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Strategies for neurotrophin-3 and chondroitinase ABC release from freeze-cast chitosan–alginate nerve-guidance scaffolds

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Abstract

Freeze casting, or controlled unidirectional solidification, can be used to fabricate chitosan–alginate (C–A) scaffolds with highly aligned porosity that are suitable for use as nerve-guidance channels. To augment the guidance of growth across a spinal cord injury lesion, these scaffolds are now evaluated *in vitro* to assess their ability to release neurotrophin-3 (NT-3) and chondroitinase ABC (chABC) in a controlled manner. Protein-loaded microcapsules were incorporated into C–A scaffolds prior to freeze casting without affecting the original scaffold architecture. *In vitro* protein release was not significantly different when comparing protein loaded directly into the scaffolds with release from scaffolds containing incorporated microcapsules. NT-3 was released from the C–A scaffolds for 8 weeks *in vitro*, while chABC was released for up to 7 weeks. Low total percentages of protein released from the scaffolds over this time period were attributed to limitation of diffusion by the interpenetrating polymer network matrix of the scaffold walls. NT-3 and chABC released from the scaffolds retained bioactivity, as determined by a neurite outgrowth assay, and the promotion of neurite growth across an inhibitory barrier of chondroitin sulphate proteoglycans. This demonstrates the potential of these multifunctional scaffolds for enhancing axonal regeneration through growth-inhibiting glial scars via the sustained release of chABC and NT-3.

Keywords

chitosan-alginate scaffold; freeze casting; microcapsule; neurotrophin-3; chondroitinase ABC; spinal cord injury

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Author contributions

All authors of this research paper participated directly in the planning, execution or analysis of the study.

Conflict of interest

The authors declare no conflicts of interest.

1. Introduction

Approximately 270 000 people are affected by spinal cord injury (SCI) in the USA, with an estimated 12 000 new cases reported each year (Center, 2012). The application of neurotrophic factors is widely used for SCI repair, since these proteins have been shown to enhance neuronal survival and promote repair and recovery of function after SCI (Nomura *et al.*, 2006). Neurotrophin-3 (NT-3) is frequently used in SCI repair strategies due to its ability to promote the growth of motor axons (Alto *et al.*, 2009). The bacterial enzyme chondroitinase ABC (chABC) has also been shown to promote axonal growth by degrading chondroitin sulphate proteoglycans (CSPGs), the predominant inhibitory molecules found at the injury site (Bradbury *et al.*, 2002; Tom *et al.*, 2009). It is not possible for these therapeutic proteins to be delivered to the spinal cord via systemic administration, since they are rapidly degraded in the blood stream. Furthermore, large proteins cannot cross the blood–cerebrospinal fluid (CSF) barrier. Local delivery of these proteins into the spinal cord has been achieved experimentally by various methods, including direct injection, osmotic mini-pumps and controlled-release platforms (Schnell *et al.*, 1994; Bradbury *et al.*, 1998; Shibayama *et al.*, 1998).

Implantation of a biomaterial scaffold at the site of spinal cord injury can provide a local source for sustained neurotrophin delivery, while simultaneously acting as a physical substrate for guidance of axon growth across the lesion cavity at the injury site. Chitosan–alginate nerve guidance scaffolds (C–A scaffolds) with longitudinally aligned channels have been fabricated using a controlled directional solidification process known as freeze casting (Francis *et al.*, 2013; Riblett *et al.*, 2012). Since scaffold formation via freeze casting occurs at low temperatures, biomolecules can be incorporated into the scaffolds without compromising their function during the fabrication process (Meghri *et al.*, 2010; Wegst *et al.*, 2010).

Chitosan and alginate are linear polysaccharides widely used as biomaterials for implantation (Kim *et al.*, 2008; Augst *et al.*, 2006). Hydrogels, such as those made from chitosan or alginate, are well suited as biomolecule delivery vehicles, due to their porous nature, with release being dependent on bio-molecule diffusion through the hydrogel matrix (Pakulska *et al.*, 2012). A drawback of the high water content and open network of hydrogels is that hydrophilic biomolecules such as proteins can diffuse out of the gel quickly, over a time span of several hours to a few days (Hoare and Kohane, 2008). Interpenetrating polymer networks (IPNs), such as those formed from chitosan–alginate polyelectrolyte complexes (PECs) present in our scaffolds, have relatively dense hydrogel networks and can moderate the effects of burst release of protein and lengthen the duration of release. These effects are due to the IPNs' ability to restrict equilibrium swelling of either or both of the interpenetrating phases according to the crosslinking density (Hoare and Kohane, 2008). Delivery of entrapped biomolecules can also be prolonged by incorporating them in microparticles, such as polymeric microspheres or lipid microtubules, held within the hydrogel (Lee *et al.*, 2010; Baumann *et al.*, 2010). The hydrogel scaffold keeps these secondary delivery vehicles in a predetermined location and limits the burst release commonly seen with microsphere-only systems (Hoare and Kohane, 2008).

We have previously demonstrated the ability of our C–A scaffolds to provide a growth-permissive substrate to promote DRG neurite growth aligned with the scaffold pores *in vitro* (Francis *et al.*, 2013). In addition to providing a substrate for orientated axon growth, here we intended to evaluate the efficacy of our C–A scaffolds as delivery vehicles for the sustained release of neurotrophic factors and chABC. In this study, NT-3, and chABC encapsulated in either alginate or chitosan microcapsules, were incorporated into our porous C–A scaffolds. The *in vitro* release profiles were examined and compared in order to determine whether the scaffolds could provide controlled, sustained biomolecule release.

2. Material and methods

2.1. Chemicals and materials

Alginate (Protanal LF200M, MW approx. 270–325 kDa; lot number S21721) was obtained as a sample from FMC Biopolymer (Philadelphia, PA, USA). Chitosan (MW approx. 50–190 kDa, 85% deacetylated), chondroitinase ABC, calcium chloride, sodium chloride, sodium hydroxide, sodium citrate, epichlorohydrin, D-(+)-trehalose dehydrate, neurotrophin-3 (NT-3), poly-L-ornithine hydro-bromide (MW 30–70 kDa), laminin-111 from EHS murine sarcoma basement membrane (L2020), decorin and mouse monoclonal anti-neurofilament 200 (phos. and non-phos.) clone N52 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol and Pierce Silver Stain Kit were purchased from Fisher Scientific (Fair Lawn, NJ, USA). NuPAGE LDS Sample Buffer, Invitrogen Reducing Agent, Novex Sharp Unstained Protein Standard, NuPAGE 4–12% Bis–Tris gradient gel (1.0 mm), NuPAGE MOPS SDS Running Buffer, tetramethylrhodamine-5-(and-6)-isothiocyanate [5(6)-TRITC] mixed isomers, B-27 supplement, Live/Dead Cell Viability/Cytotoxicity Kit for mammalian cells and AlexaFluor 488 goat anti-mouse IgG secondary antibody were purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM; with 4.5 g/l glucose, L-glutamine and sodium pyruvate), Hanks' buffered salt solution (HBSS), 0.25% trypsin EDTA and antibiotic–antimycotic solution (containing a combination of penicillin, streptomycin and amphotericin formulated to contain 10 000 U/ml penicillin G, 10 mg/ml streptomycin sulphate and 25 µg/ml amphotericin B and diluted to 1% v/v prior to use) were purchased from Mediatech (Herndon, VA, USA). Polystyrene culture plates (12- and 24-well) were purchased from Falcon, Becton Dickinson Labware (Franklin Lakes, NJ, USA). NT-3 DuoSet ELISA Development Kits were purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Preparation of chitosan microcapsules

Chitosan microcapsules were prepared by spray drying a chitosan solution (300 ml, 1.5% w/v) using a Buchi 190 Mini Spray Dryer (Buchi, Switzerland) under the following conditions: nozzle diameter, 0.5 mm; inlet temperature, 160 °C; outlet temperature, 90–95 °C; flow rate, 5 ml/min; air spray flow, 700 L/h; aspirator setting, 17. The dry chitosan microcapsules were neutralized by stirring in NaOH/ethanol (500 ml absolute ethanol containing 12.5 ml 6 M NaOH), for 2 h at 400 rpm. The neutralized microcapsules were evenly distributed into four 50 ml polypropylene tubes and centrifuged at $5000 \times g$ for 2 min (Allegra™ 21 Centrifuge, Beckman Coulter, Brea, CA, USA). Each tube of microcapsules was sequentially washed in 50 ml 100% ethanol (1×), 75% ethanol (1×), 50% ethanol (1×),

25% ethanol (1×) and deionized (DI) water (3×). After the last wash with DI water, the microcapsules were vortexed in 20 ml water, frozen in liquid nitrogen and lyophilized for approximately 72 h. Dried chitosan microcapsules were stored in a desiccator at room temperature until further use. The diameter of the dry and hydrated chitosan microcapsules was measured using ImageJ software (NIH, Bethesda, MD, USA).

2.3. Preparation of alginate microcapsules

Alginate microcapsules were prepared by spray drying an alginate solution (300 ml; 1.5% w/v) using a Buchi 190 Mini Spray Dryer under the same processing conditions as the chitosan microcapsules (nozzle diameter, 0.5 mm; inlet temperature, 160 °C; outlet temperature, 90–95 °C; flow rate, 5 ml/min; air spray flow, 700 L/h; aspirator setting, 17). The microcapsules were collected in a calcium chloride (CaCl₂) solution (90 ml, 1.3% w/v) within the product collection vessel of the spray-drying apparatus. The CaCl₂-crosslinked alginate microcapsules were evenly distributed into four 50 ml polypropylene tubes, centrifuged at 5000 × *g* for 2 min and washed once with DI water, followed by a wash in 100% ethanol. Each tube of microcapsules was resuspended in 35 ml 100% ethanol, vortexing briefly before pipetting 30 ml of the microcapsule–ethanol suspension into a 100 ml beaker.

The alginate microcapsules were covalently crosslinked using epichlorohydrin by modifying a protocol described by Sharma *et al.* (2001); epichlorohydrin (6 ml) was added to the microcapsule suspension (30 ml) and this mixture was homogenized on ice at ~6000 rpm (Brinkmann Instruments, Westbury, NY, USA) while slowly adding 10 ml 5 M NaOH. The homogenizer speed was then increased to 9500 rpm for 1 min. The crosslinking mixture was removed from the homogenizer and stirred at approximately 400 rpm on a stir plate for 24 h at room temperature. After 24 h, the mixture was adjusted to pH 7 using 1 M HCl. The covalently crosslinked microcapsules were then washed in 50 ml 100% ethanol (1×), 75% ethanol (1×), 50% ethanol (1×), 25% ethanol (1×) and DI water (3×). Residual CaCl₂ was removed from the alginate microcapsules by stirring in 55 mM sodium citrate for 10 min at room temperature. The microcapsules were then washed in DI water until the microcapsule pellet was firmly settled at the bottom of the tube after centrifugation (*ca.* five washes). After the last wash with DI water, the microcapsules were vortexed in 20 ml water, frozen in liquid nitrogen and lyophilized for approximately 72 h. Dried alginate microcapsules were stored in a desiccator at room temperature until further use. The diameters of the hydrated alginate microcapsules were imaged via differential interference contrast, using an Olympus IX81 microscope, and measured using ImageJ.

2.4. Scanning electron microscopy (SEM)

Dry microcapsules or dry C–A scaffolds with incorporated microcapsules were directly mounted onto aluminium stubs with double-sided adhesive tabs. The samples were sputter-coated with a 3 nm-thick platinum–palladium layer and imaged using a Zeiss Supra 50VP SEM (Carl Zeiss SMT Inc.) with an accelerating voltage of 6 kV and a working distance of 5 mm.

2.5. Fabrication of protein-loaded C–A scaffolds

NT-3 (10 µg) was dissolved in 26.5 µl cold (4 °C) phosphate-buffered saline (PBS). ChABC (Sigma) was resuspended in cold PBS to a concentration of 754 U/ml. These dilutions were calculated in order to give a final load of 1 µg NT-3/98 mm³ scaffold, or 2 U chABC/98 mm³ scaffold (total mass depending on chABC specific activity). An aliquot of 20 µl of the desired protein solution was added to a 1.5 ml microcentrifuge tube containing 2 mg of either dry alginate or chitosan microcapsules, or an empty tube. These tubes were kept at 4 °C while the C–A PEC mixture was prepared, as described in Francis *et al.* (2013).

Briefly, 1.5% w/v chitosan and alginate solutions were produced by dissolving in 1% acetic acid and DI water, respectively. These solutions were mixed in equal volumes and the pH was adjusted to 7.4 with 6 M sodium hydroxide. A homogeneous C–A PEC mixture was produced by sonicating in an ice bath with 90 W applied power, using a 20 kHz sonicator probe (CL4 tapped horn probe with 0.5 in tip; Misonix Inc.), pulsing for 2 min at 15 s intervals with a 3 s pause. The C–A PEC mixture was degassed at 2500 rpm for 1 min in a SpeedMixer (DAC 1510 FVZ-K, FlackTek, Landrum, SC, USA) before mixing with the protein-loaded microcapsules (total final volume of 667 µl). The C–A PEC + microcapsule mixture was pipetted into custom-made cylindrical polytetrafluoroethylene moulds (2.5 mm i.d. × 20 mm tall) with a copper bottom plate and secured onto the temperature-controlled copper cold finger of the freeze-casting apparatus (Wegst *et al.*, 2010). The cold-finger temperature was reduced at a constant rate of 1 °C/min to a final temperature of –150 °C. After the samples were completely frozen, the copper bottom plates were removed from the moulds and the frozen samples within the moulds were lyophilized for 24 h. Retaining the frozen scaffolds within the mould during lyophilization helped preserve the shape. The dried protein-loaded scaffolds were removed from the moulds using dissecting forceps and stored in a desiccator at –20 °C until further use.

2.6. Quantification of NT-3 release from C–A scaffolds

Dry scaffolds (with or without microcapsules) were crosslinked in an aqueous 0.25% calcium chloride (CaCl₂) solution for 10 min and washed in PBS. The scaffolds were then sequentially coated by adsorption with poly-L-ornithine (PLO; 0.5 mg/ml) for 6 min, rinsed three times in PBS, followed by laminin (100 µg/ml) for 1 h, and further rinsed three times in PBS prior to further testing. The NT-3-loaded scaffolds were then placed into a 1.5 ml microcentrifuge tube with an O-ring seal cap (Corning, Tewksbury, MA, USA) containing 0.5 ml release buffer (PBS containing 1 M trehalose for protein stabilization, 0.2 µm filtered). The tubes were kept at 37 °C, under gentle agitation on an end-over-end shaker. At designated time points, the total 0.5 ml release buffer was removed and kept at –20 °C until further use, and replaced with 0.5 ml fresh release buffer. To quantify the mass of NT-3 present in the collected release buffer, DuoSet ELISA Development Kits (NT-3, R&D Systems) were used according to the manufacturer's protocol. Two-way ANOVA with repeated measures was performed for statistical analysis of neurotrophin release ($n = 3$ for each scaffold formulation).

2.7. Determination of NT-3 bioactivity after release from C–A scaffolds

Bioactivity of NT-3 released from C–A scaffolds was measured by releasing into DMEM + 2% B-27 supplement + 1% antibiotic–antimycotic in the absence of trehalose, since it was found to inhibit neurite growth. Acid-washed, flamed glass coverslips (12 mm) were coated with 0.5 ml 0.1 mg/ml PLO (diluted in water) overnight. The PLO-coated coverslips were washed three times with PBS to remove any excess, unadsorbed PLO and placed into 24-well culture plates under sterile conditions. Dorsal root ganglia explants (DRGs) were isolated from embryonic day 10 (E10) chick embryos (Charles River) (Francis *et al.*, 2013) and placed on the PLO-coated coverslips. Release medium collected from the C–A scaffolds was diluted 1:1 with fresh culture medium (DMEM + 2% B-27 supplement + 1% antibiotic–antimycotic) and 200 µl of this diluted medium was added to each well of the 24-well culture plates containing DRGs. Experimental groups were: culture medium with no scaffold present and no exogenous NT-3 added (negative control); culture medium with no scaffold present and 1 ng/ml NT-3 added (positive control); NT-3 loaded scaffold + alginate capsules (SAC); scaffold + chitosan capsules (SCC); and scaffold with direct incorporation of protein (SDI); and SAC, SCC and SDI not loaded with protein (blank scaffolds). The DRGs were incubated at 37 °C and 5% CO₂ for 48 h. After 48 h, DRG neurite outgrowth was observed using an Olympus IX71 fluorescence microscope and a SPOT RT3 microscope camera. To determine the average neurite length, the distance from the edge of the explant to the tip of the neurite growth cone was measured in four places around the perimeter of the DRG, with each length measurement separated by 90° ($n = 14$ DRGs/group) (Larsson *et al.*, 2011; Comolli *et al.*, 2009). These average neurite length measurements were compared to average DRG neurite lengths using negative and positive controls of culture medium containing either no exogenous neurotrophin or 1 ng/ml NT-3, respectively. One-way ANOVA was performed for statistical analysis of neurite length measurements.

2.8. SDS–PAGE analysis of ChABC release from C–A scaffolds

To determine whether chABC maintained activity after release from the C–A scaffolds, release buffer was used in an assay with decorin as a substrate. Decorin is a low molecular weight CSPG that is susceptible to ChABC degradation. SDS–PAGE was performed to analyse the degradation of decorin in a method modified from Lee *et al.* (2010). Decorin (10 µl 0.5 mg/ml solution in PBS) was added to 250 µl release buffer collected at various time points from the scaffolds and incubated at 37 °C for 4 h. The samples were further diluted (1:1.5 with PBS) before mixing 13 µl with 5 µl NuPAGE LDS Sample Buffer and 2 µl Invitrogen Reducing Agent and heating at 70 °C for 10 min. The samples were run on a 4–12% Bis–Tris gradient gel (1.0 mm) with NuPAGE MOPS SDS Running Buffer at 200 V for 1 h, using Novex Sharp Unstained Protein Standard as a marker. Silver staining was performed using a Pierce Silver Stain Kit, according to the manufacturer's instructions. Images were taken with an Alpha Innotech FluorChem SP MultiImage Light Cabinet and AlphaEase FC software v 6.0.0 (Santa Clara, CA, USA).

2.9. Assessment of DRG neurite growth on laminin/CSPG striped substrate

DRGs were isolated from embryonic day 10 (E10) chick embryos (Charles River) (Francis *et al.*, 2013) and collected in sterile Hanks' buffered saline solution. DRGs were dissociated

by incubating the whole DRG explants in PBS for 10 min at 37 °C, followed by incubating in 0.25% trypsin–EDTA for 12 min at 37 °C. The DRGs were triturated in DMEM + 10% FBS, using a flame-polished glass Pasteur pipette. Dissociated DRGs were plated in a tissue culture plate with DMEM + 10% FBS + 1% antibiotic–antimycotic for up to 2 h in order to allow non-neuronal cells to attach. The neuronal cells were then harvested from the tissue-culture plate and centrifuged to collect the pellet.

A laminin/CSPG striped substrate was patterned on glass coverslip wells, as described by Curinga *et al.* (2007). Silicone aquarium sealant was used to glue acid-washed, flamed-glass coverslips to the underside of holes formed in the bottom of 12-well culture plates with an area of 2.4 cm². This allowed for accommodation of a larger cover slip and also facilitated subsequent microscopic examination. The coverslip wells were coated with 50 µl nitrocellulose solution (20 cm² Whatman Protran BA85 nitrocellulose dissolved in 24 ml reagent-grade methanol) and the surfaces were then air-dried and UV-sterilized. Filter paper (Whatman No. 1) was cut into strips of 1 mm width and soaked in a solution of 50 µg/ml CSPGs (Chemicon) and 4.4 µg/ml TRITC in PBS. The soaked filter paper strips were placed onto the nitrocellulose substrate in parallel, air-dried and then removed, resulting in the transfer of CSPGs and TRITC to the nitrocellulose substrate. Laminin (100 µl 25 µg/ml solution in PBS) was then spread evenly over the entire well and allowed to adhere to the surface for 2 h before washing three times with PBS to create alternating stripes of laminin and CSPG + laminin. Dissociated DRG neurons were seeded on the laminin/CSPG striped substrate at a density of 10 000 cells/well in DMEM + B27 + antibiotic–antimycotic. Protein-loaded scaffolds were placed into Netwell inserts (500 µm mesh size; Corning) and inserted into the 12-well plates. Additional culture medium was added to raise the total volume to 1 ml/well. The culture plates were incubated at 37 °C and 5% CO₂ for 48 h.

After 48 h, DRG neurons were fixed in 4% paraformaldehyde for 30 min at room temperature and washed three times in PBS. The cells were blocked in 0.1% Triton X-100 with 10% normal goat serum in PBS for 1 h at room temperature, followed by incubation overnight at 4 °C in blocking solution containing mouse monoclonal anti-NF 200 kDa (1:500). The surface was washed three times in PBS and the secondary antibody AlexaFluor 488 goat anti-mouse IgG (1:500) was applied for 1 h at room temperature. The surface was washed three times in PBS and images were captured using an Olympus IX71 fluorescence microscope and a SPOT RT3 microscope camera. The density of neurites growing on the laminin/CSPG striped substrate was measured using ImageJ. The image threshold was adjusted to black and white, using the threshold adjustment controls in ImageJ. Neurite density was quantified by measuring the integrated density value within equal-sized rectangular boxes drawn on either side of the CSPG–laminin border. Average integrated density values were calculated for each treatment group ($n = 3$ scaffolds/treatment group; five images/scaffold analysed). One-way ANOVA was performed for statistical analysis of neurite density.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad). The data were quantitatively analysed using one-way ANOVA and Tukey–Kramer posttests, with a

priori confidence intervals of 95%. The data shown in graphs represent mean $\pm$ standard error.

3. Results and discussion

Freeze casting can be used to fabricate porous C–A scaffolds with channels aligned with the longitudinal scaffold axis, which are suitable for use as nerve guidance conduits (Francis *et al.*, 2013). In this study, the C–A scaffolds were evaluated *in vitro* to determine their efficacy as biomaterial vehicles for sustained release of the therapeutic proteins NT-3 and chABC, in order to promote axon regeneration in the injured spinal cord. It was hypothesized that localizing reservoirs of neurotrophic factors within the scaffold by first encapsulating them within biopolymer microcapsules would lead to their prolonged release. Alginate and chitosan were chosen for this purpose, as microcapsules made from the same polymers as the scaffold base would integrate well without introducing different biomaterial characteristics into the scaffold. Further, incorporating proteins within alginate or chitosan microcapsules would allow the proteins to be maintained in a protected aqueous environment at physiological pH, diminishing the risk of denaturing the proteins.

3.1. Microcapsule fabrication and characterization

Hollow alginate and chitosan microcapsules were fabricated by spray drying under similar conditions. SEM images of the dry epichlorohydrin-crosslinked alginate microcapsules revealed shrivelled capsules with no apparent spherical structure, in contrast to the dry chitosan microcapsules (Figure 1). However, the alginate microcapsules swell to a diameter of $5.23 \pm 2.71 \mu\text{m}$ when hydrated, significantly larger than the hydrated chitosan microcapsules (diameter $3.13 \pm 1.39 \mu\text{m}$; $p < 0.001$) (Figure 2). Crosslinking the alginate microcapsules with epichlorohydrin and replacing the Ca^{++} increased the distance between the alginate polymer chains by inserting the $-\text{OCH}_2\text{CH}(\text{OH})\text{CH}_2\text{O}-$ crosslinker group, leading to a more open and elastic microcapsule structure. This allows these microcapsules to completely collapse while dry and regain their spherical shape when hydrated, in contrast to the chitosan microcapsules (and also the CaCl_2 -crosslinked alginate microcapsules), whose stiffer shell prevented total collapse in the dry state (Rocher *et al.*, 2010; Fundueanu *et al.*, 1999).

Both the alginate and chitosan microcapsules could be incorporated into the C–A scaffolds by simple mixing, without affecting the overall scaffold structure, as shown in SEM images of the scaffolds with incorporated alginate and chitosan microcapsules, termed SAC (scaffold + alginate capsules) and SCC (scaffold + chitosan capsules), respectively (Figure 3A, B). Higher-magnification SEM images show the presence of the microcapsules within the scaffold channel walls (Figure 3C, D). The shrivelled, dry alginate microcapsules give the scaffold wall a rough appearance (Figure 3C), while the larger, spherical, dry chitosan microcapsules can be seen visibly embedded in the channel wall (Figure 3D). These are compared to the smooth appearance obtained with native scaffold (3E). During the freeze-casting fabrication process, the microcapsules are concentrated in the spaces between ice crystals, along with polymer molecules and C–A PEC particles. This results in

microcapsules embedded within the interpenetrating network of chitosan, alginate and PECs.

3.2. NT-3 release from C–A scaffolds

Release of NT-3 from SAC, SCC and SDI (scaffold with direct incorporation of protein) during processing, and from the final loaded scaffold, was quantified using ELISA. NT-3 release during the crosslinking, PLO/laminin-coating and washing steps (prior to $t=0$; Figure 4A) and cumulative release after these preliminary steps (Figure 4B) is shown over a period of 8 weeks. During the crosslinking, coating and washing steps, a total of 101 ± 33.4 ng ($10.1 \pm 3.34\%$ of initial mass loaded), 223 ± 43.9 ng ($22.3 \pm 4.39\%$) and 66.1 ± 12.2 ng ($6.61 \pm 1.22\%$) NT-3 was released from SAC, SCC and SDI, respectively. After these preliminary steps, the amount of NT-3 released from the scaffolds over an 8 week period was 52.5 ± 17.0 ng (SAC), 70.9 ± 20.9 ng (SCC), and 82.4 ± 35.8 ng (SDI), corresponding to $5.25 \pm 1.70\%$, $7.09 \pm 2.09\%$ and $8.24 \pm 3.58\%$, respectively, of the initial mass loaded (1 μ g). The mass of NT-3 released from all scaffold treatments (SAC, SCC and SDI) during this time was not significantly different ($p > 0.05$), indicating that incorporating microcapsules as secondary delivery vehicles within the scaffold had no effect on delivery profile.

A polycation (i.e. PLO) coating on alginate microspheres has been shown to act as a perm-selective size-exclusion membrane, which can regulate the passage of biomolecules into and out of a hydrogel (Tobias *et al.*, 2001). NT-3 release from uncoated scaffolds was also examined to determine whether the surface adsorptions of PLO and/or laminin were preventing large amounts of NT-3 release. There was no significant difference in NT-3 release from SAC, SCC or SDI when comparing the effects of scaffold surface coatings (uncoated, PLO coated or PLO/laminin coated; $p > 0.05$; data not shown), indicating that the presence of a perm-selective membrane at the scaffold wall surface is not the parameter affecting NT-3 release.

The high water content and large (micron-sized) macropores of most single-polymer hydrogels usually leads to a large burst release of encapsulated proteins and rapid diffusion-mediated protein release within several hours to days (Comolli *et al.*, 2009). Calcium-crosslinked alginate gels have been shown to completely release lysozyme ($pI = 11.0$) and chymotrypsin ($pI = 9.1$) within 3 h into PBS, and over 150 h into Tris-buffered saline, indicating that the release of proteins with similarly high pI would not be hindered by electrostatic interaction with alginate in the scaffold (Wells and Sheardown, 2007). In contrast, sustained release of NT-3 from these C–A scaffolds over several weeks was observed, with large amounts of NT-3 remaining entrapped within the scaffold walls. The findings in this study indicate that the main factor affecting NT-3 release from our scaffolds would be the hydrogel mesh size, restricting diffusion out of the scaffold wall. Rather than the large macropores of a single polymer hydrogel, the C–A scaffold walls are instead tightly compacted interpenetrating networks of chitosan, alginate and C–A PECs. This tight matrix prevents the rapid diffusion of neurotrophins out of the interior of the scaffold walls, as the hydrogel network mesh size is likely close to, or smaller than, the hydrodynamic radius of the encapsulated proteins. The inability to completely release proteins from some hydrogel delivery vehicles has also been previously reported by several investigators (Han *et*

et al., 2012; Pfister *et al.*, 2008; Goraltchouk *et al.*, 2006). Another explanation offered for low protein release is that of protein aggregate formation, which would hinder release via diffusion (Pfister *et al.*, 2008). NGF tends to aggregate over time and, since NT-3 is also a member of the neurotrophin family, it is likely that this protein can also form aggregates (Eng *et al.*, 1997).

Despite low total percentages of neurotrophin release from the scaffolds, the amounts released even after the burst are in the low nanogram range, which meets specifications for daily therapeutic doses administered at the SCI site (Pfister *et al.*, 2008; Fine *et al.*, 2002). Additionally, the neurotrophin release rate from the C–A scaffolds is expected to be higher *in vivo*, due to faster scaffold degradation rates. Cell ingrowth into the scaffolds and subsequent ECM deposition, along with the presence of acidic by-products, free radicals and enzymes produced at the site of injury, would contribute to an increased rate of scaffold degradation, and thereby neurotrophin release, *in vivo* (Bolgen *et al.*, 2005).

3.3. Bioactivity of NT-3 released from C–A scaffolds

The bioactivity of NT-3 released from the C–A scaffolds was evaluated using an *in vitro* neurite outgrowth assay, culturing DRG explants in conditioned release medium collected from the scaffolds. The average neurite lengths of DRGs cultured in conditioned medium from scaffolds containing NT-3 collected at days 7 or 14 were not significantly different ($p > 0.05$) from the positive control (DRGs cultured in medium containing 1 ng/ml exogenous NT-3), indicating that NT-3 released from the scaffolds retained its bioactivity (Figure 5). DRGs cultured in conditioned medium taken from these NT-3-loaded scaffolds were also significantly different from the negative control of medium not containing exogenous NT-3 ($p < 0.001$ for SAC, $p < 0.001$ for SCC and $p < 0.05$ for SDI).

3.4. ChABC release from C–A scaffolds

Several methods were investigated to evaluate the release of chABC from the C–A scaffolds and, using SDS–PAGE to track decorin degradation via chABC present in the release buffer, was found to be the most sensitive method. Visualization of these degraded decorin bands on a SDS–PAGE gel illustrates the degradation of decorin by chABC released from the C–A scaffolds (Figure 6). Decorin, a small CSPG, is composed of one chondroitin or dermatan sulphate GAG chain with a molecular weight of approximately 50 kDa, attached to a core protein of approximately 40–45 kDa. On a SDS–PAGE gel, this CSPG appears as a broad smear between 60–80 kDa, while the core protein of degraded decorin appears at approximately 45 kDa. ChABC appears as a sharp band at approximately 100 kDa, with fainter bands seen nearby, since the enzyme is unpurified and may contain trace amounts of other chondroitinases. The chABC band is only visible in lane 4 (day 1), since the amount of chABC released after this was below the range detectable by silver staining. Although a chABC band is not visible after day 1, chABC was still being released from the scaffolds, as evidenced by the presence of a degraded decorin band at approximately 45 kDa. The presence of this band indicated that chABC was being released from the scaffolds up to day 35 (lane 7). The molecular weight, and therefore hydrodynamic volume, of chABC is much larger than that of NT-3, meaning that even less of this protein would be able to diffuse out of the IPN matrix of the scaffold walls.

3.5. Effects of protein release from scaffolds on DRG neurite growth through an *in vitro* glial scar model

The effects of the multifunctional scaffolds on DRG neurite growth were evaluated by culturing dissociated DRG neurons on a laminin/CSPG striped substrate in the presence of these protein-loaded scaffolds. The CSPG stripes represented an *in vitro* glial scar model, since CSPGs are inhibitory to neurite growth and are the main growth-inhibitory molecules present in glial scar tissue in the spinal cord. C–A scaffolds with various combinations of incorporated proteins were placed in a permeable 500 μm mesh support, suspended above the DRG neurons growing on the striped substrate. The use of this insert allowed us to evaluate the growth of DRG neurites through a CSPG barrier under the influence of therapeutic proteins actively being released from the C–A scaffolds, rather than adding conditioned media to the cells, which would effectively be a bolus application of chABC and/or neurotrophic factors. Neurite density on laminin and CSPG + laminin areas was compared in order to quantify the ability of scaffold-released proteins to promote growth through this barrier.

There was significantly greater neurite density on laminin areas compared to the CSPG + laminin stripes for DRG neurons cultured in the presence of blank scaffolds (Figure 7A) and NT-3-loaded scaffolds (Figure 7B) ($p < 0.001$). This indicated that neither blank scaffold nor NT-3 released from a scaffold had a significant effect on the penetration of neurites into the growth inhibitory area. Furthermore, normalized laminin/CSPG neurite density ratios were not significantly different when compared to positive controls of exogenous neurotrophins added at similar concentrations ($p > 0.05$).

For scaffolds loaded with chABC alone (Figure 7C) or in combination with neurotrophins (Figure 7D), neurite density on the laminin areas was not significantly different from neurite density on the CSPG + laminin areas, indicating that proteins released from these particular scaffolds were able to promote DRG neurite growth through the CSPG barrier ($p > 0.05$). A synergistic effect on neurite growth (neurite density) was not observed using C–A scaffolds loaded with a combination of chABC and NT-3. The normalized laminin/CSPG neurite density ratios were not significantly different when compared to positive controls of exogenous chABC and/or NT-3 added at similar concentrations ($p > 0.05$). These data demonstrate the potential of multifunctional scaffolds for promoting nerve regeneration through the growth-inhibiting glial scar via the release of chABC, alone or in combination with NT-3.

4. Conclusions

This study demonstrated that C–A scaffolds produced by freeze casting are capable of providing the sustained release of incorporated NT-3 and chABC over several weeks, although most of the loaded protein remained entrapped within the IPN matrix of the scaffold walls. Inclusion of microcapsules as secondary delivery vehicles within the scaffold had no effect on biomolecule delivery time. However, *in vivo*, if the matrix is more rapidly broken down, microencapsulation may have a significant influence. NT-3 and chABC released from the scaffolds retained bioactivity, promoting the growth of neurites through an *in vitro* growth-inhibitory barrier of CSPGs. Future work will involve *in vivo* testing of these

multifunctional scaffolds to evaluate their ability to enhance nerve regeneration in the injured spinal cord as an orientated growth-permissive substrate, along with a combination of chABC and NT-3 release.

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Figure 1. SEM images of microcapsules produced by spray drying: (A) epichlorohydrin-crosslinked alginate microcapsules; (B) chitosan microcapsules; scale bar = 10 μm

Figure 2. Differential interference contrast (DIC) images of rehydrated microcapsules produced by spray drying: (A) alginate microcapsules; (B) chitosan microcapsules; (C) size distribution histogram of hydrated alginate; and (D) chitosan microcapsules; scale bar = $10 \mu\text{m}$

Figure 3.

SEM images of C–A scaffolds with incorporated microcapsules: (A) C–A scaffold with alginate microcapsules; (B) C–A scaffold with chitosan microcapsules; schematic indicates cross-sectional orientation of scaffold. Higher magnification SEM images showing microcapsules incorporated into C–A scaffold walls: (C) alginate microcapsules in scaffold wall; (D) chitosan microcapsules in scaffold wall; (E) scaffold wall with no capsules: scale bars = 100 μm (A, B), 10 μm (C–E)

Figure 4.

Release of NT-3 from C-A scaffolds: (A) non-cumulative release during crosslinking, coating and washing steps; (B) cumulative release over 8 weeks after crosslinking, coating and washing steps: SAC, scaffold + alginate capsules; SCC, scaffold + chitosan capsules; SDI, scaffold with direct incorporation of neurotrophin-3; error bars, SEM; $n = 3$

Figure 5.

Bioactivity of NT-3 released from scaffolds at day 14, as determined by neurite outgrowth assay: SAC, scaffold + alginate capsules; SCC, scaffold + chitosan capsules; SDI, scaffold with directly incorporated neurotrophin; negative control indicates that no exogenous neurotrophins were added to the culture medium: * $p < 0.05$; *** $p < 0.001$; error bars, SEM; $n = 14$

Figure 6.

SDS-PAGE of decorin degradation by scaffold-released chABC. MW marker, 3.5–260 kDa; lane 1, molecular weight marker; lane 2, intact decorin; lane 3, day 1 released chABC; lane 4, day 1 released chABC + decorin; lane 5, day 7 released chABC + decorin; lane 6, day 21 released chABC + decorin; lane 7, day 35 released chABC + decorin; lane 8, day 1 released buffer from blank scaffold + decorin: arrow, presence of degraded decorin band

Figure 7.

DRG neurite growth on laminin/CSPG stripes after 48 h: (A) blank scaffolds; (B) NT-3-loaded scaffolds; (C) ChABC-loaded scaffolds; (D) ChABC/NT-3-loaded scaffolds; red (TRITC-labelled) stripes, growth-inhibitory areas of CSPG + laminin; black areas, laminin alone; DRG neurites are fluorescently labelled green (neurofilament 200; AlexaFluor 488); scale bar = 100 μ m; (E) ratio of neurite density on laminin stripe, laminin + CSPG stripe: * p < 0.05; ** p < 0.01; error bars, SEM; n = 3 scaffolds/treatment group; five images/scaffold analysed