ARTICLES: FORMULATION AND ENGINEERING OF BIOMATERIALS

Design of Protein-Releasing Chitosan Channels

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*After traumatic injury to the spinal cord, the neural tissue degenerates, resulting in lost function below the site of injury. Promoting axonal regeneration after injury remains a challenge; however, guidance channels have demonstrated some success when combined with cellular and protein therapies. One of the limitations of current guidance channels is the inability to deliver therapeutically relevant molecules in situ, within the guidance channel, to enhance regeneration. In an effort to provide a system for local and sustained drug release, poly(lactide-*co*-glycolide) (PLGA) microspheres were embedded into chitosan guidance channels by a novel spin-coating technique. The method was designed to create guidance channels with the appropriate dimensions for implantation into the spinal cord, with special attention paid to the wall thickness. The release and bioactivity of a model protein, alkaline phosphatase, was followed from the channels and compared to those from free-floating microspheres over a 90-day period. Since chitosan formulations often require the use of acidic solutions, careful attention was paid to redesign the process to minimize exposure of PLGA microspheres to acid. This was achieved as demonstrated by release and bioactivity data where alkaline phosphatase released from chitosan/microsphere channels followed a profile and bioactivity similar to those of free floating microspheres.*

Introduction

Axons in the adult spinal cord fail to regenerate after injury, translating into permanent loss of motor and sensory function. Although the clinical treatment of spinal cord injury has improved over the past several decades, promotion of regeneration of nerve tissue still eludes scientists.[1] Complete transection of the spinal cord is the most severe experimental model of spinal cord injury. While relatively little success has been achieved with this model in terms of functional recovery, it serves as a good model for studying axonal regeneration because there is no ambiguity that axons crossing the injury site have regenerated.

One promising strategy for studying and promoting axonal regeneration after spinal cord transection is entubulation by guidance channels. Guidance channels, popularized by their success in treating long-gap peripheral nerve injuries,[2,3] function as a physical substrate for directed growth of regenerating tissue and provide a permissive environment. Our laboratory and others have demonstrated that entubulation of the transected spinal cord results in tissue bridging across the injury site and supports the survival of cellular transplants using a variety of guidance channels including poly (hydroxyethylmethacralyate-*co*-methylmethacrylate) (pHEMA-MMA),[4,5] polylactic acid (PLA),[6] polyacrylonitrile/polyvinyl chloride

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(PAN/PVC),[7] and chitosan[8] Unfortunately, meaningful recovery of function was not achieved in these studies.

Combining the guidance channels with growth promoting signals is a logical extension of the regenerative strategy. Therapeutic molecules such as neurotrophic factors,[9–11] agents that neutralize growth inhibition,[12–14] and enzymes that break down scar tissue[15] are promising candidates for stimulating axon growth. Many potential drug therapies for nerve regeneration will require prolonged delivery to allow the relatively slow process of axonal regeneration to occur. Moreover, localized delivery is desired because many of these agents have limited ability to cross the blood brain barrier and may also result in unwanted side effects if delivered systemically. Minipumps/catheters have been used to achieve sustained delivery to the spinal cord; however, the release is not localized, there is no pathway on which axons can regenerate and there are complications associated with catheter placement, such as infection and/or compression of the cord.[16,17] Our hypothesis is that the guidance channel implant can be adapted to serve a dual role as drug delivery vehicle and regenerative pathway to enhance axonal regeneration.

Several groups, our own included, have reported various designs of drug delivery channels. Typically, these have involved filling channels with a loose hydrogel matrix containing drug,[5,18] direct drug encapsulation into the channel walls,[19,20] or incorporation of separate drug delivery devices such as rods[21,22] or degradable microspheres.[23,24] Of these designs, microsphere-based systems are the most desirable because they allow for the greatest flexibility in controlling release rates. Recently, our group has reported the successful release of bioactive epidermal growth factor (EGF) from poly(lactide-*co*-glycolide) (PLGA) microspheres entrapped between concentric chitin and chitosan channels.[25] However, the large wall thickness of this three-layered tube was problematic when used in the spinal cord: the tube wall occupied too much space in the spinal canal, requiring the spinal cord tissue to be raised in order to be inserted into the tube. This thick, three-layered tube design led to further injury and compression of the cord and was thus inappropriate for use in the spinal cord. This concern was reported by Nomura et al, who showed that thick-walled channels (0.6 mm) resulted in cavity formation (syrin-gomyelia) inside the inserted spinal cord stump.[26]

The present study describes the design and evaluation of a drug delivery channel specifically for use in the spinal cord. Figure 1 summarizes the design strategy. Protein-loaded PLGA microspheres were embedded into chitosan channels by a spin-coating technique, resulting in an even distribution of microspheres along the interior channel wall. These microsphere-loaded channels were tested by monitoring the release and bioactivity of a model protein, alkaline phosphatase, over a 3-month period.

Materials and Methods

Materials

Poly(lactide-*co*-glycolide) (PLGA) 50/50 with an inherent viscosity of 0.37 dL/g in HFIP was purchased from Absorbable Polymers Incorporated (Pelham, NJ). Poly(vinyl alcohol) (PVA), magnesium carbonate, and alkaline phosphatase were purchased from Sigma Aldrich (Oakville, ON, CA). Purified chitosan chloride (Protosan UP CL213) was obtained from NovaMatrix (Drammen, Norway). Glass and stainless steel tubing were purchased from McMaster-Carr (Dayton, NJ). A Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Billerica, MA) water purification unit provided distilled and deionized water for all experiments. All other reagents were purchased and used as received from Caledon Laboratories (Georgetown, ON, CA) unless otherwise stated.

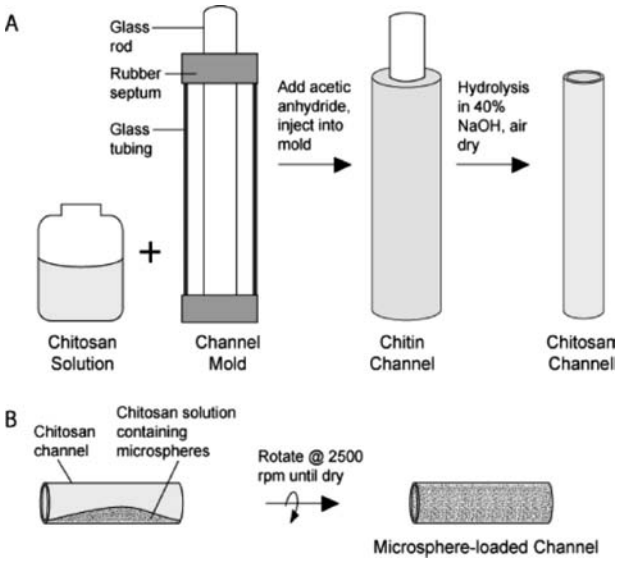

Figure 1. (A) Chitosan channels are prepared by adding acetic anhydride to chitosan then injecting the mixture into a glass mold to prepare chitin channels. The channels are converted back into chitosan by high-temperature alkaline hydrolysis. (B) Microsphere-loaded channels are prepared by spin-coating the interior of a chitosan channel with a chitosan solution containing microspheres.

Microsphere preparation

PLGA microspheres were prepared by a double emulsion (W/O/W) and solvent evaporation procedure. The organic phase consisted of 200 mg of PLGA 50/50 and 6 mg of magnesium carbonate dissolved in 1.5 mL of dichloromethane. A protein solution of 10 mg of alkaline phosphatase in 100 μL of deionized water was added to the polymer solution and the primary emulsion was created by probe sonication for 30 s at 30% amplitude on a Vibracell VCX 130 (Sonics & Materials; Newtown, CT). This mixture was then added to 25 mL of 1% PVA/10% NaCl aqueous solution under homogenization at 4500 rpm for 1 min. This secondary emulsion was poured into 250 mL of 0.1% PVA/10% NaCl solution and magnetically stirred for 3 h to allow for evaporation of the organic solvent. The formed microspheres were washed in water over a 0.2-μm nylon membrane filter, lyophilized, and stored at 4 °C. The theoretical weight percent of alkaline phosphatase in the microspheres was 4.63% based on the initial protein loaded (10 mg) and the total solids (protein, PLGA and magnesium carbonate, 216 mg).

Microsphere characterization

Microsphere mean diameter and size distribution were measured via static light scattering using a Malvern Mastersizer 2000 laser diffraction particle sizer, using refractive indices of 1.33 and 1.59 for water and PLGA, respectively. Reported values are the average of three consecutive measurements.

Microspheres were imaged under high magnification by scanning electron microscopy (SEM). Samples were gold

sputtered twice for 45 s and analyzed on a Hitachi S2500 SEM at 20 kV acceleration voltage.

The loading of alkaline phosphatase in the microspheres was measured by the microBCA protein quantification assay (Pierce; Rockford, IL). Briefly, an accurately measured weight of particles was dissolved in 0.8 mL of dichloromethane then mixed with 0.8 mL of water. The solution was mixed vigorously for 60 s then centrifuged at 10,000 rpm for 5 min. The aqueous portion was removed and the process repeated two more times. The pooled aqueous portion was assayed against a standard curve of known alkaline phosphatase concentration. Encapsulation efficiency was defined as experimental weight percent of protein in the microsphere compared to the theoretical weight percent.

Chitosan guidance channel preparation

Chitosan guidance channels were prepared by a chitosan-chitin-chitosan conversion process as previously described,[27] with minor alterations, as summarized in Figure 1. Chitosan chloride was dissolved in distilled water, then precipitated with 4% NaOH solution, filter washed, and lyophilized. The dried chitosan was dissolved as a 3% solution in 2% acetic acid, followed by 50/50 dilution in ethanol and stored at 4 °C.

Channels were prepared in 15 cm length cylindrical glass molds, made by inserting an inner glass rod (o.d. = 4 mm) into a larger glass tube (i.d. = 7 mm). The inner rod was fixed in place at both ends by rubber septa (Sigma Aldrich). The first step in the process was to convert chitosan into chitin hydrogel in order to control the shape of cast channels; 67 μL of acetic anhydride (2-fold molar excess with respect to available amines) was added to 4 mL of chitosan/ethanol solution, vortexed, evacuated to remove air bubbles, and injected into the molds. After 24 h, the chitin channels were removed from the outer mold, and washed in deionized water for an additional 24 h. The chitin channels were converted back into chitosan by two consecutive hydrolysis cycles (2 h, 110 °C) in 40 wt % NaOH solution, with rinsing between cycles. This conversion process allows for control over the final degree of deacetylation of the chitosan channels,[27] which affects many biological properties of chitosan.[28] After another 24-h wash, chitosan channels were removed from the glass rods and air-dried over stainless steel cylindrical cores (o.d. = 3.7 mm). Channels were rehydrated in water, removed from the steel core, and cut to 8 mm lengths. The degree of deacetylation of the chitosan channels was 95% as determined by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.[29]

Microsphere-loaded channels

Microspheres were embedded into the channel walls by a spin-coating method. PLGA particles were suspended at a concentration of 75 mg/mL in a 2 : 1 mixture of 75 mM phosphate buffer (pH7) and 2% chitosan in 1% acetic acid. Then 20 μL of microsphere/chitosan solution was added to a horizontally mounted chitosan channel, held inside a glass mold. The channels were rotated at 2500 rpm for approximately 30 min, until the inner coating became dry. Microsphere loading was 1.5 mg per 8 mm channel.

Channels with alkaline phosphatase dissolved directly into the secondary chitosan layer (75 μg/channel) and channels loaded with blank (non-protein containing) PLGA microspheres were used as controls for the release study.

Microsphere-loaded channels were imaged by light microscopy (Leica MZ6) using ImagePro software. High magnification images of the microsphere-embedded channel wall were taken by SEM (Hitachi S2500). Channels were gold sputter coated in two cycles of 45 s and imaged at an acceleration voltage of 20 kV.

In vitro protein release

The release of alkaline phosphatase was monitored over time and assayed for bioactivity. Free microspheres, microsphere-loaded channels, and control channels (n = 3 per group) were placed in 2-mL maximum recovery Eppendorf vials and suspended in release media (PBS with 0.01% sodium azide) and incubated at 37 °C under mild agitation. At various time points, release media was collected and replaced. Samples containing free-floating microspheres were centrifuged at 8000 rpm for 3 min before media collection and vortexed to re-suspend the microspheres after media replacement. Channel groups had full media collection and replacement at each time point.

The collected release media was analyzed for protein content using the Pierce microBCA protein quantification assay and following the manufacturer's protocol. Alkaline phosphatase was quantified by assessing the enzyme's ability to act on a substrate *para*-nitrophenylphosphate (pNPP). One pNPP (Sigma Aldrich) tablet was dissolved in 5 mL of 100 mM Tris-HCl buffer, pH 8.6 with 10 mM $MgCl_2$; 50 μL of pNPP solution was added to 50 μL for 10 min at room temperature. The reaction was stopped with 50 μL of 4% NaOH and the absorbance of free *p*-nitrophenyl was measured at 405 nm and compared against a standard curve. Both assays were read on a Molecular Devices (Sunnyvale, CA) VERSAmax plate reader.

Results and Discussion

PLGA is a well-characterized polymer used in many drug delivery applications because of its ease of preparation, biocompatibility, and tunable degradation kinetics. Here, PLGA microspheres were successfully fabricated using a standard double emulsion technique. The resultant microspheres were confirmed to be discrete spherical particles under visualization with SEM. As shown in Figure 2, particles had mostly smooth exterior surfaces with very few surface pores and had a mean volume weighted diameter of 26.5 μm with 80% of the spheres in the range of approximately 15–44 μm.

The described protocol for fabricating these microspheres was the result of several iterations where a series of process parameters (e.g., sample volumes and concentrations, homogenization speed) were adjusted to achieve high protein encapsulation efficiency and limit initial burst release. Encapsulation efficiency of alkaline phosphatase-loaded microspheres was 80.3 $\pm$ 1.3%, which is comparable to other optimized PLGA double emulsion systems.[30–33] High encapsulation efficiencies result in greater drug content in the microsphere product and minimize losses during the manufacturing process.

Burst release, which we define as the percentage of encapsulated protein released after 24 h in aqueous suspension, was another important factor in microsphere preparation. About 20% of encapsulated alkaline phosphatase was released in the burst phase, a value that is typical for PLGA systems.[25,34,35] Burst release is typically attributed to protein at or near a porous surface or adsorbed to the outside of the particl.[36] High burst values, which in other systems are as

high as 50% or greater,[31,37–39] are problematic in most drug delivery applications because dose dumping is both inefficient and potentially harmful depending on the drug and target tissue. In our case, limiting burst release is especially important because the microspheres are exposed to additional aqueous processing during the embedding procedure. Low burst will minimize losses during this step, not only from diffusion-mediated release but also because protein close to the microsphere surface may be more susceptible to denaturation from the extra processing required. To gain a greater understanding of the acidic denaturing conditions possible with PLGA[40] and chitosan formulations, we chose to work with alkaline phosphatase as a model protein because of its known sensitivity to acidic conditions.[41]

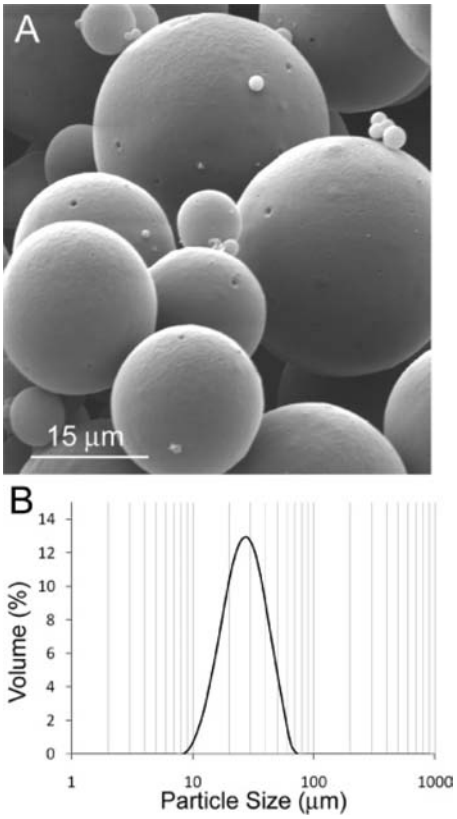

Figure 2. (A) PLGA microspheres visualized using scanning electron microscopy have smooth exterior surfaces with sporadic pores. (B) The size distribution of PLGA particles as measured by light scattering. The average volume weighted diameter of the particles was 26.5 μm as measured by static light scattering.

Our primary focus on the spinal cord presents unique requirements not satisfied by previous drug delivery channels designed for peripheral nerve injury. These include scale-up in channel diameter and a more stringent limit on wall thickness, which is important due to the space limitations of the spinal canal. Another important consideration is a simple manufacturing procedure that minimizes potential drug loss due to harsh conditions or extended exposure to aqueous environments.

The spin-coating procedure was devised as a simple method of stably incorporating microspheres into a chitosan channel without significant changes in the original channel dimensions or materials. Microspheres were suspended in a dilute chitosan solution and spun to dryness onto the interior of a preformed chitosan channel. The secondary chitosan solution was buffered such that the pH of the solution was as high as possible (~pH 6) without causing precipitation of the chitosan. This was done to minimize acidic exposure to both the PLGA polymer and the protein encapsulated within. As the microparticle/chitosan mixture dried, an even coating of microspheres embedded into the tube wall by the secondary chitosan coating resulted, as shown in Figure 3 under both light microscopy and SEM. This coating was stable under normal handling conditions throughout the 3-month study and did not delaminate even under vigorous shaking conditions.

From a design perspective, this method of incorporating microparticles improves many aspects of our previously published drug delivery channel, which was made by sandwiching microspheres between a chitosan and chitin channel.[25] The sandwich channels were primarily designed as a peripheral nerve guide, where the small inner diameter was not a limiting factor. The current design has thinner walls of approximately 200 μm (Figure 3). From our experience, a 300 μm thickness is the upper limit to prevent spinal cord distortion upon placement inside channel in an adult rat model (unpublished observation). Other advantages over the previous design include fewer material components, ability to predetermine microsphere loading, and localization of microspheres to the inner wall of the channel which should result in preferential release to target tissues in the central lumen.

The in vitro release of alkaline phosphatase was tracked and the results plotted in Figure 4. The complete release of alkaline phosphatase from free floating microspheres took approximately 90 d. The cumulative release profile of microsphere-loaded channels mirrored that of free microspheres, with a consistent difference between the two curves of approximately

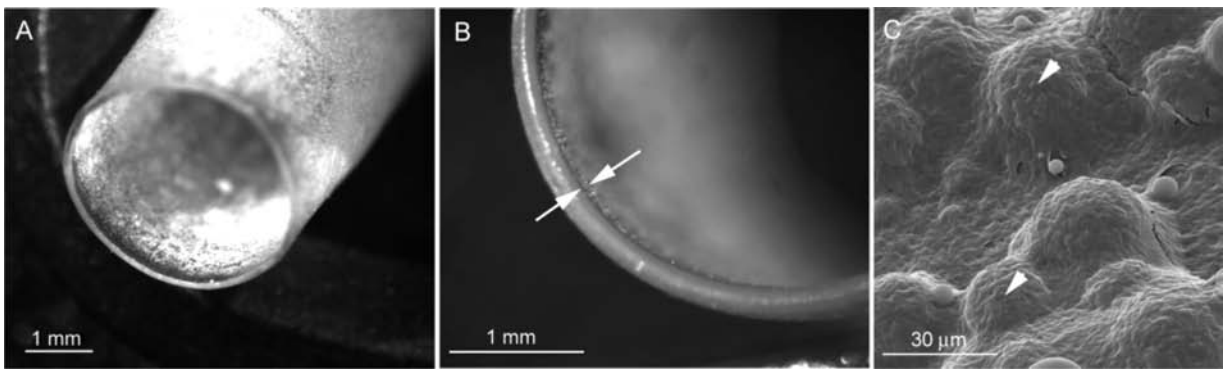

Figure 3. (A, B) Light micrographs of the microsphere-loaded chitosan channels. The thickness of the channels when hydrated is approximately 200 μm, of which the secondary chitosan layer (indicated by arrows) contributes about 20 μm. (C) Scanning electron microscopy shows microspheres (arrowheads) embedded by the secondary chitosan coating.

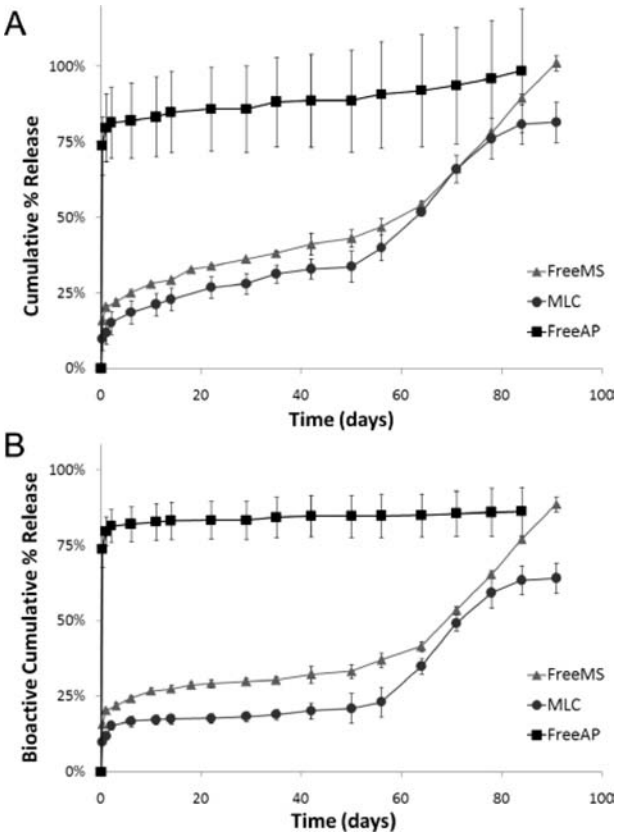

Figure 4. Cumulative release profile of (A) total and (B) bioactive alkaline phosphatase from free floating PLGA microspheres (FreeMS), microsphere-loaded channels (MLC), and dispersed directly into the channel walls (FreeAP) ($n = 3$ for all groups). The release profile of MLCs closely mirrored that of free microspheres. Alkaline phosphatase was released in bioactive form throughout the study, with 87.6 ± 0.4% (av ± SD) of released protein remained active from free microspheres compared to 78.7 ± 1.1% from MLCs.

7%, which represents protein loss during the microsphere embedding procedure. In contrast, when alkaline phosphatase was dissolved directly into the secondary chitosan coating, 80% was released in the first 24 h. Thus the secondary chitosan layer does not act as a significant barrier to protein diffusion and the release of protein from channels is primarily dependent on the properties of the microspheres themselves.

Release from microspheres and channels followed the classic PLGA triphasic profile.[42] The initial stage is attributed to burst release, which is mainly diffusion mediated. The secondary phase is characterized by a steady release rate and is attributed to a balance between increased drug mobility due to hydrolysis of PLGA chains, and the increasing distance that drugs farther from the surface must diffuse.[42] The final accelerated release phase is explained by the local build-up of lactic and glycolic acid, the PLGA degradation byproducts, which have an auto-catalytic effect on polyester degradation.[43] Eventually the PLGA chains breakdown to a critical state such that the microparticle structure starts to disintegrate.[44] Although the time frame for release in this study was 3 months, the release rates for specific drug applications can be tuned to be shorter or longer by modifying various process parameters during microsphere preparation. These include changing the polymer lactide/glycolide ratio[45] or molecular weight,[46] introducing copolymers such as polycaprolactone[47] or additives such as poly(ethylene glycol),[33] or by simply altering microsphere size.[48] PLGA microsphere systems have been shown to be capable of degradation over timescales of a few weeks[46,49] to several months.[50,51]

Protein stability is always one of the biggest concerns in drug delivery systems. As shown in Figure 4B, the majority of alkaline phosphatase remained bioactive throughout the study. Comparing the release data shows that 87.6 ± 0.4% (av ± SD) of the alkaline phosphatase released from PLGA microspheres was active. The bioactivity ratio dropped to 78.7 ± 1.1% in microsphere-loaded channels, indicating that the embedding process did not greatly denature the encapsulated protein. The concern over acidic exposure was attenuated by buffering both the chitosan solution used in the embedding procedure, and by buffering the interior of the PLGA microspheres themselves with magnesium carbonate.[52] Together, these precautionary measures resulted in a high retention of bioactivity of an acid-sensitive protein released from the drug delivery channels.

Guidance channels provide a unique opportunity to influence the local environment, which we have exploited through the integration of a drug delivery system. The microsphere-based system allows great flexibility in both the type and number of therapeutic factors to be delivered, and also the timeframes during which these drugs are released. Concurrent drug therapies can be achieved by incorporating each drug separately into microspheres, then embedding these different microspheres into the same channel. The addition of a drug delivery system capable of local and sustained release is a major and potentially significant evolution of the guidance channel, and will serve as an important tool to test the regenerative capacity of combination therapy for spinal cord injury repair. Future directions of this work will focus on incorporating relevant growth factors for promoting regeneration after spinal cord injury.

Conclusions

PLGA microspheres were formed with high encapsulation efficiency and embedded into preformed chitosan guidance channels. Spin-coating of chitosan channels resulted in the stable entrapment of PLGA microspheres along the inner channel surface. The current design results in thin-walled channels with appropriate dimensions for implantation in the spinal cord. These channels are capable of long-term release of bioactive protein and are promising for the delivery of therapeutic agents to the regenerating spinal cord.

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