haastert et al., 2006; Meyer et al., 2015). In accordance with these studies, we found an enhancement only of motor axon regeneration compared to both controls in the MP group.

On the other hand, as motoneurons do not express TrkA receptor and after sciatic nerve axotomy TrkC receptor levels remain relatively unchanged (Boyd and Gordon, 2003a), significant effects on motoneurons regeneration with the application of NGF and NT-3 were not expected. However, when encapsulated in MPs both NGF and NT-3 produced significant increase in the number of motoneurons that regenerated their axons distal to the conduit. A previous study described improvement of motor regeneration with NGF administration in a fibrin depot (Jubran et al., 2003). It may be hypothesized that the prolonged supply by the MPs might indirectly enhance axonal regeneration by stimulating Schwann cell proliferation and reactivity. Furthermore, a

possible inhibition of NGF or NT-3 via p75 receptor activation (Boyd and Gordon, 2002) could also be compensated by the upregulation of other NTFs such as BDNF secreted by stimulated Schwann cells or fibroblasts (Acheson et al., 1991; Klein et al., 1991).

In conclusion, the results of this study indicate that the use of PLGA MPs as a prolonged delivery system within a nerve conduit is a good approach to enhance nerve regeneration in comparison with a single application of NTFs that have a limited effect due to degradation, leakage outside the conduit or dilution resulting in sub-optimal concentration. Despite some NTFs are able to enhance axonal regeneration when directly applied to the injured nerve, our in vitro and in vivo results support that the therapeutic effect in terms of increasing axon growth is significantly increased by a sustained release from PLGA MPs. The lengthening of trophic support will be of further relevance for the repair of long gaps with nerve conduits, a situation in which the endogenous support is insufficient to sustain axonal regeneration, as well as for delayed repair of nerve injuries (Wood et al., 2013) that need a reactivation of the pro-regenerative environment along the distal nerve. Further research to refine the MPs supply of NTFs should address on tuning the time-concentration rate of delivery, as well as the potential contribution of several factors simultaneously or sequentially.

4. Experimental Procedure

4.1. Microsphere fabrication and characterization

The chosen material was Poly-lactic Co-Glycolic acid (PLGA, Sigma-Aldrich Co. LLC, cat# 719870), a synthetic biocompatible and biodegradable co-polymer widely used for controlled release applications (Makadia et al., 2011). Low molecular weight PLGA (Mw 24,000-38,000) was used because of its encapsulation efficiency (Blanco and Alonso, 1998) and a water-in-oil-in-water (WoW) protocol was followed, as already described (Ghaderi et al., 1996), with modifications to adapt to the application. Briefly, 30-35 mg of PLGA (50:50) were dissolved in 1 ml DCM. The first emulsion was created with 200 µl aqueous solution of the desired trophic factor (NGF, BDNF, GDNF, FGF-2 18 kDa, or NT-3, Peprotech, USA) with an immersion homogenizer. This water-in-oil emulsion was then poured in 7 ml PVA 5% aqueous solution to create the second emulsion. After 45 min in the magnetic stirrer the double emulsion was poured in 40 ml PVA 0.1% aqueous solution to allow for solidification and solvent evaporation over 16h in the magnetic stirrer at RT. The MPs were washed with distilled water twice by centrifugation at 4650 RCF at 4°C, finally resuspended in an aqueous solution with penicillin/streptomicyn, to control possible downstream bacterial contamination and sent forth for in vitro/in vivo experiments.

MP surface and size distribution assessment was performed by SEM (EVO™MA10 Scanning Electron Microscope, Zeiss, Germany). Briefly, 1-3 µl of particles dispersion were spotted on silicone stubs, metallized with a sputter coater (Quorum Q150R ES, Quorum technologies, UK) and imaged with an acceleration voltage of 10 kV. Size distribution characterization was performed by means of an inverted microscope (Eclipse Ti; Nikon Instruments, Japan) equipped with a cooled CCD camera (DS-Fi1C; Nikon Instruments, Japan). MPs were imaged immediately after synthesis in 5 µl distilled water spotted on a clean glass coverslip, and snapshots

of random fields were taken in visible light. Particle size was evaluated via image analysis with the Fiji software (Schindelin et al., 2013). Assessment of fluorescent proteins encapsulation and tracking was performed with same apparatus equipped with suitable fluorescent filters.

NGF was chosen to further characterize the performance of the microspheres system. NGF encapsulation efficiency of the fabrication protocol was calculated as the fraction between experimental load versus theoretical load. For efficiency testing, 4 µg of NGF were encapsulated in 30 mg PLGA MPs, following the above described protocol. The MPs were washed and dissolved by incubation overnight in 2 ml dissolution buffer (5% SDS, 0.1N NaOH) at RT. Following microsphere dissolution, the released NGF was quantified with an ELISA test kit (Human beta-NGF ELISA kit, Sigma-Aldrich, USA), following the manufacturer's protocol.

For NGF release assessment, a tube containing 1 ml of phosphate buffered saline (PBS) was loaded with 62.5 µg of MPs coming from batches of 30 mg total mass, equivalent to an estimated 50 ng encapsulated NGF (based on previously calculated load efficiency). The tube was incubated at 37°C and at specific time points (0, 1, 2, 3, 5, 6, 7, 8, 15, 20, 25, 33 days), the mixture was centrifuged at 4650 RCF at 4°C, the supernatant was collected and fresh PBS was then added to replace the withdrawn supernatant; the MPs were resuspended again and returned to 37°C. Supernatants were frozen for subsequent quantification. Assessment of quantity of the released NGF in each supernatant at different time points was also performed by an ELISA kit (Human beta-NGF ELISA kit SIGMA-Aldrich, USA), following the manufacturer's protocol. Release experiments were all performed in duplicate.

4.2. In vitro study on organotypic cultures

The in vitro procedures were approved by the ethical committee of the Universitat Autonoma de Barcelona in accordance with the European Communities

Council Directive 2010/63/EU. Organotypic cultures were prepared as previously described (Allodi et al., 2011). A volume of 392 µl of rat tail collagen type I solution (#354236, Corning) at a concentration of 3.83 mg/ml was mixed with 50 µl of 10× basal Eagle's medium (Gibco) and 2 µl of 7.5% sodium bicarbonate solution. Moreover, 56 µl of PBS with MPs containing FITC-BSA for biocompatibility assessment or 56 µl of PBS with free or MP encapsulated NGF or BDNF for bioactivity experiments were added to the collagen solution. The same volume of MPs with PBS vehicle was used for the control group (Col). The final concentration of the NTF was 10 ng/ml of within a 3.0 mg/ml collagen gel.

Single drops of 30 µl were deposited on poly-d-lysine (PL, 1 g/ml, Sigma) coated coverslips, which were placed in Petri dishes or 24-well multidishes (Iwaki, Asahi Technoglass, Chiba, Japan) and kept in the incubator at 37ºC and 5% CO_2 for two hours to induce collagen gel formation. Three different sets of collagen-based matrices (containing MPs loaded with PBS, NTFs or free NTFs) were prepared and used immediately or maintained for 1 week (conditioning) in the incubator to test if MPs released the factors over time and if the NTF was accumulated or eluted with time in each condition.

P7 rats were euthanised by cervical dislocation following AVMA Guidelines on Euthanasia (Leary et al., 2013), and spinal cord (SC, n=6-8/group) lumbar segments and lumbar DRG (n=14-24/group) were harvested, placed in cold Gey's balanced salt solution (Sigma) enriched with 6 mg/ml glucose and cleaned from blood and meningeal debris. SCs were cut with a McIlwain Tissue Chopper in 350 µm thick slices. SC slices and DRG explants were then placed on the gelled collagen droplets, prepared as indicated above, and covered by a second drop of 30 µl collagen matrix. The embedded samples were placed again in the incubator for 45 min before adding Neurobasal medium (NB, Invitrogen), supplemented with B27 (Invitrogen), glutamine and penicillin/streptomycin (Sigma). The medium volume delivered into Petri dishes

and wells was 1.5 ml for SC slices and 0.5 ml for DRG. After one day in culture, the medium of SC cultures was removed and changed by a penicillin/streptomycin free medium. DRG explants were cultured for 2 days, and SC slices for 4 days.

SC and DRG cultures were fixed with 4% paraformaldehyde in PBS for 30 min. Afterwards, SC and DRG samples were incubated for 48 h with primary antibody mouse RT97 (1:200, Developmental Studies Hybridoma Bank) at 4ºC. After three hours washing, the sections were incubated with secondary antibodies AF594 conjugated donkey anti-mouse (1:200, Jackson IR) overnight at 4ºC. After two washes samples were mounted on slides with Mowiol containing DAPI (1:10000, Sigma) nuclear counterstain. Cultures were visualized with an Olympus BX51 fluorescence microscope, images of different areas were taken with Cell A software (Olympus) and merged using Adobe Photoshop CS3 (Adobe System). Confocal images were obtained using a ZEISS LSM 700 microscope and the ZEN ZEISS software. Whole culture images were analyzed with the Neurite-J plug-in (Torres-Espín et al., 2014) for ImageJ software (NIH, available at http://rsb.info.nih.gov/ij/) and the number of neurites grown at different distances from the explant was compared between sets of cultures.

4.3. In vivo study of peripheral nerve regeneration

Female Sprague-Dawley rats (250–300 g) were used for the in vivo studies. They were kept on standard laboratory conditions with a light-dark cycle of 12:12 h and *ad libitum* access to food and tap water. All efforts were made to minimize pain and animal discomfort during surgery and treatments.

Animals were anaesthetized with pentobarbital sodium (40 mg/kg i.p.)., the sciatic nerve was exposed at the midthigh and sectioned 90 mm from the tip of the third toe, and a 6 mm nerve portion distal to the section was resected. A silicone tube was then sutured with 10-0 monofilament sutures to each nerve stump leaving a 6 mm gap

between both nerve ends. Animals were kept for 20 days post-operation (dpo) to allow axonal regeneration before testing.

NGF, NT-3, GDNF and BDNF free or encapsulated in MPs were added to a collagen solution to reach a final concentration of 2 µg/ml; FGF free and encapsulated was used at 10 µg/ml. Each preparation of NTF was mixed with 50 µl of 10× basal Eagle's medium (Gibco), 2 µl of 7.5% sodium bicarbonate and 187 µl of PBS, and then kindly mixed with 261 µl of collagen type I solution at 3.83 mg/ml to a reach a final collagen concentration of 2 mg/ml. Silicone tubes 8 mm long with an external diameter of 3 mm and an internal diameter of 2 mm were filled with one of the collagen mixtures containing free or encapsulated NTFs. In order to promote fibril alignment, the collagen was left to gel vertically for 12 hours before surgery (Verdú et al., 2002). Therefore, there were 10 experimental groups (n=5-6 per group), one for each NTF free and encapsulated in MPs. In addition, two control groups were implanted with tubes containing collagen alone or collagen with MPs containing only PBS. All in vivo procedures were approved by the ethical committee of the Universitat Autonoma de Barcelona in accordance with the European Communities Council Directive 2010/63/EU.

4.4. Retrograde labeling and neuronal counting

To quantify regenerated motor and sensory neurons, rats were anaesthetized at 20 days after lesion with pentobarbital sodium, the sciatic nerve was exposed and transected 8 mm distal to the distal end of the silicone tube. The tip of the severed nerve was soaked into 5 µl of Fluorogold (FG; 5%; Fluorochrome Inc.) for 1 hr in a vaseline well. After retrieval of the well, the area was rinsed with saline to clean remnants of the tracer, and the wound sutured in planes. Animals were allowed to survive for 7 days to allow accumulation of the tracer in the soma of the spinal motoneurons and the DRG sensory neurons. Then, rats were deeply anesthetized with

pentobarbital sodium overdose (200 mg/kg i.p.) following AVMA Guidelines on Euthanasia (Leary et al., 2013) and transcardially perfused with 4% paraformaldehyde in PBS. The lumbar segment (L3-L6) of the SC and L4 and L5 DRG were removed, postfixed in the same fixative solution for 1h and transferred to 30% sucrose in PBS. The SC and DRG were cut longitudinally in 40 and 20 µm thick sections, respectively, in a cryostat and mounted on slides. Sections were observed with an Olympus BX51 fluorescence microscope under UV light and the number of labeled neurons were counted in every third section following the fractionator principle (Gundersen et al., 1986).

4.5. Nerve histology

After perfusion, the regenerated nerves inside the tubes were also harvested and fixed in glutaraldehyde-paraformaldehyde (3%/3%) in cacodylate-buffer solution (0.1 M, pH 7.4) overnight at 4ºC. Then, nerves were postfixed with osmium tetroxide (2%, 2 h) and dehydrated through ethanol series prior to embedding samples in epon resin. Semithin sections (0.5 µm thick) taken 3mm distal from the proximal stump were stained with toluidine blue and examined by light microscopy. Images of the whole sciatic nerve were acquired at 10× with an Olympus DP73 camera attached to a computer. Sets of images for analysis obtained at 100× magnification were chosen by systematic random sampling of squares representing at least 30% of the nerve cross-sectional area and measurements of cross-sectional area of the whole nerve and counts of the number of myelinated nerve fibers (MF) were conducted.

4.6. Data analysis and statistics

Data are presented as mean ± SEM. Results were statistically analyzed by using GraphPad Prism (GraphPad Software, USA). One- or two-way ANOVA followed by Bonferroni's multiple comparison test was used in the in vitro studies for comparison

between groups. Student's t-test or one-way ANOVA followed by post hoc Fisher's exact test were applied in the in vivo studies. Statistical significance was considered when P value was <0.05.

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6. Acknowledgments

7. Figure legends:

Figure 1: A, B: Steps in image treatment for automated microspheres counting. A: starting, as-is, captured image of microspheres suspension in visible light; B: same image after normalization, thresholding, binarization and automatic recognition for counting. C: sample SEM micrograph of PLGA MPs showing regular surface roughness. D: statistical data distribution for MPs counted with the automated procedure from A, B.

Figure 2: Cumulative release of NGF from PLGA MPs, measured by ELISA and expressed as percentage of an estimated starting total concentration of 50 ng/ml of neurotrophin. Data expressed as mean ± SEM.

Figure 3: Neurite outgrowth stained with RT97 (red) from DRG (A) and SC (B) organotypic cultures in a collagen matrix filled with fluorescent MPs (green). Scale bar: 100 μm.

Figure 4: A-C: Neurites stained with RT97 from DRG neurons cultured within non-conditioned collagen alone (A), with added free NGF (B), and MP-NGF (C). D-F: Neurite outgrowth from DRG neurons cultured within 1 week-conditioned collagen matrix alone (D), with added free NGF (E), and MP-NGF (F). G, I: Plot of the number of neurites grown at increasing distance from the DRG body in a collagen matrix non-conditioned (G) and after 1 week conditioning with free NGF or MP-NGF (I). H, J: Measurement of the longest neurite in non-conditioned (H) and after 1 week conditioning with free NGF or MP-NGF (J). *$p < 0.05$ vs Col; \$$p < 0.05$ vs MP-NGF; #$p < 0.05$ vs free NGF, & $p < 0.05$ vs free NGF and MPNGF. Data expressed as mean ± SEM. Scale bar: 250 μm.

Figure 5: A-C: Neurites stained with RT97 from SC neurons cultured within non-conditioned collagen alone (A), with added free BDNF (B), and MP-BDNF (C). D-F: Neurite outgrowth from SC neurons cultured within 1 week-conditioned collagen matrix alone (D), with added free BDNF (E), and MP-BDNF. G, I: Plot of the number of neurites grown at increasing distance from the SC body in a collagen matrix non-conditioned (G) and after 1 week conditioning with free BDNF or MP-BDNF (I). H, J: Measurement of the longest neurite in non-conditioned (H) and after 1 week conditioning with free BDNF or MP-BDNF (J). *p<0.05 vs Col; $p<0.05 vs MP-BDNF, #p<0.05 vs free BDNF, & p<0.05 vs free BDNF and MP-BDNF. Data expressed as mean ± SEM. Scale bar: 250 µm.

Figure 6: Representative micrographs of neurons retrolabeled with FG in the lumbar SC of rats after sciatic nerve section and repair with a nerve conduit filled with collagen plus MP-BDNF (A, left) and with collagen alone (A, right). Representative micrographs of neurons retrolabeled with FG in the lumbar DRG of rats after sciatic nerve section and repair with a nerve conduit filled with collagen alone (B) and with collagen plus MP-NGF (C). Scale bar: 500 µm.

Figure 7: Histograms of the number of motor neurons in the SC (A) and of sensory neurons in the DRG (B) retrolabeled with FG after sciatic nerve section and conduit repair counted in the different groups compared, with and without NFs in free and in MP preparations. Percentage of retrolabeled motor (C) and sensory (D) neurons counted in rats with NTF loaded MPs with respect to rats with the same NTF applied as free factor in the collagen gel. Data expressed as mean ± SEM. *p<0.05 vs Col; #p<0.05 vs MP-PBS.

Fig. 8. A-F: Representative microphotographs of semithin transverse sections of the regenerated nerves in groups MP-PBS (A, D), NGF (B, E) and MP-NGF (C, F). **G:** Histogram of myelinated fibers number in the different groups compared. Data expressed as mean ± SEM. *p<0.05 vs Col; #p<0.05 vs MP-PBS. Scale bar 100 µm in A-C and 10 µm in D-F.
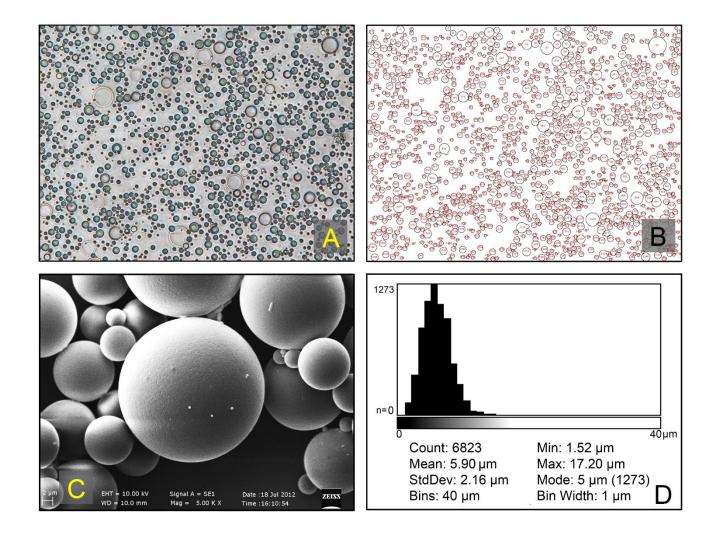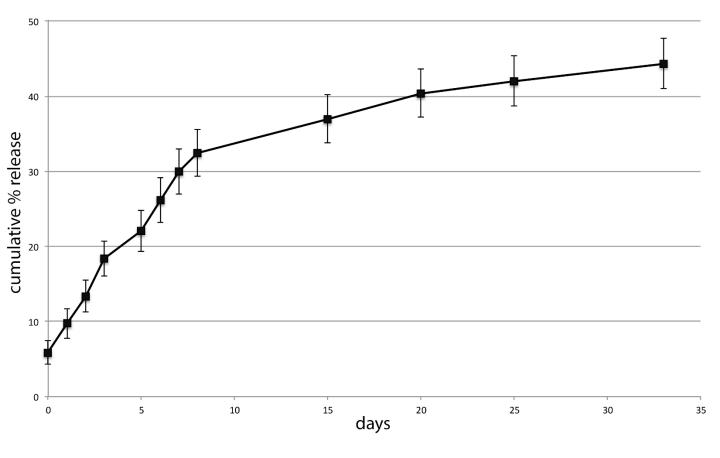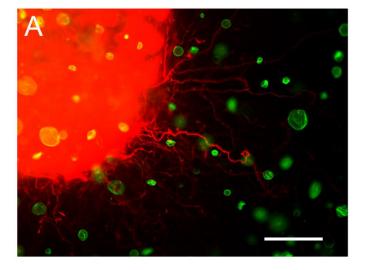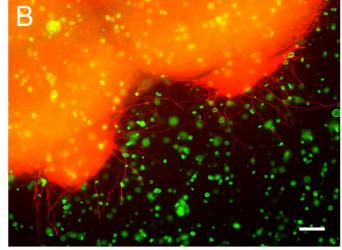
Fig. 1

A

B

C — EHT = 10.00 kV   Signal A = SE1   Date :18 Jul 2012
WD = 10.0 mm   Mag = 5.00 K X   Time :16:10:54
ZEISS
2 µm

D
Count: 6823   Min: 1.52 µm
Mean: 5.90 µm   Max: 17.20 µm
StdDev: 2.16 µm   Mode: 5 µm (1273)
Bins: 40 µm   Bin Width: 1 µm
1273
n=0
0   40 µm

Fig. 2

Fig. 3

Fig. 4

Fig. 5

G 0 weeks (no preconditioning)

H 0 weeks (no preconditioning)

I 1 week of preconditioning

J 1 week of preconditioning

Fig. 6

Fig. 7

Fig. 8

G