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10 Title:

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

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110 1.) Abstract

111 In the last decade silk fibroin has been increasingly used in peripheral nerve tissue engineering.
112 Current approaches that aimed to produce silk fibroin based nerve guidance conduits used either
113 aqueous solutions or organic solvents. Here we describe a novel procedure that uses the braided
114 tubular structure of raw *Bombyx mori* silk, subsequently degummed and treated with the ternary
115 solvent $\text{CaCl}_2/\text{H}_2\text{O}/\text{ethanol}$, formic acid and methanol to improve its mechanical and chemical
116 characteristics.

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

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

133 2.) Introduction

134 The incidence of peripheral nerve injury in traumatic wounds of the extremities is approximately 2-
135 5% . Moreover, tumor resection or congenital malformation may also lead to nerve damage.
136 Consequently, these incidences display a major burden on health care expenses, extensive absence
137 from work and chronic disability . Direct repair of nerves is one clinical option, however, this direct
138 end-to-end coaptation is limited to short-distance gaps. The current clinical gold standard for the
139 repair of longer nerve gaps is the use of autologous nerve grafts . The main advantage of autografts is
140 their morphologically native structure, which provides an ideal guide for axonal regeneration from
141 the proximal to the distal nerve stump. However, autografting carries several disadvantages such as
142 the limited number of donor sites for graft harvesting or the associated donor site morbidity,
143 including loss of nerve function, painful neuroma formation and hyperaesthesia . These negative
144 aspects have led to the search for alternative approaches. Beside nervous tissue, other autologous
145 materials, such as vein grafts or muscles have been used to bridge nerve gaps. However, the use of
146 these substances was both preclinically and clinically unsatisfactory. Recent advances in tissue
147 engineering (TE) have opened new opportunities in peripheral nerve repair. Artificial nerve guidance
148 conduits (NGCs), composed of synthetic or natural polymers, are currently being investigated for
149 bridging nerve defects . The rationale behind using a NGC is to entubulate the nerve stumps to
150 provide a protective micro-environment for the regenerating peripheral nerve. While numerous
151 synthetic and natural biomaterials have been evaluated, both preclinically and clinically, for the
152 bridging of nerve defects, their therapeutic benefits still appear unsatisfactory.

153 In the last years silk fibroin (SF) has attracted considerable interest as a biomaterial suitable for
154 applications in peripheral nerve regeneration. SF has been shown to possess characteristics that favor
155 its use as a NGC, such as mechanical stability, slow degradation rate, biocompatibility and its ability to
156 support nerve regeneration . Apart from biocompatibility, a NGC should act as a barrier for infiltrating
157 fibroblasts and provide mechanical resistance against compression and kinking by the surrounding
158 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 $\text{CaCl}_2/\text{H}_2\text{O}/\text{ethanol}$ 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.

169 3.) Materials and Methods

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

172 **3.1) Design and preparation of silk conduits**

173 White raw *Bombyx mori* silkworm fibers of 20/22 den, 250 T/m, were purchased from Testex AG
174 (Zürich, Switzerland). The tubular silk conduit was fabricated in cooperation with a commercial
175 braiding company (Edelrid GmbH, Isny/Allgäu, Germany). Six single silk fibers form a twisted yarn,
176 representing the raw material for the commercial braiding machines. Figure 1 shows the tubular
177 structure designed from six intertwined twisted yarns. The resulting raw silk conduit was degummed
178 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
179 conduits were boiled twice in 500 mL of degumming solution for 45 min, with an intermittent
180 exchange of the degumming solution. After degumming, scaffolds were thoroughly washed in ddH₂O
181 and air-dried before further processing (Fig. 1).

182 The degummed SF tubes were placed on an ABS (acrylonitrile butadiene styrene) rod of 2 mm in
183 diameter and dipped in a boiling solution of the ternary solvent calcium chloride/distilled
184 water/ethanol (CaCl₂/H₂O/ethanol) in a molar ratio of 1:2:8 for 20 seconds. Immediately after etching
185 the outer surface, the tubes were dipped in 100% of formic acid (FA) at room temperature for 20
186 seconds. The tubes were then fixed in methanol for 20 minutes and subsequently washed thoroughly
187 with ddH₂O (Fig. 1). The tubes were dried under laminar airflow and sterilized by autoclaving prior to
188 use.

189 **3.2.) Endurance and fatigue tests**

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

193 position, a piston is moved downwards by a servo motor (Modelcraft RS2 MG/BB standard servo,
194 Conrad Electronic SE, Hirschau, Germany) at a speed of approximately 5 mm/s linearly until it touches
195 the probe. The piston continually stresses the probe until a predefined force threshold is reached. A
196 force sensitive resistor (Strain gauge FSR 151, Interlink electronics, Camarillo, CA, USA), integrated
197 into the piston, acts as a sensor and a voltage divider. The resistance, and thus the applied force, is
198 constantly sampled at 50 Hz sampling frequency using the built-in 10 bit AD-converter of the
199 microcontroller (Arduino Duemilanove Controller Board with Atmega 328 μ C, Atmel Munich GmbH,
200 Garching/Munich, Germany). The system was calibrated using a laboratory scale and operates with a
201 ± 5 g accuracy (corresponding to 0.98 MPa). Once the threshold is reached, the piston is returned to
202 the top position, where it remains for a period of time set by the user.

203 In order to evaluate the effect of the various treatment components four types of tubes have been
204 evaluated (Fig. 2). The first type was produced by degumming of the raw tube followed by methanol
205 treatment. The second and third types were created either by $\text{CaCl}_2/\text{H}_2\text{O}$ /ethanol- or formic acid-
206 treatment, both fixed with methanol. The fourth type of tube was produced as described above,
207 combining all sequential treatments (Fig. 1).

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

215 **3.3) Scanning electron microscope analysis**

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

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

222

223 **3.4) Cell culture experiments**

224 NIH/3T3

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

229 Schwann cells (SCs)

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

236 Five adult male Sprague-Dawley rats were deeply anesthetized by inhalation of 3.5% isoflurane and
237 euthanized with 110 mg/kg BW ketamine hydrochlorid (Ketasol®; Dr. E. Graeub AG, Berne, Suisse)
238 and 12 mg/kg BW xylazine (Rompun® 2 %, Bayer AG, Vienna, Austria) intraperitoneally. The sciatic
239 nerves were dissected free of connective tissue and minced after removing the epineurium. Nerve
240 fragments were incubated with 0.05% collagenase for 1 hour at 37°C, subsequently filtered through a
241 40 µm cell strainer and centrifuged at 400g for 6 minutes. After washing the cell pellet in DMEM
242 containing 10% FCS, the pellet was resuspended in Schwann cell culture medium consisting of

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

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

254 **3.5) Cell permeability**

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

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

274 **3.6) Cytotoxicity assay**

275 To test cytotoxicity of the prepared SF-NGC, 1 g of dissected material of SF-NGC was immersed in 5
276 mL cell culture medium for at least 24 h. In parallel, 0.2×10^5 Schwann cells per well were seeded onto
277 24-well plates. Then the medium containing leach-out products from the dissected material was
278 filtered (0.22 µm, Rotilabo, Karlsruhe, Germany) and used to change media in the cell cultures.
279 Standard culture medium was used as a negative control. After 72 h, cell culture medium was
280 aspirated and the respective cell culture medium containing 650 mg/mL MTT [3-(4,5-dimethylthiazol-
281 2-yl)-2,5-diphenyltetrazolium] bromide was added to each well. After 1 h of incubation at 37° C and in
282 5% CO₂, medium was aspirated and MTT formazan precipitate was dissolved in DMSO by shaking
283 mechanically in the dark for 20 min. Aliquots of 100 µl of each sample were transferred to 96-well
284 plates. The absorbance at 540 nm was read immediately on an automatic microplate reader (Spectra
285 Thermo, TECAN Austria GmbH, Austria). Optical density (OD) values were corrected for unspecific
286 background.

287 **3.7) Animals and surgery**

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

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

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

316 **3.8) Tissue sampling, perfusion and immunohistochemistry**

317 Twelve weeks after surgery the animals were deeply anesthetized by inhalation of 3.5% isoflurane
318 and euthanized with 110 mg/kg BW ketamine hydrochlorid (Ketasol®; Dr. E. Graeb AG, Berne,
319 Suisse) and 12 mg/kg BW xylazine (Rompun® 2 %, Bayer AG, Vienna, Austria) intraperitoneally.
320 Animals were perfused transcardially with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH
321 7.4). The autologous transplants or the implanted SF-NGC were harvested under the operating
322 microscope along with the proximal and distal nerve stumps.

323 The nerve grafts or conduits (n=6 per group), removed from perfusion-fixed animals, were
324 immersion-fixed for 6 h in 4 % paraformaldehyde and then cryoprotected in 30% sucrose in PBS.
325 Parallel cryostat sections were cut on a Leica 1850 cryostat (Leica Microsystems, Vienna, Austria) and
326 sections were either stained with cresyl violet or processed for 200 kD neurofilament
327 immunohistochemistry. Sections were treated with a 1% milk powder solution and then incubated
328 with an anti-200 kD rabbit neurofilament antibody (Abcam Ltd, UK, Lot. No.: ab8135, rabbit, 1:1000)
329 or with biotinylated Griffonia (Bandeira) simplicifolia lectin B₄ (GSA-B₄, 1:200, Vector Labs,
330 Burlingame, CA, USA) overnight at 4°C. The sections were then processed for incubation with an anti-
331 rabbit Alexa 546 or secondary antibody (1:400) or with streptavidin Alexa 488 (to visualize 200 kD
332 neurofilament or Griffonia simplicifolia isolectin, respectively) for 2 h at room temperature, protected
333 with a coverslip and investigated under an epifluorescence microscope (Olympus FX-50, Olympus Ltd,
334 Tokyo, Japan).

335 The sciatic nerve segment distal to the graft was transferred into a 2.5% phosphate-buffered
336 glutaraldehyde solution after perfusion and immersion fixed for 24 h. Remnants of fixative were
337 carefully washed out from the nerve, and the tissue was treated in 1% OsO₄ in PBS (Agar Scientific,
338 Stansted, UK) for 1 h, dehydrated in a graded ethanol series and propylene oxide and then embedded
339 in Durcupan (Fluka, Switzerland). Semithin sections (0.4 µm thick) were cut 2 mm distal to the graft
340 on a Leica Ultracut-R ultramicrotome and stained according to R deberg .

341 **3.9) Quantification of the Schwann cell-like cell densities**

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

350 **3.10) Semiautomated gait analysis (CatWalk™)**

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

359 **3.11) Electrophysiology**

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

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

368 **3.12) Statistical analysis**

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

381

382 4.) Results

383 4.1) Structural changes during processing of silk tubes

384 Figure 1 A-C shows the raw SF-NGC consisting of braided single silk fibers. After degumming, the silk
385 tube was treated with $\text{CaCl}_2/\text{H}_2\text{O}/\text{ethanol}$ and subsequently with FA for 20 seconds each, followed by
386 fixation with methanol. This treatment results in a fusion of the outer single silk fibers to a closed
387 layer with a varying thickness ranging from 40 to 75 μm (Fig. 1D-F). Treatment with
388 $\text{CaCl}_2/\text{H}_2\text{O}/\text{ethanol}$ shorter than 20seconds results in thinner outer layer (Fig. 1G). In contrast, the
389 luminal wall of the tube which was not treated with various solvents, preserves its original braided
390 structure (Fig. 1F). Figure 2 shows the time-dependent effects of $\text{CaCl}_2/\text{H}_2\text{O}/\text{ethanol}$ and FA on the SF
391 fibers. SF fibers solely treated with $\text{CaCl}_2/\text{H}_2\text{O}/\text{ethanol}$ dissolve and precipitate, especially after 40
392 seconds. In contrast, FA-treatment alone disorganizes the original braided structure in a time-
393 dependent manner.

394 4.2) Cytotoxicity and viability assays

395 To investigate whether cytotoxic residuals were left in the SF-NGCs during the preparation procedure
396 a MTT assay was performed. Dissected pieces of the SF-NGCs and the unprocessed raw silk scaffold
397 were incubated in cell culture media, to remove cytotoxic molecules from the constructs. Treatment
398 of cultured Schwann cells with these wash-out media resulted in no significant difference in the cell
399 viability of Schwann cells in any treatment group (Fig. 3A). Next we tested the viability and adhesion
400 pattern of Schwann cells cultured on the luminal surface of the silk tubes processed to various
401 extents during the SF-NGC preparation procedure. Treatment with $\text{CaCl}_2/\text{H}_2\text{O}/\text{ethanol}$ and the full
402 treatment procedure including incubation with FA induced significantly more Schwann cells to adhere
403 to the luminal surface of the silk tubes than degumming only (Fig. 3B-F). On the other hand,
404 propidium iodide staining did not reveal any apoptotic Schwann cells on the luminal surface of these
405 cultured silk tubes (Fig. 3C).

406 4.3) Endurance test

407 Implanted silk tubes have to resist to external pressure originating from the surrounding tissues and
408 organs during movements. To verify whether ready-to-use SF-NGCs are able to withstand external
409 forces, mechanical compression tests were performed with a custom-made system (Fig. S1).
410 Degummed tubes were seriously challenged during the compression test, they remained compressed
411 and flat after 1000 cycles of compression (Fig 3G). FA or CaCl₂/H₂O/ethanol alone improved the
412 elastic properties of the SF-NGCs resulting in a moderate preservation of the lumen, whereas full
413 treatment (degumming, CaCl₂/H₂O/ethanol followed by FA treatment) helped improving the elastic
414 properties of these SF-NGCs to become resistant to external forces (Fig. 3G).

415 **4.4) Cell permeability**

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

423 **4.5) Short term in vivo studies - general observations**

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

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

436 **4.6) Axonal regeneration**

437 Our results showed that a short gap of 8 mm in the rat sciatic nerve could be bridged by implanting
438 an SF-NGC in the gap. To compare the axon growth promoting capacity of the three conduits used in
439 the study we looked at the axon outgrowth from the proximal nerve stump into the conduits ten days
440 after grafting by using neurofilament staining. Autologous nerve grafts were already populated with
441 regenerated axons along their whole length at this time-point and the axons approached the distal
442 coaptation site (Fig. 6A). In contrast, the silk tubes were able to promote only limited outgrowth of
443 the axons at this stage. The regenerating axons have grown to a distance of approximately 2 mm in
444 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
445 group) without considerable difference between them (Fig. 6B, C). The autologous nerve grafts 10
446 days after postoperatively are well vascularized (Fig. 6A). A similar range of vascularization of the silk
447 tubes could be observed on day 10 after surgery. No considerable number of macrophages were
448 seen in the implanted silk tubes (Fig. 6B,C).

449 Three months after transplantation the course of regenerated axons throughout the lumen of the
450 implanted SF-NGCs and autologous nerve grafts was clearly visible (Fig. 6D-F). Although the axon
451 bundle was present in the empty silk tubes the immunohistochemical analysis did not reveal a
452 significant staining pattern for neurofilament 200kD in the distal portion of the tubes. On the other
453 hand, myelinated axon counts showed significantly less myelinated axons in the empty SF-NGC as
454 well as the collagen-filled SF-NGC compared to the autologous nerve graft (empty SF-NGC: 1274
455 ± 146 , collagen-filled SF-NGC: 1678 ± 303 , autologous nerve graft: 6252 ± 474 ; Fig. 7). Accordingly,
456 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: $22,8 \pm 7,5$; NCV: $49,2 \pm 14,2$)
472 compared to both silk tube groups (empty SF-NGC, CNAP: $6,5 \pm 3,1$; NCV: $23,9 \pm 6,6$. Collagen-filled SF-
473 NGC, CNAP: $9,7 \pm 4,4$; NCV $25,9 \pm 7,3$; 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.

476 5.) Discussion

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

481 The nature of the aggressive chemical treatment to transform a braided structure to a
482 mechanically resistant, flexible tube, non-permeable for externally invading cells made it
483 necessary to investigate cytotoxicity and cellular viability prior to *in vivo* implantation. The
484 mixture of CaCl₂/H₂O/ethanol dissolves native silk fibers , while methanol induces the formation
485 of β-sheets, leading to a crystalline-like structure of the silk fibroin . Formic acid functions as a
486 solvating and crystallizing agent. Um et al. reported that formic acid induces an ordered structure
487 and molecular arrangement. As the end result, this combination treatment resulted in a
488 homogenous crystalline-like outer layer of the nerve conduit wall. In addition, by controlling the
489 incubation times we were able to design the structure and thickness of the outer crystalline layer.
490 All together the treatment steps resulted in the generation of a mechanically stable tubular
491 conduit.

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

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

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

507 It is evident that a chemically inert silk tube bears several of the above mentioned features still is
508 unlikely to guide significant number of degenerating axons over long distances. The longest
509 distances that can still be bridged by artificial or natural conduits are frequently called “critical
510 gap”, and they are thought to range between 2 and 6cm in humans (38-40). Gaps longer than
511 6cm can only be reconnected by using autologous nerve grafts or nerve allografts (40,41). The
512 intriguing question is raised how nerve conduits should be altered in order to make them suitable
513 for grafting in long nerve defects. The silk tube conduit presented in this study is likely to undergo
514 a number of further modifications to suit these requirements. It could be argued that by making
515 the silk tubes permeable for growing vessels and modifying their luminal environment in order to
516 foster axonal regeneration the silk tube conduits would be transformed into structures with
517 features closely resembling peripheral nerve grafts. Such conduits should carry features normally
518 present in an intact or freshly degenerated peripheral nerve e.g. the presence of axonal growth
519 promoting cells (like Schwann cells or Schwann cell like cells) (43-45) and the extracellular matrix
520 compounds produced by these cells. Recently advances in experimental bridging of larger nerve
521 defects have been made including the strategies outlined above. We suggest that the next
522 generation of biologically inert silk tube conduits could possibly include treatment with the
523 peripheral nerve specific extracellular matrix molecules fibronectin and laminin along with
524 sequential transplantation of Schwann cells or Schwann cell like cells into the conduit.

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

528

529 6.) Conclusion

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

536

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543 8.) Disclosure statement

544 No competing financial interests exist.

545 9.) References

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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 (1st treatment step: degumming in
648 boric acid; 2nd treatment step: CaCl₂/H₂O/ethanol for 20 sec, >70°C; 3rd 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 CaCl₂/H₂O/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 (CaCl₂/H₂O/ethanol) or formic acid and the effect of the combined
655 treatment (CaCl₂/H₂O/ethanol + FA), respectively. In the case of the combined treatment, SF constructs were treated with
656 CaCl₂/H₂O/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 CaCl₂/H₂O/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 μ m.

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 \pm 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 values recorded one week before surgery. All
699 data are means of 6 animals \pm 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 \pm SD. * indicates significant difference of
705 $p < 0.005$ between the autologous grafting and both silk tube groups.

706 Supplementary Fig. 1 Custom-made compression testing machine. The test sample (1) is mounted on a silicone mat that fits
707 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
708 manner until it touches the probe. The piston continually stresses the probe until a predefined force threshold is reached. A
709 force sensitive resistor (4), which is integrated into the piston, works as a sensor and is part of a voltage divider. The
710 resistance and thus the applied force is constantly sampled at 50 Hz sampling frequency using the built-in 10 bit ADC
711 of the microcontroller (5). Once the threshold is reached, the piston is returned to the top position, where it remains for a
712 time set by the user.