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Title:

A new preparation method for anisotropic silk fibroin nerve guidance conduits and its evaluation *in vitro* and in a rat sciatic nerve defect model

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1.) Abstract

In the last decade silk fibroin has been increasingly used in peripheral nerve tissue engineering. Current approaches that aimed to produce silk fibroin based nerve guidance conduits used either aqueous solutions or organic solvents. Here we describe a novel procedure that uses the braided tubular structure of raw *Bombyx mori* silk, subsequently degummed and treated with the ternary solvent $CaCl_2/H_2O$/ethanol, formic acid and methanol to improve its mechanical and chemical characteristics.

These conduits are proved to be mechanically resistant and flexible and showed no signs of cytotoxicity. Moreover, they were impermeable for fibroblasts placed on the external surface whereas Schwann cells have readily attached to the luminal surface of the silk tubes. Short term *in vivo* studies revealed that the conduits implanted in a nerve gap of the rat sciatic nerve did not induce host inflammatory reactions one and three weeks after implantation. On the other hand, limited ingrowth of regenerating axons from the proximal nerve stump into the conduit was observed ten days after implantation compared with an autologous nerve graft. In case of longer survival (12 weeks) morphological and functional reinnervation of the distal targets have been achieved. Axon counts distal to the graft revealed significant numbers of axons in both the collagen-filled and empty silk tube groups (1678 ±303 *vs* 1274 ±146). In contrast, many more fibres were found to regenerate through an autologous nerve graft (6252 ±474). Nerve compound action potentials recorded in animals treated with collagen-filled and empty silk tubes (9,7 ±4,4 *vs* 6,5 ±3,1) were significantly lower than the ones in animals receiving autologous nerve grafts (22,8 ±7,5).

The present structure of silk tube conduits is proven to support axonal regeneration in a relatively short nerve gap. However, further improvements and the use of extracellular matrix molecules and Schwann cells is suggested to enable silk tube-based conduits to bridge long distance nerve gaps.

2.) Introduction

The incidence of peripheral nerve injury in traumatic wounds of the extremities is approximately 2-5% . Moreover, tumor resection or congenital malformation may also lead to nerve damage. Consequently, these incidences display a major burden on health care expenses, extensive absence from work and chronic disability . Direct repair of nerves is one clinical option, however, this direct end-to-end coaptation is limited to short-distance gaps. The current clinical gold standard for the repair of longer nerve gaps is the use of autologous nerve grafts . The main advantage of autografts is their morphologically native structure, which provides an ideal guide for axonal regeneration from the proximal to the distal nerve stump. However, autografting carries several disadvantages such as the limited number of donor sites for graft harvesting or the associated donor site morbidity, including loss of nerve function, painful neuroma formation and hyperaesthesia . These negative aspects have led to the search for alternative approaches. Beside nervous tissue, other autologous materials, such as vein grafts or muscles have been used to bridge nerve gaps. However, the use of these substances was both preclinically and clinically unsatisfactory. Recent advances in tissue engineering (TE) have opened new opportunities in peripheral nerve repair. Artificial nerve guidance conduits (NGCs), composed of synthetic or natural polymers, are currently being investigated for bridging nerve defects . The rationale behind using a NGC is to entubulate the nerve stumps to provide a protective micro-environment for the regenerating peripheral nerve. While numerous synthetic and natural biomaterials have been evaluated, both preclinically and clinically, for the bridging of nerve defects, their therapeutic benefits still appear unsatisfactory.

In the last years silk fibroin (SF) has attracted considerable interest as a biomaterial suitable for applications in peripheral nerve regeneration. SF has been shown to possess characteristics that favor its use as a NGC, such as mechanical stability, slow degradation rate, biocompatibility and its ability to support nerve regeneration . Apart from biocompatibility, a NGC should act as a barrier for infiltrating fibroblasts and provide mechanical resistance against compression and kinking by the surrounding tissue. The majority of current approaches to create tubular structures use electrospinning, as this

159 process can be well controlled . Other techniques include dipping , gel spinning or molding . All the
160 above-mentioned preparation processes are based on dissolved SF that is then used to create a
161 tubular construct. For the first time, to our knowledge, our study attempts to use textile-engineered
162 raw silk constructs as the starting material for bridging a nerve defect. To improve the mechanical
163 properties favoring its use as a NGC these braided tubular structures are further processed by
164 treatment in a ternary solvent system of a $CaCl_2/H_2O$/ethanol solution and then re-stabilized with
165 formic acid.

166 The aim of this study was to develop a novel method to generate a silk fibroin-based NGC with
167 distinct mechanical and anisotropic properties, and to prove its biocompatibility and functionality *in*
168 *vitro* and *in vivo* in a rat sciatic nerve injury model.

3.) Materials and Methods

Unless otherwise noted, all reagents were obtained from Sigma (Vienna, Austria) and of analytical grade.

3.1) Design and preparation of silk conduits

White raw *Bombyx mori* silkworm fibers of 20/22 den, 250 T/m, were purchased from Testex AG (Zürich, Switzerland). The tubular silk conduit was fabricated in cooperation with a commercial braiding company (Edelrid GmbH, Isny/Allgäu, Germany). Six single silk fibers form a twisted yarn, representing the raw material for the commercial braiding machines. Figure 1 shows the tubular structure designed from six intertwined twisted yarns. The resulting raw silk conduit was degummed by boiling in 0.2 M boric acid in a 0.05 M sodium borate buffer (pH = 9.0) . Batches of 2 g of silk conduits were boiled twice in 500 mL of degumming solution for 45 min, with an intermittent exchange of the degumming solution. After degumming, scaffolds were thoroughly washed in ddH_2O and air-dried before further processing (Fig. 1).

The degummed SF tubes were placed on an ABS (acrylonitrile butadiene styrene) rod of 2 mm in diameter and dipped in a boiling solution of the ternary solvent calcium chloride/distilled water/ethanol ($CaCl_2/H_2O$/ethanol) in a molar ratio of 1:2:8 for 20 seconds. Immediately after etching the outer surface, the tubes were dipped in 100% of formic acid (FA) at room temperature for 20 seconds. The tubes were then fixed in methanol for 20 minutes and subsequently washed thoroughly with ddH_2O (Fig. 1). The tubes were dried under laminar airflow and sterilized by autoclaving prior to use.

3.2.) Endurance and fatigue tests

To test the elasticity of the SF-NGC in comparison to the unprocessed initial tubular SF-scaffold, a custom-made compression test machine was built (Suppl. Fig. 1). This device was designed for repeated compression of a test specimen with constant maximum pressure. Starting from the top

position, a piston is moved downwards by a servo motor (Modelcraft RS2 MG/BB standard servo, Conrad Electronic SE, Hirschau, Germany) at a speed of approximately 5 mm/s linearly until it touches the probe. The piston continually stresses the probe until a predefined force threshold is reached. A force sensitive resistor (Strain gauge FSR 151, Interlink electronics, Camarillo, CA, USA), integrated into the piston, acts as a sensor and a voltage divider. The resistance, and thus the applied force, is constantly sampled at 50 Hz sampling frequency using the built-in 10 bit AD-converter of the microcontroller (Arduino Duemilanove Controller Board with Atmega 328 µC, Atmel Munich GmbH, Garching/Munich, Germany). The system was calibrated using a laboratory scale and operates with a ±5 g accuracy (corresponding to 0.98 MPa). Once the threshold is reached, the piston is returned to the top position, where it remains for a period of time set by the user.

In order to evaluate the effect of the various treatment components four types of tubes have been evaluated (Fig. 2). The first type was produced by degumming of the raw tube followed by methanol treatment. The second and third types were created either by $CaCl_2/H_2O$/ethanol- or formic acid-treatment, both fixed with methanol. The fourth type of tube was produced as described above, combining all sequential treatments (Fig. 1).

Prior to testing, respective samples were hydrated in PBS overnight. For testing, the conduits were fixed in a Sylgard-plated Petri dish (Sylgard® 184, Dow Corning Europe S.A., Seneffe, Belgium) and covered with PBS. The mechanical test regimen consisted of 1,000 cycles of compression (300 ms duration and 58.8 MPa load) and release. After testing the tubes were air-dried overnight at room temperature and an approximately 1 mm thick slice was cut out from them at the impression site for morphological analysis. The deformity remaining after the compressions was assessed by scanning electron microscope analysis.

3.3) Scanning electron microscope analysis

Samples were fixed in 2.5% glutaraldehyde in cacodylate buffer overnight at room temperature, then washed and dehydrated through graded ethanol changes followed by treatment with hexamethyldisilazane, and allowed to air-dry in a fume hood. Coating with Pd-Au was performed

through the use of a Polaron SC7620 sputter coater (Quorum Technologies Ltd. East Grinstead, United Kingdom) and the samples were examined under a JEOL JSM-6510 scanning electron microscope (JEOL Ltd., Tokyo, Japan) at 15 kV.

3.4) Cell culture experiments

NIH/3T3

NIH/3T3 cell line was purchased from ECACC (European Collection of Cell Cultures, UK). NIH/3T3 cells were cultured in DMEM containing 10% fetal calf serum (FCS, Lonza Ltd., Basel, Switzerland) supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin in plates coated with 0.2 % gelatin solution.

Schwann cells (SCs)

Schwann cells were isolated from rat sciatic nerves as described by Kaekhaw et al. . All animals were housed in the facilities of the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology in a temperature-controlled environment. Animals were provided with food and water ad libitum. All experimental protocols were approved by the City Government of Vienna, Austria, in accordance with the Austrian law and Guide for the Care and Use of Laboratory Animals as defined by the National Institute of Health.

Five adult male Sprague-Dawley rats were deeply anesthetized by inhalation of 3.5% isoflurane and euthanized with 110 mg/kg BW ketamine hydrochlorid (Ketasol®; Dr. E. Graeub AG, Berne, Suisse) and 12 mg/kg BW xylazine (Rompun® 2 %, Bayer AG, Vienna, Austria) intraperitoneally. The sciatic nerves were dissected free of connective tissue and minced after removing the epineurium. Nerve fragments were incubated with 0.05% collagenase for 1 hour at 37°C, subsequently filtered through a 40 µm cell strainer and centrifuged at 400g for 6 minutes. After washing the cell pellet in DMEM containing 10% FCS, the pellet was resuspended in Schwann cell culture medium consisting of

DMEM-D-Valine (PAA, Austria) supplemented with 10% FCS, 2 mM L-glutamin, 1% antibiotics, N2 supplement, 10 µg/ml bovine pituitary extract and 5 µM forskolin. Cell suspension was seeded on 6-well plates coated with poly-l-lysin and laminin.

Schwann cells were seeded on the inside wall of the SF-NGC at a concentration of 10^5cells/mL. Three groups were set: in group 1 only degummed (boric acid treatment) silk tubes were used, in group 2 the silk tubes were further treated with $CaCl_2$ (degumming + $CaCl_2$), whereas group 3 tubes received a full treatment completed with formic acid (degumming + $CaCl_2$ + FA). As a final step all tubes were fixed with methanol. After 2 hours, cells were supplied with Schwann cell culture medium. Schwann cell attachment to the inner wall structure of the SF-NGC was evaluated after 48 hours with Calcein AM staining (Invitrogen, Vienna, Austria). In order to evaluate whether the viability of Schwann cells attached to the various types of tubes a propidium iodide staining was performed.

3.5) Cell permeability

A cell migration assay was designed to verify the cell impermeability of the SF-NGC. A 100 µl fibrin clot (Tisseel, Baxter International Inc., Deerfield, IL, USA) containing PDGF-AA (Peprotech Austria, Vienna, Austria) was used to induce cell migration. Prior to the addition of 250 Units/mL of thrombin to induce polymerization, 10 ng of PDGF-AA was thoroughly mixed in fibrinogen. The resulting solid fibrin structure provides a slow release of PDGF-AA. This fibrin clot was then placed inside the investigated tubes and the assembled constructs were pinned in silicone-coated (Sylgard® 184, Dow Corning Europe S.A., Seneffe, Belgium) 12-well plates. Besides allowing the possibility of fixing the constructs to a Petri dish, Sylgard® 184 is known to discourage cell adhesion as a result of its hydrophobic character and therefore prevents cell migration from one clot to the other over the surface of the cell culture plate. Thus, the cells had only their way to move from one clot to the other through the wall of the SF-NGC and thereby we could investigate the cell permeability of the SF-NGC. A second 100 µl fibrin clot containing $2.5x10^5$ NIH/3T3 fibroblasts was placed on top of the tube. For this clot, cells were suspended in fibrinogen and then the polymerization was initiated with 2

units/mL thrombin. The generated loose fibrin structure allows fibroblasts to migrate from the clot towards the chemotactic stimulus. As a positive control, the fibrin clot with cells was separated from the clot containing PDGF-AA using the nylon mesh of a cell strainer with a 100 µm pore size. (Becton Dickinson Ltd., Schwechat, Austria). The constructs were completely covered with cell culture medium. On day 6, cell migration was evaluated by staining the PDGF-AA-containing fibrin clot with Calcein AM (Invitrogen, Vienna, Austria).

3.6) Cytotoxicity assay

To test cytotoxicity of the prepared SF-NGC, 1 g of dissected material of SF-NGC was immersed in 5 mL cell culture medium for at least 24 h. In parallel, $0.2x10^5$ Schwann cells per well were seeded onto 24-well plates. Then the medium containing leach-out products from the dissected material was filtered (0.22 µm, Rotilabo, Karlsruhe, Germany) and used to change media in the cell cultures. Standard culture medium was used as a negative control. After 72 h, cell culture medium was aspirated and the respective cell culture medium containing 650 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide was added to each well. After 1 h of incubation at 37° C and in 5% CO_2, medium was aspirated and MTT formazan precipitate was dissolved in DMSO by shaking mechanically in the dark for 20 min. Aliquots of 100 µl of each sample were transferred to 96-well plates. The absorbance at 540 nm was read immediately on an automatic microplate reader (Spectra Thermo, TECAN Austria GmbH, Austria). Optical density (OD) values were corrected for unspecific background.

3.7) Animals and surgery

All animals were housed in the facilities of the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology in a temperature-controlled environment. Animals were provided with food and water ad libitum. All experimental protocols were approved by the City Government of Vienna, Austria in accordance with the Austrian law and Guide for the Care and Use of Laboratory Animals as defined by the National Institute of Health.

A total of 28 female Sprague-Dawley rats (Animal Research Laboratories, Himberg, Austria), weighing between 350 – 450 g were used in the experiments. Eighteen animals were randomly assigned into three different treatment groups: autologous grafting (n = 6), SF-NGC (n = 6) and collagen-filled SF-NGC (n = 6) for 12 weeks observation. The animals were weighed and anesthetized in a fume box with 3.5% isoflurane (Forane®, Abbott, Vienna, Austria) at a flow rate of 800 mL/min. Subsequent anesthesia throughout the surgical procedure was maintained using 2.5% isoflurane via a nosepiece. At right mid-thigh level, the surgical area was shaved and disinfected with povidone-iodine (Betaisodona®, Mundipharma, Vienna, Austria). All the following surgical procedures were carried out under an operating microscope (Leica M651, Leica Microsystems, Vienna, Austria). The sciatic nerve was exposed and an 8 mm segment of the sciatic nerve was excised resulting in a 10 mm gap. In the autologous grafting group, the excised 8 mm segment of the sciatic nerve was rotated 180° and then sutured to the proximal and distal stumps using Ethilon 8/0 epineurial sutures (Ethicon-Johnson & Johnson, Brussels, Belgium). In both SF-NGC groups, the conduit was implanted by insertion of the proximal and distal nerve stumps into the 12 mm tube and coaptated to the conduit by two epineurial sutures. In the SF-NGC-collagen group, the lumen of the SF-NGCs was filled with 8 microliters of collagen solution (Type I, 2.5 mg/ml, Millipore, Vienna, Austria). Afterwards the wound was closed in anatomical layers. The analgesic treatment was administered in form of 0.75 mg/kg bodyweight (BW) meloxicam (Metacam®, Boehringer Ingelheim, Ingelheim/Rhein, Germany) and 1.25 mg/kg BW butorphanol (Butamidor®, Richter Pharma AG, Wels, Austria) immediately before the surgical procedure and for two days thereafter.

To monitor the possible adverse effects against the implanted SF-NGCs and the initial axon outgrowth into these tubes, animals with short survival times (7, 10 and 21 days, n=2, 6, 2, respectively) were sacrificed.

3.8) Tissue sampling, perfusion and immunohistochemistry

Twelve weeks after surgery the animals were deeply anesthetized by inhalation of 3.5% isoflurane and euthanized with 110 mg/kg BW ketamine hydrochlorid (Ketasol®; Dr. E. Graeub AG, Berne, Suisse) and 12 mg/kg BW xylazine (Rompun® 2 %, Bayer AG, Vienna, Austria) intraperitoneally. Animals were perfused transcardially with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4). The autologous transplants or the implanted SF-NGC were harvested under the operating microscope along with the proximal and distal nerve stumps.

The nerve grafts or conduits (n=6 per group), removed from perfusion-fixed animals, were immersion-fixed for 6 h in 4 % paraformaldehyde and then cryoprotected in 30% sucrose in PBS. Parallel cryostat sections were cut on a Leica 1850 cryostat (Leica Microsystems, Vienna, Austria) and sections were either stained with cresyl violet or processed for 200 kD neurofilament immunohistochemistry. Sections were treated with a 1% milk powder solution and then incubated with an anti-200 kD rabbit neurofilament antibody (Abcam Ltd, UK, Lot. No.: ab8135, rabbit, 1:1000) or with biotinylated Griffonia (Bandeira) simplicifolia lectin B_4 (GSA-B_4, 1:200, Vector Labs, Burlingame, CA, USA) overnight at 4°C. The sections were then processed for incubation with an anti-rabbit Alexa 546 or secondary antibody (1:400) or with streptavidin Alexa 488 (to visualize 200 kD neurofilament or Griffonia simplicifolia isolectin, respectively) for 2 h at room temperature, protected with a coverslip and investigated under an epifluorescence microscope (Olympus FX-50, Olympus Ltd, Tokyo, Japan).

The sciatic nerve segment distal to the graft was transferred into a 2.5% phosphate-buffered glutaraldehyde solution after perfusion and immersion fixed for 24 h. Remnants of fixative were carefully washed out from the nerve, and the tissue was treated in 1% OsO_4 in PBS (Agar Scientific, Stansted, UK) for 1 h, dehydrated in a graded ethanol series and propylene oxide and then embedded in Durcupan (Fluka, Switzerland). Semithin sections (0.4 µm thick) were cut 2 mm distal to the graft on a Leica Ultracut-R ultramicrotome and stained according to Rüdeberg .

3.9) Quantification of the Schwann cell-like cell densities

To quantify the cellular area on the luminal surface of tubes treated to various extents we randomly selected three (500 µm x 500 µm) area and photographed Schwann cell-like cells at a 10-fold primary magnification, using a Olympus BX50 epifluorescence microscope. Using ImageJ Software (NIH), we measured the relative density of Calcein AM stained Schwann cell-like cells. The background/autofluorescence of unstained samples as reference intensity was then subtracted from the intensity of Calcein AM stained samples. The cellular area occupied on the total surface divided by the total area and multiplied by 100. Furthermore, automatic thresholding was performed for each image by using the NIH ImageJ software to determine the threshold for the specific signal.

3.10) Semiautomated gait analysis (CatWalk™)

To evaluate the functional recovery of the animals we used the Catwalk (version 7.1, Noldus, Wageningen, The Netherlands) gait analysis system. This method allows an objective quantification of multiple static and dynamic gait parameters . The animals were pre-trained to use the runway for 3 weeks before surgery. The animals were tested postoperatively once a week from week 4 to week 12 for all groups. Various parameters for locomotor functional recovery including print area, maximum intensity, stance time and duty cycle were determined. The intensity of the right hind paw was expressed as a percentage of the contra-lateral left hind paw. The Catwalk experiments were performed in a blinded fashion.

3.11) Electrophysiology

At the end of the defined regeneration period, electrophysiological analysis (NeuroMax-XLTEK, Oakville, ON, Canada) was carried out before sacrificing the animals. Stimulation electrodes were placed 2 mm proximal and 2 mm distal to the graft for calculation of the nerve conduction velocity. A needle electrode was placed as a recording electrode into the tibialis anterior muscle, and the sciatic nerve was stimulated for 0.05 ms first proximally and then distally to the graft, so as to achieve the supramaximal stimulation amplitude. The compound action potential, the amplitude and the nerve

conduction velocity were determined. All measurements were carried out at a body temperature between 38 and 39° C.

3.12) Statistical analysis

Statistical analysis was performed using Graph Pad Prism software (Graph Pad Software Inc., San Diego, CA, USA). Normal distribution of data was tested with the Kolmogorov-Smirnov test. One-way ANOVA followed by Tukey´s post hoc test was used to assess statistical significance and p-values below 0.05 were considered statistically significant. All graphs in this study are shown as mean ± standard deviation (SD).To evaluate the functional recovery of the animals we used the Catwalk (version 7.1, Noldus, Wageningen, The Netherlands) gait analysis system. This method allows an objective quantification of multiple static and dynamic gait parameters . The animals were pre-trained to use the runway for 3 weeks before surgery. The animals were tested postoperatively once a week from week 4 to week 12 for all groups. Various parameters for locomotor functional recovery including print area, maximum intensity, stance time and duty cycle were determined. The intensity of the right hind paw was expressed as a percentage of the contra-lateral left hind paw. The Catwalk experiments were performed in a blinded fashion.

4.) Results

4.1) Structural changes during processing of silk tubes

Figure 1 A-C shows the raw SF-NGC consisting of braided single silk fibers. After degumming, the silk tube was treated with $CaCl_2/H_2O$/ethanol and subsequently with FA for 20 seconds each, followed by fixation with methanol. This treatment results in a fusion of the outer single silk fibers to a closed layer with a varying thickness ranging from 40 to 75 µm (Fig. 1D-F). Treatment with $CaCl_2/H_2O$/ethanol shorter than 20seconds results in thinner outer layer (Fig. 1G). In contrast, the luminal wall of the tube which was not treated with various solvents, preserves its original braided structure (Fig. 1F). Figure 2 shows the time-dependent effects of $CaCl_2/H_2O$/ethanol and FA on the SF fibers. SF fibers solely treated with $CaCl_2/H_2O$/ethanol dissolve and precipitate, especially after 40 seconds. In contrast, FA-treatment alone disorganizes the original braided structure in a time-dependent manner.

4.2) Cytotoxicity and viability assays

To investigate whether cytotoxic residuals were left in the SF-NGCs during the preparation procedure a MTT assay was performed. Dissected pieces of the SF-NGCs and the unprocessed raw silk scaffold were incubated in cell culture media, to remove cytotoxic molecules from the constructs. Treatment of cultured Schwann cells with these wash-out media resulted in no significant difference in the cell viability of Schwann cells in any treatment group (Fig. 3A). Next we tested the viability and adhesion pattern of Schwann cells cultured on the luminal surface of the silk tubes processed to various extents during the SF-NGC preparation procedure. Treatment with $CaCl_2/H_2O$/ethanol and the full treatment procedure including incubation with FA induced significantly more Schwann cells to adhere to the luminal surface of the silk tubes than degumming only (Fig. 3B-F). On the other hand, propidium iodide staining did not reveal any apoptotic Schwann cells on the luminal surface of these cultured silk tubes (Fig. 3C).

4.3) Endurance test

Implanted silk tubes have to resist to external pressure originating from the surrounding tissues and organs during movements. To verify whether ready-to-use SF-NGCs are able to withstand external forces, mechanical compression tests were performed with a custom-made system (Fig. S1). Degummed tubes were seriously challenged during the compression test, they remained compressed and flat after 1000 cycles of compression (Fig 3G). FA or $CaCl_2/H_2O$/ethanol alone improved the elastic properties of the SF-NGCs resulting in a moderate preservation of the lumen, whereas full treatment (degumming, $CaCl_2/H_2O$/ethanol followed by FA treatment) helped improving the elastic properties of these SF-NGCs to become resistant to external forces (Fig. 3G).

4.4) Cell permeability

A cell migration assay was applied to test the impermeability of the SF-NGC wall to invading cells. The test was based on the chemotactic properties of PDGF-AA embedded in a fibrin clot (Fig. 4A). The efficacy of NIH/3T3 fibroblasts to penetrate and pass through the wall of the silk tube was tested. Degummed silk tube walls were suitable structures for the fibroblasts to migrate through their braided structure similar to positive controls, where the cells were able to pass through the mesh of a cell strainer (100 µm pore size) (Fig. 4B-C). In contrast SF-NGC with a completely closed outer surface did not support the penetration of fibroblast into the wall of the conduit (Fig. 4C).

4.5) Short term in vivo studies - general observations

The implanted SF-NGCs were explored and thoroughly checked under the operating microscope one and three weeks after surgery. Figure 5A shows the proximal and distal nerve stumps coaptated to the SF-NGC by two epineurial sutures at the time of surgery. One week after implantation, visual inspection revealed that the SF-NGC did not exhibit substantial degradation (Fig. 5B). Furthermore, no signs of inflammatory reaction or neuroma formation at the coaptation sites could be detected. The entire outer surface of the implanted graft was covered by a thin layer of connective tissue. Interestingly, the proximal as well as the distal end of the implanted SF-NGC shows a partial integration of the nervous tissue with the SF-NGC (Fig. 5B). Moreover, the thin layer of connective

tissue on the surface of the SF-NGC contained small blood vessels (Fig. 5C-D). At 3 weeks after implantation the lumen of the SF-NGC was completely filled with regenerated tissue (Fig. 5E). Careful dissection of the SF-NGC (Fig. 5F) revealed a complete reconnection of the proximal and distal nerve stumps.

4.6) Axonal regeneration

Our results showed that a short gap of 8 mm in the rat sciatic nerve could be bridged by implanting an SF-NGC in the gap. To compare the axon growth promoting capacity of the three conduits used in the study we looked at the axon outgrowth from the proximal nerve stump into the conduits ten days after grafting by using neurofilament staining. Autologous nerve grafts were already populated with regenerated axons along their whole length at this time-point and the axons approached the distal coaptation site (Fig. 6A). In contrast, the silk tubes were able to promote only limited outgrowth of the axons at this stage. The regenerating axons have grown to a distance of approximately 2 mm in both conduits (1.7 and 1.8 mm in empty tubes and 2 and 2.1 mm in collagen-filled tubes, n=2 in each group) without considerable difference between them (Fig. 6B, C). The autologous nerve grafts 10 days after postoperatively are well vascularized (Fig. 6A). A similar range of vascularization of the silk tubes could be observed on day 10 after surgery. No considerable number of macrophages were seen in the implanted silk tubes (Fig. 6B,C).

Three months after transplantation the course of regenerated axons throughout the lumen of the implanted SF-NGCs and autologous nerve grafts was clearly visible (Fig. 6D-F). Although the axon bundle was present in the empty silk tubes the immunohistochemical analysis did not reveal a significant staining pattern for neurofilament 200kD in the distal portion of the tubes. On the other hand, myelinated axon counts showed significantly less myelinated axons in the empty SF-NGC as well as the collagen-filled SF-NGC compared to the autologous nerve graft (empty SF-NGC: 1274 ±146, collagen-filled SF-NGC: 1678 ±303, autologous nerve graft: 6252 ±474; Fig. 7). Accordingly, filling the silk tubes with collagen did not influence the short and long term regeneration of axons.

457 **4.7) Functional recovery – Catwalk analysis**

458 Twelve weeks after surgery the functional recovery parameters (Fig. 8A-D) including mean stance
459 time, mean print area, mean duty cycle and the mean maximally exerted intensity of the right hind
460 limb were evaluated. In three out of four parameters (excluding limb print area) there was a
461 significant difference between the extent of recovery of the autologous nerve grafts compared to the
462 empty silk tube, whereas in the case of duty cycle the animals receiving an autologous nerve graft
463 performed significantly better in comparison to both silk tube groups. A minor, statistically not
464 significant difference was found between the two silk tube groups in case of all parameters (Fig. 8A-
465 D). It should be noted that animals treated with autologous nerve grafts displayed functional
466 parameters approaching but never closely reaching the pre-training values.

467 **4.8) Electrophysiology**

468 The results of the electrophysiological analysis strongly correlate with the functional data described
469 above. Electrophysiological recordings were carried out twelve weeks after transplantation.
470 Compound nerve action potential (CNAP) and nerve conduction velocity (NCV) values were
471 significantly improved in the autologous nerve grafting group (CNAP: 22,8 ±7,5; NCV: 49,2 ±14,2)
472 compared to both silk tube groups (empty SF-NGC, CNAP: 6,5 ±3,1; NCV: 23,9 ±6,6. Collagen-filled SF-
473 NGC, CNAP: 9,7 ±4,4; NCV 25,9 ±7,3; Fig. 9). No difference could be detected between the groups
474 receiving the various silk tubes, although the animals with empty silk tubes displayed slightly
475 impaired electrophysiological data.

5.) Discussion

In this study we have investigated the mechanical properties and biocompatibility of a novel nerve guidance conduit manufactured from a braided tubular structure of silk fibroin fibers. Moreover, the ability of this novel conduit to bridge a peripheral nerve gap and support the regeneration of injured rat sciatic nerve axons has been tested.

The nature of the aggressive chemical treatment to transform a braided structure to a mechanically resistant, flexible tube, non-permeable for externally invading cells made it necessary to investigate cytotoxicity and cellular viability prior to *in vivo* implantation. The mixture of $CaCl_2/H_2O$/ethanol dissolves native silk fibers , while methanol induces the formation of β-sheets, leading to a crystalline-like structure of the silk fibroin . Formic acid functions as a solving and crystallizing agent. Um et al. reported that formic acid induces an ordered structure and molecular arrangement. As the end result, this combination treatment resulted in a homogenous crystalline-like outer layer of the nerve conduit wall. In addition, by controlling the incubation times we were able to design the structure and thickness of the outer crystalline layer. All together the treatment steps resulted in the generation of a mechanically stable tubular conduit.

Apart from the favorable mechanical properties the question remained whether this construct maintained its biocompatibility, was able to prevent invasion of connective tissue cells from the environment and provided a supportive luminal surface for proliferating Schwann cells. According to our findings these conduits fulfilled all these requirements.

Indeed, our short term in vivo studies have provided evidence that the implanted silk tube conduits were able to integrate into the host environment without generating significant inflammatory reactions and on the other hand could successfully bridge an eight millimeter long nerve defect. These features may enable this type of silk tube conduit to act as a strong candidate for nerve repair. From a practical point of view, the best available nerve conduit is an autologous

nerve graft, frequently regarded as the gold standard for experimental and clinical use of nerve grafting. There is, however, an urging need for nerve conduits in the clinical use when autologous nerve grafts are not available. These conduits should preferably fulfill a number of requirements: they should be biocompatible, long enough to bridge large defects, able to support Schwann cell proliferation followed by rapid axonal regeneration and accept external vascular ingrowth, while they resist to invasion of external cell populations especially that of fibroblast.

It is evident that a chemically inert silk tube bears several of the above mentioned features still is unlikely to guide significant number of degenerating axons over long distances. The longest distances that can still be bridged by artificial or natural conduits are frequently called “critical gap”, and they are thought to range between 2 and 6cm in humans (38-40). Gaps longer than 6cm can only be reconnected by using autologous nerve grafts or nerve allografts (40,41). The intriguing question is raised how nerve conduits should be altered in order to make them suitable for grafting in long nerve defects. The silk tube conduit presented in this study is likely to undergo a number of further modifications to suit these requirements. It could be argued that by making the silk tubes permeable for growing vessels and modifying their luminal environment in order to foster axonal regeneration the silk tube conduits would be transformed into structures with features closely resembling peripheral nerve grafts. Such conduits should carry features normally present in an intact or freshly degenerated peripheral nerve e.g. the presence of axonal growth promoting cells (like Schwann cells or Schwann cell like cells) (43-45) and the extracellular matrix compounds produced by these cells. Recently advances in experimental bridging of larger nerve defects have been made including the strategies outlined above. We suggest that the next generation of biologically inert silk tube conduits could possibly include treatment with the peripheral nerve specific extracellular matrix molecules fibronectin and laminin along with sequential transplantation of Schwann cells or Schwann cell like cells into the conduit.

These novel methodological approaches may open new horizons in the field of peripheral nerve regeneration and repair and may contribute to better treatment opportunities of large human nerve defects.

6.) Conclusion

In this study we describe the production of a novel nerve guidance conduit based on raw silk textile tubular structures. The chemical treatment of the raw silk tube resulted in a biocompatible and mechanically stable conduit which was able to bridge relatively short gaps in the rat sciatic nerve. It can be concluded that these silk tube conduits are subject to further studies and modifications in order to produce cellularised bioartificial conduits that would support long distance nerve regeneration.

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8.) Disclosure statement

No competing financial interests exist.

9.) References

1. Haftek J. Autogenous cable nerve grafting instead of end to end anastomosis in secondary nerve suture. Acta Neurochir. (Wien). **34**(1-4), 217, 1976
2. Madduri S, Papaloizos M, Gander B. Trophically and topographically functionalized silk fibroin nerve conduits for guided peripheral nerve regeneration. Biomaterials. 2009/12/17 ed. **31**(8), 2323, 2010
3. Millesi H, Meissl G, Berger A. The interfascicular nerve-grafting of the median and ulnar nerves. J Bone Jt. Surg Am. 1972/06/01 ed. **54**(4), 727, 1972
4. Millesi H. Bridging defects: autologous nerve grafts. Acta Neurochir. Suppl. **100**, 37, 2007
5. Battiston B, Geuna S, Ferrero M, Tos P. Nerve repair by means of tubulization: literature review and personal clinical experience comparing biological and synthetic conduits for sensory nerve repair. Microsurgery. 2005/06/04 ed. **25**(4), 258, 2005
6. Chalfoun CT, Wirth G a, Evans GRD. Tissue engineered nerve constructs: where do we stand? J. Cell. Mol. Med. **10**(2), 309, 2006
7. Bellamkonda R V. Peripheral nerve regeneration: an opinion on channels, scaffolds and anisotropy. Biomaterials. **27**(19), 3515, 2006
8. Chiu DT, Janecka I, Krizek TJ, Wolff M, Lovelace RE. Autogenous vein graft as a conduit for nerve regeneration. Surgery. **91**(2), 226, 1982
9. Walton RL, Brown RE, Matory WE, Borah GL, Dolph JL. Autogenous vein graft repair of digital nerve defects in the finger: a retrospective clinical study. Plast. Reconstr. Surg. **84**(6), 944, 1989
10. Itoh S, Shinomiya K, Samejima H, Ohta T, Ishizuki M, Ichinose S. Experimental study on nerve regeneration through the basement membrane tubes of the nerve, muscle, and artery. Microsurgery. **17**(10), 525, 1996
11. Geuna S, Nicolino S, Raimondo S, Gambarotta G, Battiston B, Tos P, et al. Nerve regeneration along bioengineered scaffolds. Microsurgery. 2007/06/29 ed. **27**(5), 429, 2007
12. Ichihara S, Inada Y, Nakamura T. Artificial nerve tubes and their application for repair of peripheral nerve injury: an update of current concepts. Injury. 2008/09/23 ed. **39 Suppl 4**, 29, 2008
13. Nectow AR, Marra KG, Kaplan DL. Biomaterials for the development of peripheral nerve guidance conduits. Tissue Eng. Part B. Rev. **18**(1), 40, 2012
14. Kameswaran N, Cullen DK, Pfister BJ, Ranalli NJ, Huang JH, Zager EL, et al. A novel neuroprosthetic interface with the peripheral nervous system using artificially engineered axonal tracts. Neurol Res. 2008/12/17 ed. **30**(10), 1063, 2008

15. Kingham PJ, Terenghi G. Bioengineered nerve regeneration and muscle reinnervation. J Anat. 2006/09/29 ed. **209**(4), 511, 2006

16. Yang Y, Chen X, Ding F, Zhang P, Liu J, Gu X. Biocompatibility evaluation of silk fibroin with peripheral nerve tissues and cells in vitro. Biomaterials. 2006/12/26 ed. **28**(9), 1643, 2007

17. Soffer L, Wang X, Zhang X, Kluge J, Dorfmann L, Kaplan DL, et al. Silk-based electrospun tubular scaffolds for tissue-engineered vascular grafts. J. Biomater. Sci. Polym. Ed. **19**(5), 653, 2008

18. Zhang X, Baughman CB, Kaplan DL. In vitro evaluation of electrospun silk fibroin scaffolds for vascular cell growth. Biomaterials. **29**(14), 2217, 2008

19. Marelli B, Alessandrino A, Farè S, Freddi G, Mantovani D, Tanzi MC. Compliant electrospun silk fibroin tubes for small vessel bypass grafting. Acta Biomater. Acta Materialia Inc.; **6**(10), 4019, 2010

20. Park SY, Ki CS, Park YH, Lee KG, Kang SW, Kweon HY, et al. Functional recovery guided by an electrospun silk fibroin conduit after sciatic nerve injury in rats. J. Tissue Eng. Regen. Med. 2012

21. Lovett M, Cannizzaro C, Daheron L, Messmer B, Vunjak-Novakovic G, Kaplan DL. Silk fibroin microtubes for blood vessel engineering. Biomaterials. 2007/08/31 ed. **28**(35), 5271, 2007

22. Ghaznavi AM, Kokai LE, Lovett ML, Kaplan DL, Marra KG. Silk fibroin conduits: a cellular and functional assessment of peripheral nerve repair. Ann. Plast. Surg. 2011/01/26 ed. **66**(3), 273, 2011

23. Lovett ML, Cannizzaro CM, Vunjak-Novakovic G, Kaplan DL. Gel spinning of silk tubes for tissue engineering. Biomaterials. Elsevier Ltd; **29**(35), 4650, 2008

24. Lovett M, Eng G, Kluge JA, Cannizzaro C, Vunjak-Novakovic G, Kaplan DL. Tubular silk scaffolds for small diameter vascular grafts. Organogenesis. 2011/01/12 ed. **6**(4), 217, 2010

25. Yang Y, Ding F, Wu J, Hu W, Liu W, Liu J, et al. Development and evaluation of silk fibroin-based nerve grafts used for peripheral nerve regeneration. Biomaterials. 2007/09/22 ed. **28**(36), 5526, 2007

26. Teuschl AH, van Griensven M, Redl H. Sericin Removal from Raw Bombyx mori Silk Scaffolds of High Hierarchical Order. Tissue Eng. Part C. Methods. **20**(5), 431, 2014

27. Kaewkhaw R, Scutt AM, Haycock JW. Integrated culture and purification of rat Schwann cells from freshly isolated adult tissue. Nat. Protoc. **7**(11), 1996, 2012

28. Ai H, Lvov YM, Mills DK, Jennings M, Alexander JS, Jones S a. Coating and selective deposition of nanofilm on silicone rubber for cell adhesion and growth. Cell Biochem. Biophys. **38**(2), 103, 2003

29. Rüdeberg C. A rapid method for staining thin sections of vestopal W-embedded tissue for light microscopy. Experientia. **23**(9), 792, 1967

30. Deumens R, Bozkurt A, Meek MF, Marcus M a E, Joosten E a J, Weis J, et al. Repairing injured peripheral nerves: Bridging the gap. Prog. Neurobiol. Elsevier Ltd; **92**(3), 245, 2010

31. Bozkurt a, Deumens R, Scheffel J, O'Dey DM, Weis J, Joosten E a, et al. CatWalk gait analysis in assessment of functional recovery after sciatic nerve injury. J. Neurosci. Methods. **173**(1), 91, 200

32. Bessa PC, Balmayor ER, Azevedo HS, Nürnberger S, Casal M, van Griensven M, et al. Silk fibroin microparticles as carriers for delivery of human recombinant BMPs. Physical characterization and drug release. J. Tissue Eng. Regen. Med. **4**(5), 349, 2010

33. Bessa PC, Balmayor ER, Hartinger J, Zanoni G, Dopler D, Meinl A, et al. Silk fibroin microparticles as carriers for delivery of human recombinant bone morphogenetic protein-2: in vitro and in vivo bioactivity. Tissue Eng. Part C. Methods. **16**(5), 937, 2010

34. Vepari C, Kaplan DL. Silk as a Biomaterial. Prog. Polym. Sci. **32**(8-9), 991, 2007

35. Rockwood DN, Preda RC, Yücel T, Wang X, Lovett ML, Kaplan DL. Materials fabrication from Bombyx mori silk fibroin. Nat. Protoc. **6**(10), 1612, 2011

36. Um IC, Kweon HY, Park YH, Hudson S. Structural characteristics and properties of the regenerated silk fibroin prepared from formic acid. Int. J. Biol. Macromol. **29**(2), 91, 2001

37. Um IC, Kweon HY, Lee KG, Park YH. The role of formic acid in solution stability and crystallization of silk protein polymer. Int. J. Biol. Macromol. 2003/11/11 ed. **33**(4-5), 203, 2003

38. Siemionow M, Brzezicki G. (2009) Chapter 8: Current techniques and concepts in peripheral nerve repair. Int Rev Neurobiol., 87:141-72. doi: 10.1016/S0074-7742(09)87008-6. Review. PubMed PMID: 19682637.

39. Battiston B, Raimondo S, Tos P, Gaidano V, Audisio C, Scevola A, Perroteau I, Geuna S. (2009). Chapter 11: Tissue engineering of peripheral nerves. Int Rev Neurobiol, 87:227-49

40. Tseng CY, Hu G, Ambron RT, Chiu DT. Histologic analysis of Schwann cell migration and peripheral nerve regeneration in the autogenous venous nerve conduit (AVNC). J Reconstr Microsurg. 2003 Jul;19(5):331-40.

40. Um IC, Kweon HY, Lee KG, Park YH. The role of formic acid in solution stability and crystallization of silk protein polymer. Int. J. Biol. Macromol. 2003/11/11 ed. **33**(4-5), 203, 2003

41. Sridharan R, Reilly RB, Buckley CT. Decellularized grafts with axially aligned channels for peripheral nerve regeneration. J Mech Behav Biomed Mater. 2015 Jan;41:124-35.

42. Rinker B, Vyas KS. Clinical applications of autografts, conduits, and allografts in repair of nerve defects in the hand: current guidelines. Clin Plast Surg. 2014 Jul;41(3):533-50.

43. di Summa PG, Kingham PJ, Raffoul W, Wiberg M, Terenghi G, Kalbermatten DF.Adipose-derived stem cells enhance peripheral nerve regeneration. J Plast Reconstr Aesthet Surg. 2010 Sep;63(9):1544-52.

44. Georgiou M, Golding JP, Loughlin AJ, Kingham PJ, Phillips JB. Engineered neural tissue with aligned, differentiated adipose-derived stem cells promotes peripheral nerve regeneration across a critical sized defect in rat sciatic nerve. Biomaterials. 2015 Jan;37:242-51.

639 45. Berrocal YA, Almeida VW, Gupta R, Levi AD. Transplantation of Schwann cells in a collagen tube for the repair of
640 large, segmental peripheral nerve defects in rats. J. Neurosurg. **119**(3), 720, 2013
641

642 **Figure legends**

643 **Figure legends**

644 Figure 1. Overview of the preparation method of the silk fibroin (SF) nerve guidance conduit (NGC). (A) shows a scheme of
645 the treatment steps that modify the tubular braided structure based on SF fibers and generate a NGC. Scanning electron
646 micrographs of the raw tubular structure of SF-based NGCs are shown before (B; C indicates the initial braiding design of the
647 raw unprocessed tube) and after (D-F) degumming and subsequent treatment steps (1^{st} treatment step: degumming in
648 boric acid; 2^{nd} treatment step: $CaCl_2/H_2O$/ethanol for 20 sec, >70°C; 3^{rd} treatment step: formic acid [FA] for 20 sec at room
649 temperature and a final fixation step with methanol [MeOH], for 20 min at room temperature). E: cross-sectional view of SF-
650 NGC, D) Lateral view of SF-NGC, F) enlarged view of the framed area in E). In G) the SF structure was only treated for 10
651 sec in $CaCl_2/H_2O$/ethanol and FA, resulting in a thinner outer layer.

652 Figure 2. Effects of the treatment steps on the surface of the silk fibroin (SF) fiber based nerve guidance conduits (NGC).
653 Panels show scanning electron micrographs of the degummed samples treated with the single treatments of either calcium
654 chloride, ethanol and water in a molar ratio of 1:2:8 ($CaCl_2/H_2O$/ethanol) or formic acid and the the effect of the combined
655 treatment ($CaCl_2/H_2O$/ethanol + FA), respectively. In the case of the combined treatment, SF constructs were treated with
656 $CaCl_2/H_2O$/ethanol and FA subsequently for 20 sec each, resulting in a total treatment time of 40 sec. For comparison the
657 single step treatments were carried out for 40 sec.

658 Figure 3. Results of the cytotoxicity, Schwann cell viability and compression tests of the various conduits. (A) The primary
659 Schwann cells cultured in the leach-out medium of either fully treated or degummed silk structures did not show signs of
660 cytotoxic damage. (All data are means ± SD of 8 independent experiments) (B-E) Fluorescent micrographs of Schwann cell-
661 like cells showed good adherence and viability on the inside of the silk fibroin nerve guidance conduits, where the
662 propidium iodide staining (in C) revealed no dead cells on the internal wall of the tubes. (F) Schwann cell-like cell densities
663 (cellular area occupied on the total surface) on the luminal surface of tubes treated to various extents. Note the increasing
664 attachment with the treatment steps. Asterisks indicate significant ($p<0.005$) difference between the tubes receiving
665 degumming and the tubes treated further. (G) Compression test by using a custom-made test system in order to prove the
666 improved elasticity of modified degummed tubular structures show that the $CaCl_2/H_2O$/ethanol–FA–MeOH modification is
667 most resistant to mechanical compression load. Red circles indicate the initial lumen (2 mm in diameter). Scale bars indicate
668 500 µm.

669 Figure 4. Results of the cell permeability assay using a fibrin clot containing NIH/3T3 fibroblasts and a second clot loaded
670 with PDGF-AA as chemoattractant (A) in the various experimental groups (B). Fibroblasts passed through different spacers,
671 including a cell strainer mesh of 100 µm pore size (C, positive control, panel top row) and the unprocessed tubular
672 silk structure (C, panel middle row), but were not able to penetrate the applied SF-NGC (C, panel bottom row). Columns 1,
673 2, and 3 represent the view from the initial cell containing fibrin clot, the opposite side of the used spacer and the initial

674 fibrin clot containing PDGF-AA, respectively. All samples were stained for residual or invaded cells with Calcein AM staining.
675 Scale bar is 500 µm.

676 Figure 5. Integration of the silk-fibroin conduit in the defect site. (A) Photograph of a silk-fibroin nerve guidance
677 conduit (SF-NGC) immediately after implantation between the sciatic nerve stumps. (B) Proximal side of the SF-NGC 1
678 week after implantation. Arrow indicates a thin layer of newly formed connective tissue capping the end of the SF-
679 NGC. (C) Area of peripheral nerve surgery (1 week survival), showing small blood vessels in the thin layer of
680 connective tissue around the conduit. (D) Enlarged photograph of the framed area in C. Note the fine network of blood
681 vessels. (E) At 3 weeks after implantation the lumen of the SF-NGC was completely filled with newly formed tissue.
682 (F) Dissection of the SF-NGC in E revealed a reconnection of the distal and proximal nerve stumps.

683 Figure 6. Axonal regeneration and vascular ingrowth in the various experimental groups. (A) Regenerating axons (green)
684 reach the distal coaptation site (indicating by broken lines) in the autologous nerve grafts 10 days postoperatively and the
685 nerves are also well vascularized as shown with the GSA-B4 lectin histochemistry (red). Note the lack of macrophages in
686 these grafts. (B and C) The regenerating axons extend approximately 2 mm into the silk tubes with a similar range of
687 vascularization on day 10 after surgery. No considerable number of macrophages were seen in the implanted silk tubes.
688 Broken lines indicate the proximal coaptation zone. (D) Representative examples of axon growth through various conduits
689 12 weeks after surgery. Note the robust regeneration via the autologous nerve graft, although numerous axons are able to
690 regenerate through the silk tube-based conduits, too. Axons were stained via neurofilament 200 kDa immunostaining,
691 which failed to label the regenerating axons in the distal one third of the empty silk tube (asterisk).

692 Figure 7. Numbers of myelinated axons in the various experimental groups, 12 weeks after implantation. Arrows point to
693 well myelinated axons in the distal stumps (A-D). Note the significant differences between the myelinated axon
694 numbers found in autologous nerve graft and the silk tube-based conduits (E). All data are means of 6 animals ± SD. *
695 indicates significant difference of $p<0.005$.

696 Figure. 8 Quantitative CatWalk gait analysis of locomotor functional recovery 12 weeks after implantation, including
697 print area, intensity exerted at maximum floor contact area, stance duration (time) and duty cycle of the operated right
698 hind limb relative to the unoperated left hind limb. Pretraining data show intact vales recorded one week before surgery. All
699 data are means of 6 animals ± SD. * indicates significant difference ($p<0.005$) between the autologous grafting and the
700 empty silk tube groups, whereas ** indicates significant difference between the autologous grafting and both silk tube
701 groups.

702 Fig 9 Electrophysiological analysis of the effect of axonal regeneration through the various conduits. The compound
703 nerve action potential area (CNAP) and nerve conduction velocity (NCV) values were significantly improved in cases of
704 autologous nerve grafts compared to silk conduits. All data are means of 6 animals ± SD. * indicates significant difference of
705 $p<0.005$ between the autologous grafting and both silk tube groups.

Supplementary Fig. 1 Custom-made compression testing machine. The test sample (1) is mounted on a silicone mat that fits in a petri dish via pins. A piston (2) is moved downwards via a servo motor (3) at a speed of approximately 5 mm/s in a linear manner until it touches the probe. The piston continually stresses the probe until a predefined force threshold is reached. A force sensitive resistor (4), which is integrated into the piston, works as a sensor and is part of a voltage divider. The resistance and thus the applied force is constantly sampled at 50 Hz sampling frequency using the built-in 10 bit ADC of the microcontroller (5). Once the threshold is reached, the piston is returned to the top position, where it remains for a time set by the user.