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Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders

Xudong Cao[a], Molly S. Shoichet[a, b, *]

[a]Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ontario, M5S 3E5 Canada
[b]Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario, M5S 3H6 Canada

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Abstract

Nerve growth factor (NGF) may enhance axonal regeneration following injury to the central nervous system (CNS), such as after spinal cord injury. The release profile of NGF, co-encapsulated with ovalbumin, was tailored from biodegradable polymeric microspheres using both polymer degradation and protein loading. Biodegradable polymeric microspheres were prepared from PLGA 50/50, PLGA 85/15, PCL and a blend of PCL/PLGA 50/50 (1:1, w/w), where the latter was used to further tailor the degradation rate. The amount of protein loaded in the microspheres was varied, with PCL encapsulating the greatest amount of protein and PLGA 50/50 encapsulating the least. A two-phase release profile was observed for all polymers where the first phase resulted from release of surface proteins and the second phase resulted predominantly from polymer degradation. Polymer degradation influenced the release profile most notably from PLGA 50/50 and PLGA 85/15 microspheres. The amount and bioactivity of released NGF was followed over a 91 d period using a NGF-ELISA and PC12 cells, respectively. NGF was found to be bioactive for 91 d, which is longer than previously reported.

Keywords: Biodegradation; Polymer; Drug delivery; Nerve growth factor; Controlled release; Central nervous system

1. Introduction

Traumatic injury to the central nervous system (CNS), such as in spinal cord injury (SCI), results in axonal degeneration, neuronal necrosis, gliosis, and proliferation of reactive astrocytes. Following SCI, victims are left paralyzed, with only palliative measures available but no therapy to restore function.

To overcome SCI, a pathway must be provided to guide axons across the site of injury; once across, the myelin inhibitory factors must be neutralized to permit reconnection of axons above and below the site of injury. The suitable pathway must be permissive to axons, guiding them toward their targets. In development, axons are guided to their targets by a combination of contact-mediated and diffusible cues that are either attractive or repulsive. For example, neurotrophic factors have been implicated as diffusible cues in axon guidance.

Of the numerous neurotrophic factors, nerve growth factor (NGF) has been the best characterized. NGF has been reported to promote both the growth and survival of axons of the peripheral and central nervous systems [1]. The delivery of axon-specific growth factors may promote axonal regeneration following SCI. NGF has been shown to augment axonal growth after injury; however, alone, it has not enhanced functional recovery following SCI [2]. NGF has also been investigated for its involvement in the neuropathy of Alzheimer's disease and its administration to the brain for a prolonged period has been suggested as a potential treatment [3].

Controlled release of growth factors can be achieved by many methods [4], such as delivery from polymeric microspheres or discs [5], mini-pumps [6], or transplanted cells engineered to produce NGF [7]. Using biodegradable polymeric microspheres, delivery increases with protein loading, with polymer degradation rate and inversely with the size of the microspheres [8].

Degradable polymers, such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL), are used in medical applications including surgical sutures, drug

* Corresponding author. Tel.: 416 978 1460; fax: 416 978 8605; e-mail: molly@ecf.utoronto.ca

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delivery devices, and reconstructive implants [9]. These polymers degrade by hydrolytic cleavage of the backbone ester bond to alcohol and carboxylic acid, the latter of which catalyzes further degradation. To overcome the precipitous polymer mass loss and increased local acidity, a blend of PLAs with different molecular weights was previously described [10].

In this paper, we describe the encapsulation, delivery and bioactivity of NGF over a prolonged period. We demonstrate controlled release of bioactive NGF for 91 d, which is more than twice as long as previously reported [11]. The importance of both polymer degradation and protein loading are elucidated using polymeric microspheres as the vehicle to encapsulate and deliver proteins. Polymeric microspheres, prepared by a modified solvent evaporation technique, were composed of one of the four polymers: PLGA 50/50, PLGA 85/15, PCL and a blend of PCL/PLGA 50/50 (1:1, w/w). The blend of PCL and PLGA 50/50 was prepared to better control degradation behaviour. The delivery of ovalbumin (OVA) served as a model for NGF release because both proteins have similar molecular weights of 45 000 g mol^{-1} and 26 000 g mol^{-1} for the β-subunit, respectively. By encapsulating a protein powder, as opposed to a protein solution, the proteins (OVA and NGF) were exposed to a reduced shear stress during encapsulation; however, large or irregular protein particles may have been incompletely encapsulated. NGF was co-encapsulated with OVA at three mass ratios of NGF:OVA of 1:2000, 1:10 000 and 1:100 000, with OVA serving as a pore-forming agent for NGF release and possibly a stabilizing agent for prolonged NGF activity [12]. By varying the NGF to OVA ratio, we were able to control the amount of NGF released while determining the minimum amount required for prolonged bioactivity. NGF release was followed by a NGF-enzyme linked immunosorbent assay (ELISA). The bioactivity of released NGF was followed with pheochromocytoma (PC12) cells which differentiate to a neuronal phenotype in the presence of bioactive NGF [13, 14].

2. Materials and methods

All chemicals were purchased from Sigma (St. Louis, MO) and used as received unless otherwise indicated. Analytical reagent grade sodium chloride, calcium chloride, sodium carbonate and sodium bicarbonate were purchased from BDH (Toronto, ON) and magnesium chloride was purchased from APC Chemical Inc. (Montreal, Que.). Poly(D,L-lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL) were purchased from Birmingham Polymers, Inc. (Birmingham, AL). For each polymer, the intrinsic viscosity measured in chloroform at 30°C is indicated in parentheses: (1) PLGA 50/50 (0.53 dl g^{-1}); (2) PLGA 85/15 (1.11 dl g^{-1}); and (3) PCL (1.21 dl g^{-1}). Poly(vinyl alcohol) (PVA, M_w = 6000 g mol^{-1}, 80% hydrolyzed) was purchased from Polysciences Inc. (Warrington, PA). Mouse nerve growth factor-β (NGF) was purchased from Cedarlane Laboratory (Hornby, ON) and the reagents for the NGF-ELISA were purchased from Boehringer Mannheim, Germany unless otherwise indicated. The adrenal rat PC12 cell line was purchased from American Type Culture Collection (ATCC) (Rockville, MD). Deioinized distilled water was obtained from Milli-RO 10 Plus and Milli-Q UF Plus system (Bedford, MA) and used at 18 MΩ resistance.

2.1. Preparation and characterization of microspheres

Microspheres were fabricated by a modified solvent evaporation process [15, 16]. Briefly, 0.2 g of finely ground OVA powder was dispersed in 4 ml of a 25% (w/v) solution of PLGA 85/15 in chloroform using a Polytron homogenizer for 90 s at 7500 rpm (PT3000, Brinkman, Westbury, NY). The protein–polymer dispersion was added to 20 ml of an aqueous 1% PVA solution and homogenized for an additional 90 s at 7500 rpm, thereby forming an emulsion. The emulsion was added to 300 ml of 0.1% PVA solution that was continuously stirred for 3 h at room temperature (RT) to evaporate the organic solvent. The microspheres were centrifuged, washed repeatedly with distilled water and then freeze-dried (Labconco, Kansas City, MO) for 48 h to obtain free flowing microspheres. Using the same methodology, microspheres were prepared from PLGA 50/50, PCL and a blend of PCL/PLGA 50/50 (1:1, w/w). Microspheres were stored desiccated at −20°C prior to use. The microspheres used for degradation studies were prepared identically except without the protein powder.

NGF was co-encapsulated with OVA in PLGA 85/15 microspheres only as described above for OVA, using the same total protein to polymer loading of 20% (w/w). The release of OVA serves as a model for NGF release from all four types of microspheres whereas the bioactivity of NGF released from PLGA 85/15 serves as a model for NGF bioactivity released from the other polymeric microspheres. Three NGF:OVA (w/w), ratios were compared in terms of release and bioactivity: 1:2000 (NGF-1), 1:10 000 (NGF-2) and 1:100 000 (NGF-3). Control microspheres were loaded with OVA alone.

Microspheres were characterized for size and distribution using a Coulter® counter (Model ZM, Coulter Electronics Limited, England) and for surface morphology by a scanning electron microscope (SEM, HITACHI Model S-4500). For Coulter® counting, approximately 0.1 g of microspheres was suspended in 20 ml of a balanced electrolyte solution (Isoton® II, Coulter corporation, Miami, FL), gently vortexed and transferred to a counting chamber. The relative number of microspheres was counted within increments of 5 μm, starting from 0.2 μm up to 100 μm. SEM micrographs were taken, at an acceleration

voltage of 1.0 kV, of samples mounted on metal studs and sputter-coated with gold for 15 s.

2.2. Degradation [17, 18]

Using a Sartorius MC5 microbalance (Germany), 10 mg of one microsphere type (PLGA 50/50, PLGA 85/15, PCL or blend of PCL/PLGA 50/50) was sealed in a nylon mesh bag (8 μm mesh size, Spectrum, Houston, TX), immersed in 20 ml of a phosphate-buffered saline solution (PBS, pH 7.4) with 1% penicillin–streptomycin (P/S) and incubated at 37°C. At one week intervals, for up to 12 weeks, 3 samples from each microsphere type were collected, washed repeatedly with deionized water and freeze-dried for 48 h prior to analysis. The PBS was maintained at a constant pH 7.4.

Degraded samples were characterized over time by mass loss, gel permeation chromatography (GPC, Varian 5000) and SEM. Mass loss was calculated according to Eq. [1] by comparing the initial mass (W_0) to that at a given time point (W_t). Measurements were performed in triplicate, and the results are presented as the mean ± standard deviation.

$$\text{Mass loss } \% = [(W_0 - W_t)/W_0] \times 100\% \quad (1)$$

Polymer molecular weight and distribution were determined using a GPC equipped with a refractive index detector (Waters R-400) and ultrastyragel columns (500 and 100 000 Å). The columns were calibrated with monodisperse polystyrene standards ($M_p = 4000$, 20 000, 63 000, and 200 000, Polysciences, Warrington, PA) using tetrahydrofuran as the mobile phase. To further ensure constant column conditions, toluene was added to each solution of dissolved microspheres thereby serving as an internal standard between samples.

2.3. Protein distribution within microspheres

Protein-encapsulated PLGA 85/15 microspheres were embedded in Tissue-Tek 4538 (Miles, IN), frozen to −20°C and then serially sectioned in a cryostat at a thickness of 6 μm. The sections were dried at RT and the proteins were stained with Ladd's multiple stain (Ladd Industries, Burlington, VT). The stained, cross-sectioned microspheres were mounted (EM Lab, Oshawa, ON) and photographed under a light microscope (Leitz, Germany).

2.4. Efficiency of encapsulation

To determine the amount of protein encapsulated in the microspheres, 0.2 g of OVA-encapsulated microspheres were dissolved in 100 ml of chloroform to which 150 ml of deionized water was added. Using a separatory funnel, three layers resulted, consisting of an aqueous (top layer), an organic (bottom layer) and a milky middle layer. The chloroform from the middle layer was removed by rotary evaporation (Brinkman, Westbury, NY) at 37°C and the remaining aqueous solution was combined with the aqueous (top) layer from the separatory funnel. The total amount of protein recovered was calculated by comparing the absorbance at 280 nm determined by ultraviolet-visible spectrophotometry (UV-vis, Hewlett Packard 8452A) to a calibration curve. The efficiency of encapsulation was calculated according to Eq. (2) by comparing the amount of protein recovered ($W_{\text{recovered}}$) to the amount of protein originally dispersed in the polymer solution ($W_{\text{theoretical}}$). All measurements were done in triplicate and the results are presented as the mean ± standard deviation.

$$\text{efficiency of encapsulation} = (W_{\text{recovered}}/W_{\text{theoretical}}) \times 100\% \quad (2)$$

This method was validated by adding a 0.2 g of OVA powder to 4 ml of a 25% polymer solution in chloroform in a Teflon® mold. The organic solution was removed under vacuum, resulting in a polymeric disc. The OVA was recovered by dissolving the polymeric disc in chloroform and extracting the protein into the aqueous phase as described above. This was done in triplicate for PLGA 85/15 and PCL. Given the relatively low amount of NGF added (1:2000, w/w was the highest mass ratio of NGF:OVA used), the efficiency of encapsulating OVA was assumed to be representative of that for OVA and NGF.

2.5. Protein release: OVA

For each polymer, 0.5 g of OVA-encapsulated microspheres were added to 10 ml of PBS (pH 7.4) containing 1% P/S and incubated at 37°C. At each time point, the microspheres were centrifuged (10 min at 1000 rpm), 5 ml of supernatant was collected and replaced with 5 ml of fresh PBS. Microspheres were re-suspended by vortexing for 5 min. The supernatant was analyzed by UV-vis for absorbance at 280 nm. Control samples of polymeric microspheres alone (i.e. without encapsulated proteins) were treated identically, with the supernatant analyzed by absorbance at 280 nm for any polymer degradation products. The amount of protein present in the supernatant was then calculated by comparison to a calibration curve after subtracting the absorbance resulting from any polymer degradation products.

2.6. Protein release: NGF

For PLGA 85/15, 0.5 g of microspheres (i.e., NGF-1, NGF-2, NGF-3 and OVA) were immersed in 10 ml of PBS (pH 7.4, 1% P/S) and incubated at 37°C. At each

time point, the suspension of microspheres was centrifuged (10 min, 1000 rpm), 5 ml of the supernatant was collected and replaced with 5 ml of fresh PBS followed by vortexing. The supernatant samples were stored at −80°C prior to analysis. The concentration of NGF released from microspheres over time was measured, in triplicate, by the NGF-ELISA [19] in comparison to a calibration curve; absorbance was measured at 640 nm using a Thermo Spectro III plate reader (Labinstruments, Australia).

2.7. Bioactivity of released NGF

The bioactivity of NGF released from PLGA 85/15 microspheres was assessed using the PC12 single clonal cell line [20] over a 91 d period. PC12 cells are known to differentiate to a neuronal phenotype in response to NGF. Of the 5 ml of stored supernatant, 1.5 ml from each sample was sterile-filtered through a Millex®-GV filter unit (0.22 μm, Millipore) and incubated with PC12 cells that had been plated on poly(L-lysine) (PLL) pre-coated 6-well plates with a concentration of 1.0×10^4 cells cm^{-2}. The number of neurites extending per cell body and the number of cells having one or more neurites greater than the cell body length (for 50 cell bodies) were counted under an optical microscope for every sample after 24 h of incubation. Controls consisted of PC12 cells incubated with medium alone (Blank) or medium supplemented with the supernatant released from OVA-loaded microspheres (OVA). The positive control consisted of PC12 cells incubated with the 25 ng ml^{-1} (or 1 ng ml^{-1}) of NGF-supplemented cell culture medium (NGF).

2.8. PC12 cell culture

PC12 cells were maintained in T-25 cell culture flasks (Falcon, Franklin Lakes, NJ) at 1.0×10^5 cells ml^{-1} in medium. The cell culture medium consisted of 84% RPMI 1460, 10% heat-inactivated horse serum, 5% fetal bovine serum and 1% P/S. Cells were incubated at 37°C in a 5% CO_2 in air atmosphere, [14, 21]. Cell medium was changed every other day and cells were subcultured once per week. All cells used in the bioactivity studies were within 3 passages.

2.9. Statistics

Data obtained from the NGF bioactivity study were analyzed by SAS (SAS Institute Inc., Cary, NC). One way ANOVA with 6 different levels was employed (for NGF-1, NGF-2, NGF-3, Blank, OVA, and NGF). Statistical analysis was carried out with a 95% confidence level with Duncan multiple comparisons.

3. Results

3.1. Characterization of microspheres

The microspheres that were prepared by the solvent evaporation technique, with and without OVA encapsulated, were compared by size and distribution using the Coulter® counter. Given the relatively low amount of NGF used, we assumed that OVA-encapsulated microspheres were representative of OVA/NGF-encapsulated microspheres. As shown in Table 1, the average size of all microspheres was similar. The protein release profile is affected by microsphere size with the smaller microspheres having the highest surface area to volume ratio and thus both the fastest release and degradation profiles.

The surface morphology of microspheres was further characterized by SEM. As shown in Fig. 1, empty microspheres had a smooth outer surface morphology whereas protein-encapsulated microspheres had a rough outer surface morphology. The porous outer surface morphology of protein-encapsulated microspheres (cf. Fig. 1c) likely resulted from the dissolution of the protein which was either on or interconnected to the outer surface during the wash-up process. To determine the distribution of proteins encapsulated in the microspheres, microspheres were cryo-sectioned and stained with Ladd's reagent. As shown in Fig. 2, the encapsulated proteins were distributed throughout the microspheres. The relative volume percent of protein to polymer in the microspheres was estimated, from the two-dimensional cross-sectional areas, to be 17.5%.

3.2. Efficiency of encapsulation

The amount of protein encapsulated in each of the polymeric microspheres was further determined by calculating the amount of protein that could be recovered after dissolving microspheres in chloroform and extracting the proteins into water. The validation method indicated that 95.7 ± 4.6% of the total protein could be recovered. As shown in Table 2, the efficiency of encapsulation differed for each polymer investigated, with PCL having

Table 1
Average size (± standard deviation) of microspheres ($n > 1500$)

Microsphere	Microsphere diameter (μm)
PLGA50/50	17.0 ± 4.3
PLGA 85/15	16.8 ± 5.0
PCL	20.1 ± 4.7
Blend: PCL/PLGA 50/50	17.7 ± 4.9
PLGA 50/50 + OVA	22.3 ± 5.2
PLGA 85/15 + OVA	22.7 ± 5.4
PCL + OVA	18.2 ± 5.3
Blend + OVA	19.4 ± 5.6

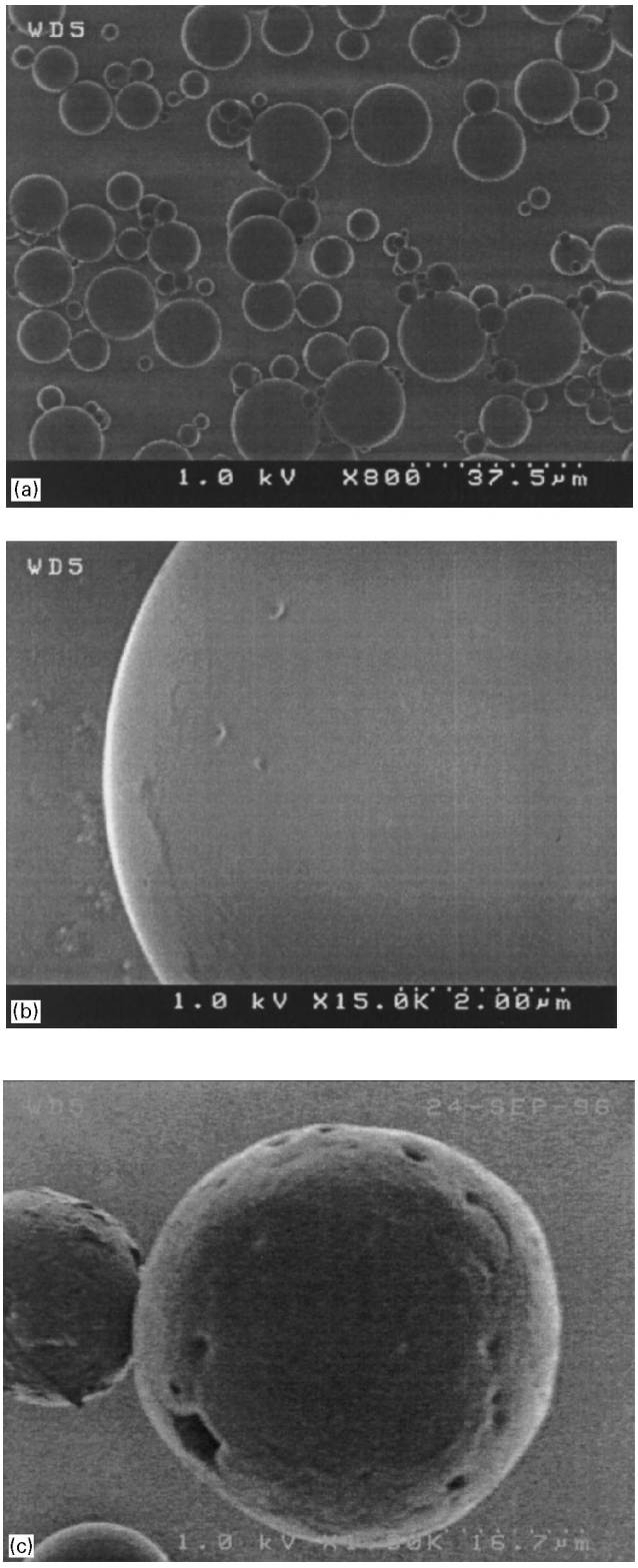

Fig. 1. Scanning electron micrographs demonstrate the (a) size distribution of microspheres, (b) smooth outer surface morphology of empty microspheres and (c) rough outer surface morphology of protein-encapsulated microspheres.

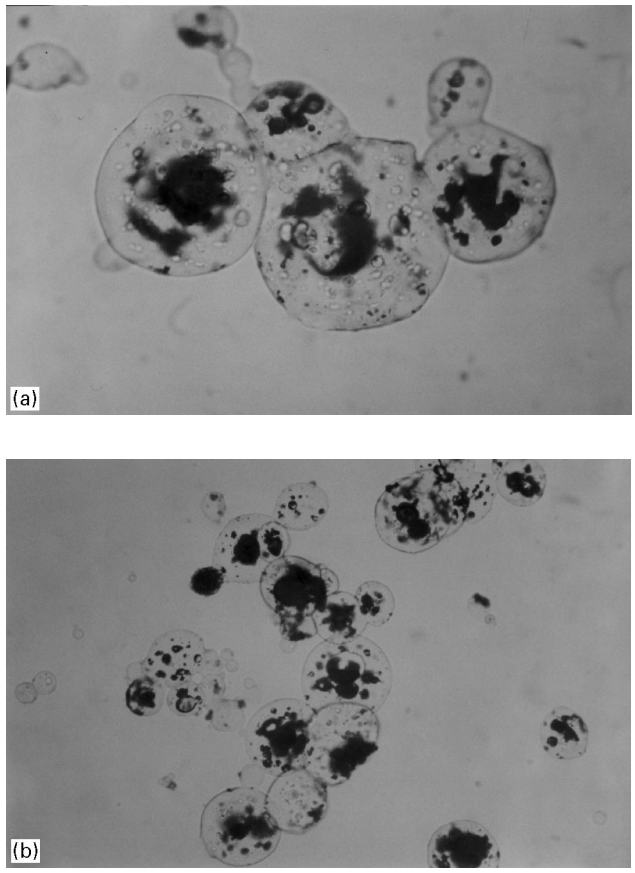

Fig. 2. Optical light micrographs demonstrate the distribution of proteins in cryo-sectioned PLGA 85/15 microspheres at a magnification of (a) ×400 and (b) ×150.

the highest efficiency of encapsulation and PLGA 50/50 having the lowest. Differences in protein loading reflect differences in the rate of polymer precipitation and impact the protein release profile.

3.3. Degradation of microspheres

Empty microspheres were immersed in PBS at 37°C, maintained at a constant pH 7.4 and characterized for 84 d in terms of mass loss, change in molecular weight by GPC and change in morphology by SEM. As shown in Fig. 3, after 84 d PLGA 50/50 microspheres degraded completely, PLGA 85/15 by 80% and PCL by less than 30% relative to initial mass. As expected, the blend of PCL/PLGA 50/50 microspheres degraded, at a rate between PCL and PLGA 50/50, by 56% relative to initial mass. All of the microspheres (except PCL) showed an increase in rate of mass loss between 28 and 50 d, as demonstrated by an increase in slope. The change in slope was particularly notable for PLGA 50/50 between 28 and 42 d and can be attributed to the autocatalytic hydrolytic degradation mechanism where released carboxylic acids contribute to an increased rate of hydrolysis along the polymer backbone. Within this 14 d period,

Table 2
Efficiency of encapsulation

Microsphere	Percentage of protein encapsulated (%)
PLGA 50/50	14.9 ± 1.5
PLGA 85/15	23.6 ± 1.8
PCL	26.8 ± 2.2
PCL/PLGA 50/50	19.8 ± 2.0

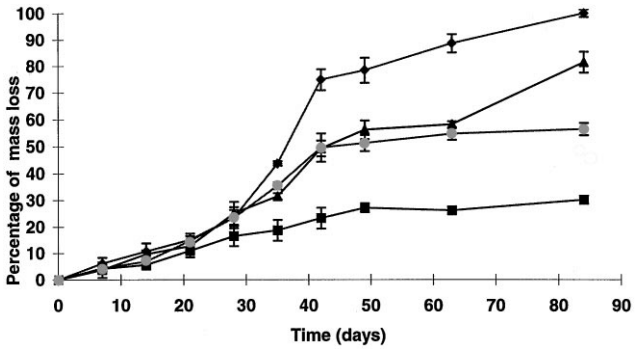

Fig. 3. The degradation profile of microspheres was characterized by mass loss over time for (◆) PLGA 50/50, (▲) PLGA 85/15, (●) the blend of PCL/PLGA 50/50; and (■) PCL.

PLGA 50/50 lost 50% of its mass whereas the blend of PCL/PLGA 50/50 lost only 25% of its mass. Thus by blending polymers with inherently different degradation rates, the degradation profile and amount of acid released to the surrounding aqueous media over time can be controlled. The mass loss data were further supported by the change in molecular weight data obtained by GPC. After 84 d, PLGA 50/50 microspheres degraded completely, PLGA 85/15 degraded by 90% relative to initial molecular weight, PCL by 25% and the blend of PCL/PLGA 50/50 by 30%. A sharp decrease in molecular weight was observed for PLGA 50/50 microspheres between 28 and 42 d, from 12.5 to 7.1 kg mol^{-1}.

The degradation of polymeric microspheres was confirmed by SEM micrographs. The initially smooth empty microspheres (cf. Fig. 1a and b) had a different surface morphology after 35 and 70 d of incubation in PBS. As shown in Fig. 4, after 35 d of incubation, all of the microspheres retained their spherical shape except those of the polymer blend which showed a dramatically different morphology (cf. Fig. 4d). The morphology changes of the blend likely resulted from the degradation of the PLGA 50/50 component and may reflect phase separation in the blend. SEM micrographs of PLGA 50/50 and PLGA 85/15 microspheres showed several pores on the outer surface, likely resulting from the leaching out of water soluble degradation products. PCL microspheres showed very little, if any, change in outer surface morphology after 35 d. After 70 d of incubation, a more dramatic change in microsphere shape was observed, as shown in Fig. 5. PLGA 50/50 microspheres no longer retained their spherical shape, having a hollow structure and a highly porous skin that resulted from heterogeneous degradation (cf. Fig. 5a). PLGA 85/15 microspheres also lost their spherical shape and seemed to aggregate together (cf. Fig. 5b). Unlike the other polymeric microspheres, those of PCL maintained the same spherical shape having a smooth outer surface morphology (cf. Fig. 5c). The blend of PCL/PLGA 50/50 showed more of the same heterogeneous change in outer surface morphology at 70 d as was observed at 35 d (cf. Figs. 4d and 5d). The morphology data are consistent with those of mass loss and molecular weight and impact the interpretation of protein release profiles.

3.4. Release profile of OVA

OVA is a model protein for the 2.5S subunit of NGF because they have similar molecular weights of 45 000 and 26 000 g mol^{-1}, respectively. OVA was encapsulated in each of the four polymeric microspheres and its diffusion followed over time into a constant volume of PBS, maintained at a constant pH 7.4 and 37°C. Figure 6 summarizes the cumulative protein release profile normalized to the initial amount encapsulated in the polymeric microspheres while taking any polymer degradation into account. Overall, the greatest normalized cumulative release of OVA was observed from PLGA 50/50 followed by PLGA 85/15, PCL and then by the blend of PCL/PLGA 50/50. Protein was released in two phases. Given that immediate polymer degradation was not significant, as shown by the mass loss data (cf. Fig. 3), in the first phase (i.e. within the first 14 d) the protein released was likely on or near the surface, allowing facile aqueous dissolution. The plateau region, which preceded the second release phase, was indicative of a depletion in the protein on or near the surface and the lack of other protein release mechanisms. In the second phase, between 26 and 44 d, the accelerated release profile was consistent with the accelerated degradation profile between 28 and 42 d. This effect was particularly notable for PLGA 50/50 and PLGA 85/15. Release in the second phase resulted from microsphere degradation which in turn enabled the percolation mechanism by providing increased porosity. PCL microspheres released 58% of their encapsulated proteins while losing only 30% of their mass. By calculating the total protein released (as opposed to normalized protein released of Fig. 6), the greatest amount of protein was released from PCL microspheres, which corresponds to the data showing that PCL microspheres had the greatest amount of protein encapsulated (cf. Table 2). Release from PCL likely depended upon both degradation and loading.

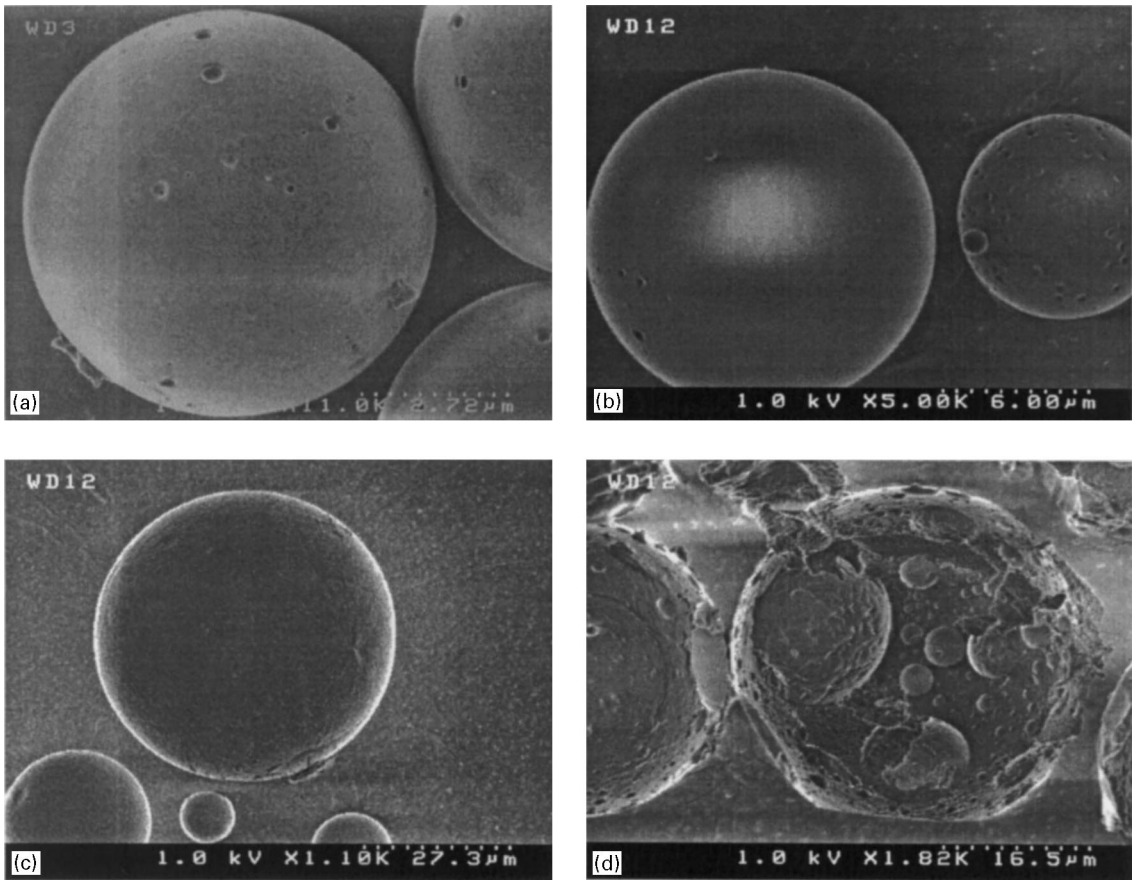

Fig. 4. Scaning electron micrographs of polymeric microspheres after 35 d of degradation: (a) PLGA 50/50, (b) PLGA 85/15, (c) PCL, (d) blend of PCL/PLGA 50/50.

3.5. Release profile of NGF

The release profile of NGF was followed from PLGA 85/15 microspheres, from which NGF and OVA were co-encapsulated at 3 mass ratios, and followed by the NGF-ELISA. As shown in Fig. 7, most of the NGF was released within the first 12 d, with a large initial burst, as was observed for the release of OVA from PLGA 85/15 microspheres. The amount of NGF released at each time point after 12 d varied between 0 and 1 ng ml^{-1} for all samples.

3.6. Bioactivity of released NGF

The bioactivity of NGF released from PLGA 85/15 biodegradable microspheres over time was followed by adding the supernatant in which microspheres were maintained to the medium in which PC12 cells were cultured. PC12 cells respond to bioactive NGF by differentiating to a neuronal phenotype. The number and relative length of neurites per cell body, averaged over 50 cells, were measured as an indicator of bioactivity and compared to a positive control of NGF-supplemented medium and to two other controls consisting of medium alone and medium supplemented with the supernatant from OVA-encapsulated microspheres. The latter served as a control for both released OVA on neurite outgrowth and any potential cytotoxic effects of the PLGA degradation products.

As shown in Fig. 8, the number of neurites per cell body was followed over time for each group. Initially, NGF-1 samples (i.e. NGF : OVA $= 1:2000$, w/w) had the same effect as the NGF-supplemented group in stimulating neurite outgrowth ($P < 0.0001$). At 48 d and beyond, the NGF released from NGF-1 samples promoted less neurite outgrowth than the NGF-supplemented group indicating that either significantly less NGF was released by the microspheres than was supplemented into the medium by the positive control (i.e. 25 ng ml^{-1}) or the released NGF was inactive. To determine why the released NGF effected less neurite outgrowth than the NGF 25 ng ml^{-1} positive control, PC12 cell cultures were supplemented with 1 ng ml^{-1} NGF, a concentration closer to that observed by the NGF-ELISA at 48 d. PC12 cells supplemented with 1 ng ml^{-1} of NGF extended an average of 1.8 ± 0.6 neurites per cell body ($n = 50$). By comparing the number of neurites per cell body data for NGF-1 samples, NGF (1 ng ml^{-1})

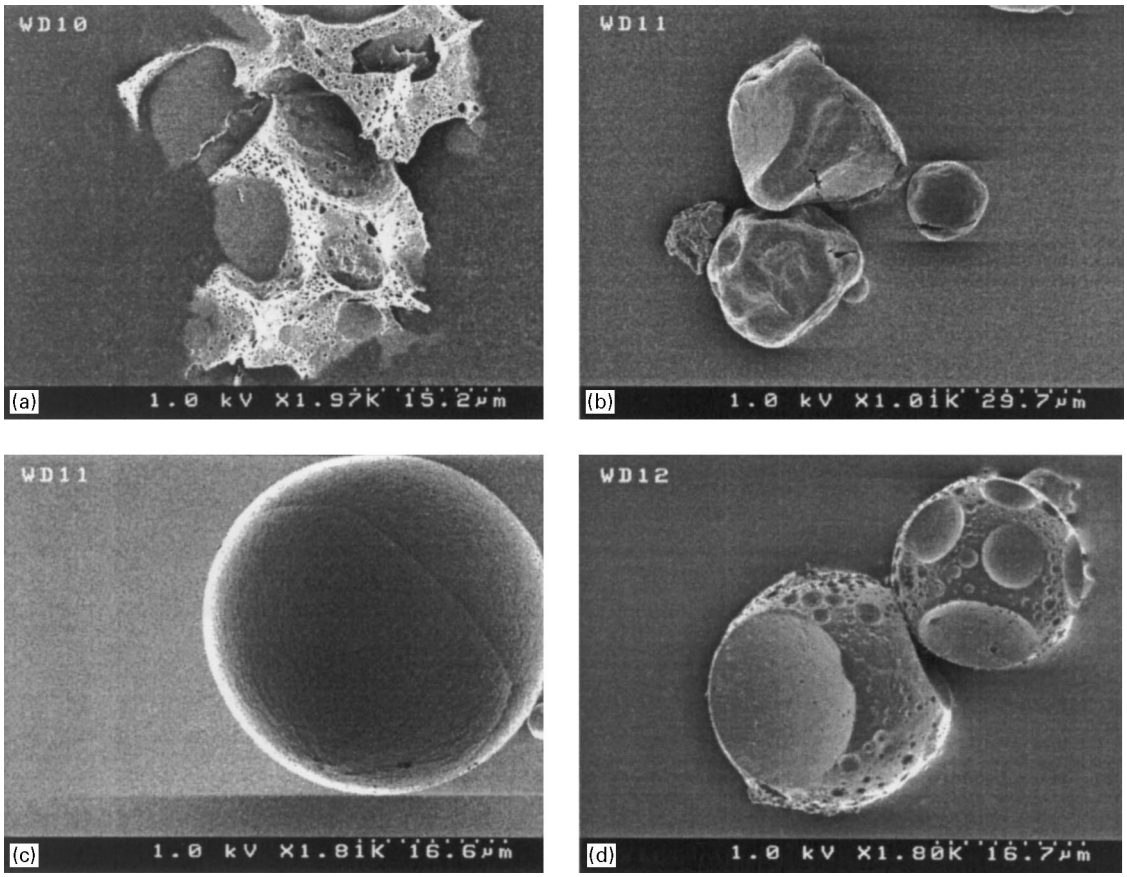

Fig. 5. Scanning electron micrographs of polymeric microspheres after 70 d of degradation: (a) PLGA 50/50, (b) PLGA 85/15, (c) PCL, (d) blend of PCL/PLGA 50/50.

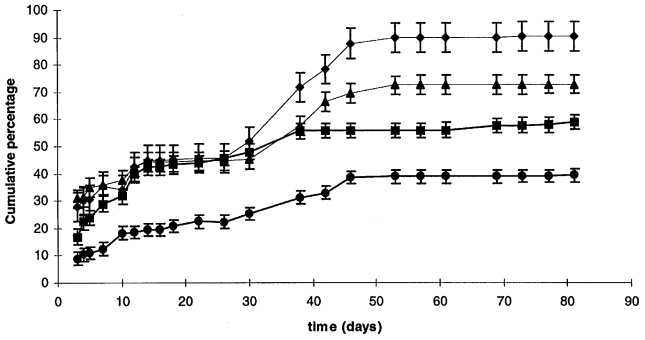

Fig. 6. Normalized cumulative release profile of OVA from biodegradable polymeric microspheres with respect to the initial amount of OVA encapsulated for (◆) PLGA 50/50, (▲) PLGA 85/15, (●) the blend of PCL/PLGA 50/50; and (■) PCL.

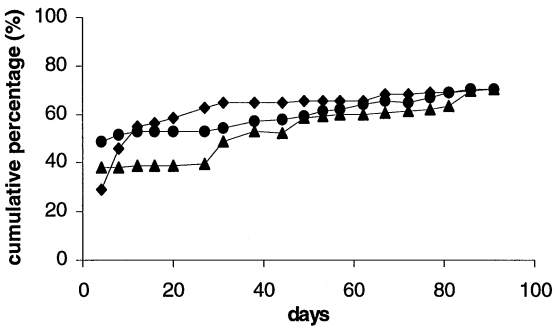

Fig. 7. Normalized cumulative release profile of NGF from PLGA 85/15 microspheres, with respect to the total amount of NGF encapsulated, as determined by the NGF-ELISA. Protein loading was compared for NGF : OVA, w/w (●) 1 : 2000, NGF-1; (◆) 1 : 10 000, NGF-2; (▲) 1 : 100 000, NGF-3.

supplemented controls, Blank controls and OVA controls, no significant difference was observed for the 91 d period of NGF release from NGF-1 and NGF (1 ng ml^{-1}) controls ($P < 0.05$); however, at 91 d of release, significantly more neurite outgrowth was observed in NGF-1 samples [and NGF (1 ng ml^{-1}) controls] than both Blank and OVA controls ($P < 0.05$). Together, this indicates that the NGF released from PLGA 85/15 microspheres was bioactive for 91 d. Initially, NGF-2 and NGF-3 samples had a significantly greater effect on neurite outgrowth than those of the Blank and OVA controls, suggesting that NGF released from these samples was bioactive, but at a concentration lower than

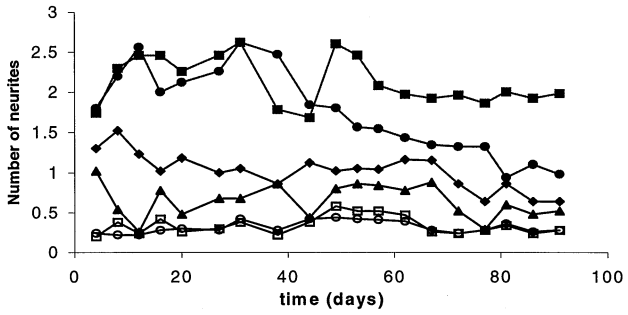

Fig. 8. Bioactivy of NGF was assessed after 24 h of incubating released NGF with PC12 cells by assessing the average number of neurites per cell body for (●) NGF-1, (◆) NGF-2, (▲) NGF-3, (■) NGF, (○) OVA and (□) Blank. (For each point, $n = 50$ and the standard deviation is ± 0.5).

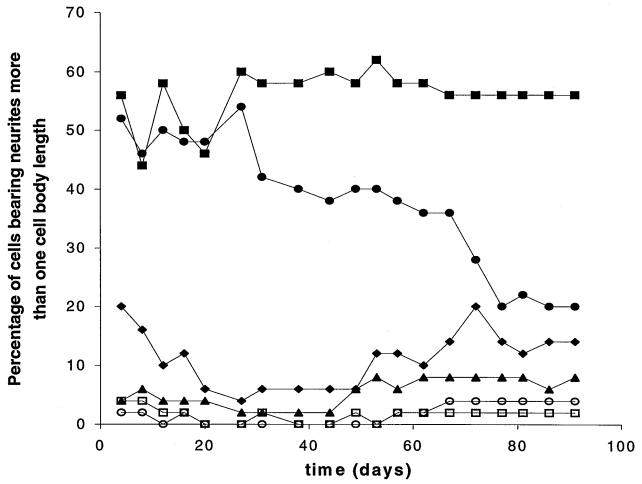

Fig. 9. Bioactivty of NGF over time was assessed after 24 h of incubating released NGF with PC12 cells by calculating the percentage of cells bearing one or more neurites that exceeded the cell body length for (●) NGF-1, (◆) NGF-2, (▲) NGF-3, (■) NGF, (○) OVA and (□) Blank.

both NGF-1 and the NGF (25 ng ml^{-1}) control. This is consistent with the lower amount of NGF released as determined by the NGF-ELISA assay. Compared to the Blank and OVA controls, an insignificant amount of neurite outgrowth was induced by NGF released from NGF-2 and NGF-3 after 24 and 20 d, respectively.

NGF activity was also followed over time by calculating the percentage of cells bearing one or more neurites longer than the cell body length for the three samples and three controls. As shown in Fig. 9, these data confirmed those in Figs. 7 and 8. The NGF released from NGF-1 samples showed the same bioactivity as the 25 ng ml^{-1} NGF-supplemented control for the first 28 d and the same bioactivity as the 1 ng ml^{-1} NGF-supplemented controls, which had 40% of cells with neurites longer than cell body length, up to 66 d. NGF-1 samples maintained greater activity than the Blank and OVA controls up to 91 d. NGF-2 samples demonstrated bioactivity for the 91 d period, but had a significantly lower response than the 25 or 1 ng ml^{-1} NGF-supplemented controls. NGF-3 samples demonstrated a PC12 cell response only slightly higher than those of the Blank and OVA controls.

4. Discussion

The release profiles of OVA and NGF from polymeric microspheres were controlled by a combination of polymer degradation and protein loading where a pathway for release was provided by both the degraded polymer and dissolved protein (i.e. percolation). By comparing the degradation profiles to the release profiles, the mechanism for release can be further probed, given that the protein is insoluble in and cannot diffuse through the polymer phase. By the degradation-controlled mechanism, proteins diffuse through pores that are formed as a result of degradation. By the percolation (diffusion controlled) mechanism, proteins diffuse through water-filled pores that form interconnected channels within the polymer matrix [27].

A pathway for protein release was provided by microsphere degradation where water-soluble degradation products (i.e. monomers and oligomers) leave the microsphere matrix for the surrounding aqueous medium. Since oligomers close to the surface can leach out faster than those located deeper within the matrix, carboxylic acid oligomers trapped within the matrix autocatalyze further ester bond hydrolysis, resulting in the increased rate of mass loss shown in Fig. 3. The mass-to-volume ratio of microspheres to PBS was sufficiently low not to affect the pH in the studies described herein; however, in vivo, the accelerated rate of polymer degradation, accompanied by a greater release rate of carboxylic acid groups, may result in an acidic local environment that may, in turn, induce an inflammatory response. The microspheres that were prepared from a blend of PCL and PLGA 50/50 demonstrated a degradation profile intermediate between those of PCL and PLGA 50/50, thereby providing a facile method to control degradation. As shown by the SEM micrographs in Figs. 4 and 5, the microspheres prepared from the blend of PCL/PLGA 50/50 and from PLGA 50/50 degraded heterogeneously. Given that PLGA 50/50 microspheres had an average diameter of 17.0 $\pm$ 4.3 μm, the hollow sphere structure observed after 70 d of incubation in PBS (cf. Fig. 5a) disproves Vert's [18] prediction that devices smaller than 200 μm degrade homogeneously.

The accelerated polymer degradation observed between 28 and 42 d, particularly for PLGA 50/50 and PLGA 85/15, corresponded to an increased rate of OVA release. Others have also shown a correlation between increased protein release and polymer degradation [22].

The pronounced effect of degradation on the release of OVA from PLGA 50/50 and PLGA 85/15 suggests that the degradation mechanism played a dominant role in the second phase of its the release.

The percolation mechanism is determined by the method and amount of protein loading and the additional porosity created upon degradation. By encapsulating protein powder, a rough outer surface morphology resulted, as shown in Fig. 1c, indicating that some of the encapsulated proteins were on the surface. The initial OVA diffusion preceded significant polymer degradation, indicating that proteins on the surface were solubilized, thereby providing a pathway for the release of additional proteins near the surface. For example, within the first 10 d, 18–35% of OVA was released from all polymeric microspheres while less than 5% of mass loss was observed. Since OVA's release from these polymeric microspheres by partition diffusion was minimal, proteins diffused from water filled pores.

The amount of protein encapsulated was determined quantitatively by the efficiency of encapsulation results. The efficiency of encapsulating OVA depended upon the chemical structure/hydrophobicity of the polymer, with the faster precipitating polymers (i.e. PCL) having the highest efficiency of encapsulation (cf. Table 2). Others have shown that the amount of protein encapsulated depends upon (1) hydrophobicity [23] and solubility [24] of the protein and (2) viscosity of both the aqueous and organic phases, the latter of which is affected by polymer concentration [25] and molecular weight [26]. Consistent with the protein loading data, PCL released the greatest amount of protein. Given that the relative volume percent of protein to polymer was estimated at 17.5% (cf. Fig. 2), initially, protein-encapsulated microspheres had insufficient porosity for a percolating porous network [27].

4.1. Bioactivity of NGF

The stability of proteins at physiological conditions is limited while that of encapsulated proteins may be further reduced by exposure to organic solvents, shear, and acidic degradation products. To assess bioactivity, the response of PC12 cells to released NGF was monitored over a 91 d period. Since PC12 cells respond reversibly to NGF by induction of a neuronal phenotype, a correlation exists in the amount of NGF present and neurite outgrowth. Since the positive control had 25 ng ml^{-1} of NGF supplemented to the medium, which was significantly more NGF than was released from the microsphere samples, the results may be misinterpreted. For example, NGF-1 samples induced less differentiation than the positive control after 48 d, indicating that either the released NGF was inactive or more simply had a lower concentration than the positive control. The ELISA data clearly demonstrated that the concentration of released NGF was less than 1 ng ml^{-1} after 12 d of release. By comparing the PC12 cell response to supplemented medium containing 1 ng ml^{-1} NGF to the NGF-1 samples, it is clear that the released NGF is active for at least 62 d; in comparison to the Blank and OVA controls, NGF released from NGF-1 is active for 91 d.

The prolonged stability of encapsulated NGF is substantially longer than previously reported [11] and may result from a combination of factors. For example, co-encapsulation of OVA may stabilize NGF by providing some buffering capacity [12]. In addition, NGF may experience a lower than normal shear in the processing step because it was dispersed as a powder and not as a dissolved solution.

5. Conclusions

The protein release profile of OVA and NGF from polymeric biodegradable microspheres was found to depend predominantly upon the degradation mechanism. Released NGF remained bioactive for a prolonged period of 91 d, which is longer than previous reports. The induction of neurite outgrowth observed with PC12 cells lays the foundation for future studies aimed at following the potential of NGF, and other important trophic factors, to guide axons for use in regeneration studies.

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