Silk fibroin matrices for the controlled release of nerve growth factor (NGF)

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Abstract

Nerve conduits (NC) for peripheral nerve repair should guide the sprouting axons and physically protect the axonal cone from any damage. The NC should also degrade after completion of its function to obviate the need of subsequent explanation and should optionally be suitable for controlled drug release of embedded growth factors to enhance nerve regeneration. Silk fibroin (SF) is a biocompatible and slowly biodegradable biomaterial with excellent mechanical properties that could meet the above stated requirements. SF material (films) supported the adherence and metabolic activity of PC12 cells, and, in combination with nerve growth factor (NGF), supported neurite outgrowth during PC12 cell differentiation. NGF-loaded SF-NC were prepared from aqueous solutions of NGF and SF (20\%, w/w), which were air-dried or freeze-dried (freezing at \(-20\) or \(-196\) \(\text{C}\)) in suitable molds. NGF release from the three differently prepared SF-NC was prolonged over at least 3 weeks, but the total amount released depended on the drying procedure of the NC. The potency of released NGF was retained within all formulations. Control experiments with differently dried NGF-lactose solutions did not evidence marked protein aggregation (SEC, HPLC), loss of ELISA-reactivity or PC12 cell bioactivity. This study encourages the further exploitation of SF-NC for growth factor delivery and evaluation in peripheral nerve repair.

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Keywords: Silk fibroin; Nerve conduits; Nerve growth factor; Bioactivity; PC12 cells; SEC

1. Introduction

Peripheral nerve injury is a serious issue affecting 2.8\% of trauma patients, many of whom will be subjected to lifelong disability [1]. For successful nerve regeneration, axons from the proximal stump of a severed nerve have to bridge the gap towards the distal stump, where they should follow the idiotypic Schwann cell tubes, replicating the original receptor innervation pattern and finally leading to restoration of function of sensory and/or motor targets [2]. Throughout this critical process, the axons are supported by neurotropic factors, such as the nerve growth factor (NGF), which are released from in-growing Schwann cells or cells from the distal stump [3,4]. Axonal growth is guided through physical (contact guidance) and chemotactical (neurotropism) mechanisms. For the bridging of small nerve gaps, microsurgical end-to-end sutures of the nerve stumps are often used [5,6]; for longer gaps, autologous nerve grafts [5,7] have to be implanted. Despite the well-developed microsurgical techniques used, the functional motor and sensory regeneration is often unsatisfactory because of neuroma and scar formation at the suture site, axonal loss, or failure of target reinnervation.

The limited availability of donor tissue for nerve autografts with the related morbidity at the donor site motivated the use of synthetic nerve conduits (NC) [8]. NC made of non-bioresorbable materials [9,10] such as S-hydrophilic nitrocellulose (ShNC), S-hydrophobic poly (vinylidene fluoride) (PVDF) [11], or silicone showed promising results, but caused sometimes inflammation or compression of the nerve [12]. NC made of bioreposable materials such as bioglass [13,14], poly(l-lactide-co-ethylene

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oxide) (p(LA-co-EO)) [15,16], poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) (p(HEMA-co-MMA)) [17], and poly(glycolic acid) (PGA) [18,19] have been successfully used in small gaps. However, the outgrowth of sensory axons after nerve transection was compromised in the case of a synthetic PGA NC [12]. NC have also been successfully produced from natural materials such as collagen and collagen/agarose blends [20,21], although the physical stability of pure collagen NC has to be increased by cross-linking [22]. Cross-linked collagen NC has produced inflammatory reactions and shown complete bioerosion within 2 months after implantation [23].

To promote the bridging of gaps exceeding the critical size of 10 mm, NC have been combined with neurotrophic factors [24]. Such NC drug delivery systems promoted efficaciously nerve guidance and target reinnervation [25,26] both in vitro and in vivo [27–29]. The sustained delivery of GDNF or NGF from NC enhanced nerve regeneration over long gaps in vivo [30]. A general problem in using growth factors in combination with drug delivery systems resides in the inherent instability of such proteins, which tend to degrade or aggregate in solution or during processing [31,32]. The stability of neuronal factors during processing and formulation might be reduced when using mild processing conditions, e.g. through avoiding organic solvents, aqueous/organic interfaces, or chemical reagents. Indeed, the fabrication of parenteral delivery systems frequently requires one or several of these stress factors either for dissolving or shaping the matrix materials or for rendering water-soluble matrix materials insoluble by cross-linking. The latter point is of particular interest for silk fibroin (SF). Thanks to its solubility in water, SF can be processed in aqueous media by freeze-drying [33,34] or air-drying, and subsequently be rendered insoluble by physical induction of β-sheet formation. This qualifies SF as a promising biomaterial for the fabrication of protein delivery systems, in particular long nerve guidance conduits, as it is biocompatible, of high resilience, and slowly biodegrading [35–38]. Silk has been used for decades as suture material and has a long-standing clinical history [39,40]. Moreover, silk fibers are mechanically strong and can absorb high amounts of energy before break [38]. These properties are important for NC materials. The use of SF as a substrate for neuronal cells [41] as well as a biomaterial for the delivery of proteins [42] has been well documented by our group as well as their biocompatibility in vitro and in vivo [36,37].

The present study investigated SF derived from the silk worm *Bombyx mori* as a biomaterial for the preparation of NGF-loaded NC. The NGF-loaded SF-NC were prepared from aqueous solutions of NGF and SF and subsequent drying of this solution in suitable molds according to three different protocols (air-drying, freeze-drying after freezing at −20°C or −196°C). An initial experiment revealed that PC12 cells adhered well to SF, remained metabolically active, and showed neurite outgrowth during cell differentiation when NGF was supplemented, which is in agreement with findings in the literature [43–45]. The different processing conditions used for preparing NGF-loaded SF NC did not destroy the NGF bioactivity. The release of bioactive NGF over 4 weeks from both freeze-dried and air-dried formulations substantiated the potential of NGF-loaded SF materials for use in peripheral nerve defects.

2. Materials and methods

2.1. Materials

Cocoons from *B. mori* were obtained from Trudel, Zurich, Switzerland, and processed in-house to obtain SF, as described below. Collagen 1 (Col-1) (calf skin) and laminin (natural mouse) were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant human beta-NGF (NGF) was kindly provided by Genentech (South San Francisco, CA). All cell culture materials including the medium and medium supplements were purchased from Invitrogen (Carlsbad, CA, USA), unless otherwise stated. The ATP bioluminescent assay kit, MTT assay kit, Triton-X, paraformaldehyde, and EDTA were from Sigma-Aldrich (St. Louis, MO, USA), and the Pico green assay kit from Molecular Probes Europe BV (Leiden, The Netherlands). The duo-set ELISA kit for NGF was from R&D systems (Minneapolis, MN).

2.2. Purification of silk fibroin

SF was prepared as described previously [46]. Briefly, cocoons from *B. mori* were boiled in ultra purified water (UPW) containing 0.02 M Na2CO3, rinsed with water, and dissolved in 9 M LiBr at 55°C to obtain a 10% SF (w/v) solution. This solution was dialyzed (Pierce, MWCO 2000 g/mol) against UPW for 3 days to obtain a 4% (w/v) SF solution.

2.3. Preparation of SF films, collagen I films and laminin coatings for PC12 cell assays SF films

SF films were prepared from 2 ml of sterile filtered (0.2 μm pore size filter) aqueous SF solution (3%, w/w) that was filled into 22 mm diameter flat Teflon molds. SF films were obtained upon evaporation under laminar airflow overnight and subsequently treated with 90% methanol (v/v) for 30 min to induce insolubility in aqueous media.

2.4. Collagen (Col-1) films

Calf skin Col-1 films were prepared in 12-well non-tissue culture polystyrene plates (Techno Plastic Products, TPP, Trasadingen, Switzerland) by pipetting 1 ml of a collagen solution (0.5 g/l in 0.1% (v/v) acetic acid) into each well. Col-1 films were obtained upon drying overnight, neutralizing with 2 ml of 0.1 M Na2HPO4 for 1 h, and washing twice with PBS.

2.5. Laminin (LN) coating

LN coating was applied to 12-well non-tissue culture polystyrene plates (TPP) by pipetting 640 μl of a LN solution (1 μg/ml in PBS of pH 7.4) into each well. After 2 h, the solution was withdrawn, and the wells were washed twice with 1 ml of PBS of pH 7.4.

2.6. Preparation of NGF-loaded SF films and SF tubes

SF films were prepared as described above; NGF was embedded by adding 16 μg NGF to 2 ml of 4% (w/v) SF solution, which was then poured into Teflon molds for evaporation of water. Films were then dried...
under laminar airflow. SF tubes were prepared as follows. The 3% (w/v) SF solution as prepared above was dia lyzed against PEG 6000 (Pierce, MWCO 2000 g/mol) to yield a viscous solution with a final concentration of 20% (w/v) SF. Then, 40 μg NGF was added to 1 ml of the 20% SF solution, which was shaken for 10 min at 4 °C to achieve homogeneity. This resulting solution was poured into tubular molds designed to obtain SF tubes with an outer and inner diameter of 3 and 1 mm, respectively. The filled molds were deep frozen at −20 °C during 1 h (Lyo-20) or shock-frozen at −196 °C in liquid nitrogen (Lyo-196) before they were freeze-dried at −30 °C during 36 h (LYOVAC GT2, FINN-AQUA, Hurr, Germany). Dried films and tubes (300 mg) were immersed in 90% (v/v) methanol in UPW (20 ml) for 30 min to induce β-sheet formation and water insolubility of the SF matrices.

2.7. Scanning electron microscopy (SEM)

SF films and SF tubes were cut into slices of 1–2 mm thickness, dried, platinum-coated and analyzed by scanning electron microscopy (SEM; Leo 1530, Carl Zeiss, Trasadingen, Germany).

2.8. Fourier transformed infrared spectroscopy (FTIR)

For FTIR, 5 mg of SF film or SF tube were ground with mortar and pestle, mixed with 300 mg KBr, and compressed with 10 tons (diameter of compact = 13 mm) under vacuum during 1 min before analysis (2000 FT-IR Spectrometer, V3.01, Perkin Elmer, Boston, MA).

2.9. Differential scanning calorimetry (DSC)

DSC was performed with 3 mg of SF film or SF tube at a heating rate of 10 °C/min and nitrogen flushing at 100 ml/min in two subsequent scans from 25 to 200 °C, and 25 to 260 °C, respectively (DSC822, Mettler Toledo, Greifensee, Switzerland). The thermal transitions of SF were determined from the second scan, after evaporation of the initial moisture.

2.10. Size exclusion chromatography (SEC) of processed NGF

The effect of the processing (air-drying, freeze-drying) on the stability of NGF was examined by SEC to detect high molecular weight aggregates. To exclude potential effects of SF, lactose was used as a surrogate excipient for SF, at a NGF: lactose ratio of 1:5500. Aqueous lactose solutions (20%, w/v), 5% sucrose, 0.05% NaN3 in PBS (300 μl/well) and the samples and the biotinylated antibody, appropriately diluted in a 1% BSA-TBST solution were sequentially added and incubated for 2 h each. The streptavidin-HPR was diluted in a 5% BSA (w/v) PBS solution and added and incubated for 20 min. Reactant volumes were 100 μl/well. A tablet of TMB (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.05 m phosphate-citrate buffer of pH 5.0 containing 0.02% hydrogen peroxide 30% and placed in the wells for 20 min. The reaction was stopped by the addition of 50 μl of 1 m H2SO4. Absorbance was read at 450–570 nm using a microplate reader (Molecular Devices, Bucher Biotec, Basel, Switzerland).

2.12. NGF in vitro release and quantification by ELISA

Pieces of tubes or films (~10 mg, n = 6) were immersed in 50 ml acetate buffer of pH 5.0 containing 0.02% Tween 40 and 0.05% sodium azide. Samples were taken at regular intervals during 4 weeks, and the liquid was replaced with fresh buffer. NGF concentration was assayed by a duo-set ELISA kit for NGF following the manufacturer’s protocol. Briefly, flat-bottom 96-well polystyrene plates (Nalge Nunc, Roskilde, Denmark) were washed with a 0.05% Tween 20 PBS solution of pH 7.4 between every step of the assay and 3 times at each step (300 μl/well). Microplates were initially coated overnight with a purified mouse monoclonal IgG1 anti hFGF-NGF in PBS (100 μl/well), and non-specific sites were blocked for 1 h using a solution of 1% BSA (w/v). For the MTT-assay, PC12 cells were seeded on the different substrates (see below), 5 × 104 cells/well were seeded in a 12-well plate in DMEM containing 1% FCS and 1% P/S.

2.13. Expansion and differentiation of PC12 cells

PC12 cells were cultured on Petri dishes for tissue culture (TPP) using DMEM containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S) solution. The cells were split every 2–3 days. For assessing PC12 proliferation and differentiation on different substrates (see below), 5 × 104 cells/well were seeded in a 12-well plate in DMEM containing 1% FCS and 1% P/S.

2.14. Proliferation and differentiation of PC12 on LN, Col-1 and SF substrates

PC12 cell proliferation on the various substrates was assessed by total DNA quantification and a MTT assay. For the DNA assay, cells were seeded in quintuplicate at 5 × 104 cells/well on the different substrates inserted into the wells of 12-well plates and incubated at 37 °C and 5% CO2. After 24 h, the DNA content in each well was assessed by the PicoGreen assay according to the manufacturer’s protocol. Supernatant was soaked off, and the cells were disrupted by adding 1 ml of lysis buffer (0.2% Triton-X and 5 mg/ml MgCl2) to each well and incubated in the dark for 48 h. The lysates were transferred into tubes and centrifuged at 3000 g and RT for 10 min. Then, 41 μl of supernatant were transferred into a new tube and complemented with 166.6 μl of diluted PicoGreen solution (PicoGreen dimethylsulfoxide stock solution diluted 200 times in TE assay buffer). After vortexing for 3 min, 100 μl of the solution were transferred to a 96-well plate and incubated for 5 min in the dark. The fluorescence of the standard and sample solutions was measured in a FluoroCount™ plate reader with λex and λem of 485 and 530 nm, respectively (Packard Instrument, Downers Grove, IL). For the MTT-assay, PC12 cells were seeded on the different substrates and incubated for 24 h as described above. MTT dissolved in PBS (5 mg/ml) was sterile filtered and diluted 10 times with DMEM before use. For the assay, the cell medium was soaked off, and 2.0 ml of the MTT solution was added to the cells, which were then incubated at 37 °C and 5% CO2 for 2 h. Then, the solution was soaked off, and 1 ml of acidic isopropanol (500 μl isopropanol + 3.5 ml 6 M HCl) was added to dissolve the formed crystals. After 10 min incubation at RT, the tubes were
centrifuged (300 g, 1 min, 4 °C), and 100 μL of the supernatant were transferred to a 96 well plate. The absorbance was measured at 570 nm and the background (630 nm) subtracted using a Thermomax microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

For assessing the differentiation of PC12 cells, 5 × 10⁴ cells per well were seeded in triplicate on the different substrates (SF, Col-1, LN) and incubated for 24 h as described above. Then, the medium was replaced by DMEM containing 1% FCS and 100 ng/ml of NGF. Neurite outgrowth from PC12 cells was subsequently monitored during 3 days. Cells exposing neurites exceeding double the length of their cellular body were considered as fully differentiated. The extent of differentiation was quantified on the basis of 100 cells per specimen.

2.15. Assessment of bioactivity of released NGF from SF matrices

For assessing the bioactivity of released NGF, PC12 cells were used as an in vitro assay system. Films or freeze-dried tubes (10 mg pieces) were incubated in triplicate in acetate buffer at 37 °C on an orbital shaker (80 rpm). After 0, 1, 2, 3 and 4 weeks of incubation, the SF matrices were taken out and rinsed with PBS before they were added (n = 5) to plated PC12 cells (5 × 10⁴ cell per well) in DMEM containing 1% FCS on LN-coated 12-well plates (TPP), DMEM containing 1% FCS and 100 ng/ml of NGF was used as control for all experiments. Pictures of PC12 cells were taken after 4 days of culture using an Axiovert 35 microscope equipped with an Axio Cam MRC5 digital camera (Carl Zeiss, Jena, Germany). For quantification, cells were considered fully differentiated when the outgrowth of the exposed neurites exceeded double the size of their cellular body; average and standard deviation of 100 differentiated cells per specimen were calculated.

2.16. Statistical analysis

Statistical data analysis was performed by one-way analysis of variance (ANOVA) and Tukey-HST procedure for post hoc comparison using Minitab® Release 14 for Windows. Values with p < 0.05 were considered statistically significant.

3. Results

3.1. Proliferation and differentiation of PC12 cells on LN, SF and Col-1 substrates

Prior to the seeding of PC12 cells on the three substrates SF, Col-1 and LN, the static contact angle (θ) of purified water on the materials and the tissue culture plates (TCP) was assessed at 22 °C using a goniometer (NRL C, Ramé-Hart, Mountain Lakes, NJ, USA). All three substrates were more hydrophilic than the TCP, with SF being the most hydrophilic followed by Col-1 and LN (θSF = 43.0 ± 1.4°; θCol-1 = 58.3 ± 0.7°; θLN = 62.1 ± 0.4°; θTCP = 77.2 ± 0.8°).

The metabolic activity (MTT assay; Fig. 1A) and proliferation (DNA content) of adherent PC12 cells did not reveal any significant differences between the cells grown on SF, Col-1, and LN (n = 5) after a cultivation time of 24 h. Hence, the metabolic cell activity normalized to the DNA content of the cultures (ODMTT/DNA) was also comparable between the cells grown on the different substrates (Fig. 1B).

The differentiation of adherent PC12 cells grown on the various substrates was assessed after addition of 100 ng/ml NGF in terms of neurite outgrowth (Fig. 2). Microscope images demonstrated that all substrates supported the outgrowth of neurites from differentiated PC12 cells (Fig. 2A). The percentage of differentiated cells increased gradually on all the three substrates during the 3 days of incubation (Fig. 2B). Statistical differences in the numbers of differentiated cells grown on the three substrates were observed only at day 2, whereas the percentage of differentiated PC12 cells was comparable on all substrates at days 1 and 3 of culture.

3.2. Porosity and crystallinity of air-dried and freeze-dried SF matrices

SEM revealed a porous morphology of freeze-dried SF tubes and an essentially non-porous structure of air-dried SF films (Fig. 3, column 3–5). SF solutions slowly frozen at −20 °C (Lyo-20) prior to freeze-drying at −30 °C yielded a porous matrix exposing a connected meshwork of unordered lamellar-like pores of sizes between 20 and 100 μm (Fig. 3, Lyo-20, row 1); the inner wall of the tube (Fig. 3, column) was smooth and also porous. In contrast, SF solutions shock-frozen at −196 °C (Fig. 3, Lyo-196, row 2) prior to freeze-drying at −30 °C yielded matrices with smaller pores of about 5–20 μm in diameter; the inner wall of the tube was smooth and displayed few small pores. Finally, the air-drying of SF solutions at ambient temperature and pressure resulted in 20–40 μm thick films that were even and smooth (Fig. 3, films, row 3). The measurement of total porosity, ε, by helium pycnometry...
(n = 5; Accupyc 1330, Micromeritics, Moenchengladbach, Germany) corroborated the SEM observations in that the air-dried SF-films were essentially non-porous, whereas the freeze-dried SF tubes featured a high porosity, which was essentially independent of the mode of initial freezing temperature and rate: ε = 84.03 ± 1.98% for Lyo-196; ε = 87.39 ± 2.69% for Lyo-20).

The induction of crystallinity in the different SF matrices by methanol treatment was examined by DSC and FTIR (Fig. 4). DSC thermograms did not show any crystallization or melting before the matrices started to decompose at 190–200 °C, irrespective of the preparation method of the SF matrices (data not shown). FTIR measurement revealed β-sheet conformation in all formulations, as evidenced by a shoulder at 1262 cm⁻¹, corresponding to the amide III band shift (for random coil at 1235 cm⁻¹), and an absorption band at 1630 cm⁻¹, corresponding to the amide I band shift (for random coil at 1660 cm⁻¹).

Fig. 2. Neurite outgrowth of PC12 cells grown on laminin (LN), collagen 1 (Col-1) and silk fibroin (SF). Neurite outgrowth was induced by addition of 100 ng/ml of nerve growth factor (NGF). (A) Phase contrast micrographs of adherent PC12 cells at different days after addition of NGF; scale bar = 20 μm. (B) Percentage of differentiated PC12 cells showing neurite outgrowth at different days after addition of NGF.
3.3 Stability and bioactivity of NGF under process conditions for SF matrix preparation

In view of preparing NGF-loaded SF matrices, NGF stability was assessed upon air-drying of the NGF/SF solution at room temperature and upon slow and shock freezing (−20 °C for 1 h or −196 °C for 1 min) with subsequent freeze-drying (n = 3). In this preliminary experiment, SF was replaced by lactose to avoid any potential interference of SF in the analysis of NGF. After drying, the solid product was re-dissolved in acetate buffer and analyzed by SEC, HPLC and in a PC12 cell bioassay; a fresh solution of equivalent amounts of NGF and lactose was used as control.

SEC chromatograms (Fig. 5) of the control (Control) and freeze-dried (Lyo-20 and Lyo-196) NGF/lactose showed a main peak at a retention time (RT) of 9.5 min, corresponding to the NGF dimer, and a minor peak at RT of 5.6 min, most probably corresponding to a high molecular weight aggregate of NGF. The air-dried NGF/lactose showed enlarged main and minor peaks with shoulders between 10.5 and 13 min and between 5.1 and 5.3 min, respectively. Data integration revealed statistically similar amounts of air-dried, freeze-dried (Lyo-20 and Lyo-196), and control NGF (data not shown), which was also confirmed by ELISA (Fig. 6A). The percentage of high molecular aggregates was calculated from the ratio of areas under the curve (AUC) of the peaks at RT = 5.6 and 9.5 min. The thus calculated percentages of high molecular weight aggregates did not differ significantly between NGF control, air-dried or freeze-dried samples (Fig. 6B). Hence, the AUC-values were not sufficiently sensitive to discriminate between the observed differences in the shape of the main peak. Consequently, we calculated the ratio of the AUC (at RT of 9.5 min) over the corresponding peak height. This ratio was significantly (p < 0.05) increased, i.e., broader peak, for air-dried NGF/lactose in comparison to the control NGF and the Lyo-20 freeze-dried NGF/lactose; it was also significantly (p < 0.05) increased for Lyo-196 freeze-dried samples in comparison to the control NGF (Fig. 6C).

The testing of the various NGF/lactose formulations and control NGF in a PC12 cell bioassay evidenced a loss of NGF-bioactivity upon air-drying and freeze-drying of
NGF/lactose solutions (Fig. 6D). At a nominal NGF concentration of 2.5 ng/ml, none of the processed NGF/lactose blends triggered a significant differentiation of PC12 cells, in contrast to the control NGF. At a NGF concentration of 5 ng/ml, the freeze-dried samples (Lyo-20, Lyo-196) performed significantly ($p < 0.05$) better than the air-dried samples, although all processed samples triggered significantly ($p < 0.05$) less PC12 differentiation than the control NGF. The decreased bioactivity of the processed samples was no longer distinguishable at NGF concentrations of 10 ng/ml (freeze-dried samples) or 100 ng/ml (air-dried samples).

### 3.4. NGF content and bioactivity of rehydrated SF matrices

The amount and bioactivity of NGF that was recoverable from the rehydrated SF matrices were assessed by ELISA and PC12 cell bioassay, respectively (Fig. 7). In general, more than 50% of NGF remained unrecoverable from the SF formulations (control SF solution or matrix) when detected by ELISA, suggesting a strong binding between NGF and SF. The amount of recoverable NGF did not significantly differ between the NGF/SF control solution (33.0±6.6%) and SF matrices (Lyo-20: 49.6±13.3%; Lyo-196: 55.2±36.5%; film: 30.7±7.5%) (Fig. 7B). In the bioassay, NGF eluted from freeze-dried matrices (Lyo-20 and Lyo-196) induced low (<20%) but significant PC12 differentiation at a dilution that corresponded nominally to 2.5 ng/ml NGF, which was not observed with comparable NGF dilutions from the NGF/SF control solution and NGF/SF film (Fig. 7B). At a nominal concentration of 5 ng/ml, the elutions from freeze-dried samples yielded a similar bioactivity as the control NGF/SF solution, which was comparable to the bioactivity of the eluted freeze-dried samples diluted to 2.5 ng/ml. At a nominal NGF concentration of 10 ng/ml, all matrix elutions as well as the NGF/SF control solution triggered a significant PC12 differentiation, although the bioactivity of the NGF/SF film elutions was significantly lower (48.6±25.0) than the NGF control (90.6±4.5) and elutions of the matrices frozen at −196°C and subsequently

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Fig. 4. Fourier transformed infrared spectra (FTIR) of silk fibroin matrices prepared by freeze-drying after shock-freezing at −196°C (Lyo-196), freeze-drying after slow freezing at −20°C (Lyo-20), or by air-drying (film).

Fig. 5. Size exclusion chromatograms (SEC) of NGF after processing in blends with lactose. Solutions of NGF and lactose in acetate buffer of pH 5.5 were either freeze-dried after shock-freezing at −196°C (Lyo-196), freeze-dried after slow freezing at −20°C (Lyo-20), or air-dried (air-dried). NGF in acetate buffer (pH 5.5) stored at 4°C served as control (NGF control).
freeze-dried (Lyo-196; 87.2% ± 5.8). At NGF dilutions of 100 ng/ml, all matrix elutions as well as the NGF/SF control solution induced comparable differentiation in the order of 80% of all adherent cells.

3.5. In vitro release kinetics and bioactivity of NGF released from SF matrices

The three NGF/SF matrix types released significantly different absolute amounts, but comparable relative amounts of NGF within the first 4 days (Fig. 8D), with Lyo-20 releasing 0.37 ng ± 0.03 or 78% of total (as measured after 22 d), Lyo-196 releasing 2.607 ng ± 0.69 or 73% of total, and film releasing 12.8 ng ± 1.33 or 66% of total). During this initial phase, both freeze-dried matrices released substantially less than 1 ng NGF per mg scaffold and day (Fig. 8A, B), whereas the SF film released between 2 and 4.5 ng NGF per mg scaffold and day (Fig. 8C). After 5 days, the daily NGF release from all matrices remained very low, though it continued until the end of the experiment.

The absolute cumulative release after 22 days differed significantly between the formulations and attained 0.49 ± 0.09 ng/mg (Lyo-20 tube), 3.57 ± 0.78 ng/mg (Lyo-196 tube), and 23.40 ± 8.64 ng/mg (air-dried film). Therefore, the SF film released 49 and 6.5 times more NGF than the Lyo-20 and Lyo-196 SF tubes, respectively. Relative to the total NGF dose incorporated into the matrices, the percentages of NGF released after 22 days amounted to 0.27 ± 0.04%, 2.0 ± 0.4%, and 13.0 ± 4.8% for the Lyo-20, Lyo-196, and air-dried SF matrices, respectively.

NGF loaded SF matrices (n = 3) were pre-incubated under release conditions for different time periods prior to transferring the matrix samples to adherent PC12 cultures and assessing bioactivity. Co-incubation of matrix samples with PC12 cells lasted for 4 days. At time zero, i.e., without pre-incubation, all matrices induced a similar extent of PC12 differentiation (approx. 55%), which was slightly lower than that induced by 100 ng/ml NGF (approx. 70%; control) (Fig. 9). The pre-incubated air-dried NGF/SF film and freeze-dried Lyo-196 SF tube sustained sufficient NGF release during the entire 4-week period to trigger a consistently high PC12 cell differentiation. On the contrary, the freeze-dried Lyo-20 NGF/SF tubes induced significantly less cell differentiation already after 1 week of pre-incubation (41.7 ± 16.5%), and the bioactivity of the Lyo-20 samples dropped further to 2–20% at weeks 2–4 of pre-incubation. In conclusion, the bioactivity results were consistent with the profiles of the daily NGF release (Fig. 8A–C).

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Fig. 6. Stability and bioactivity of NGF after processing in blends with lactose and assessed by ELISA (A), SEC (B, C), and PC12 cell differentiation (D). Solutions of NGF and lactose in acetate buffer of pH 5.5 were either freeze-dried after shock-freezing at −196 °C (Lyo-196), freeze-dried after slow freezing at −20 °C (Lyo-20), or air-dried (air-dried); a solution of NGF-lactose in acetate buffer of pH 5.5 stored at 4 °C served as control (Control). (A) NGF quantification by ELISA; the data shows the percentage of recovered NGF relative to the initial amount. (B) SEC-derived percentage of high molecular aggregates of NGF after processing. (C) SEC-derived ratio of AUC/peak height of the NGF homodimer peak. (D) PC12 cell differentiation-derived bioactivity of NGF after processing; note that the different gray shadings of the bars in panel D correspond to those shown in panels A–C.
4. Discussion

Several authors have reported that NC releasing neurotrophic factors can enhance nerve regeneration across long nerve gaps [30,47–49]. Maximal benefit from neurotrophic factors can, however, only be achieved with effective and prolonged growth factor delivery [29]. Recent approaches aimed at embedding or encapsulating neurotrophic factors into a polymer [48,50] or protein [30] matrix, which was subsequently integrated into pre-existing NC with adequate mechanical strength. Depending on the materials, the direct embedding of neurotrophic factors into polymeric NC may suffer from the use of organic solvents or heat; further, single materials generally do not afford optimal properties for both prolonged drug release kinetics and adequate mechanical strength. Consequently, we considered SF for manufacturing NC with appropriate physical and growth factor release properties. SF had already demonstrated excellent biocompatibility both in vitro and in vivo [36,37,51], good mechanical properties [38], and slow biodegradation. The beneficial mechanical properties of SF over those of native and cross-linked collagen have been shown in bone and cartilage tissue engineering [52,53]. Recent studies have also demonstrated the multifunctionality of SF for drug delivery in the context of tissue engineering [54,55] and for controlled release of bioactive proteins in general [42]. Thus, in the present work, we evaluated technologies for the embedding of NGF into a SF matrix to produce subsequently a mechanically adequate NC. The SF matrices (films, tubes) were prepared under relatively mild conditions to respect the bioactivity of the embedded NGF and to achieve controlled release of bioactive NGF over several weeks.

Our study demonstrates that SF may indeed be a suitable material for NC, as it supported the proliferation of PC12 cells with subsequent cell differentiation as evidenced by neurite outgrowth. SF supported neurite outgrowth as efficiently as Col-1 or LN, materials that were successfully used for NC [56–60]. The suitability of SF as a substrate for cell proliferation and differentiation has recently been demonstrated also in the differentiation of embryonic stem cells into glial precursor cells [41], cartilage, bone, and ligament cells [52,53,61].

SF was also adequate for co-processing with NGF in aqueous medium to produce NGF/SF matrices. Upon freeze-drying, but not upon air-drying, the proportion of high molecular aggregates of NGF remained comparable to that of the NGF stock solution (Fig. 6). An important observation concerned the strong interaction between NGF and SF, as compared to NGF and lactose (Fig. 6). SF co-dissolved with NGF lowered the availability of the protein to a concentration of 50–70% of the actual amount (180 ng/mg). This interaction affected of course also the availability of NGF from the SF films and tubes (Fig. 7A, B). We assume the observed interaction being governed largely by ionic attractions, because NGF carries a positive (pI = 4.3 [63]) and SF a negative net charge (pI = 9.3 [62]) at isohydric pH.

The NGF/SF ionic interaction must have also affected the NGF release from the SF matrices, which was prolonged over more than 3 weeks, but remained largely incomplete, i.e., 0.3–13% within 22 days. By comparison, insulin-like growth factor-1 (IGF-1) with a lower net charge at isohydric pH (pI = 8.3) and a lower molecular weight (7.4 kDa for IGF-1 versus 26 kDa for the NGF homodimer) was released more completely from freeze-dried SF matrices, i.e., 20–25% of the actual dose (unpublished data). These findings demonstrate that growth factor release kinetics from SF matrices is likely influenced by both ionic charge and molecular weight of the protein. In a previous study, we showed that dextrans...
with increasing molecular weight are released slower and less complete \[42\]. Interestingly, NGF release did not cease entirely even after 3 weeks (Fig. 8A, Fig. 9).

Despite the assumed strong molecular interaction between NGF and SF, the differently prepared NGF/SF matrices exhibited different release profiles. We can only speculate that the freezing of the NGF/SF solution might have temporarily changed the conformation of either or both of the compounds so that a larger number of functional ionic, polar or H-bonding groups became exposed and available for NGF/SF interaction. While the conformation of NGF during and after the processing was not analyzed, all SF matrices revealed indistinguishable \(\beta\)-sheet conformation (Fig. 4).

5. Conclusions

This study evidenced that SF should be a suitable material for NC designed to deliver neurotrophic factors. First, SF was found suitable for supporting PC12 cell proliferation and maturation upon stimulation with NGF. SF was readily shaped into matrices for future use as NC and loaded with NGF. The SF matrices were rendered water-insoluble through treatment with methanol, which induced \(\beta\)-sheet formation. The used methanol treatment did not appear to alter the NGF bioactivity. NGF-release from the SF matrices lasted for more than 3 weeks, but the total fraction released remained generally very low, i.e., between 0.3\% and 13.0\%, depending on the preparation method. This incomplete release could not be related to NGF-aggregation. The released NGF remained fully bioactive over the entire release experiment of 4 weeks and afforded PC12 cell differentiation with neurite outgrowth. Further work will encompass the optimization of mechanical and release properties of the matrices, for example through blending of SF with other biomaterials.

Fig. 8. NGF in vitro release from SF matrices in acetate buffer of pH 5.5. (A–C) Daily release of NGF per mg of SF matrix prepared by freeze-drying a NGF-SF solution after slow freezing at \(-20^\circ\text{C}\) (A; Lyo-20), or by freeze-drying a NGF-SF solution after shock-freezing at \(-196^\circ\text{C}\) (B; Lyo-196), or by air-drying a NGF-SF solution (C; Film). (D) Cumulative release per mg of SF matrix prepared by the aforedescribed processes.

Fig. 9. PC12 cell differentiation-derived bioactivity of 10mg NGF-SF matrices that had previously been pre-incubated in acetate buffer (pH 5.5) at 37\(^\circ\)C for 0, 1, 2, 3, and 4 weeks. At each time point, the pre-incubated NGF-SF matrices were carefully rinsed and incubated with PC12 cells for 4 days, after which the percentage of PC12 cells showing neurite outgrowth was determined. A solution of NGF in the release medium (100 ng/ml) served as control (NGF). The NGF-SF matrices were prepared as described before.
such as collagen or LN. Finally, growth factor loaded SF-NC will be tested in vivo for their biocompatibility in a peripheral nerve gap and their nerve growth stimulating capacity.

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