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Stimulation of neurite outgrowth by neurotrophins delivered from degradable hydrogels

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Abstract

Degradable hydrogels are useful vehicles for the delivery of growth factors to promote the regeneration of diseased or damaged tissue. In the central nervous system, there are many instances where the delivery of neurotrophins has great potential in tissue repair, especially for treatment of spinal cord injury. In this work, hydrogels based on poly(ethylene glycol) that form via a photoinitiated polymerization were investigated for the delivery of neurotrophins. The release kinetics of these factors are controlled by changes in the network crosslinking density, which influences neurotrophin diffusion and subsequent release from the gels with total release times ranging from weeks to several months. The release and activity of one neurotrophic factor, ciliary-neurotrophic factor (CNTF), was assessed with a cell-based proliferation assay and an assay for neurite outgrowth from retinal explants. CNTF released from a degradable hydrogel above an explanted retina was able to stimulate outgrowth of a significantly higher number of neurites than controls without CNTF. Finally, unique microsphere/hydrogel composites were developed to simultaneously deliver multiple neurotrophins with individual release rates.

Keywords: Neurotrophins; Drug delivery; Biomaterials; Hydrogel

1. Introduction

Disruption of the central nervous system (CNS) tissue such as the spinal cord and optic nerve can severely affect a patient's motor, sensory, and autonomic functions, and depending on the severity of the injury, the patient's quality of life can decline dramatically. Unfortunately, current clinical treatment options are severely limited and are not able to restore complete function to these individuals. In the spinal cord, one reason for the lack of sufficient treatments is the extremely complex cascade of events (e.g., inflammation, glial scarring, release of inhibitory molecules) that occurs after injury that must be addressed to restore functional recovery to the patients [1,2]. One promising therapy is the delivery of neurotrophins that can influence the local function of cells within and surrounding the injured site.

Specifically, neurotrophins have been widely investigated for their influence on cell mortality, differentiation, and function in the CNS [3]. In the spinal cord, neurotrophins can potentially promote axonal growth, neuronal survival, and plasticity after injury [4]. Lu and coworkers [5] recently illustrated the ability of NT-3 in combination with cAMP to induce regeneration of sensory axons past a spinal cord lesion. Additionally, the overexpression of neurotrophins after injury induced sprouting of corticospinal tract axons past the injury site [6]. Neurotrophins such as ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) are being investigated for a wide-range of neurological conditions of the CNS,

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including the spinal cord. Techniques such as gene therapy and delivery via stem cells, and recently, polymeric delivery vehicles are being investigated for the supplementation of neurotrophins to the injured spinal cord [3].

Biodegradable polymers have a long history of use as a method for the delivery of growth factors [7] and, thus, could be used for the controlled delivery of neurotrophins to the CNS to tissues such as the spinal cord and retina. Several polymeric systems have previously been investigated for the delivery of neurotrophins. As an example, Benoit and coworkers [8] used degradable poly(lactide-*co*-glycolide) microspheres for the delivery of several molecules, including nerve growth factor (NGF), intracranially to avoid the blood–brain barrier that had prevented previous application of the neurotrophins. Their work illustrated the ability to encapsulate NGF that was released over several months and remained active upon release from the microspheres.

In this work, we have investigated the controlled delivery of neurotrophins from an alternate synthetic polymer system, namely photopolymerizable PEG hydrogels. Specifically, the ability to alter release profiles by changing the network structure, the activity of the released factors using assays for both neurotrophin stimulated proliferation and outgrowth of neurites from retinal explants, and the introduction of a unique composite delivery system are presented.

2. Materials and methods

2.1. Macromer synthesis and hydrogel formation

The degradable macromer (acrylated PLA-*b*-PEG-*b*-PLA) was synthesized in a two-step reaction as described previously [9,10]. First, degradable lactic acid units were added to the hydroxyl end groups of poly(ethylene glycol) (PEG, MW 4000 Da, Polysciences) via a ring opening polymerization of D,L-lactide (Polysciences) by the mixture of these components at 140 °C in the presence of a catalytic amount (1/200 molar ratio to D,L-lactide) of stannous 2-ethyl hexanoate (Sigma) for 6 h under vacuum. Next, the intermediate product was cooled to room temperature and dissolved in methylene chloride. For the addition of acrylate functional groups, triethylamine was added while stirring on ice with the subsequent addition of acryloyl chloride (mixed 1:10 in methylene chloride), and continued stirring for 36 h. The final product was precipitated in cold ethyl ether, filtered, and dried under vacuum. The macromer structure was characterized with ^{1}H-NMR and showed ~100% acrylation and ~2.7 lactic acid units per side. All materials were obtained from Aldrich unless noted otherwise.

For polymerization, the macromer (10, 20, or 30 wt%) was added to phosphate-buffered saline (PBS) containing 0.05 wt% of the photoinitiator 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (I2959, Ciba Geigy). Fifty microliters of the solution was pipetted into a cylindrical mold (5 mm diameter) and exposed to ~10 mW/cm^2 ultraviolet light (365 nm, Blackray) for 10 min. These conditions were previously determined to be compatible for cell encapsulation [11].

2.2. In vitro drug release and quantification

Human CNTF, BDNF, and NT-3 (R&D Systems, Minneapolis, MN) were obtained in lyophilized form and stored at a concentration of 25 μg/mL in PBS supplemented with bovine serum albumin according to the manufacturer's protocols. The various neurotrophins were added to the macromer solutions prior to photopolymerization. After polymerization, the hydrogels ($n = 3$ per composition) were placed in eppendorf tubes containing 1 mL of PBS and placed on an orbital shaker. The PBS containing the released neurotrophins and degradation products was removed at various time points and stored at −20 °C. After the polymer had completely degraded, the neutrophin concentration was determined in all of the samples using DuoSet ELISA development kits (R&D Systems) and reported as either a cumulative amount of neurotrophin release or a fractional release with degradation time.

2.3. CNTF activity assay

The activity of CNTF released from degradable PEG hydrogels was assessed through a proliferation assay using a TF-1 cell line (ATCC) transfected with the α-subunit of the human CNTF receptor. TF-1 cells were placed in media (RPMI 1640, ATCC) supplemented with 5% fetal calf serum (Invitrogen) and CNTF at a concentration of 25 pg/mL. The CNTF was used either as received (day 0) or after 2, 5, 11, or 15 days of release from a 10 wt% hydrogel (quantified with ELISA). The released CNTF added to the culture media represents release in the 24 h prior to the stated time point (e.g., release between days 14 and 15 for the 15 day time point). Proliferation was assessed with a commercially available MTT viability assay (ATCC, 30–1010 K) after 48 h of culture without changing the media. For this assay, 100 μL of the MTT reagent (tetrazolium salt solution) was added directly to the wells and the plate was placed in an incubator at 37 °C for 4 h. The purple formazen produced by active mitochondria was solubilized by addition of 1 mL of the provided detergent solution and orbital shaking for 2 h. The absorbance of these solutions was then read at 570 nm (Molecular Devices SpectraMax 384). Results are reported as the mean ($n = 3$) and standard deviation of the absorbance normalized to the absorbance of cells cultured without CNTF in the culture media.

2.4. Neurite outgrowth from retinal explants

Globes were harvested from 5 day old B6 mice and placed in Hanks balanced salt solution containing 3% penicillin/streptomycin. The retinas were extracted and placed in Neurobasal culture media (Invitrogen) supplemented with 2% B27 (Invitrogen), 1% penicillin/streptomycin (Sigma), and 2 mM glutamine (Sigma), and cut into small squares (~0.5 mm^2). The squares were placed (4 per coverslip) on

precoated poly D-lysine/laminin glass coverslips (12 mm^2, BDBiosciences) in 5 μL of media in 24-well plates. After incubation overnight, 1.5 mL of media was added to each well and subsequently, (i) 100 μL of PBS, (ii) 100 μL of PBS containing CNTF (150 ng), (iii) a hydrogel (10 wt% PEG macromer), or (iv) a hydrogel (10 wt% PEG macromer) containing encapsulated CNTF (150 ng/hydrogel) was added to each well. This system was designed so that the hydrogel was suspended in the media above the explants using transwell inserts, so that released neurotrophins and degradation products were released directly into the media without any direct interaction between the gel and the explants.

After 7 days of culture, the coverslips were fixed in 4% paraformaldehyde for 15 min and washed in PBS followed by blocking with 1% BSA and 0.2% Triton X-100 for 30 min. The coverslips were then incubated overnight with rabbit anti-neurofilament-200 IgG (1:1000 in PBS) at 0 °C, washed (3 × PBS for 15 min each), and incubated with goat anti-rabbit cy3 secondary antibody (1:1000 in PBS) for 1 h at room temperature. The coverslips were then mounted and visualized on a fluorescent microscope. Both the number of neurites and the neurite length (for neurites > 50 μm) were determined using NIH Imaging Software for a minimum of 20 explants per culture condition.

2.5. Microsphere incorporation

Degradable microspheres based on poly(lactic-*co*-glycolic acid) (503 H, Boehringer Ingelheim) incorporating neurotrophins were prepared using a double emulsion process [12]. Microspheres were mixed with the macromer solution and polymerized to produce hydrogels (10 wt% macromer concentration) without neurotrophins, neurotrophins only in the microspheres, and neurotrophins in both the hydrogel phase and the microspheres, to illustrate the ability to deliver multiple factors simultaneously at independent release rates. The morphology and size range of the microspheres were analyzed on a JEOL 6320FV scanning electron microscope (SEM).

2.6. Statistical analysis

Statistical analysis was performed using a Student's *t*-test with a minimum confidence level of 0.05 for statistical significance. All values are reported as the mean and standard deviation of the mean.

3. Results and discussion

To investigate the feasibility of a hydrogel platform for the replacement and delivery of neurotrophins to the CNS, acrylated PLA-*b*-PEG-*b*-PLA macromers were synthesized and used as vehicles for neurotrophin delivery. A schematic of the photopolymerization and degradation of these networks is shown in Fig. 1. These hydrogels have been extensively investigated for applications such as cartilage tissue engineering [13,14], restenosis [15], and growth factor delivery [16]. One of the primary benefits of this biomaterial is that hydrogel properties are easily modified by changes in the macromer chemistry and the macromer solution. For instance, the degradation and swelling of the hydrogels are dictated by the number of lactic acid repeat units, the acrylation efficiency, the molecular weight of the PEG core, and the concentration of macromer in the prepolymer solution [17]. Although not investigated in this work, the type of degradable unit (e.g., lactic versus caproic acid) can also alter network degradation [18].

CNTF, BDNF, and NT-3 were suspended in a 10 wt% macromer solution and encapsulated with the addition of low-intensity ultraviolet light in the presence of a non-toxic photoinitiator to produce transparent hydrogels. An additional benefit of this technique is that complete encapsulation and molecule distribution is possible, something that is difficult to achieve with growth factor encapsulation in other types of polymers. When the hydrogels were degraded under physiological conditions on an orbital shaker, the neurotrophins were released from the network and quantified with an ELISA assay. The results for this study are shown in Fig. 2. The general release profile consisted of a burst of neurotrophin release over the first 1–2 days of degradation, followed by near-linear release up to complete degradation by 3 weeks. The burst (~38%) of neurotrophin at early degradation times could indicate that the size of the neurotrophin is smaller than the critical mesh size of the network, leading to rapid release of molecules at or near the hydrogel/buffer interface. As shown in Fig. 1, the network begins to degrade as the ester units in the lactic acid blocks in the crosslinks hydrolyze and the mesh size changes throughout the

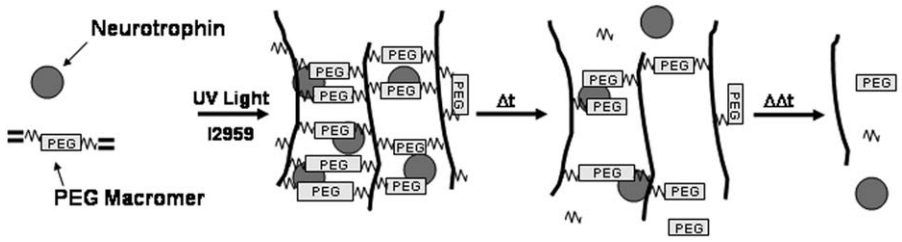

Fig. 1. Schematic of neurotrophin photoencapsulation, hydrogel degradation, and neurotrophin release. The thick black lines represent kinetic chains that are formed by the radical polymerization of the acrylate groups on the starting macromers.

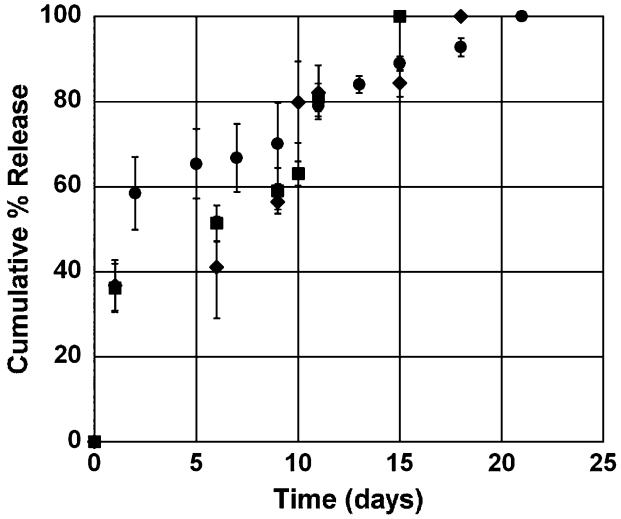

Fig. 2. Cumulative release of CNTF (●), BDNF (◆), and NT-3 (■) from 10 wt% PEG hydrogels.

hydrogel. This temporal change in crosslinking density allows for further diffusion of the neurotrophin from the hydrogel and eventually the network completely degrades. Overall, molecule release is dictated by diffusion through the network, with diffusion changing as the network degrades [19]. The results indicate that there are only slight changes in the release profiles based on the neurotrophin investigated, which is potentially explained by differences in the neurotrophin confirmation and subsequent diffusion through the network.

To investigate the influence of network structure on neurotrophin release, CNTF was encapsulated in networks formed with various concentrations (i.e., 10, 20, and 30 wt%) of macromer in the precursor solution. The release profiles for these networks are shown in Fig. 3A. The macromer concentration directly influences the crosslinking density of the hydrogel and, subsequently, molecule diffusion through the network. For instance, increasing the macromer concentration decreases the network mesh size due to an increase in both cyclization and kinetic chain lengths during network formation. Again, each of the profiles consists of a burst of neurotrophin followed by a near-linear release until complete degradation. The burst is much larger (~58%) for the lowest macromer concentration network than the 20 (only ~18%) and 30 (only ~9%) wt% hydrogels. It should be noted that there was no significant change between the 20 and 30 wt% hydrogels as was seen between the 10 and 20 wt% hydrogels. This is potentially attributed to the relative size of the release neurotrophin compared to the mesh size of these networks.

For the more loosely crosslinked hydrogel (i.e., lower macromer concentration), the size of the neurotrophin is closer to the network mesh size, which leads to a more

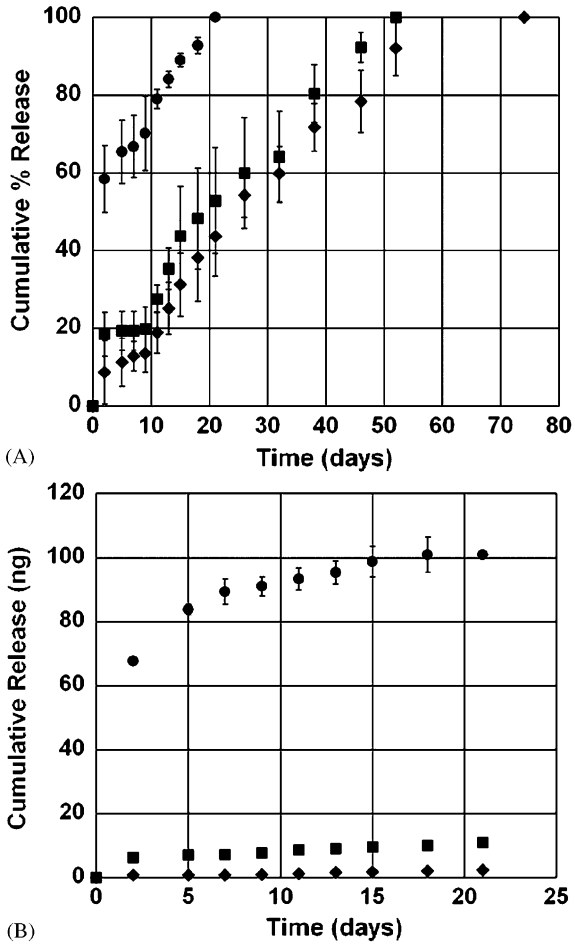

Fig. 3. Release of CNTF from PEG hydrogels. (A) Cumulative CNTF release from hydrogels fabricated with 10 (●), 20 (■), and 30 (◆) wt% macromer, illustrating the control over neurotrophic factor release kinetics with changes in hydrogel structure. (B) Cumulative release from hydrogels loaded with 100 (●), 10 (■), or 1 (◆) ng of CNTF.

rapid diffusion and release of the CNTF. As expected, the changes in network crosslinking density (i.e., macromer concentration) also led to changes in the overall time for degradation. For example, the network formed from the lowest macromer concentration degrades in ~21 days, but the degradation time increases to ~74 days when the macromer concentration is increased 3-fold. This is due to the greater number of crosslinks (i.e., lactic acid blocks) with the greater macromer concentration that need to be cleaved for network degradation. Thus, this becomes a simple technique to alter neurotrophin release profiles, which can then be tailored for the intended application to the CNS. Additionally, there was little change in the overall release profiles when the concentration of neurotrophin was altered in the networks. These results are shown in Fig. 3B. Although the overall amount of neurotrophin changed, there was little change in the overall time for complete neurotrophin delivery.

Although desirable release profiles can be demonstrated with these networks, it is important to investigate the activity of the released factors, especially since the neurotrophins are encapsulated in a potentially damaging free radical environment. One neurotrophin, CNTF, was used in these studies. Initially, the ability of the released CNTF to stimulate the proliferation of a TF-1 cell line that was transfected with the α-subunit of the CNTF receptor was investigated. In this assay, active CNTF induces cell proliferation and, thus, gives an indication of activity after release from the hydrogels. Results for the release of CNTF after 2, 5, 11, and 15 days from a 10 wt% hydrogel are shown in Fig. 4. Proliferation was normalized to controls with no CNTF added to the media, so values greater than one are indicative of active CNTF. Overall, all of the released CNTF investigated was able to stimulate proliferation of the TF-1 cells. Although the unreleased CNTF had a higher value, there was no statistical difference between any of the release times and this control, indicating similar proliferation levels between all samples. These results are evidence that active CNTF is released from the hydrogel networks, even for samples obtained after 15 days of hydrogel degradation/neurotrophin release.

As an additional measure of neurotrophin activity, hydrogels containing CNTF were investigated for their ability to enhance neurite outgrowth from retinal explants. Investigators have previously shown [20] the ability of neurotrophins to stimulate neurite outgrowth from these explants. In these studies, the explants had no contact with the hydrogels, but were exposed to the released neurotrophins and degradation products of the hydrogels over a week of culture. Fluorescent images of neurite outgrowth from the various culture conditions are shown in Fig. 5. Although there is neurite outgrowth under all conditions from the retinal explants, the images indicate greater outgrowth from the conditions where the media is supplemented with CNTF or when CNTF is released into the media from a hydrogel.

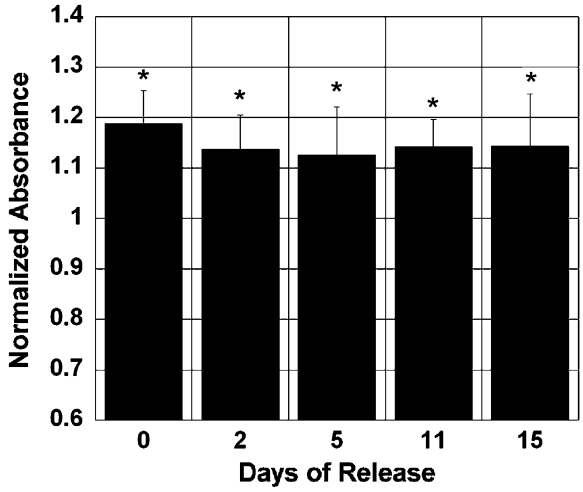

Fig. 4. Stimulation of proliferation of TF-1 cells by CNTF (day 0) or CNTF delivered from a 10 wt% PEG hydrogel after 2, 5, 11, and 15 days. Values are normalized to TF-1 cell proliferation in normal media, so values >1 indicate stimulated proliferation and CNTF activity. * denotes statistical significance compared to cells cultured without CNTF. Also, there was no statistical difference between the released CNTF and the control of unreleased CNTF added directly to the cultures.

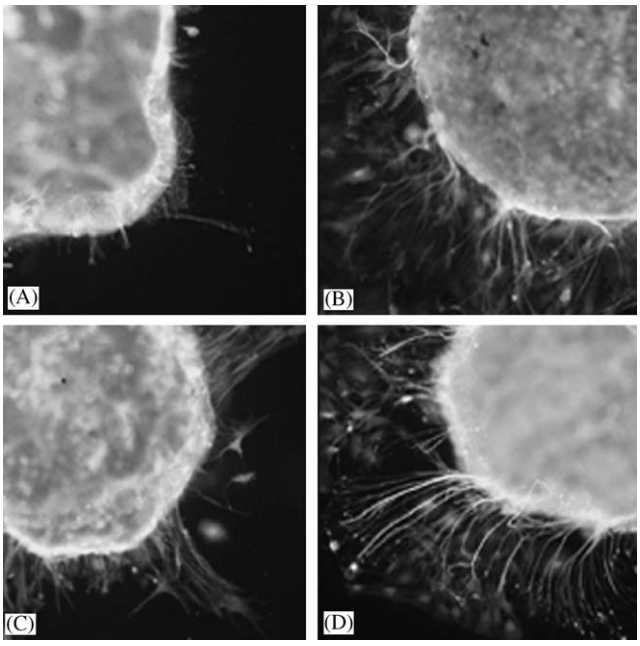

Fig. 5. Neurite outgrowth (stained with neurofilament-200) from retinal explants cultured in control media without any CNTF (A), CNTF supplemented media (B), in the presence of 10 wt% PEG hydrogels without CNTF (C), and in the presence of 10 wt% PEG hydrogels releasing CNTF (D).

Quantification of neurite outgrowth under various conditions is shown in Fig. 6. First, quantification of the number of neurites (>50 μm) extending from the explants (normalized to the perimeter length of the explant) indicates greater stimulation when CNTF was present in the media, both added initially and when released from the hydrogel. For instance, 2.29 ± 1.14 and 2.26 ± 0.83 neurites/mm were measured for the control media and unloaded hydrogel conditions, respectively, but increased to 5.36 ± 1.35 neurites/mm in CNTF supplemented media and 4.92 ± 2.32 neurites/mm for the CNTF releasing hydrogel. A statistically significant difference was found between the CNTF releasing hydrogel and unmodified media and unloaded hydrogel controls, but not the CNTF supplemented media. The ability of CNTF to support the survival and regeneration of axotimized retinal ganglion cells in the explants is the most likely reason for this observation. The neuroprotective effects of CNTF have been well documented [21,22].

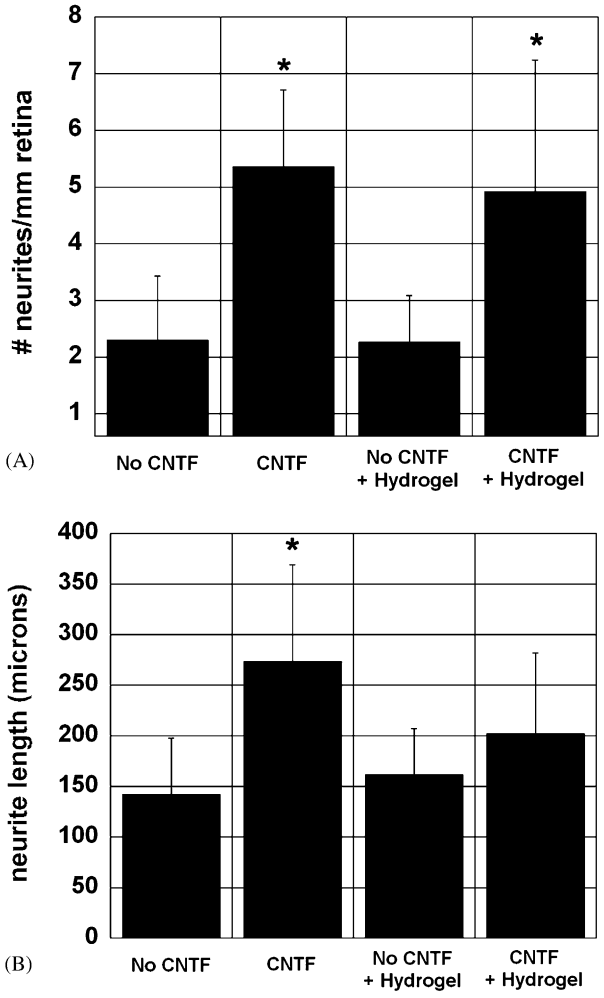

Fig. 6. Quantification of neurite outgrowth from retinal explants cultured for 7 days in control media, CNTF modified media, in the presence of 10 wt% PEG hydrogels without CNTF, and in the presence of 10 wt% PEG hydrogels releasing CNTF. (A) Overall number of neurites normalized to perimeter of explant. (B) Length of neurites growing from explants. * denotes statistical significance compared to the no CNTF control samples.

The average length of neurites was measured and found to be 142.3 ± 55.2 and $161.4 \pm 45.7\,\mu m$ for the control media and unloaded hydrogel conditions, respectively, but increased to $202.2 \pm 79.6\,\mu m$ for the CNTF releasing hydrogel and to $273.5 \pm 95.4\,\mu m$ for the CNTF supplemented media. The values for the CNTF releasing hydrogel were not statistically significant compared to any of the other culture conditions. It should be noted that the hydrogels were not completely degraded at the 7 day time point, so there was less CNTF introduced to the explants overall than for the CNTF modified media where a bolus of CNTF was introduced immediately. From the previous in vitro release investigation, only ~65% of the CNTF should be delivered at this point. Additionally, the ability to deliver low concentrations of neurotrophins to specific compartments of the CNS has the potential to overcome a significant hurdle to clinical application of neurotrophin therapy, namely, the potential toxicity and systemic and local side effects of high dose, bolus neurotrophin injections.

The neurite outgrowth experiments show several important results in the development of these hydrogel networks. First, the ability of the released CNTF to stimulate an increased number of neurites from the retinal explants further confirms the activity of CNTF upon release from the photopolymerized constructs. Additionally, the control hydrogels gave similar values to explants in unmodified media, indicating that the degradation products were not substantially toxic to the cells, which is especially important with the static culture environment used for these experiments. Although these in vitro experiments present an investigation of neurite outgrowth from the retina, it serves as a model for spinal cord injury, and also introduces potential application for glaucoma and optic nerve injury. Although these hydrogels have great potential as drug delivery vehicles, no neurite extension has been observed by cells encapsulated within the hydrogels at the investigated crosslinking densities (unpublished observations). This is potentially due to the hydrogels being covalently crosslinked and lacking the appropriate enzymatically degradable sequences.

As a final investigation of these photopolymerizable hydrogels for neurotrophin delivery, a technique for the simultaneous delivery of multiple neurotrophins from one explant was developed. To accomplish this, NT-3 was encapsulated in degradable microspheres of poly (lactic-*co*-glycolic acid) using a double emulsion technique [12]. An SEM image of the microspheres is shown in Fig. 7A and shows spherical particles with a size range of 1–10 μm. These microparticles were then mixed in the precursor solution and photopolymerized to produce constructs containing NT-3 in the microsphere phase and CNTF in the hydrogel phase. Light microscopy revealed evenly distributed microspheres throughout the hydrogels. The release profiles show very rapid release of the neurotrophin in the hydrogel phase and a slow continuous release from the degradable microspheres. It should be noted that neurotrophin release from the hydrogels could be very different in the presence of the microspheres since this will alter the diffusion process through the gel. These profiles could be tailored by changing properties of the microspheres such as the polymer chemistry (e.g., ratio of lactic to glycolic acid), polymer molecular weight, and microsphere size. Additionally, changes in the concentration of microspheres and concentration of neurotrophins in the microspheres would add to potential variations in release profiles. Overall, this technique would be applicable to situations where the release of several factors at individual rates from the same injectible implant is desirable.

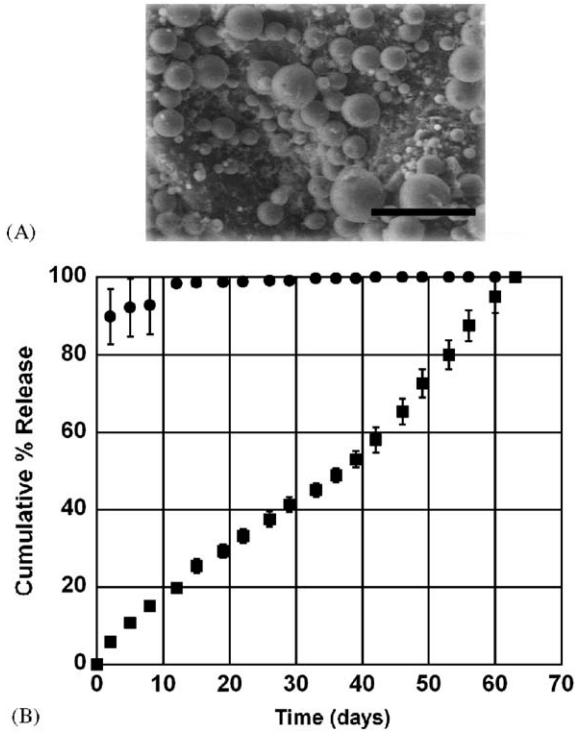

Fig. 7. Microsphere/hydrogel composites for releasing multiple growth factors. (A) SEM image of PLGA microspheres used for the delivery of neurotrophic factors, bar = 20 μm. (B) Cumulative release of CNTF (●, hydrogel phase) and NT-3 (■, microsphere phase) from the same implant, illustrating the potential for simultaneous delivery of multiple neurotrophins with individual release rates.

4. Conclusions

Neurotrophins were successfully photoencapsulated and released in a controlled manner from degradable PEG hydrogels. The ability of released CNTF to stimulate proliferation in a TF-1 cell line transfected with the α-subunit of the CNTF receptor and to stimulate outgrowth of a greater number of neurites from retinal explants than control culture conditions confirmed activity of the released CNTF and the lack of toxicity of the hydrogel degradation components. Additionally, a hydrogel/microsphere composite system was able to deliver multiple neurotrophins simultaneously with individual release profiles. These results support the development of photopolymerizable hydrogels for delivery of neurotrophins to the damaged CNS.

Acknowledgements

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