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Nerve guidance channels as drug delivery vehicles

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Abstract

Nerve guidance channels (NGCs) have been shown to facilitate regeneration after transection injury to the peripheral nerve or spinal cord. Various therapeutic molecules, including neurotrophic factors, have improved regeneration and functional recovery after injury when combined with NGCs; however, their impact has not been maximized partly due to the lack of an appropriate drug delivery system. To address this limitation, nerve growth factor (NGF) was incorporated into NGCs of poly(2-hydroxyethyl methacrylate-*co*-methyl methacrylate), P(HEMA-*co*-MMA). The NGCs were synthesized by a liquid–liquid centrifugal casting process and three different methods of protein incorporation were compared in terms of protein distribution and NGF release profile: (1) NGF was encapsulated (with BSA) in biodegradable poly(D,L-lactide-*co*-glycolide) 85/15 microspheres, which were combined with a PHEMA polymerization formulation and coated on the inside of pre-formed NGCs by a second liquid–liquid centrifugal casting technique; (2) pre-formed NGCs were imbibed with a solution of NGF/BSA and (3) NGF/BSA alone was combined with a PHEMA formulation and coated on the inside of pre-formed NGCs by a second liquid–liquid centrifugal casting technique. Using a fluorescently labelled model protein, the distribution of proteins in NGCs prepared with a coating of either protein-loaded microspheres or protein alone was found to be confined to the inner PHEMA layer. Sustained release of NGF was achieved from NGCs with either NGF-loaded microspheres or NGF alone incorporated into the inner layer, but not from channels imbibed with NGF. By day 28, NGCs with microspheres released a total of 220 pg NGF/cm of channel whereas those NGCs imbibed with NGF released 1040 pg/cm and those NGCs with NGF incorporated directly in a PHEMA layer released 8624 pg/cm. The release of NGF from NGCs with microspheres was limited by a slow-degrading microsphere formulation and by the maximum amount of microspheres that could be incorporated into the NGCs structure. Notwithstanding, the liquid–liquid centrifugal casting process is promising for localized and controlled release of multiple factors that are key to tissue regeneration.

Keywords: Nerve guidance channel; Microspheres; Drug delivery; Hydrogel; HEMA; PLGA

1. Introduction

The current gold standard for repair of a transected peripheral nerve is the autograft, which is used to bridge the proximal and distal nerve tissue. Synthetic nerve guidance channels (NGCs) have been investigated as alternatives to autografts in order to eliminate the associated drawbacks, such as the creation of a secondary injury and donor site morbidity. Although promising results have been observed, and some channels are used clinically for repair of short-gap defects, regeneration over longer gaps remains incomplete and elusive [1]. Similarly, NGCs have shown promise in promoting regeneration after spinal cord injury; however, functional recovery in the inhibitory central nervous system (CNS) environment has been limited [2,3].

Axonal regeneration through NGCs, tissue integration, and consequently functional recovery, can be improved by the delivery of various therapeutic agents. Neurotrophic factors, which promote neuronal survival and axonal outgrowth, have been shown to improve regeneration in

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both the peripheral nervous system (PNS) and the CNS, while intracellular signalling molecules, antibodies to inhibitory proteins, and digestive enzymes of the glial scar have been shown to improve regeneration in the CNS [1,4]. However, safe and effective use of these molecules requires their localized, controlled and sustained delivery to the site of injury. Systemic administration is not feasible due to the short half-lives and high potency of many biomolecules, and delivery to the CNS is further limited by the blood–brain barrier [5,6]. Currently, osmotic mini-pumps are the preferred drug delivery devices for sensitive therapeutics, but problems exist with infections or tissue damage, pump failure due to catheter dislodgement or occlusion, as well as drug instability in the pump reservoir [7].

To locally administer neurotrophins, NGCs have been filled with either growth factor solutions, or matrices saturated with growth factors, at the time of implantation [8–12]. However, since growth factors lose bioactivity in solution, and can also easily leak from the channel during the implantation procedure, these techniques do not allow for the growth factor concentrations to be maintained within the NGC, which is necessary to achieve significant and long-term regeneration [11,13]. An alternative strategy is to use the NGC as a delivery vehicle. Entrapment of biomolecules within the polymer matrix can protect them from degradation, while the drug release rate can be controlled by the choice of polymer and properties, drug loading or device design. The concept of drug-releasing NGCs has been investigated in a few studies, where neurotrophic factors were incorporated into the polymer structure of NGCs prepared by dip-coating or solution casting from organic polymer solutions containing growth factors dispersed as a solid powder [14–16]. NGCs releasing neurotrophins have also been prepared by incorporating into one side of the channel wall a rod extruded at 55 °C from a polymer/growth factor mix [17–19]; however, this design can result in the creation of a non-symmetric growth factor concentration profile within the lumen of the NGC.

Additionally, growth factor-loaded biodegradable microspheres have been dispersed in the lumen of NGCs to serve as drug delivery devices. Microsphere technology is well established, and biodegradable microspheres have been shown to release bioactive neurotrophic factors in a controlled manner for prolonged periods of time [20]. Xu et al. dispersed nerve growth factor (NGF)-loaded poly (phosphoester) microspheres inside NGCs and found greater regeneration across a 10 mm gap in a rat sciatic nerve than in channels filled with bare NGF solution [21]. Rosner et al. proposed dispersing NGF-loaded PLGA 85/15 microspheres in magnetically aligned collagen gels placed within NGCs, but found that increased amounts of microspheres compromised magnetic alignment of the collagen fibrils [22]. Dispersing microspheres loosely in NGCs may lead to uneven microsphere distribution within the channels, while dispersing them within scaffolds may interfere with guided axonal extension into the scaffolds. A more suitable approach may be to prepare a composite tissue engineering device by incorporating microspheres directly into the NGC structure.

We have developed a liquid–liquid centrifugal casting process which enables us to easily form NGCs from acrylate-based hydrogels such as poly(hydroxylethyl methacrylate-*co*-methylmethacrylate) or P(HEMA-*co*-MMA) [23]. The centrifugal casting process relies on phase separation of the polymer phase from the monomer formulation during polymerization in a rotating cylindrical mold; the denser polymer phase is pushed to the periphery by centrifugal forces, where it gels forming a tube. The resulting NGCs are semi-permeable, soft and flexible, and match the modulus of the nerve or spinal cord [23,24]. This is in contrast to the many hydrophobic NGCs investigated, which are often stiff and may compress the regenerating tissue. Through a series of studies in both the PNS and CNS, we have demonstrated that our P(HEMA-*co*-MMA) NGCs are biocompatible and promote nerve regeneration [3,25]. It is anticipated that if the channels are combined with a drug delivery strategy, regeneration and functional improvement could be further increased.

Here we compare three methods for incorporation of NGF into the walls of P(HEMA-*co*-MMA) NGCs in order to enable them to serve as drug delivery vehicles: (1) NGF was encapsulated in biodegradable PLGA 85/15 microspheres, which were combined with a PHEMA polymerization formulation and coated on the inside of pre-formed NGCs by a second liquid–liquid centrifugal casting technique; (2) pre-formed NGCs were imbibed with a solution of NGF and (3) NGF was combined with a PHEMA formulation and coated on the inside of pre-formed NGCs by a second liquid–liquid centrifugal casting technique. The latter two methods are based on the commonly used approaches for loading of proteins into hydrogel networks, where the hydrogel is either soaked in protein solution after formation, or is initially formed in the presence of the protein solution [26,27]. NGF was chosen as the model biomolecule for release because it is a well-characterized neurotrophic factor that can maintain neuronal survival and induce axonal regeneration or sprouting in sensory neurons [1]. PLGA was chosen for microencapsulation because it is a biocompatible polymer whose degradation rate can be controlled by composition, thus allowing for control of drug release rate.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma Aldrich (Oakville, Ont., CA) and used as received, unless otherwise stated. Glass molds with an inner diameter of 3.4 mm were purchased from Kimble Kontes (Vineland, NJ, US). Biomedical grade poly(D,L-lactide-*co*-glycolide) 85/15 (PLGA 85/15, intrinsic viscosity = 0.64 dl/g) was purchased from Birmingham Polymers (Birmingham, AL, US). Poly(vinyl alcohol) (PVA, $M_w = 6000$ g/mol, 80% hydrolysed) was purchased from PolySciences Inc. (Warrington, PA, US). Mouse NGF (2.5S) was purchased from Cedarlane Laboratories Ltd.

(Hornby, Ont., CA). A *ChemiKine*™ NGF Sandwich ELISA kit was purchased from Chemicon International Inc. (Temecula, CA, US). Sterile phosphate-buffered saline (PBS, pH = 7.4) and penicillin/streptomycin (P/S) were purchased from Invitrogen Corporation (Burlington, Ont., CA). Coomassie Plus protein assay reagent was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). Deionized water was obtained from Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA, USA) and used at 18 MΩ resistance.

2.2. Preparation of microspheres

Microspheres loaded with NGF and bovine serum albumin (BSA) were prepared by a customized double emulsion (W/O/W) solvent evaporation method [28]. BSA was co-encapsulated in the microspheres in order to serve as both a stabilizer/carrier for NGF and as a pore-forming agent [29,30]. A total of 36 mg of protein (NGF:BSA = 1:150 w/w) was dissolved in 300 μl of deionized water, and 600 mg of PLGA 85/15 was dissolved in 3 ml of dichloromethane. The protein solution was emulsified in the polymer solution using a homogenizer (Polytron PT3000, Brinkman Instruments, Westbury, NY, USA) at 10,000 rpm for 1.5 min. The resulting primary emulsion was poured into 25 ml of a 1% aqueous PVA solution and homogenized for 1.5 min at 7500 rpm to form a secondary emulsion, which was then poured into 300 ml of a 0.1% PVA solution and stirred for 3 h to evaporate the organic solvent. The hardened microspheres were collected by centrifugation, washed 5 times with deionized water, freeze-dried, and stored in a desiccator at −20 °C until use. The same procedure was used to prepare microspheres loaded with fluorescein isothiocyanate-labelled BSA (FITC-BSA), which were used to characterize the distribution of microspheres within the NGC wall structure.

2.3. Characterization of NGF/BSA-loaded microspheres

The loading and encapsulation efficiency of NGF/BSA in the microspheres was determined by protein extraction. Five milligrams of microspheres were dissolved in 300 μl of dichloromethane, to which 300 μl of PBS was added and vortexed vigorously for 1 min. The mixture was centrifuged at 10,000 rpm for 10 min and the aqueous PBS phase was separated. The extraction was repeated 2 more times and the separated PBS fractions were pooled for analysis. The amounts of BSA and NGF recovered were determined using the Coomassie Plus protein assay and the NGF Sandwich ELISA, respectively, and based on these results, the protein loading in the microspheres (mass of protein/mass of microspheres) and the encapsulation efficiency were calculated ($n = 3$).

The morphology of the microspheres was assessed by scanning electron microscopy (SEM). Small quantities of microspheres were scattered on metal stubs covered with adhesive carbon tape, sputter-coated with gold 3 times for 50 s, and imaged on a Hitachi S-2500 SEM at a working distance of 15 mm and an acceleration voltage of 20 kV.

The average size and size distribution of the microspheres was determined by a laser diffraction particle sizer (Malvern Mastersizer 2000, Spectra Research Corporation, Mississauga, ON, CA), using a sample concentration yielding a 15–17% obscuration of the laser light. Data was collected for 10 s, in triplicate, and analysed using the Mastersizer 2000 software. The 'general purpose' mode based on the Mie Theory was employed, with a refractive index of 1.33 for water as the dispersion medium and 1.59 for the microsphere particles.

2.4. Release of NGF from NGF/BSA-loaded microspheres

To examine the release of NGF from PLGA 85/15 microspheres, 15 mg of freeze-dried microspheres was weighed out into polyethylene microcentrifuge vials and suspended in 1.5 ml of sterile PBS buffer containing 1% P/S and 0.1 mg/ml BSA. The samples were prepared in triplicate. The vials were inverted to prevent the microspheres from settling into a pellet and were placed on a shaker in an incubator at 37 °C. At regular intervals, the microspheres were centrifuged at 1000–3000 rpm for 3 min and the release buffer was completely removed and replaced with fresh buffer. The collected buffer was stored frozen at −80 °C until analysis by the NGF Sandwich ELISA.

2.5. Preparation of NGCs

P(HEMA-*co*-MMA) channels were fabricated by the liquid–liquid centrifugal casting process described previously [23]. Briefly, a polymerization formulation consisting of 23 wt% hydroxyethyl methacrylate (HEMA) monomer, 2 wt% methyl metharcrylate (MMA) monomer, 0.1 wt% ethylene glycol dimethacrylate (EDMA) cross-linker, 0.5 wt% ammonium persulfate (APS) initiator, 0.4 wt% sodium metabisulphite (SMBS) accelerator (the latter three components expressed as weight percentages of the monomer) and 75 wt% deionized water, was injected into glass molds (3.4 mm inner diameter) capped at both ends with rubber septa. The molds were completely filled, ensuring there were no air bubbles inside, inserted into the chuck of a horizontally mounted variable-speed stirrer, and spun around their horizontal axis at 5000 rpm for 5 h, which resulted in the formation of hydrogel channels with an outer diameter of 3.4 mm (matching the inner diameter of the cylindrical glass mold) and an inner diameter of 2.8 mm.

2.6. Incorporation of NGF/BSA into NGCs

With the P(HEMA-*co*-MMA) channels still in the molds after fabrication, the solution remaining in the lumens of the channels was withdrawn using a syringe. Then, three different methods were used to load NGF into the walls of the NGCs: (1) NGF/BSA-loaded PLGA 85/15 microspheres were entrapped in a polymer layer coated on the inner surface of the channel wall; (2) NGF/BSA was imbibed into the inner portion of the channel wall; and (3) NGF/BSA alone was directly entrapped in a polymer layer coated on the inner surface of the channel wall. In all cases, BSA was used as a stabilizer for NGF and steps were taken to load protein only into the inner part of the channel wall in order to enable preferential protein release to the lumen of the channels.

Specifically, to prepare NGCs with microspheres incorporated into the channel wall, a new polymerization formulation consisting of 5 wt% HEMA, 0.1 wt% EDMA, 1 wt% APS, 0.4 wt% SMBS (the latter three components expressed as weight percentages of the monomer), 95 wt% deionized water, and 1 or 2 wt% pre-hydrated microspheres in suspension (reported as weight percentage of total formulation mass) was introduced into molds containing pre-fabricated channels. The molds were then spun at 4000 rpm for 5 h in order to polymerize a thin PHEMA layer entrapping microspheres on the inner surface of the channel wall. Formulations with a higher monomer concentration, specifically 10 wt% HEMA, 0.1 wt% EDMA, 0.5 wt% APS, 0.2 wt% SMBS, 90 wt% deionized water, and 1 or 2 wt% microspheres, were also investigated.

To prepare NGCs with protein imbibed into the channel wall, the lumens of pre-fabricated channels in molds were rinsed with deionized water, and a 5 mg/ml solution of protein (NGF:BSA = 1:150 w/w) in PBS was injected into the molds, which were then placed in an incubator at 37 °C for 3 days.

To prepare NGCs with protein alone entrapped in the channel wall, a new polymerization formulation consisting of 5 wt% HEMA, 95 wt% water, 0.1 wt% EDMA, 1 wt% APS, 0.4 wt% SMBS (the latter three components expressed as weight percentages of the monomer), and 5 mg/ml protein (NGF:BSA = 1:150 w/w) was injected into molds containing pre-fabricated channels. The molds were then spun at 4000 rpm for 5 h in order to polymerize a thin PHEMA layer entrapping the protein directly on the inner surface of the channel wall.

2.7. Characterization of NGF/BSA-loaded NGCs

The morphology of the NGF/BSA-loaded NGCs was characterized by SEM as described for microsphere samples. NGC samples for imaging

were cut into small pieces and either freeze-dried to preserve the hydrogel structure or air-dried to collapse the hydrogel structure for facilitated visualization of the microspheres.

To visualize the protein distribution in the NGCs prepared with the three different loading techniques, representative samples of the channels were prepared according to the methods described in Section 2.6, but using FITC-BSA instead of NGF/BSA. After preparation, the channels were rinsed in deionized water and sliced into thin sections, which were then dried of excess water and visualized using a fluorescent microscope (Axiovert S100, Zeiss, Toronto, Ont., CA).

2.8. Release of NGF from NGF/BSA-loaded NGCs

A release study was conducted to compare the delivery of NGF from the three different types of NGF/BSA-loaded NGCs. Samples of each channel type were prepared in triplicate and each individual sample consisted of three 1 cm sections of the channel immersed in 1 ml of PBS buffer containing 1% P/S and 0.1 mg/ml BSA. Each channel section was rinsed in buffer for 10 min prior to immersion in the release buffer at the start of the study in order to remove any loosely adsorbed protein. The samples were placed on a shaker in an incubator at 37 °C and the release buffer was exchanged with fresh buffer at regular intervals. The collected release buffer was stored frozen at −80 °C until analysis by the NGF Sandwich ELISA.

3. Results and discussion

3.1. NGF/BSA-loaded microspheres

The commonly used W/O/W solvent evaporation method was chosen to encapsulate NGF/BSA into PLGA 85/15 microspheres because it can yield good encapsulation efficiencies and is effective in encapsulating neurotrophic factors for sustained release applications [20]. For example, release of bioactive NGF, as confirmed by a cellular bioassay, has been demonstrated for 35 days from PLGA 75/25 microspheres and 70 days from polyphosphoester microspheres prepared using the W/O/W method [31,32].

The microspheres prepared for this study had an average (±SD) total protein loading of 5.63±0.11 wt% and an NGF loading of $(1.65\pm0.04)\times10^{-2}$ wt% (reported as a percentage of the total microsphere mass). The encapsulation efficiency was 99.2±2.0% for BSA and 73.8±1.9% for NGF, as measured using the Commassie Plus assay and the NGF ELISA, respectively. The lower NGF encapsulation efficiency possibly indicates some growth factor immunoreactivity loss or denaturation during the encapsulation or extraction procedure, both of which required organic solvents and mechanical agitation. A similar extraction procedure has been previously shown to underestimate the encapsulation efficiency of NGF due to growth factor denaturation incurred in the process [22]. The release of bioactive NGF from the microspheres was confirmed by a PC12 bioassay (data not shown). The microspheres had a smooth surface morphology, with no visible pores, as shown in Fig. 1. The average microsphere size (±SD) was 36.42±13.07 μm (volume-weighted) or 25.01±8.94 μm (number-weighted). The size distribution was unimodal, with approximately 80% of the microspheres being between 15 and 35 μm in diameter (number-weighted).

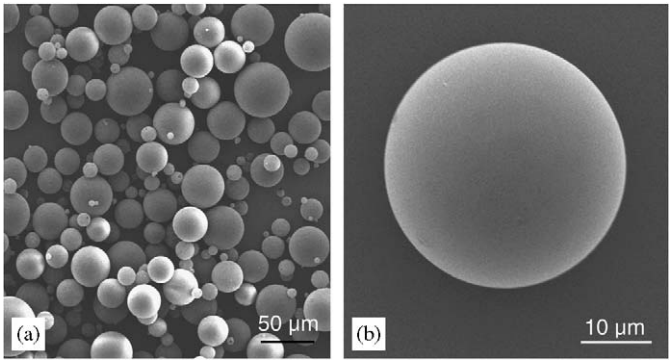

Fig. 1. PLGA 85/15 microspheres loaded with NGF/BSA have a smooth outer surface morphology as viewed by scanning electron microscopy (a) at 300× magnification and (b) a close-up of (a) at 2000× magnification.

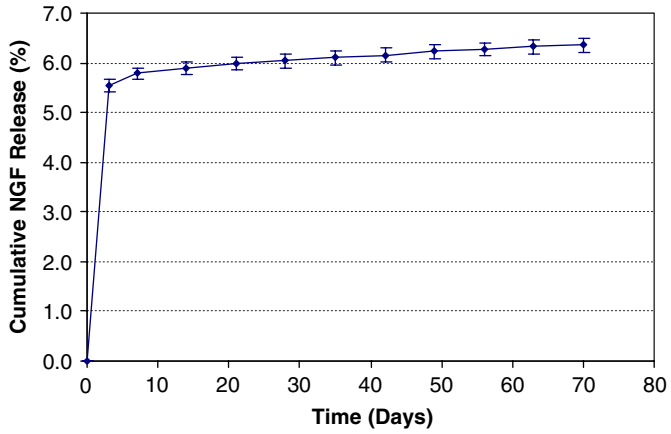

Fig. 2. The cumulative release profile of NGF from PLGA 85/15 microspheres over a 70-day period demonstrates a small burst release and then a near-linear release profile (mean±standard deviation, $n=3$).

The release profile of NGF from PLGA 85/15 microspheres, quantified by NGF ELISA, is shown in Fig. 2. After an initial burst, where 5.5% of the encapsulated NGF was released, the release continued at a relatively steady rate for the remaining 70 days of the study, at the end of which only 6.4% of the encapsulated NGF had been released. The concentration of NGF in the buffer at each sampling point after 1 week varied between 700 and 2000 pg/ml.

Protein release from PLGA microspheres is governed by the relative contributions of diffusion- versus degradation-mediated release mechanisms. The initial burst release results from desorption of loosely attached or encapsulated proteins near the surface of the microspheres. The leaching out of surface-bound proteins creates an interconnecting porous network in the polymer matrix through which further protein diffusion can take place. At later stages of incubation, polymer degradation and mass loss facilitate greater protein release [33,34]. Since the PLGA 85/15 microspheres exhibited no erosion during the 70-day study,

the observed NGF release was mainly due to diffusion of the growth factor out of the polymer matrix. The use of a highly concentrated polymer solution and a low ratio of internal aqueous phase volume to organic phase volume when manufacturing the microspheres led to the formation of microspheres with a smooth surface morphology and likely a dense inner core [35]. Although the low porosity promoted a high encapsulation efficiency and minimized the magnitude of the initial burst release, it also significantly limited the subsequent diffusion of protein out of the polymer matrix and resulted in slow protein release.

3.2. Incorporation of microspheres into the NGC wall

The centrifugal casting process was investigated as a means to incorporate microspheres into the inner wall structure of P(HEMA-*co*-MMA) NGCs. The microspheres, being heavier than the polymerization formulation, were pushed to the periphery during spinning and became entrapped by a newly formed PHEMA layer deposited on the inner surface of pre-fabricated channels. Air-drying the channel samples caused the 'spongy' hydrogel morphology of the PHEMA layer to collapse and the hard polyester microspheres underneath it to be visualized by SEM. Figs. 3a and b show the lumen of a channel containing an inner layer made with 1 wt% microspheres and 5 wt% HEMA, where it can be seen that the microspheres are coated by a thin polymer layer. The thickness of this layer could be increased by using a higher monomer concentration, as shown qualitatively in Fig. 3c, where a 10 wt% PHEMA layer was used to entrap 1 wt% microspheres. The thickness of the covering layer was difficult to quantify because the original NGC wall inner surface had a similar morphology to that of the coating layer, making the interface between them difficult to distinguish by SEM. The ability to control the thickness of the covering layer may offer another level of control over the drug release rate from the microspheres.

To increase the amount of protein loaded into the NGCs, an inner layer was fabricated with 2 wt% microspheres and 5 wt% HEMA. As shown qualitatively in Fig. 4, increasing the microsphere concentration in the formulation resulted in more microspheres embedded in the channel wall. Even with the use of smaller microspheres (shown in Fig. 4 relative to those shown in Fig. 3), at these microsphere concentrations, the PHEMA layer was prone to de-lamination, and using a higher monomer concentration (10 wt% HEMA) for the inner layer did not remedy this effect. These results imply that the microspheres do not become embedded within the matrix of the newly formed layer but instead are trapped beneath the layer, as if trapped underneath a 'blanket', that is attached to the channel wall primarily by mechanical interlocking. A high concentration of microspheres obscures a significant portion of the channel surface area and prevents the inner PHEMA layer from effectively adhering to the channel wall. Therefore, further characterization was pursued with NGCs fabricated with 1 wt% microspheres.

3.3. Protein distribution in NGCs

In order to determine the protein distribution within the NGCs prepared by the three different loading methods described in Section 2.6, FITC-BSA was loaded into the channels instead of NGF/BSA and visualized using fluorescent microscopy. In NGCs with FITC-BSA microspheres, shown in Figs. 5a and b, the protein was mainly localized within the microspheres, which were embedded in the inner structure of the channel wall. In NGCs which had their lumens soaked in FITC-BSA solution, shown in Figs. 5c and d, the protein appeared to be dispersed throughout the channel wall. In NGCs which were coated with an inner PHEMA layer polymerized in the presence of

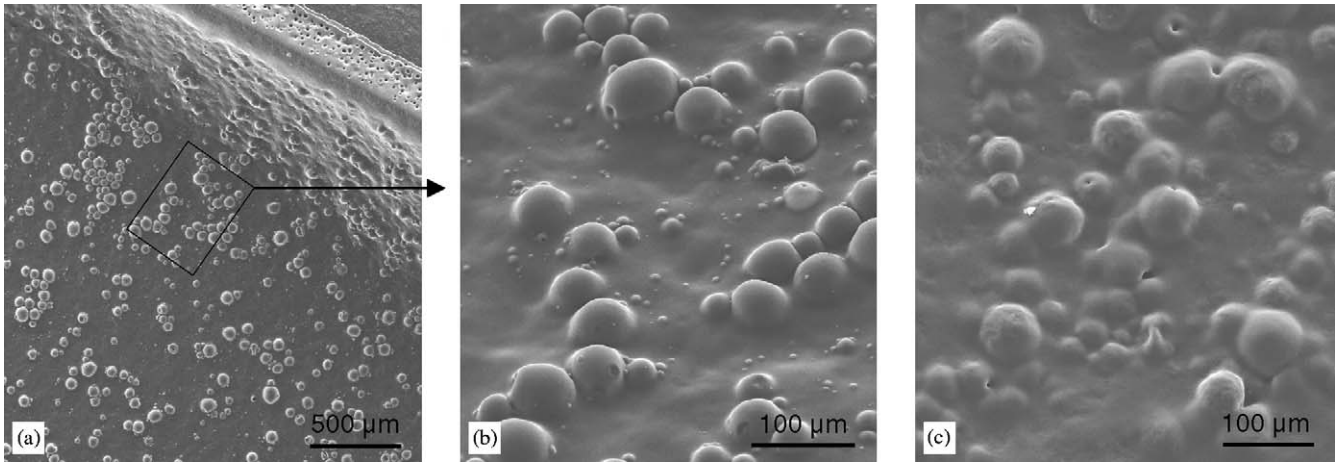

Fig. 3. Scanning electron micrographs of the air-dried, inner lumen surfaces of P(HEMA-*co*-MMA) channels coated with an inner layer of PHEMA and 1 wt% PLGA 85/15 microspheres: (a) at 40 × magnification for a 5 wt% PHEMA layer; (b) a close-up of (a) at 220 × magnification for a 5 wt% PHEMA layer; and (c) at 200 × magnification for a 10 wt% PHEMA layer.

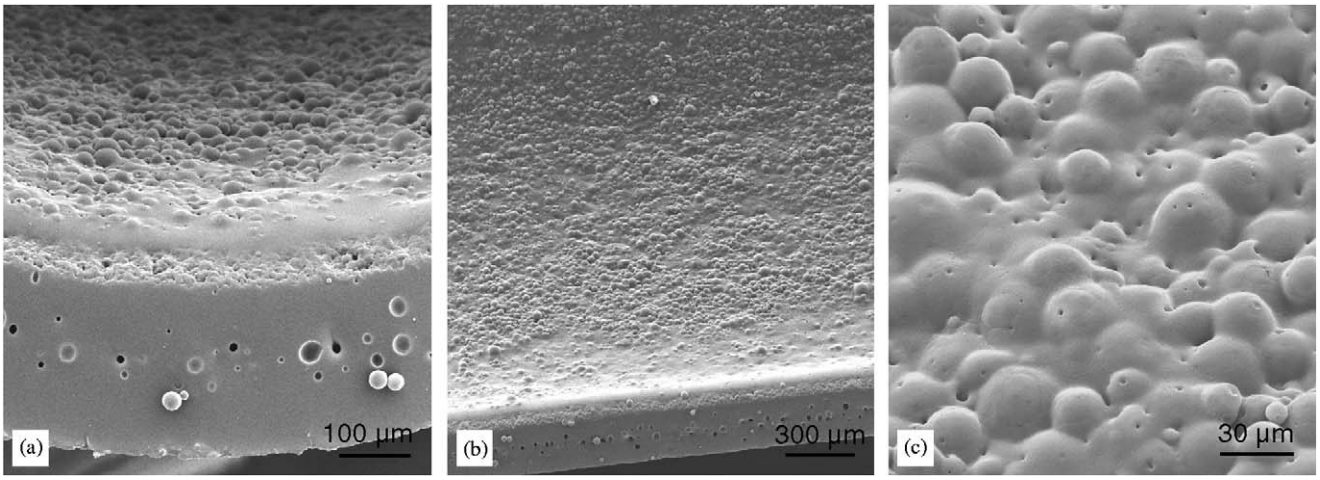

Fig. 4. Scanning electron micrographs of the air-dried, inner lumen surfaces of the P(HEMA-*co*-MMA) channels coated with an inner layer of 5 wt% PHEMA and 2 wt% PLGA 85/15 microspheres as viewed (a) in cross-section, where the full wall morphology is evident, (b) in a longitudinal section at 50× magnification and (c) in a close-up of (b) at 500× magnification.

FITC-BSA solution, shown in Figs. 5e and f, most protein was confined to the inner layer. Thus, spatial control of protein loading in the channel wall was achieved by incorporating protein into an inner PHEMA layer either directly or in microspheres; soaking the channel lumen in protein solution did not allow for such control. For nerve regeneration purposes, the goal is to release the growth factor preferentially to the lumen of the channel where regeneration occurs. P(HEMA-*co*-MMA) channels prepared using the centrifugal casting technique have a unique bi-phasic morphology consisting of a gel-like outer layer and a spongy inner layer [23]. Localization of the protein source in the 'spongy' inner portion of the channel wall should facilitate delivery to the lumen of the channels, since the gel-like outer layer can provide resistance to the outward diffusion of protein [24]. In vivo, this may lead to growth factor accumulation within the channel and enhanced regenerative capacity.

3.4. Release of NGF from NGF/BSA-loaded NGCs

Release of NGF from the three different types of NGF/BSA-loaded P(HEMA-*co*-MMA) channels was compared over a 28-day period. Fig. 6a shows the cumulative mass of NGF released per centimetre section of an NGC with microspheres. Although the release was sustained, only 220 pg of NGF/cm were released by day 28. The release was limited by the slow release obtained from the microspheres, and by the total amount of microspheres incorporated into the channel wall. The initial burst release from the channels was less pronounced than that observed from the PLGA microspheres alone (as can be qualitatively confirmed by comparing the release profiles of Fig. 2 vs. Fig. 6a), possibly because some NGF was lost during the processing of the microspheres into the NGCs, or because the microspheres were coated with an additional layer of PHEMA. The reduction in the magnitude of the burst release may be advantageous, since large initial doses of growth factor can sometimes have down-regulatory or cytotoxic effects on cells. For example, Barras et al. found that with poly(EVAc) channels containing a GDNF-releasing rod in the channel wall, the channels had to be immersed in buffer for 3 days prior to implantation in order to eliminate the high burst release observed, otherwise no regeneration occurred [18].

NGF release from NGCs soaked in NGF/BSA solution for 3 days is shown in Fig. 6b. Most of the imbibed NGF, or specifically 990 pg/cm of NGC, was released in a large burst by day 1, with only 50 pg of additional NGF released until day 28. Thus, soaking is not an effective means of loading growth factor into the channels for sustained or controlled release applications, and likely offers no advantage over simply filling the channels with growth factor solution at the time of implantation, as has been investigated [8]. Low protein loadings and fast release are commonly associated with imbibed hydrogels, since large molar mass proteins do not partition easily into the cross-linked polymer network and may only diffuse into the macropores of the hydrogel or adsorb to the surface [26,36,37]. Swelling the polymer network can sometimes be employed to overcome these limitations, but most swelling solvents are detrimental to growth factor stability. Additionally, when soaking a hydrogel in protein solution, a long time may be required to achieve equilibrium saturation, which is problematic for growth factors that are unstable in solution.

NGF release from NGCs with NGF/BSA entrapped directly in an inner PHEMA layer during polymerization is shown in Fig. 6c. The release rate was faster initially and then decreased with time, yielding a release profile that is characteristic of diffusion-based release from hydrogels; a total of 8624 pg of NGF was released over 28 days, which is bioacceptable and non-cytotoxic. Of the three types of NGCs examined, these channels had the least pronounced

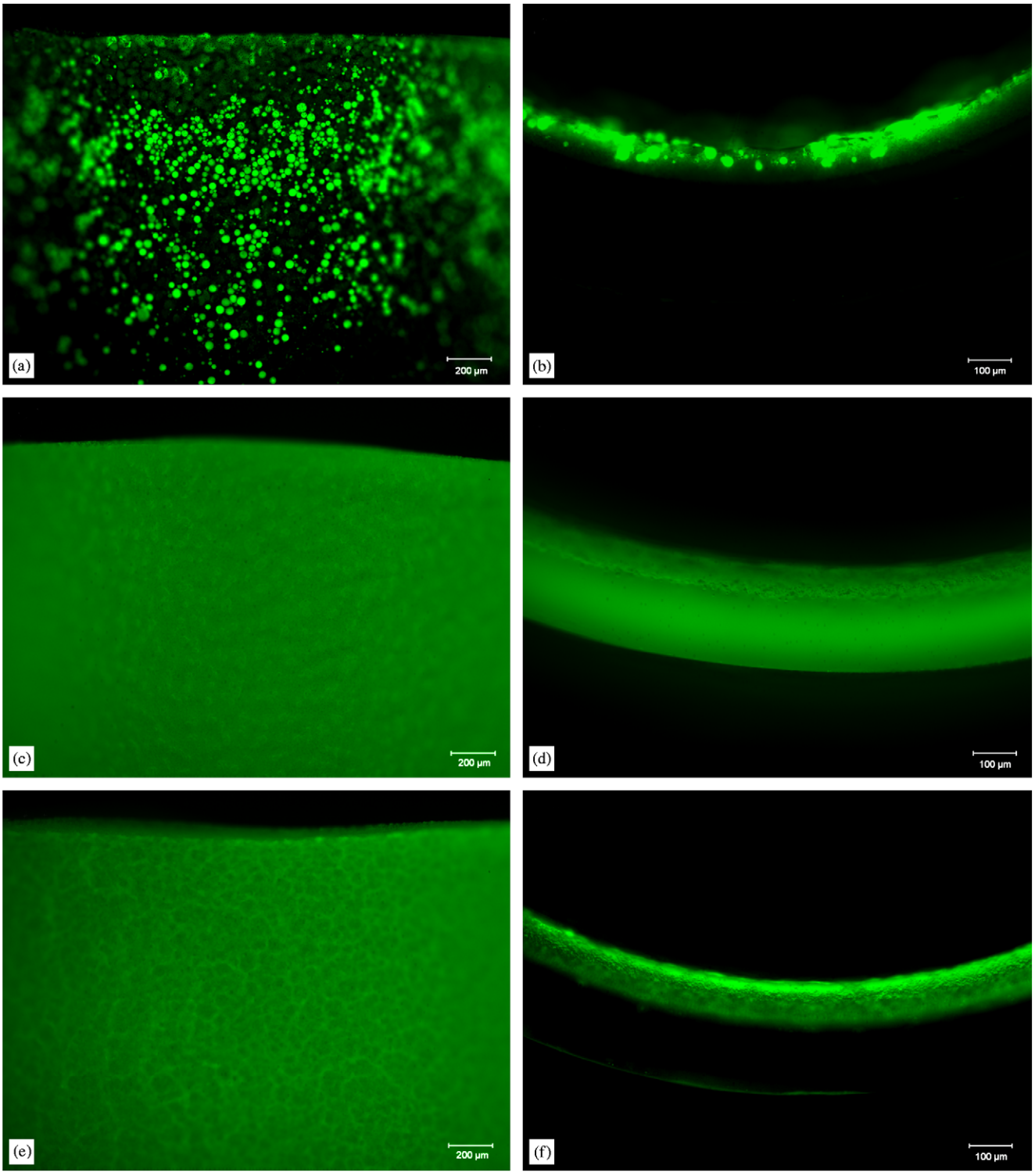

Fig. 5. The distribution of protein in the three different NGC types was visualized by fluorescent microscopy using FITC-BSA: (a), (b) NGCs with FITC-BSA microspheres incorporated into an inner PHEMA layer (longitudinal and cross-sectional perspectives, respectively) had protein localized in the microspheres within the inner layer; (c), (d) NGCs with lumens soaked in FITC-BSA solution (longitudinal and cross-sectional perspectives, respectively) had protein distributed throughout the wall; and (e), (f) NGCs with FITC-BSA entrapped directly in an inner PHEMA layer (longitudinal and cross-sectional perspectives, respectively) had protein localized within the inner layer.

burst of NGF initially, and delivered the most NGF over the 28-day period. Interestingly, these channels continued to release measurable amounts of NGF for at least 70 days (data not shown), indicating that this method of loading growth factors into NGCs can result in sustained release for an extended period of time.

For proteins incorporated into a non-degradable cross-linked hydrogel during its formation, if the mesh size is

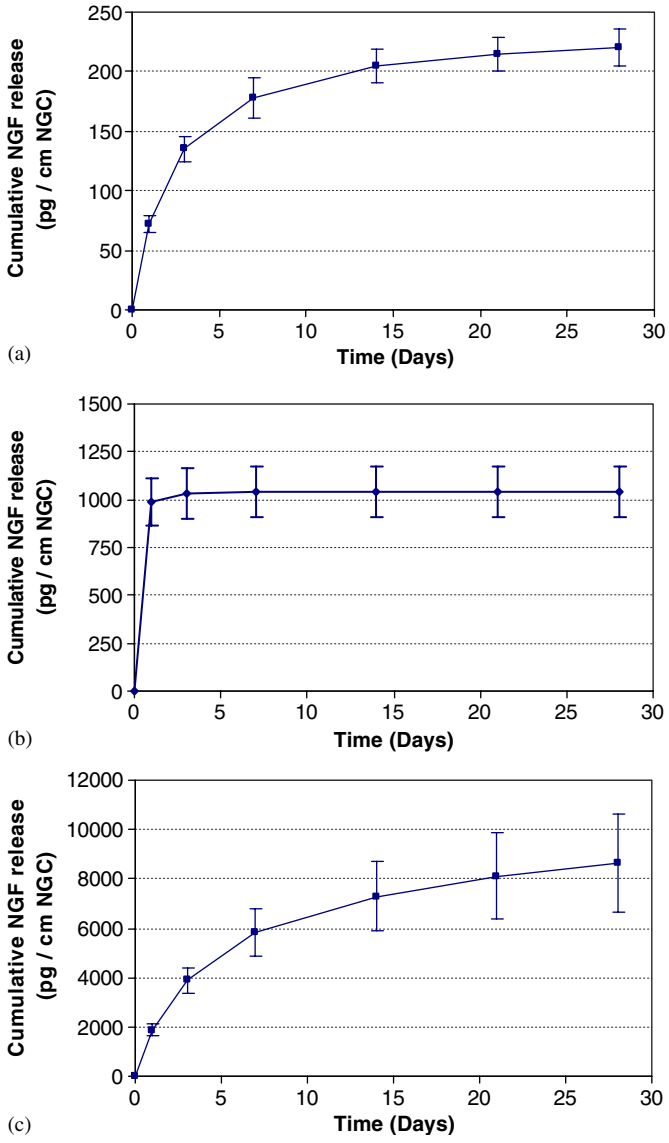

Fig. 6. Cumulative release profile of NGF (pg/cm of NGC) over a 28-day period from NGCs (a) coated with an inner layer of PHEMA containing NGF-loaded PLGA 85/15 microspheres; (b) imbibed with NGF; and (c) coated with an inner layer of PHEMA containing directly entrapped NGF (mean ± standard deviation, $n = 3$).

larger than the hydrodynamic diameter of the protein, release will proceed by diffusion, with the rate being faster for proteins that are substantially smaller than the mesh size. Entrapped proteins larger than the mesh size will not be released [38,39]. Thus, the fraction of protein available for release and the release profile can vary significantly depending on the properties of the hydrogel (i.e., cross-link density, water content, etc.), the size of the protein and the protein loading [38]. Sustained release of NGF from our P(HEMA-*co*-MMA) channels was likely facilitated by the low monomer concentration (5 wt% HEMA) used for the inner layer, enabling the formation of a porous hydrogel network with high water content, and by the relatively high total protein concentration used for the inner layer, enabling the large amount of BSA to act as a pore-forming agent. The prolonged release of BSA from non-degradable hydrogels has previously been attributed to a percolation mechanism, where protein from inside the hydrogel matrix slowly diffuses through interconnected channels formed by release of proteins closer to the surface [40].

Overall, the results indicate that NGCs with growth factor incorporated into a polymer layer on the inner surface of the channel wall, either in the form of microspheres or directly, are capable of delivering growth factors to the regenerating peripheral nerve or spinal cord in a localized and sustained manner. Although in this study the NGCs with microspheres released limited amounts of NGF, the delivery from these NGCs could be improved in future studies by optimizing the microsphere formulation (for example, by using a faster degrading PLGA, such as PLGA 50/50) to release more growth factor at a faster rate, thus yielding a more suitable release profile. Alternatively, release from these NGCs could be increased by multi-layering successive PHEMA coatings with microspheres in order to increase the number of microspheres incorporated into the NGC structure. There is an interest in pursuing the microsphere approach for loading of therapeutic agents into the NGCs because it can allow for simultaneous incorporation of microspheres with different release kinetics into one NGC, thereby allowing the delivery of multiple therapeutic agents at different rates to the regenerating nerve or spinal cord. NGCs capable of such versatile delivery are likely to be more successful in regenerating functional tissue. Current approaches investigated for incorporation of proteins into the NGC structure, such as by dip-coating of channels from organic polymer solutions containing dispersed protein particles, do not lend themselves equally well to achieving precise control over the protein delivery rates. Similarly, achieving precise control over the delivery rates of unencapsulated proteins entrapped directly in a PHEMA layer during polymerization may be more difficult.

Microspheres provide the additional benefit of protecting growth factors from denaturation or cross-linking during the polymerization of NGCs. Since growth factor bioactivity can be maintained after encapsulation into microspheres, the centrifugal casting process can be used to incorporate microencapsulated growth factors into the NGC structure without further bioactivity loss. Additionally, utilizing microspheres ensures that all the incorporated growth factor is available for release, instead of some of it inevitably becoming permanently entrapped in the cross-linked PHEMA network [41].

In order to maximize regeneration in vivo, the loading in the NGCs may need to be adjusted to deliver the required quantities of growth factor. However, due to the localized nature of the delivery, the doses required are likely to be much lower than those used in mini-pump delivery. Enhanced nerve regeneration has been observed with low delivery rates within NGCs [16,18,21,42], and modelling studies confirm that low doses may be sufficient to achieve

therapeutic concentrations. For example, Rosner et al. used a mathematical model to evaluate the NGF concentration within NGCs containing either Schwann cells or microspheres as drug delivery devices, and specified that delivery rates of 2.78 and 44.8 ng/ml/day were required to maintain an NGF concentration of 1 ng/ml for 2 months within channels used for human median nerve repair and rat sciatic nerve repair, respectively [22] (1 ng/ml is the minimum concentration required to sustain survival and neurite outgrowth of dorsal root ganglion cells). Based on dimensions of the P(HEMA-*co*-MMA) channels and the release data obtained, it can be calculated that the various NGCs studied here (which had a lower permeability [24] than the channels modeled by Rosner et al.) released NGF at rates ranging from 0.013 to 30.5 ng/ml/day. The magnitude of these rates implies that the channels can be optimized to meet the required release rates for NGF or other growth factors.

4. Conclusions

The liquid–liquid centrifugal casting process can be used to incorporate protein, either directly or in microspheres, into the inner wall structure of P(HEMA-*co*-MMA) NGCs, thereby allowing for localized and sustained protein delivery to the lumen of the channels. Conversely, soaking NGCs in protein solution is not an effective means of incorporating protein into the channels for localized and sustained release applications. NGCs capable of serving a dual purpose as bridging implants and drug delivery vehicles offer a method to overcome the limitations of current delivery techniques, and are likely to be more successful in regenerating functional tissue.

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