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European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 563–572
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Controlled nerve growth factor release from multi-ply alginate/chitosan-based nerve conduits

Lukas A. Pfister [a], Eva Althera [a], Michaël Papaloïzos [b], Hans. P. Merkle [a], Bruno Gander [a,*]

[a] Institute of Pharmaceutical Sciences, Zurich, Switzerland
[b] Center for Hand Surgery and Therapy, Geneva, Switzerland

Received 10 August 2007; accepted in revised form 15 January 2008
Available online 19 January 2008

Abstract

The delivery kinetics of growth factors has been suggested to play an important role in the regeneration of peripheral nerves following axotomy. In this context, we designed a nerve conduit (NC) with adjustable release kinetics of nerve growth factor (NGF). A multi-ply system was designed where NC consisting of a polyelectrolyte alginate/chitosan complex was coated with layers of poly(lactide-co-glycolide) (PLGA) to control the release of embedded NGF. Prior to assessing the *in vitro* NGF release from NC, various release test media, with and without stabilizers for NGF, were evaluated to ensure adequate quantification of NGF by ELISA. Citrate (pH 5.0) and acetate (pH 5.5) buffered saline solutions containing 0.05% Tween 20 yielded the most reliable results for ELISA active NGF. The *in vitro* release experiments revealed that the best results in terms of reproducibility and release control were achieved when the NGF was embedded between two PLGA layers and the ends of the NC tightly sealed by the PLGA coatings. The release kinetics could be efficiently adjusted by accommodating NGF at different radial locations within the NC. A sustained release of bioactive NGF in the low nanogram per day range was obtained for at least 15 days. In conclusion, the developed multi-ply NGF loaded NC is considered a suitable candidate for future implantation studies to gain insight into the relationship between local growth factor availability and nerve regeneration.

Keywords: NGF; Release kinetics; Nerve conduit; Nerve regeneration

1. Introduction

Injuries to peripheral nerves remain a clinical challenge, and functional regeneration after axotomy is often disappointing despite highly refined microsurgical methods [1,2]. Strategies to improve the current situation include treatment with growth factors or the use of nerve conduits (NCs). NCs are tubular structures that are used to bridge the gap of a severed nerve, thereby acting as a guide for the regenerating axons and as a barrier against the ingrowth of scar-forming tissue. NCs have been made of various materials of biologic (vein, collagen, and chitosan) or synthetic origin (silicone, poly(glycolide) (PGA), and poly(hydroxbutyrate)), as reviewed in [3–7]. Commercially available biodegradable NCs made of collagen [8,9] (NeuraGen; Integra LifeSciences, Plainsboro, NJ) and PGA [10] (GEM Neurotube; Synovis Micro Companies Alliance, Birmingham, AL) have performed therapeutically in selected cases as well as did autografts, the gold standard for bridging severed nerves. Both products have been approved for clinical use in humans.

Physical nerve guidance by a NC may, however, not be sufficient to foster optimal recovery. Growth factors are also required to assure the survival of the cell bodies and to support the regeneration of the axons toward specific target organs. Many growth factors have been identified that may improve nerve regeneration, including nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4/5, glial cell line-derived

* Corresponding author. Institute of Pharmaceutical Sciences, HCI J392.4, ETH Zurich, Wolfgang-Pauli-Strasse 10, CH-8093 Zurich, Switzerland. Tel.: +41 44 633 73 12; fax: +41 44 633 1314.
E-mail address: bruno.gander@pharma.ethz.ch (B. Gander).

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doi:10.1016/j.ejpb.2008.01.014

neurotrophic factor (GDNF), the fibroblast growth factors 1 and 2, platelet-derived growth factor, vascular endothelial growth factor, ciliary neurotrophic factor, or insulin-like growth factor I [11–14]. Although all of these factors have been shown to improve nerve regeneration under certain conditions, little is known about the impact of their delivery kinetics on peripheral nerve regeneration [15]. This is partly due to the limitations of the so far used microparticulate or single molded delivery systems to control the release kinetics of the incorporated growth factors [16].

The aim of this study was thus to develop a NC with integrated delivery system that provides adjustable release kinetics of NGF. Prior to the design of the NC and, in particular, of the delivery system, appropriate conditions to preserve the stability of NGF during NC manufacturing and *in vitro* release testing had to be developed. The analytical sensitivity required to monitor the release of clinically relevant quantities of NGF (pico- to nanomoles) from the NC necessitated the use of an enzyme-linked immunosorbent assay (ELISA).

The basic design of the NGF-releasing NC consisted of a porous supporting hollow cylinder, which was made of a polyelectrolyte complex of alginate and chitosan subsequently coated with concentric layers of release-modifying poly(lactide-co-glycolide) (PLGA) type polymers. NGF was embedded at different radial positions of this multi-ply NC. The flexible design of the multi-ply NC was expected to accommodate different controlled release kinetics for NGF through variation in the PLGA layers. This variation in PLGA layers was not expected to alter unfavorably the mechanical properties of the NC, which are provided primarily by the hydrogel-forming alginate/chitosan support structure, representing the main body of the NC. A soft hydrogel inner surface of the cylindrical NC was preferred for the direct contact with regenerating axons over a more hydrophobic and acid-producing PLGA material. Further, the materials used in this study, alginate, chitosan and the PLGA polymers, are all biocompatible and widely used in biomedical applications [17–19].

2. Materials and methods

2.1. Materials

NGF was a generous gift from Genentech (South San Francisco, CA, USA). Medium molecular weight chitosan (degree of deacetylation 82.5%) was from Sigma–Aldrich (Buchs, Switzerland), and sodium alginate (Manugel GMB, ISP alginates) was a kind gift from Staerkle und Nagler (Zurich, Switzerland). The end-group uncapped and capped poly(lactide-co-glycolide) 50:50 (PLGA) of a M_w of approx. 35 kDa (Resomer RG503H, RG503) were purchased from Boehringer-Ingelheim (Ingelheim, Germany). Polysorbate 20 and 40 (Tween 20, Tween 40), bovine serum albumin (BSA), D(+)-trehalose-dihydrate, as well as all buffer salts and solvents were from Fluka (Buchs, Switzerland) or Hänseler (Herisau, Switzerland). NGF was assayed by ELISA (R&D Systems, Minneapolis, MN, USA) using a monoclonal mouse IgG (MAB256) for NGF capture and a biotinylated polyclonal IgG from goat (BAF256) for detection.

2.2. Stability of NGF in aqueous media

In view of selecting an appropriate medium for the *in vitro* release testing of the NGF-loaded NC, we assessed the stability of NGF in different aqueous media.

Preliminary experiments were performed to test whether the treatment of NGF samples with DMSO (25%) [20] or HCl [21] prior to assay may help to maintain a uniform and consistent ELISA activity of NGF. It is known that ELISA activity of NGF can vary substantially depending on the incubation conditions, and DMSO [20] and HCl [21] were reported to dissociate aggregates of NGF. Prior to these treatments, NGF (5 μg/ml) was incubated at 37 °C on a horizontal shaker in acetate buffered saline (ABS; 20 mM, 136 mM NaCl, pH 5.5), either supplemented with 0.05% Tween 20, a common protein stabilizer, or left without Tween 20. On days 0, 1, and 2, samples were taken and kept at −20 °C until analysis by ELISA. Prior to analysis by ELISA, the samples were either left untreated or subjected to DMSO (25%) [20] or HCl treatment [21]. For the latter, 1.5 μl of HCl (1 N) was added to 100 μl of NGF solution, incubated for 20 min and subsequently neutralized with 1.5 μl NaOH (1 N). The samples were then diluted to a final NGF concentration of 1 ng/ml with ELISA diluent (Tris-buffered saline with Tween 20 [TBST] containing 0.1% BSA).

In a second series of experiments, the effect of common protein stabilizers (0% and 0.1% BSA; 0% and 5% trehalose [22]; 0% and 0.05% Tween 20 or Tween 40) on the ELISA activity of NGF was examined by using a 2^4 fractional factorial design. NGF (5 μg/ml) was incubated in citrate buffered saline (CBS; 10 mM, 150 mM NaCl, pH 5.0) containing the indicated excipients for 24 h at 37 °C in Eppendorf tubes. The test solutions were subsequently kept at −20 °C until analysis by ELISA.

In a third series of experiments, the time course of ELISA activity of NGF (10 μg/ml) was assessed upon incubation in CBS (pH 5.0), ABS (pH 5.5), and phosphate buffered saline (PBS; 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), each in the presence or absence of 0.05% Tween 20. The solutions were incubated for 8 days in screw-top Eppendorf tubes at 37 °C on a horizontal shaker. Samples were withdrawn daily and kept at −20 °C until analysis by ELISA.

2.3. Fabrication of the alginate/chitosan tubes

Tubes consisting of a polyelectrolyte complex of alginate and chitosan were fabricated by spinning mandrel technology as detailed elsewhere [23] with small modifications. Briefly, alginate was dissolved in water, and chitosan in 1% acetic acid. Ethanol was added to both solutions (50%

with respect to the total amount of liquid), before the two fluids were mixed and homogenized for 1 min (Polytron, Kinematica, Lucerne, Switzerland) to obtain a fine dispersion of the alginate/chitosan complex. The alginate-to-chitosan ratio (1.03, w/w) used corresponded to an equal number of carboxyl- and amino-groups, and the total polymer concentration was 1% (w/w). For the fabrication of the tubes the dispersion of the complex was administered via a syringe onto a spinning steel mandrel (diameter: 1.2 mm) that was fixed on a sideways reciprocating apparatus. The solvent was evaporated under a laminar stream of air. The resulting tube was placed in 0.1 M di-sodium hydrogen phosphate for 1 h to neutralize residual acetic acid. The swollen tube was then transferred onto a Teflon mandrel, dried, and kept at 4 °C until further use. The alginate/chitosan tubes served as basis for the subsequent coating of NGF and release-controlling PLGA layers.

2.4. Coating of alginate/chitosan tubes with NGF and release-controlling PLGA layers

Different numbers and types of PLGA layers were coated onto alginate/chitosan tubes to control the release of NGF. For this, PLGA in ethyl acetate (5%, w/w) was sprayed with an ultrasonic spray nozzle at a rate of 7.5 ml/h and an energy of 1.6 W (US1, Lechler, Metzingen, Germany) onto the alginate/chitosan tubes that were installed on a spinning and sideways-reciprocating Teflon-coated mandrel. For each PLGA layer, 1.18 ml of PLGA solution was spray-coated over a distance of 90 mm and dried under laminar air flow. NGF was added to the alginate/chitosan matrix at different radial positions (top panels of Figs. 5 and 6).

For the 1st generation NC, NGF in ABS containing 0.5% alginate was applied uniformly over the entire length of the alginate/chitosan tube (78 mm), prior to the cutting of the tube into individual NC (Fig. 1). Layers of PLGA were spray-coated across the entire length of the NC. Finally, the NC was swollen in 50% ethanol, cut into individual NC (6 mm), and dried at 200 mbar for 16 h.

For the 2nd generation NC, NGF in ABS (2 μg per NC) was pipetted onto the middle part (2–3 mm) of previously cut, 6 mm long NC (Fig. 1). Layers of PLGA were applied by spray-coating over the entire length of the NC, and dried. The NC was swollen in 50% ethanol, removed from the mandrel, and dried at 200 mbar for 16 h. A centered NGF deposition ensured a precisely defined amount of NGF per NC and, through the PLGA coating, lowered the risk of leakage of NGF at the ends of the NC.

2.5. Swelling of PLGA-coated alginate/chitosan NC

The swelling of PLGA-coated NC (1st generation NC only) was assessed at room temperature in CBS (pH 5.0), ABS (pH 5.5), and PBS (pH 7.4). NCs were placed upright in the different buffer solutions. Photographs were taken at regular intervals during 3 h under a stereomicroscope (Leica MS 5, camera Leica DC 200, Leica, Glattbrugg, Switzerland). Inner and outer NC diameters were measured with an imaging software (ImageJ, US National Institutes of Health, Bethesda, Maryland, USA).

2.6. In vitro release of NGF from NC

The *in vitro* release of NGF was measured by incubating the 1st generation NC in ABS (pH 5.5) containing 0.05% Tween 20, and the 2nd generation NC in CBS (pH 5.0) containing 0.05% Tween 20. The samples were incubated at 37 °C in an overhead shaker, and the buffer solutions exchanged daily. Withdrawn NGF samples were kept at −20 °C until analysis by ELISA.

2.7. In vitro NGF bioactivity testing

Rat PC12 cells were co-incubated with NC (2nd generation NC only) to examine the bioactivity of released NGF. PC12 cells differentiate in response to NGF into a neuronal phenotype by neurite extension. The cells were seeded at a density of 13,000 cells per cm^2 on laminin-coated glass inserts in 12-well plates and incubated in high glucose DMEM, supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptavidin in a humid 5% CO_2 atmosphere. After 1 day, the medium was replaced by a similar DMEM medium, which contained only 1% FCS instead of 10%. NCs were added to the wells, and photographs were taken daily under a microscope (Axiovert 35 with camera AxioCam MRc5 from Zeiss, Göttingen, Germany). For control, the PC12 cells were also incubated in medium containing 0, 1, 5, 10, 20, and 50 ng/ml NGF. For quantification, cells were considered fully differentiated when the outgrowth of the exposed neurites exceeded double the size of their cellular body.

2.8. Statistical analysis

Data are presented, unless stated otherwise, as means ± SD. Statistical analyses were performed using the General Linear Model (GLM) followed by Fisher's post hoc-test, and statistical significance was set at $p < 0.05$.

3. Results

3.1. Stability of NGF in buffer

In the preliminary experiments, the treatment of NGF solutions in buffer with DMSO or HCl prior to analysis by ELISA resulted in a general significant ($p < 0.05$) lowering of the NGF concentrations detectable by ELISA (data not shown). Treatment with DMSO compromised almost entirely the ELISA activity of NGF, while the treatment with HCl lowered the ELISA activity to a lesser extent (i.e., 70–80% of untreated NGF). Addition of Tween 20 significantly increased the amount of ELISA-active NGF to levels above the 100% initially added, whereas in the

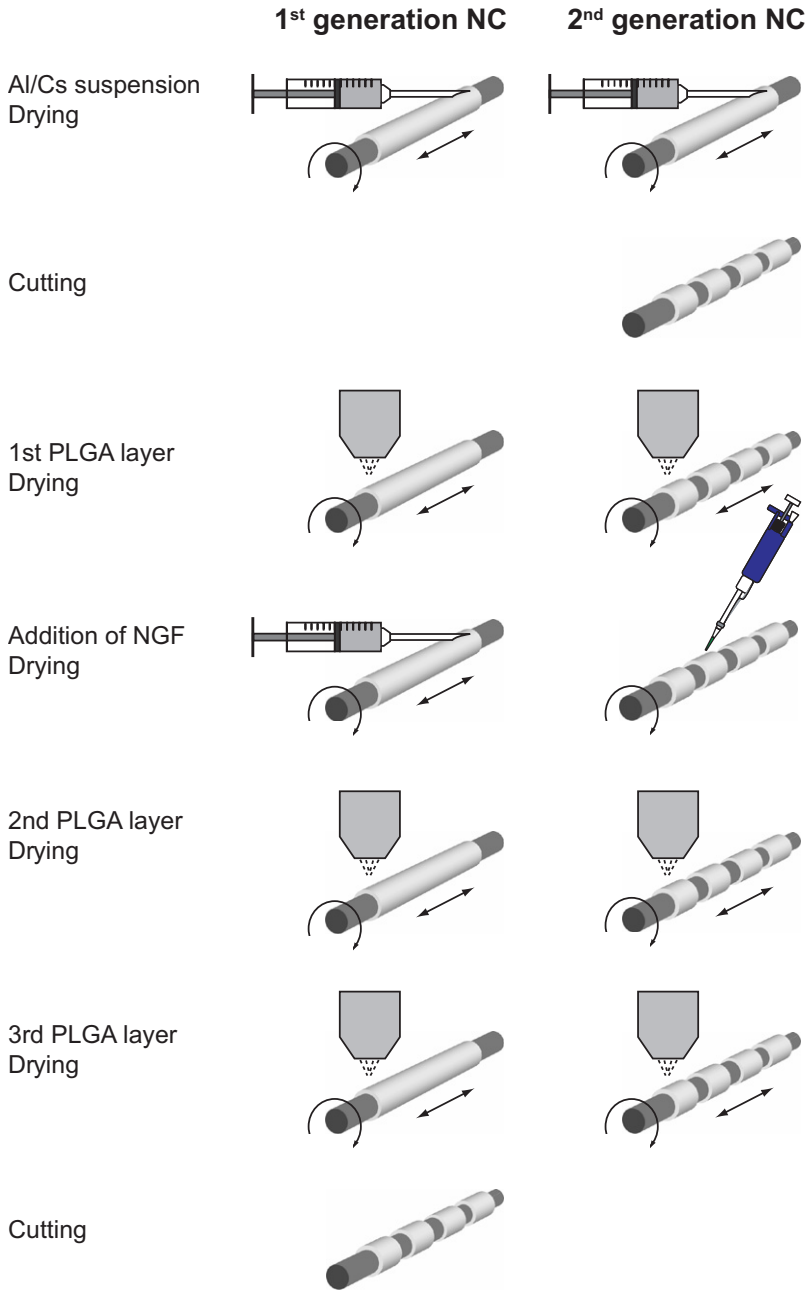

Fig. 1. Schematic representation of the two fabrication methods for the incorporation of NGF into NC. Please refer to Section 2 for details.

absence of Tween 20, the detectable amounts of NGF fell below 50% of the initial NGF amount. During a 2-day incubation, the amount of ELISA-active NGF did not alter in the presence of Tween 20. Altogether, these preliminary experiments revealed a beneficial effect of Tween 20 in the NGF solution and an adverse effect of sample pre-treatment with DMSO and, to a minor extent, also with HCl prior to the ELISA measurement. Therefore, the two sample pre-treatments were not considered for the further experiments.

The testing of putative protein stabilizers (0.1% BSA, 5% trehalose, 0.05% Tween 20 or Tween 40) on the NGF ELISA activity (5 μg/ml) revealed a statistically significant ($p < 0.05$) positive effect of both Tween 20 and Tween 40, whereas trehalose exerted a negative and BSA an ambiguous effect (Fig. 2). The effects persisted when the experiments were repeated at low concentration (10 ng/ml, data not shown) or when the samples were subjected to repeated freezing and thawing cycles (data not shown).

Based on the obtained results, NGF was incubated in different buffer solutions, with and without 0.05% Tween 20, over a period of 8 days (Fig. 3). In the absence of Tween 20, ELISA active NGF concentrations dropped to 50% or less of the initial content during the first 2 days of incubation. In the presence of Tween 20, the ELISA active NGF concentration remained within 60–80% of the initial

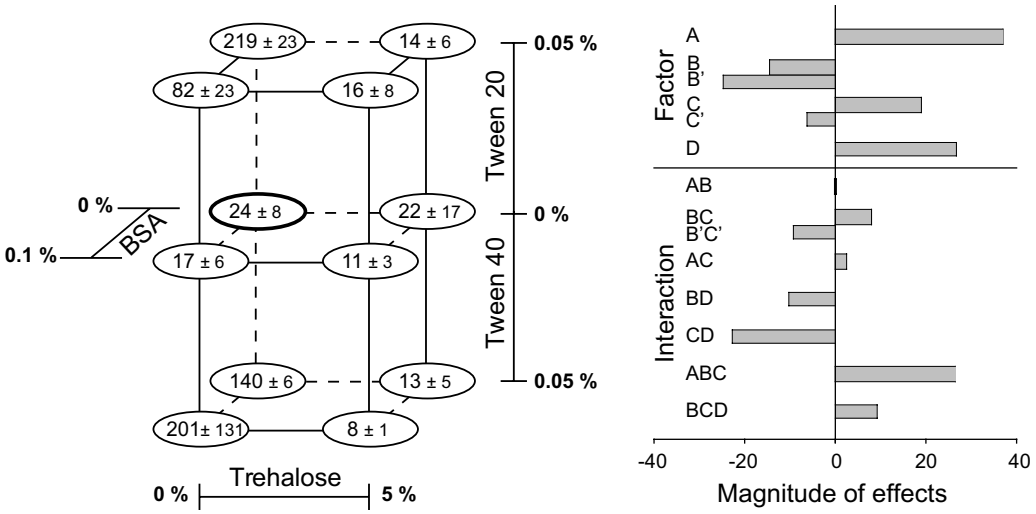

Fig. 2. Effect of excipients on ELISA active NGF (5 μg/ml) 1 day after incubation in citrate buffer saline (CBS, pH 5.0); samples were frozen for storage and thawed before analysis. Left panel: Relative ELISA active NGF concentrations (%) in a 2^4 fractional factorial design. Data represent means ± standard deviations ($n = 3$). Right panel: Magnitude of effects of excipients and interactions between factors as calculated [28] from the values shown in the left panel. The effects of the four factors are denoted by characters: A, 0.05% Tween 20; B, 5% Trehalose; C, 0.1% BSA; and D, 0.05% Tween 40. Note that the calculation of the effects yields separate values for the upper and lower cube, which leads to two values for the factors and interaction of B and C that are located in the common basis of the upper (B and C) and lower (B′ and C′) cube.

concentration over the entire study period of 8 days. The effect of the buffer system and related pH did not exert a statistically significant effect on the NGF stability and quantification, although the measured NGF concentrations varied the least in CBS (pH 5.0).

3.2. Swelling of the NC

The swelling of the PLGA-coated alginate/chitosan NC in CBS (pH 5.0), ABS (pH 5.5), and PBS (pH 7.4) did not affect the inner diameter of the NC, which remained between 1.0 and 1.2 mm (Fig. 4). By contrast, the water uptake of the NC increased the outer diameter of the tubes from approx. 1.6 mm in the dry state to 2.2 and 2.8 mm in the equilibrium swollen state in CBS and PBS, respectively, which was achieved after approx. 30–60 min. In ABS, however, swelling equilibrium was achieved only after 5–6 h, with the outer NC diameter attaining a value of 5.0 mm.

3.3. In vitro NGF release from the 1st generation NC

The 1st generation NCs were prepared by Method 1 illustrated in Fig. 1. Here, the NGF was uniformly distributed over the entire length of the NC and exposed to the environment at both ends of the NC. The *in vitro* release testing of these NC-types was performed in ABS of pH 5.5, because this buffer system corresponded to the vehicle in which the NGF was provided by the manufacturer. Furthermore, ABS provides a higher buffer capacity toward lower pH as compared to PBS and, therefore, maintains the pH at a more uniform level during the acidic degradation of the PLGA layers. Finally, ABS as well as CBS, both supplemented with Tween 20, maintained very well the ELISA reactivity of NGF during the first few days of incubation (Fig. 3), which was relevant considering that the release medium was exchanged daily. NGF release from all 1st generation NC-types extended over the entire 15-day period of testing (Fig. 5). With one exception, all release profiles show biphasic kinetics with a fast release over the first 7 days and subsequent slower release rates. The NC-type that contained the NGF directly in the alginate/chitosan matrix did not show such biphasic kinetics, but an almost constant release after a minimal burst during the first day of incubation. The fastest and quantitatively highest release was observed when NGF was directly deposited onto the alginate/chitosan matrix and coated with two PLGA layers toward the exterior of the NC (Fig. 5, design B). The release was significantly diminished when additional PLGA diffusion barriers were interposed in-between the alginate/chitosan matrix and the NGF (Fig. 5, designs C and D); variations in the PLGA layer thickness exerted a negligible influence on the NGF release kinetics (data not shown). Surprisingly, when the NGF was embedded directly within the alginate/chitosan matrix, both the total amount and rate of release were greatly diminished. The direct embedment of NGF into the alginate/chitosan matrix during fabrication of the alginate/chitosan tube may have suffered from the necessity of neutralizing the matrix with 0.1% Na_2HPO_4 after preparation. During the 1-h neutralization, some NGF was eluted from the tube, as experimentally determined. The treatment with Na_2HPO_4 may have even destroyed or diminished the ELISA reactivity of the NGF. Irrespective of their design, the various NCs released only a small fraction (1.5–7.5%) of the initial total loading (approx. 800 ng per NC) during

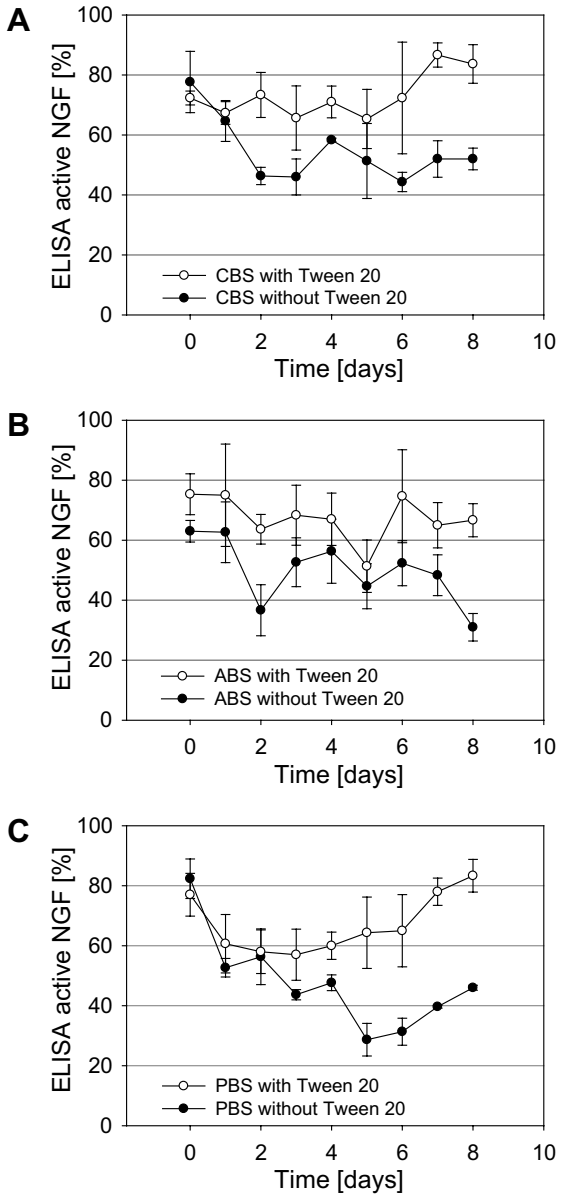

Fig. 3. NGF (10 μg/ml) stability in different incubation buffers over time with (open circles) and without Tween 20 (filled circles). (A) Citrate buffered saline (CBS, pH 5.0); (B) acetate buffer saline (ABS, pH 5.5); (C) phosphate buffer saline (PBS, pH 7.4). Data represent means ± standard deviations ($n = 3$).

the 15 days of testing. A second problem with the 1st generation NCs and their release-testing consisted in the observed instability of the NC coating. Indeed, during release testing, the PLGA coating of some NC detached from the ends and retracted toward the middle of the NC upon the important swelling of the alginate/chitosan support structure in ABS.

3.4. In vitro NGF release from the 2nd generation NC

On the basis of the above described release and swelling results with the 1st generation NC, a 2nd generation NC was fabricated that contained the NGF only in the mid-segment of the NC so that the NGF was not exposed to the envi-

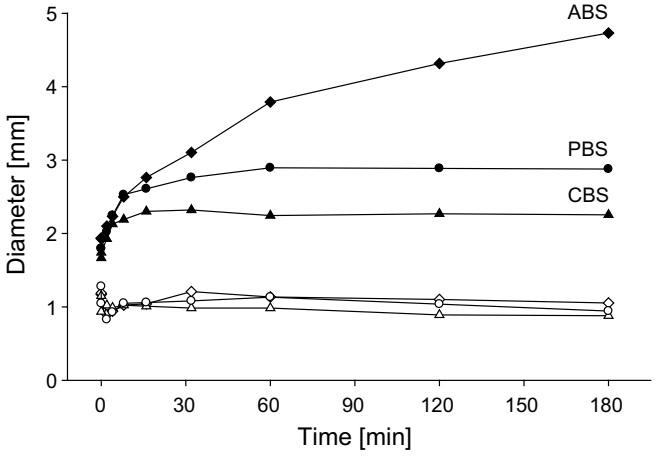

Fig. 4. Outer (filled symbols) and inner (open symbols) diameters of PLGA-coated NC (1st generation) upon swelling in different buffers. Citrate buffer saline (CBS, pH 5.0), acetate buffer saline (ABS, pH 5.5), and phosphate buffer saline (PBS, pH 7.4).

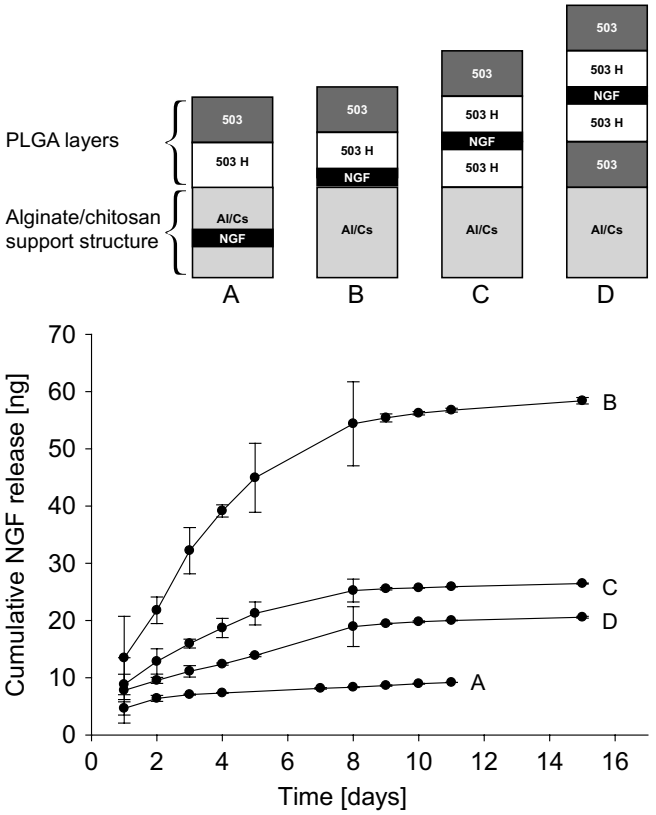

Fig. 5. Effect of the stacking sequence of the PLGA layers and deposition of NGF on the release kinetics of NGF from 1st generation NC. Schematic representation of the NC design (top panel) and the corresponding cumulative release in acetate buffer saline (bottom panel). Please note that the total PLGA layer thickness is actually only about 10% of that of the alginate/chitosan (Al/Cs) matrix. The PLGA types differ in their end groups. RG 503 is end-group capped (dark gray segments), whereas RG 503H has free carboxylic end-groups (open segments). Means with standard deviations relative to the respective time interval ($n = 4$).

ronment at both NC ends (Fig. 1; Method 2). Further, CBS (pH 5.0) rather than ABS (pH 5.5) was used here as release test medium to restrict the NC swelling and avoid damage to the PLGA coating due to excessive swelling of the alginate/chitosan support structure. Indeed, the PLGA layers remained intact in CBS during the entire incubation time of 15 days. NGF release from the 2nd generation NC was continuous and steady over the 15 days of testing (Fig. 6). The release profiles were smooth and regular, showed no initial burst release, and the measured values varied little. The data further demonstrate that the release rate could be controlled by the PLGA layers inserted between the NGF and the alginate/chitosan matrix. Very importantly, the total amount of NGF released during the 15 days amounted to approx. 250–500 ng corresponding to 12.5–25% of the total dose; this amount is considerably higher than that released from the 1st generation NC (Figs. 5 and 6).

3.5. Bioactivity of the 2nd generation NC

When PC12 cells were co-incubated with a 2nd generation NC, the cells differentiated and exhibited outgrowth of neurites (Fig. 7). At day 3 of co-incubation, most of the cells exhibited neurites; at day 7, a dense interconnected network of neurites reaching several millimeters in length had developed. This suggests that bioactive NGF was continuously released over the time period of incubation. When the incubation time was prolonged further (i.e., 15 days, matching the *in vitro* release time), cells overgrew each other so that their individual morphology became indistinguishable. In the control cells without NC and NGF, no differentiation was observed, and the cells appeared increasingly impaired at later time points. Neurite outgrowth in the presence of NGF-loaded NC was not different to that observed with the positive control cells (incubated with 50 ng/ml NGF; Fig. 7), but superior to that seen with the control cells incubated with 1 ng/ml NGF solution (data not shown).

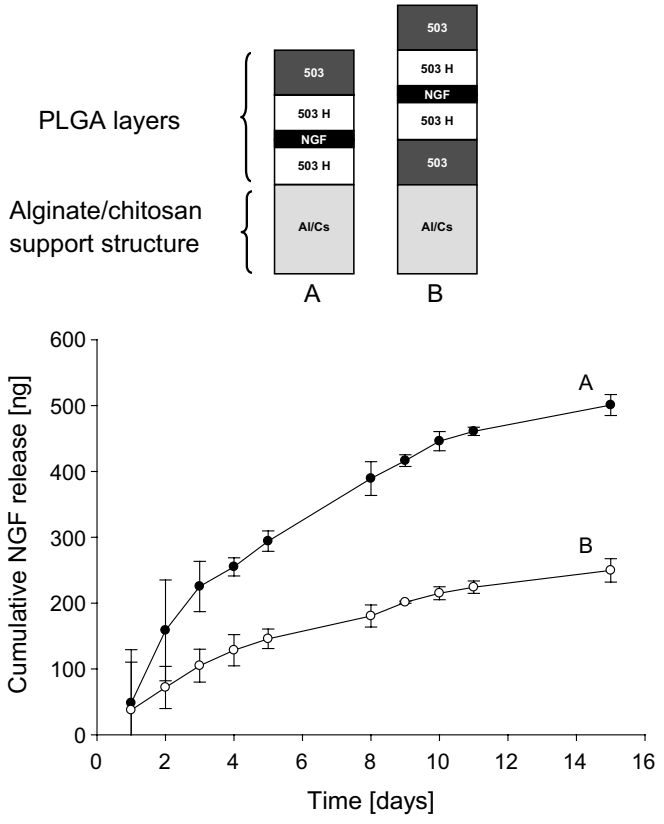

Fig. 6. Schematic representation of NC design (top panel) and corresponding cumulative NGF release (bottom panel) from 2nd generation NC. Release was measured in citrate buffer saline. It should be noted that the total PLGA layer thickness is actually only about 10% of that of the alginate/chitosan (Al/Cs) matrix. The PLGA types differ in their end groups. RG 503 is end-group capped (dark gray segments), whereas RG 503H has free carboxylic end-groups (open segments). Means with standard deviations relative to the respective time interval ($n = 4$).

4. Discussion

The aim of this study was to integrate a delivery system for NGF into a nerve conduit in a way to tailor distinct release kinetics. A multi-ply system was designed that consisted of an alginate/chitosan hydrogel NC [23] and additional layers of release controlling PLGA. This assembly offered maximum design flexibility as the alginate/chitosan tube serves as structural support and biocompatible contact surface for axons, whereas the PLGA layers allow an independent control over the NGF release.

Prior to the NC development, the assaying and stability of NGF during NC manufacturing and *in vitro* release had to be considered. NGF was analyzed by ELISA for reasons of method sensitivity and convenience as concentrations in the nanogram range had to be quantified according to the intended and clinically relevant release rate. Reports in the literature indicated, however, that ELISA data of NGF may be affected by the chemical environment causing conformational changes and restricting the accessibility of reactive epitopes [25,26]. Preliminary tests revealed that pre-treatment of NGF samples with DMSO [20] or HCl [21] prior to ELISA measurement did not produce any beneficial effect on the responsiveness and consistency of the NGF ELISA. On the contrary, addition of Tween 20 to the NGF in acetate buffer saline (ABS) improved both the responsiveness and consistency of the NGF assay. The same effect was also observed in citrate buffered saline (CBS) and phosphate buffered saline (PBS). The positive effect of Tween 20 may be related to the hindering or slowing down of NGF aggregate formation as reported in the literature [27]. ABS was the solvent for NGF as provided by the manufacturer; ABS was reported to afford best long-term storage stability of NGF when kept at 5 °C [27]. For *in vitro* release testing, we first used ABS, the medium provided by the manufacturer, and added 0.05% of Tween 20. As ABS mediated an extensive swelling of the alginate/chitosan support structure that occasionally caused damage to the PLGA coating, we had to replace ABS by CBS for testing the second generation of NC. Both

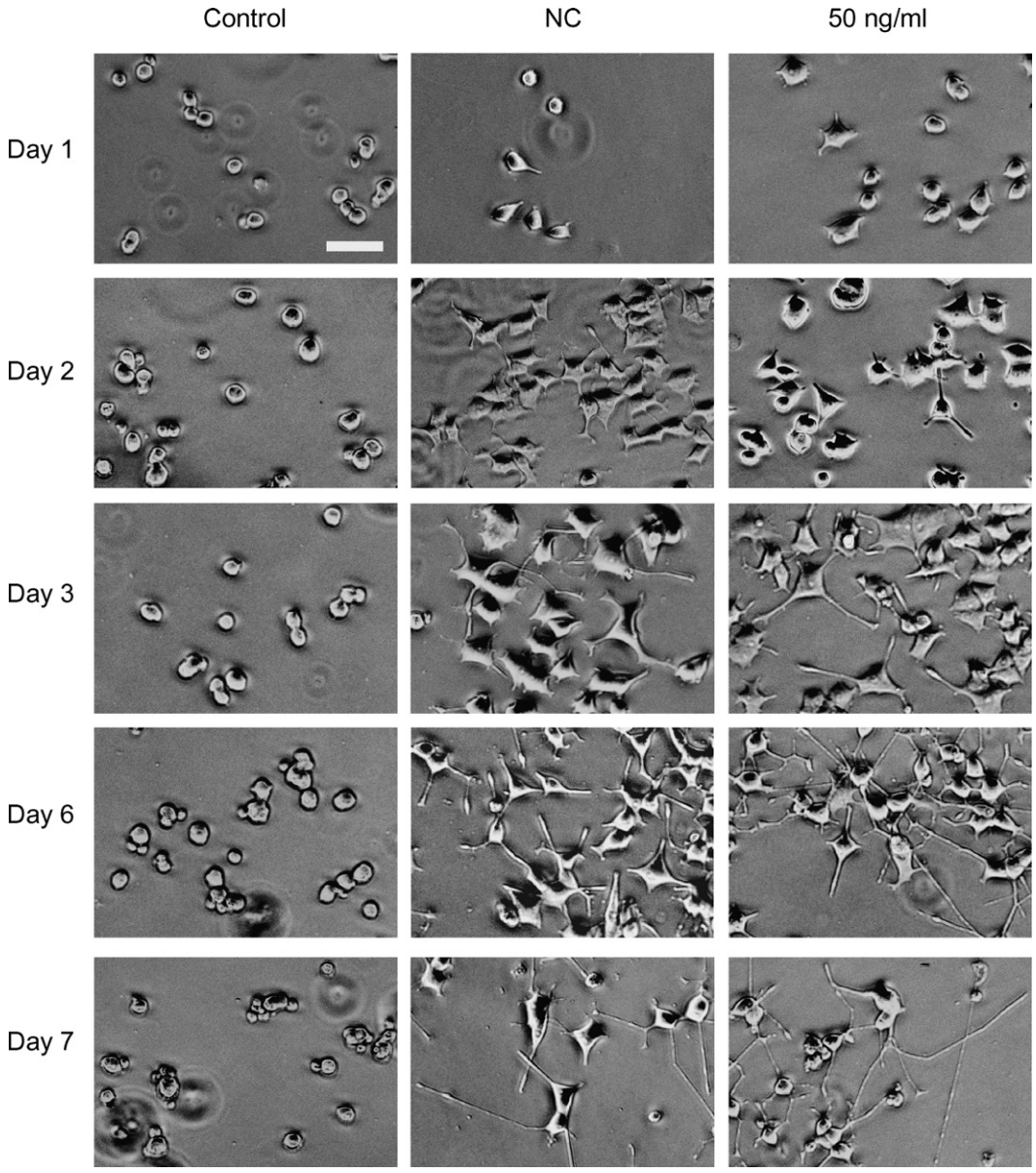

Fig. 7. Differentiation of PC 12 cells by neurite outgrowth in response to different formulations. Control: cells incubated without NGF and NC; NC: cells incubated with NGF-loaded NC; 50 ng/ml: cells incubated with 50 ng/ml of NGF dissolved in the culture medium. Scale bar: 50 μm.

ABS and CBS, when supplemented with Tween 20, maintained very well the ELISA reactivity of NGF during the first few days of incubation (Fig. 3), which was relevant considering that the release medium was exchanged daily.

The 1st generation NC mediated a NGF release of a few nanograms per day that could be efficiently controlled by the radial location of NGF within the NC (Fig. 5). However, only a small fraction of the total NGF dose was released over the 15-day test period. This might be ascribed to the fabrication method with NGF being applied along the entire length of a long tube (8 cm) before it is cut into individual NC. To obtain a uniform distribution of the NGF solution along the long tube by minimizing droplet formation of the deposited NGF solution, we increased the viscosity of the solution by adding 0.5% of alginate. However, the negatively charged alginate (p*K*s of approx. 3.5) may have strongly bound the positively charged NGF (p*I* of 9) in the coating solution (ABS, pH 5.5). Alternative attempts to avoid droplet formation of the deposited NGF solution encompassed the addition of BSA, chitosan, or the surfactants poloxamer or Tween 20, which all failed to yield the desired effect. In addition to the NGF binding by electrostatic interactions, NGF also tends to aggregate over time [27]; aggregates would very likely remain trapped in the device until the device is fully degraded.

Another fabrication-related critical factor was the cutting of the tube into individual NC, which exposed the NGF to the environment at both ends of the NC. Through this exposure, NGF could leak from both ends of the tubes resulting in an important and variable burst release within the initial 24 h. The cutting further gave free access to medium to penetrate into the Al/Cs-PLGA interface, thereby

accelerating the excessive swelling of the alginate/chitosan support structure in ABS (Fig. 4). The swelling entailed the detachment of some of the PLGA coating and its retracting from the ends toward the mid segment of the NC. Nonetheless, the NGF release from the 1st generation NC provided evidence that the control of the release kinetics is feasible through embedding NGF into different PLGA layers.

The limited duration of release testing (15 days) was motivated by the instability of NGF upon extended incubation times [27] and in view of the expected *in vivo* performance. *In vivo*, nerve growth stimulation at the site of injury is indeed the most important during the first 10–15 days of repair, until the growth cone of the proximal nerve stump reaches the distal nerve stump, which provides further guidance and growth stimulation.

In the second stage of NC development, NGF was incorporated into the NC by an alternative method to improve reproducibility and release kinetics of NGF. Here, the NGF solution, without adding alginate, was accurately deposited in the middle part of a pre-cut NC by means of a pipette. This precluded a putative NGF-alginate interaction and ensured an accurate dose of NGF per NC. The NGF was further completely sealed by the subsequent PLGA layers that were sprayed over the entire length of the NC. Generally, NGF release from these 2nd generation NCs resulted in much higher total amounts of NGF (33 and 17 ng per day) than did the 1st generation NCs (1–5 ng per day, compare Figs. 5 and 6). The achieved release rates meet the specifications for the daily therapeutic dose of locally administered growth factors, which are generally in the low nanogram range [24]. Very importantly, the released NGF was bioactive as confirmed by neurite outgrowth from PC 12 cells (Fig. 7).

5. Conclusions

We proposed a multi-ply delivery system for NGF that permits to adjust the release kinetics of NGF by embedding the growth factor between different layers of the NC. The 1st generation NC afforded good control over the release kinetics, but yielded only low total amounts of released NGF and poor reproducibility of the 24 h burst release. This was ascribed to the leakage of NGF from the ends of this type of NC. In the 2nd generation NC, embedding NGF in the coating layers of the NC was more efficient, so that leakage of the factor at the ends of the NC could be prevented. Such 2nd generation NC yielded a sustained release of bioactive NGF for at least 15 days in citrate buffer. These 2nd generation NCs thus represent promising candidates to explore further different release kinetics of growth factors and their influence on nerve regeneration.

Acknowledgements

We thank Lorenz Uebersax (Institute of Pharmaceutical Sciences, ETH Zurich) for his help with the cell culture. The authors further thank Genentech (South San Francisco, CA, USA) for kindly providing NGF. We also acknowledge financial support by ETH Zurich and SUVA (Swiss National Accident Insurance Fund, Lucerne, Switzerland).

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