Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Controlled Release 110 (2006) 400 – 407
journal of controlled release
www.elsevier.com/locate/jconrel

Incorporation of protein-eluting microspheres into biodegradable nerve guidance channels for controlled release

Alex Goraltchouk [a,c], Vanessa Scanga [d], Cindi M. Morshead [d], Molly S. Shoichet [a,b,c,*]

[a] Department of Chemical Engineering and Applied Chemistry, University of Toronto 200 College Street, Toronto, ON, Canada M5S 3E5
[b] Department of Chemistry, 80 St. George St., Toronto, ON, Canada M5S 1AH
[c] Institute of Biomaterials and Biomedical Engineering, University of Toronto 4 Taddle Creek Road, Room 407, Toronto, ON, Canada M5S 3G9
[d] Department of Surgery, University of Toronto, 1 King's College Circle–1182, Toronto, ON, Canada M5S 1A8

Received 14 July 2005; accepted 25 October 2005
Available online 2 December 2005

Abstract

Nerve guidance channels (NGCs) promote axonal regeneration after transection injury of the peripheral nerve or spinal cord, yet this regeneration is limited. To enhance regeneration further, we hypothesize that localized delivery of therapeutic molecules combined with the NGC is required. In an attempt to achieve such an NGC, we designed and synthesized a novel NGC in which protein-encapsulated microspheres were stably incorporated into the tube wall. Specifically, poly(lactide-co-glycolide) (PLGA 50/50) microspheres were physically entrapped in the annulus between two concentric tubes, consisting of a chitosan inner tube and a chitin outer tube. Taking advantage of the extensive shrinking that the outer chitin tube undergoes with drying, >15 mg of microspheres were loaded within the tube walls. Using BSA-encapsulated microspheres as the model drug delivery system, BSA was released from microsphere loaded tubes (MLTs) for 84 days, and from freely suspended PLGA microspheres for 70 days. An initial burst release was observed for both MLTs and free microspheres, followed by a degradation-controlled release profile that resulted in a higher release rate from MLTs initially, which was then attenuated likely due to the buffering effect of chitin and chitosan tubes. Epidermal growth factor (EGF), co-encapsulated with BSA in PLGA 50/50 microspheres in MLTs, was released for 56 days with a similar profile to that of BSA. Released EGF was found to be bioactive for at least 14 days as assessed by a neurosphere forming bioassay.

Keywords: Drug delivery; Nerve guidance channel; Microspheres; Chitin; PLGA

1. Introduction

Tubular structures have been used as nerve guidance channels (NGCs) to bridge the gap created by transection nerve injury [1,2]. NGCs have been implanted alone or in combination with cells which have been shown to enhance axonal regeneration [3]. Recent results from our laboratory have shown that synthetic hydrogel NGCs can promote regeneration of axons across the gap resulting from a transected spinal cord model [4]; however entubulation alone has failed to provide sufficient benefits to consistently promote functional recovery after spinal cord injury (SCI).

Recent developments have helped to identify a number of biomolecules capable of promoting nerve regeneration both in vitro and in vivo, and these approaches have been thoroughly reviewed [5,6]. Various neurotrophic factors such as NGF, BDNF and NT-3 have been shown to improve regeneration after SCI [7,8]. Neurotrophic factors have been postulated to both enhance neuronal survival and rescue axotomized neurons after nerve injury. Our group has shown that neurotrophic factors can also play a role in axon guidance [9].

Current methods for delivering therapeutic agents such as these are inadequate for local delivery. For example, oral, and intravenous administration of drugs have limitations of dose control [10], premature drug degradation, and non-specific action [11] that could lead to undesirable side-effects and/or systemic toxicity. One approach to achieve delivery to the site of injury has been to combine biomolecules with NGCs. In initial attempts, the inner lumen of the NGC was filled with either a soluble form of the therapeutic agent [12–14] or a cell

* Corresponding author. Department of Chemical Engineering and Applied Chemistry, University of Toronto 200 College Street, Toronto, ON, Canada M5S 3E5. Tel.: +1 416 978 1460; fax: +1 416 978 4317.
E-mail address: molly@ecf.utoronto.ca (M.S. Shoichet).

0168-3659/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2005.10.019

adhesive matrix embedded with the therapeutic molecule, both of which showed greater regeneration than empty channels [15–18]. These systems provided a means to locally deliver the therapeutic molecule; however, they lacked sustained and controlled release rates that can be provided by a drug delivery system (DDS) [19]. Incorporating a DDS into the NGC achieves controlled temporal and spatial release. In one study, the DDS comprised a polymeric rod that was filled with a drug and embedded into one side of the wall of the NGC, thereby providing controlled release localized to the injury site [20]; however the device design did not enable even distribution of the therapeutic agent within the NGC.

In this study we report a novel approach to creating a DDS filled NGC where protein-encapsulated microspheres are entrapped in the annulus between two concentric tubes. The innovation depends on the selective, drying-induced shrinkage of the outer tube after microsphere-loading, thereby securing the microspheres within the wall of the NGC. This results in the stable entrapment of large numbers of microspheres evenly distributed in the NGC wall and enabling localized, controlled release in a spatial and temporal fashion. The concept was demonstrated with a chitin outer tube, chosen because of its ability to undergo extensive shrinking upon drying, a cell adhesive chitosan inner tube [21] and a clinically accepted PLGA 50/50 microsphere system [22]. The release and bioactivity of two model proteins, bovine serum albumin (BSA) and recombinant human epidermal growth factor (rhEGF) were studied in order to determine the ability of these DDS loaded NGCs to release bioactive protein in a sustained manner.

2. Materials and methods

All reagents used were purchased from Sigma Aldrich (Oakville, ON, CA), unless otherwise stated. Glass moulds with inner diameter of 5 mm and 1.5–1.8 × 100 mm capillary tubes were obtained from Kimble Kontes (Vineland, NJ, USA). Stainless steel metal moulds with outer diameter of 2, 1.6 and 1.4 mm were obtained from McMaster-Carr (Dayton, NJ, USA). Biomedical grade PLGA 50/50 (inherent viscosity of 1.13dL/g in HFIP) was purchased from Absorbable Polymers International (Pelham, AL, USA). Poly(vinyl alcohol) (PVA) with a molecular weight of 6000 D hydrolyzed to 80% was purchased from PolySciences Inc (Warrington, PA, USA). Recombinant human epidermal growth factor (rhEGF) and rhEGF ELISA development kits were purchased from PeproTech Inc. (Rocky Hill, NJ, USA) and Sigma Aldrich (Oakville, ON, CA). The MicroBCA Protein Assay Kit was purchased from Pierce (Rockford, IL, USA). Deionized water was obtained from Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA, USA) water purification units at 18 MΩ resistance.

2.1. Preparation of microspheres

PLGA 50/50 microspheres were prepared by a standard double emulsion / solvent evaporation technique [23,24]. Briefly, 1 g of PLGA 50/50 was dissolved in 5mL of dichloromethane to which 0.5 mL of an aqueous protein solution containing 60 mg BSA alone or in combination with 0.1 mg rhEGF. The mixture was homogenized for 1.5 min using a Brinkman Polytron homogenizer at 6000 rpm. The homogenized mixture was then added to 25 mL of a previously homogenized 1wt.% PVA aqueous solution and homogenized for another 1 min. Finally the entire volume was added to 1000 mL of a 0.1 wt.% solution of PVA and stirred for 3 h (Caframo Stirrer Type RZR, speed setting 3.0). After complete solvent evaporation, the microspheres were washed by centrifugation in distilled water three times. After the final centrifugation, the wet microspheres were freeze dried and stored dry under aseptic conditions prior to use.

2.2. Characterization of microspheres

Microspheres were characterized for protein encapsulation efficiency, morphology and size distribution ($n=3$ batches). Encapsulation efficiency was calculated by extracting the encapsulated protein from a known mass of microspheres in a 2 mL maximum recovery eppendorf tube. One mL of water and 1 mL of dichloromethane (DCM) was added into the eppendorf tube sequentially to minimize protein-DCM contact and protein denaturation. The mixture was vortexed and centrifuged at 15,000 rpm for 5 min, and the aqueous phase removed and pooled after repeated protein extraction of the organic phase. The pooled volume was analyzed by the BCA protein assay for BSA and rhEGF ELISA for rhEGF. Encapsulation efficiency (EE) was determined according to Eq. (1):

$$\mathrm{EE} = \frac{[C_{\ \mathrm{protein}}] \times \mathrm{DF} \times V_{\ \mathrm{pooled}}}{m_{\mathrm{spheres}} \times m_{\mathrm{theoretical}}} \times 100\%. \quad (1)$$

Where $m_{\mathrm{theoretical}}$ is 60 mg / 1g PLGA or 0.1 mg / 1g PLGA for BSA or rhEGF, respectively; m_{spheres} is the mass of PLGA microspheres used for extraction; C_{protein} is the concentration of protein measured in mg/ml; DF is the post-extraction dilution factor and V_{pooled} is the total pooled eluant volume.

Microsphere morphology was analyzed by the Hitachi S-570 and S-2500 scanning electron microscope (SEM) at 20 kV acceleration voltage. Samples were prepared on double-sided tape and were coated with gold for 45 s.

Microspheres were analyzed for average size and size distribution using a Malvern Mastersizer 2000 laser diffraction particle sizer. Up to 60 mg of microspheres were dispersed in 100 mL of double distilled water and introduced into the particle sizer. Average sizes of each batch are reported averages of three 15 s readings using a refractive index of 1.33 for water and 1.59 (a polymer estimate) for PLGA microspheres. Size averages were compared for homogeneity.

2.3. Preparation of tubes

Chitin tubes of varying degrees of acetylation were prepared utilizing reversible acetylation chemistry previously described [21].

Chitosan tubes were synthesized by acetylation of chitin tubes. 3.2 mL of 70% ethanol was added to 3.12 g of chitosan (2/3 working solution), and the mixture was briefly vortexed.

After allowing the mixture to cool at 4 °C for 20 min, 110 μL of acetic anhydride was added, the mixture was vortexed for 8 s and sonicated for 30 s. The solution was then rapidly withdrawn into a 10 mL syringe and injected through the septum into the glass mould: a 32 cm long glass tube (IDS = 5 mm) was covered with an 8 mm OD rubber septum on one end and a 7 mm OD rubber septum on the other end. A 2 mm metal mould was inserted through the 8 mm OD rubber septum into the glass mould until contact with the 7 mm OD septum was made. The solution was allowed to gel overnight at room temperature after which the tube was removed, washed and left overnight in distilled water.

The washed chitin tubes were removed from the 2 mm metal mould and cut to <10 cm in length. The tubes were then affixed to 10 cm capillary tubes and placed in a solution of 40 wt.% NaOH at 110 °C for 6 h, the solution was changed two times at 2 h intervals. After the final hydrolysis step the chitosan tubes were removed from the capillary tubes and washed with water until completely neutralized. The washed tubes were placed on a 1.4 mm OD metal rod and air-dried for 1.5–2 h after which they were rewetted, removed from the metal moulds and stored in water at room temperature.

2.4. Preparation and characterization of microsphere loaded tubes (MLTs)

Wet chitin tubes ($L = 7$ mm) and air dried chitosan tubes ($L = 5$ mm) (on 1.4 mm metal moulds) were prepared as previously described. Dried chitosan tubes were weighed with the metal cores inside and mass was recorded as m_{dry}. Wet chitin tubes were blotted dry to remove the surface water and mass was recorded as m_{wet}. Soon after weighing, the chitin tubes were filled with microspheres of PLGA 50/50 using a cone-tip spatula and a chitosan tube on a metal core was inserted into the center. The microsphere loaded tubes (MLT) were washed with water to remove surface microspheres and mass measurements recorded as m_{tot}. After weighing, the MLT's were allowed to air dry for 24 h. Following drying they were removed from the metal core and stored in desicators at −20 °C for 3–4 days prior to initiating release studies.

The mass of microspheres incorporated was calculated as: $m_{microsphere} = m_{tot} - m_{wet} - m_{dry}$.

In the case of the rhEGF release study, MLTs were prepared as above, yet only those tubes containing 15 ± 0.5 μg microspheres were used for ELISA analysis. Coated tubes were prepared by submerging the MLTs into stock solutions of 1, 10 and 15 wt.% PDLLA-CL in acetone for 3 s while on the metal core.

MLTs were analyzed using a Hitachi S-2500 and S-450 SEMs at an acceleration voltage of 20 kV, and using optical microscopy with a Leica MZ6 optical microscope to visually verify the physical entrapment of microspheres.

Average layer thickness and concentricity was measured by analyzing 5 MLTs at 4 random points on the circumference. Concentricity (CC) was determined as follows:

$$CC = \left(1 - \frac{\sigma_w}{\bar{x}_w}\right) \times 100\%$$

where σ_w is the standard deviation in layer thickness and x_w is the average layer thickness. Tube coatings were analyzed by comparing uncoated chitosan tubes with 1 wt.%, 10 wt.% and 15 wt.% coated tubes in longitudinal sections. Optical microscopy used to observe microspheres under the transparent outer chitin layer of longitudinal sections and cross-sections of wet-cut MLTs.

2.5. BSA release from microspheres and MLTs

PLGA 50/50 microspheres, with 61.7% encapsulation efficiency and 3.24% protein loading, were used to study BSA release from microspheres and MLTs. For release from microspheres, 15 mg of microspheres were weighed into 6 maximum recovery Eppendorf tubes and suspended in 1.75 mL PBS with 0.01 wt.% sodium azide. The eppendorf tubes were sealed and placed on its side on a shaker plate in a 37 °C incubator for the duration of the study. At regular intervals of 1, 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 d, the Eppendorf tubes were removed, vortexed for 10 s and centrifuged at 15,000 rpm for 5 min 3 × 150 μL of the supernatant was removed as samples for the micro BCA protein assay, the remaining 550 μL was removed and discarded. 1 mL of new PBS with 0.01 wt.% sodium azide was introduced into each Eppendorf tube, which was vortexed and returned to the incubator.

For release from MLTs, five MLTs were placed into a 2 mL maximum recovery eppendorf tube with 1 mL PBS and 0.01 wt.% sodium azide. The Eppendorf tubes were placed on a shaker-plate inside an incubator. At regular intervals of 1, 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77 and 84 days, 450 μL of PBS was removed, replaced by 450 μL of new PBS with 0.01 wt.% sodium azide and vortexed. The removed eluant was analyzed using the micro BCA protein assay. Assay protocol and microplate procedure were followed, readings were taken using the VERSAmax, Molecular Devices (Sunnyvale, CA, USA) ELISA plate reader at 562 nm.

2.6. Release of rhEGF from MLTs

Dried 5 mm chitosan tubes on metal cores were autoclaved for 2 h at 121 °C. Wet chitin tubes, propeller apparatus and glassware were disinfected by washing thoroughly in 70% ethanol and dried in an oven for 4 h.

Controlled loading MLTs with rhEGF were prepared as previously described and placed in maximum recovery Eppendorf tubes with 1 mL of sterile PBS supplemented with 5 wt.% penicillin/streptomycin (pen/strep) and placed in a 37 °C incubator for the duration of the study. At regular intervals of 1, 7, 14, 28 and 56 days the Eppendorf tubes were removed and vortexed, 1000 μL of eluant was removed and replaced by 1000 μL of new sterile PBS. The removed eluant was stored in two separate volumes of 250 and 750 μL for ELISA and bioactivity assays, respectively, at −80 °C until analysed. ($n = 3$ for this study).

The concentration of rhEGF in the release buffer was determined using the PeproTech rhEGF ELISA kit according to the protocol provided. Specifically, block buffer and dilutant

solutions were spiked with 0.01 wt.% sodium azide and Costar High Binding ELISA plates were used. Readings were taken using the VERSAmax, Molecular Devices (Sunnyvale, CA, USA) ELISA plate reader at 562 nm.

Isolation of neural stem cells was achieved by dissection of forebrain germinal zones in adult mice (CD1 strain) (Charles River) as previously described [25]. The cells were maintained in serum-free media (SFM) and 1% pen/strep containing epidermal growth factor (EGF, 20 ng/mL), basic fibroblast growth factor (FGF, 20 ng/mL) and heparin (7.32 ng/mL) for no longer than 4 weeks (P4) in culture. Under these baseline conditions, clonally derived colonies of cells (or neurospheres) develop in culture and were used as a measure of bioactivity of elutants from days 1, 7, 14 and 21. Briefly, 5000 single (dissociated) neurosphere-derived cells were plated in a suspension of SFM+5% pen/strep for 9 days at 37 °C with 50 μL elutant from days 1, 7, 14 and 21. We examined the numbers of neurospheres that formed relative to positive control cultures using Peprotech rhEGF prepared with an equivalent concentration to the eluants as determined by ELISA at the respective time points (e.g., 56 ng/ml at day 1, 8.6 ng/ml at day 7, 7.1 ng/ml at day 14, 6.2 ng/ml at day 21). These concentrations were added to the cell culture media, resulting in the following rhEGF eluant concentrations being tested for bioactivity (and fresh rhEGF added to positive controls): 3.73, 0.57, 0.47 and 0.41 ng/ml of rhEGF at days 1, 7, 14 and 21, respectively. Neurosphere derived cells plated in the absence of rhEGF served as negative controls.

2.7. Statistical analysis

Statistical analysis was performed using SigmaStat statistical software. Student's *t*-test was used to analyze statistical significance of means from two groups. Single and multi-factor repeated measurement ANOVA tests were used to compare means of three or more factors at various levels. Tukey's post hoc test was used to determine specifically the different groups following ANOVA analysis. All results were considered statistically significant when $p<0.05$, unless otherwise specified.

3. Results

The goal of this study was to develop a biodegradable tubular construct for sustained release of bioactive molecules. To achieve this goal, BSA and rhEGF encapsulated PLGA50/50 microspheres were entrapped between two concentric tubes consisting of an outer chitin tube and an inner chitosan tube. This three-layered tubular structure took advantage of the drying-induced shrinkage of chitin, such that the microspheres were physically entrapped between the inner chitosan tube and the outer chitin tube.

3.1. Morphology and structure of MLTs

To determine the structure and distribution of microspheres in the MLT, tubes were analyzed longitudinally and in cross-section. As shown qualitatively in the longitudinal light micrograph of the MLT (Fig. 1A and B), microspheres (white) were easily visualized through the transparent chitin and chitosan tubes to be distributed along the entire length of the tube, with no obvious voids. Two concentric wall structures of chitin (outside) and chitosan (inside) can be seen in Fig. 1C and D, with a densely packed layer of PLGA 50/50 microspheres in-between, resulting in a 3-layered wall structure. The inner chitosan tube diameter was kept constant at 1.4 mm and the average chitosan wall thickness was 52.0±6.3 μm, with a corresponding concentricity of 88.3±4.0%. The outer chitin tube wall had an average thickness of 53.6±8.3 μm and a concentricity of 91.1±4.3%. The wall thickness and concentricity of chitin and chitosan tubes were not statistically different ($p>0.2$). The thickness and concentricity of the microsphere layer were 91.5±14.6 μm and 90.1±6.4%, respectively. The wall thickness and concentricity of the entire MLT were 197.1±21.2 μm and 89.8±5.3%, respectively. The chitin-microsphere-chitosan construct was held together by the compression forces applied resulting in the drying-induced shrinkage of the outer chitin tube. This construct maintained its three-layer structure in both dry and wet states because the shrinkage of chitin tubes was irreversible.

3.2. Release of BSA from PLGA 50/50 microspheres

PLGA 50/50 microspheres prepared by the double emulsion/solvent evaporation technique were used as the model DDS in this study. The average encapsulation efficiency was 61.70±2.1% as determined by DCM/water extraction and the protein loading was 3.24%. The average particle size was 74.12 μm in diameter with 90% of the particles being between 23 and 180 μm, as determined by

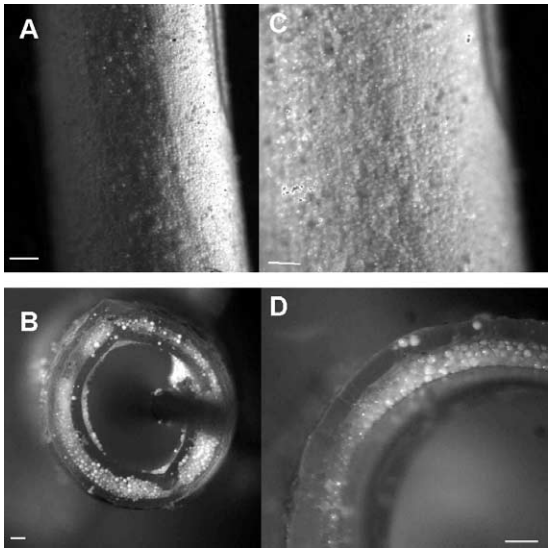

Fig. 1. Light micrographs of MLTs: (A) longitudinal view shows microspheres visualized through the transparent chitin tube and (B) shows a higher magnification of (A); (C) cross-sectional view shows the three layered structure of the tube – the outermost chitin tube, middle microsphere (white) and the innermost chitosan tube – and (D) is a higher magnification of (C). (scalebar is 100 μm).

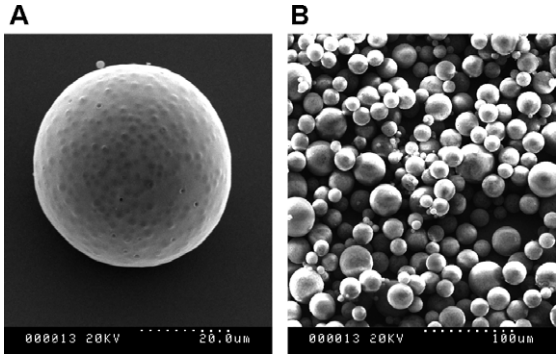

Fig. 2. PLGA 50/50 microspheres used as a model microsphere system for incorporation into the MLT: (A) shows dimpled morphology of one microsphere and (B) provides some insight into the dispersity of the microsphere sizes used.

laser diffraction particle size analyzer. Fig. 2 shows the representative morphology of a single microsphere and a population of microspheres loaded with BSA. The PLGA 50/50 microspheres were found to have a microporous morphology and to be spherical.

Fig. 3 shows the cumulative BSA release from freely suspended PLGA 50/50 microspheres and MLTs. From freely suspended microspheres BSA was released continuously for over 6 weeks, with no lag time from the PLGA 50/50 microspheres in PBS at 37 °C. The release profile was biphasic as expected with a predominantly degradation controlled mechanism. At day 1 a burst of 81.4 μg or 14.7% of the total BSA was released followed by a levelling off to day 14, during which 48.2 μg or 8.7% more BSA was released. A second burst was observed from days 14 to 42, where the majority of the BSA (291.8 μg or 52.5%) was released in a nearly linear fashion. A plateau in release was observed between days 42 to 70 where only 7.7 μg or 1.4% of BSA was released. After 70 days, 4.3 μg of protein was extracted from the remaining solids, leaving approximately 20% of BSA unaccounted for. Some BSA may have been lost due to adsorption to glassware or may reflect inaccuracies in the analytical detection assay.

The BSA release rates from MLTs were monotonic in nature. After the initial burst at day 1 where 19.6±3.5% of BSA was released, there was a progressively slower BSA release rate, as reflected by the decreasing slope. Interestingly, the BSA release profile from MLTs differed from that of freely suspended microspheres. The MLTs released a significantly larger amount of BSA than did microspheres alone throughout the first 28 days of the study; however, by day 42 of the study, the freely suspended PLGA 50/50 microspheres released 75.8±1.1% which was 8.3% more than the MLTs which had released 67.5±5.3% BSA. Significantly larger cumulative release values were observed for the day 42 and 49 groups of the freely suspended microspheres than for the MLTs. The total amount of BSA released from MLTs (82.5±2.1%,) was greater than the total amount released by microspheres (77.2±0.51%), notwithstanding that BSA was released for a longer period of time from MLTs (84 d) than from microspheres (70 d).

3.3. Release and bioactivity of rhEGF from MLTs

In order to assess the ability of the constructs to deliver controlled doses of bioactive protein, PLGA 50/50 microspheres encapsulated with rhEGF and BSA (1:600 w/w) were embedded into the MLTs and release profiles measured. Fig. 4 shows the cumulative percent and mass release of rhEGF from MLTs. A total of 8.8±0.2% of rhEGF initially embedded into the MLT was released over the 56 day study, with just over half of that released on day 1 (4.6±0.2%) and 5.8±0.4% or 71.4±4.5 ng rhEGF released by day 14.

The bioactivity of rhEGF released from MLTs was assessed using an in vitro neurosphere-forming assay. As was determined by rhEGF ELISA, the concentrations of rhEGF eluted were 56 ng/ml at day 1, 8.6 ng/ml at day 7, 7.1 ng/ml at day 14 and 6.2 ng/ml at day 21. Due to dilutions in cell culture media,

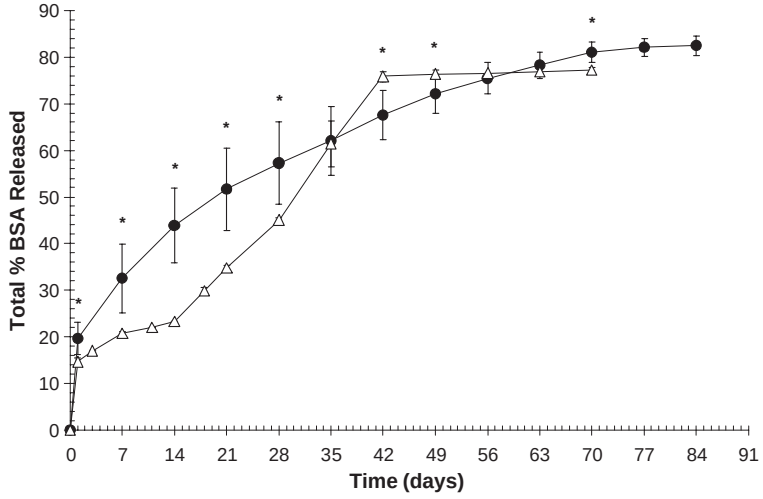

Fig. 3. The cumulative % release profile of BSA from: (Δ) PLGA 50/50 freely suspended microspheres compared to (●) MLTs over 84 days. The release profile from MLTs differed from that from microspheres which had the typical degradation-controlled release profile. The release profile from MLTs was monotonic. Sample points with (*) indicate significant differences by a student's *T*-test ($p<0.05$) ($n=4-5$).

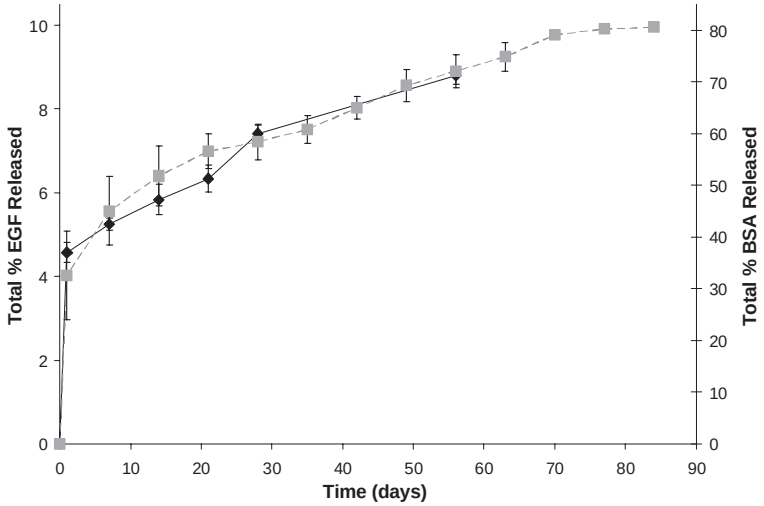

Fig. 4. Cumulative % release profile for (♦) rhEGF and (■) BSA from coated MLTs. (Note different *y*-axes for rhEGF and BSA). The release profiles of EGF and BSA were similar yet the amounts released were significantly different.

the following concentrations were tested: 3.73, 0.57, 0.47 and 0.41 ng/ml of rhEGF at days 1, 7, 14 and 21, respectively. Positive controls had an equivalent concentration of rhEGF added at the respective time-points and negative controls had 0 ng/ml rhEGF. As shown in Fig. 5, the positive controls for days 1, 7 and 14 had 17±10, 22±13 and 38±10 neuro-10 neurospheres formed, respectively, and the release buffers for days 1, 7 and 14 had 28±16, 47±36, 43±12 neurospheres formed, respectively, none of which were significantly different ($p>0.05$), demonstrating release of bioactive rhEGF for 14 days. (As is evident by the large standard deviations, this is not an exact assay and although the eluant EGF promotes greater numbers of neurospheres, these differences are not statistically different.) By day 21 however, the positive control had 31±14 neurospheres formed which was significantly different from the day 21 eluant samples which consistently showed no neurosphere formation even though both rhEGF concentrations were equivalent as analyzed by the rhEGF-ELISA. Neurospheres consistently failed to form in the negative control medium (without rhEGF). Together these data demonstrate that bioactive rhEGF was released for at least 14 days and less than 21 days.

4. Discussion

Chitosan and chitin were specifically chosen for the NGCs described in this report. The inner chitosan tube provides a cell-adhesive surface which is advantageous for stem cell interaction and guided regeneration through the tube and the outer chitin tube provides a non-cell-adhesive surface, which is important for reduced cell and tissue interaction with the outer tube [21]. Chitin also shrinks upon drying allowing a large number of

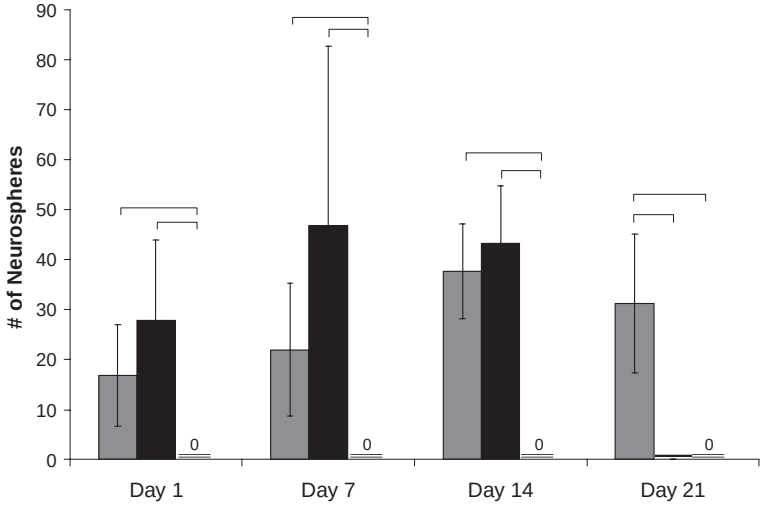

Fig. 5. The bioactivity of (■) released rhEGF was compared to (▨) positive (fresh rhEGF) and (□) negative (no rhEGF) controls by counting the number of neurospheres formed after 7 days in culture of neural progenitor cells from the sub-ventricular zone of mice. No neurospheres formed when rhEGF was not present (i.e., in negative controls). There was no statistical difference in the number of neurospheres formed between positive controls and eluant rhEGF from days 1, 7 and 14; however, at day 21, no neurospheres were observed in eluant samples indicating that the released rhEGF was not bioactive. One-Way ANOVA, Tukey *T*-test ($p<0.05$), tie-bars indicate statistical difference ($n=3$).

microspheres to be entrapped in the annulus between the two concentric tubes. Interestingly, chitin does not swell significantly after re-wetting because the original chitin honeycomb wall structure collapses upon drying [21], thereby stably entrapping microspheres in both wet and dry states. The microspheres are distributed evenly in the NGC wall, both in cross-section and longitudinally, as evidenced by the concentricity and wall thickness data. This new method for protein delivery is advantageous over other methods, such as the DDS rod incorporated into the tube lumen [20], which results in an inhomogeneous release profile due to limitations inherent in the design. According to the MLT release data, 524 μg of BSA was incorporated into a 1 cm long MLT prepared by the shrinking entrapment method using the double emulsion/solvent evaporation PLGA 50/50 microsphere encapsulation technique. This amount is limited by protein loading into microspheres, which was 3.24%, w/w. Simply doubling the percent protein encapsulated would double the protein loading [23]. Alternatively, using a newly developed spray/dry microsphere system, which boasts protein loadings of ~90% [26], the shrinkage entrapment method for MLTs could theoretically deliver 15 mg of protein/cm of NGC in a temporal–spatial manner. Similarly, changing the microsphere properties will impact release profile. For example, polymer molecular weight, composition and microsphere size and morphology will impact degradation rate and release profile [24].

The release profile of BSA from PLGA 50/50 microspheres differed from that of MLTs. The release profile from PLGA 50/50 reflects the bulk hydrolytic degradation profile that has been reported [22] where the initial burst is due to the diffusion of proteins at the microsphere surface and the second large release is due to polymer degradation [24,27]. The release profile from the MLTs differed from that of microspheres alone. The initial burst release from MLTs was greater than that from microspheres alone. Since MLTs were stored for 3–4 days before testing, the initial burst is likely greater than that from microspheres alone due to the diffusion of surface proteins on the microspheres. Up to day 14, the release profile from MLTs was greater than that from microspheres alone likely due to the high packing density of microspheres within the tube wall, causing some autocatalytic degradation process. However, from days 14 to 42, the release rate from microspheres increased relative to that from MLTs suggesting that either the presence of less water in the densely packed microsphere layer within the tube wall or the buffering effect of basic chitosan amine groups on acidic PLGA degradation [28] reduced the autocatalytic effect. This buffering effect of chitin/chitosan tubes may explain the greater time associated with the release profile from MLTs than microspheres alone. Interestingly, a greater percentage of BSA was detected from MLTs (82.5 ± 2.1%) than from microspheres alone (77.2 ± 0.5%) likely because the chitosan/chitin tubes decreased the rate of PLGA degradation or acidic build-up, thereby providing a less denaturing environment and ultimately allowing more BSA to be released and detected.

The release profile of rhEGF closely resembled that of BSA but significantly less rhEGF was released. The release profile of both rhEGF and BSA are similar likely because the mechanism of release is dependent upon the encapsulation methodology and not the protein encapsulated. The low 8.8% of rhEGF released by day 56 relative to the higher 72.1% of BSA released may be explained by the differences in assay specificities. The BCA analytical technique, used to measure BSA, is non-specific and unaffected by bioactivity whereas the ELISA, used to measure rhEGF, is highly specific and requires active binding of rhEGF epitopes [29]. Unlike the BCA colorimetric assay, the rhEGF ELISA is sensitive to protein denaturation and specific to rhEGF. Given the relatively low amounts of EGF encapsulated (compared to BSA), any loss would have had a significant impact on the overall amount of rhEGF detected (and only a marginal effect on BSA). Thus the difference in amount of BSA vs rhEGF detected is likely due to lost bioactivity of rhEGF over time.

The bioactivity of released rhEGF was tested with SVZ-derived adult stem cells which require rhEGF to form neurospheres [30]. The released rhEGF was found to be bioactive for 14 days, relative to positive controls; however, by day 21, the released rhEGF was unable to promote neurosphere formation, indicating that rhEGF bioactivity had been lost between days 14 and 21. Interestingly, rhEGF was found to be bioactive for 14 days using a completely different drug delivery system [31], suggesting an inherent limited bioactivity of rhEGF. The amount of released rhEGF was sufficient to promote neurosphere formation in vitro, suggesting that therapeutically relevant concentrations of bioactive proteins can be released and tested in vivo.

5. Conclusions

Shrink entrapment of microspheres is a promising approach for combination therapy of guidance channels and local drug delivery system. While this method was designed with a nerve regeneration application in mind, it may be useful in other applications, such as vascular or urological grafts that require a combined device–drug delivery approach. These newly formulated microsphere-loaded tubes are promising for prolonged localized delivery to the site of implantation where both temporal and spatial release control are required. In ongoing studies we are investigating the utility of these MLTs as stem cell delivery vehicles for spinal cord injury repair.

Acknowledgments

We thank the Natural Sciences and Engineering Research Council of Canada (MSS, CMM), the Ontario Graduate Scholarship in Science and Technology (AG), Ontario Neurotrauma Foundation (VS) and the Crother's Family Scholarship (AG, VS) for financial support of this work.

References

[1] G.R.D. Evans, Peripheral nerve injury: a review and approach to tissue engineered constructs, The Anatomical Record 263 (2001) 396–404.

[2] M.F. Meek, K. Jansen, R. Steendam, W. van Oeveren, P.B. van Wachem, M.J. van Luyn, In vitro degradation and biocompatibility of poly(DL-lactide-e-caprolactone) nerve guides, Journal of Biomedical Materials Research 68 (2004) 43–51.
[3] X.M. Xu, V. Guenard, N. Kleitman, M.B. Bunge, Axonal regeneration into Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord, Journal of Comparative Neurology 351 (1995) 145–160.
[4] E.C. Tsai, P.D. Dalton, M.S. Shoichet, C.H. Tator, Synthetic hydrogel guidance channels facilitate regeneration of adult rat brainstem motor axons after complete spinal cord transection, Journal of Neurotrauma 21 (2004) 789–804.
[5] S.G. Waxman, Gifts from the molecular revolution: protection and repair of the injured spinal cord, Journal of Spinal Cord Medicine 27 (2004) 304–310.
[6] F.A.I. Yakovlel A.G., Molecular strategies in CNS injury, Journal of Neurotrauma 12 (1995) 767–777.
[7] M.H. Tuszynski, D.A. Peterson, J. Ray, A. Baird, Y. Nakahara, F.H. Gage, Fibroblasts genetically modified to produce growth factor induce robust neuritic ingrowth after grafting to the spinal cord, Experimental Neurology 126 (1994) 1–14.
[8] P. Diener-Ostfield, P.S. Distefano, M.M. Mcatee, B.S. Bregman, Trophic and tropic influence of exogenous NT-3 and BDNF after neonatal spinal cord lesions, in: Sc. Neurosci. Abstr., vol. 19, 1993, pp. 1105.
[9] T.A. Kapur, M.S. Shoichet, Immobilized concentration gradients of nerve growth factor guide neurite outgrowth, Journal of Biomedicine and Biotechnology 68 (2004) 235–243.
[10] M.E. Emborg, J.H. Kordower, Delivery of therapeutic molecules into the CNS, Neural Plasticity and Regeneration 128 (2000) 323–332.
[11] M.F. Haller, W.M. Saltzman, Nerve growth factor delivery systems, Journal of Controlled Release 53 (1998) 1–6.
[12] K.M. Rich, et al., Nerve growth-factor enhances regeneration through silicone chambers, Experimental Neurology 105 (1989) 162–170.
[13] A. Derby, et al., Nerve growth-factor facilitates regeneration across nerve gaps—morphological and behavioural-studies in rat sciatic-nerve, Experimental Neurology 119 (1993) 176–191.
[14] R. Midha, C.A. Munro, P.D. Dalton, C.H. Tator, M.S. Shoichet, Growth factor enhancement of peripheral nerve regeneration through a novel synthetic hydrogel tube, Journal of Neurosurgery 99 (2003) 555–565.
[15] M.R. Wells, et al., Gel matrix vehicles for growth factor application in nerve gap injuries repaired with tubes: a comparison of biomatrix, collagen, and methylcellulose, Experimental Neurology 143 (1997) 70–81.
[16] J.H. Ye, J.D. Houlde, Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons, Experimental Neurology 143 (1997) 70–81.
[17] S.E. Sakiyama-Elbert, A.J. Hubbell, Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix, Journal of Controlled Release 69 (2000) 149–158.
[18] E.C. Tsai, P.D. Dalton, M.S. Shoichet, C.H. Tator, Matrix Modification within Synthetic Hydrogel Channels Improves Specific Supraspinal and Local Axonal Regeneration After Complete Spinal Cord Transection. Biomaterials 27 (2006) 519–533.
[19] V.R. Sinha, A. Trehan, Biodegradable microspheres for protein delivery, Journal of Controlled Release 90 (2003) 261–280.
[20] F.M. Barras, P. Pasche, N. Bouche, P. Aebischer, A.D. Zurn, Glial cell line-derived neurotrophic factor released by synthetic guidance channels promotes facial nerve regeneration in the rat, Journal of Neuroscience Research 70 (2002) 746–755.
[21] T. Freier, R. Montenegro, H. Shan Koh, M.S. Shoichet, Chitin-based tubes for tissue engineering in the nervous system, Biomaterials 22 (2005) 4624–4632.
[22] U. Edlund, A.C. Albertsson, Degradable polymer microspheres for controlled drug delivery, Degradable Aliphatic Polyesters 157 (2002) 67–112.
[23] A. Piotrowicz, M.S. Shoichet, Incorporation of microspheres into nerve guidance channels for drug delivery purposes, Applied Chemistry and Chemical Enginnering, University of Toronto, Toronto, 2005, p. 108.
[24] X. Cao, M.S. Shoichet, Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders, Biomaterials 20 (1999) 329–339.
[25] V. Tropepe, M. Sibilia, B.G. Ciruna, J. Rossant, E.F. Wagner, D. van der Kooy, Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon, Developments in Biologicals 208 (1999) 166–188.
[26] B. Sjoblom, et al., Method to Obtain Particles, AstraZeneca, U.S., 2004, p. 6.
[27] M.J. Mahoney, W.M. Saltzman, Controlled release of proteins to tissue transplants for the treatment of neurodegenerative disorders, Journal of Pharmaceutical Sciences 85 (1996) 1276–1281.
[28] D.B. Burns, A.L. Zydney, Buffer effects on the zeta potential of ultrafiltration membranes, Journal of Membrane Science 172 (2000) 39–48.
[29] B.A. Sampoli Benitez, E.A. Komives, Disulfide bond plasticity in epidermal growth factor, Proteins, Structure, Function, and Genetics 40 (2000) 168–174.
[30] C.M. Morshead, B.A. Reynolds, C.G. Craig, M.W. McBurney, W.A. Staines, D. Morassutti, S. Weiss, D. van der Kooy, Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells, Neuron 13 (1994) 1071–1082.
[31] M.C. Jimenez Hamann, E.C. Tsai, C.H. Tator, M.S. Shoichet, Novel intrathecal delivery system for treatment of spinal cord injury, Experimental Neurology 182 (2003) 300–309.