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Correlating the secondary protein structure of natural spider silk with its guiding properties for Schwann cells

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1. Introduction

Nerve damage from severe trauma cannot regenerate spontaneously and may result in a functional deficit of the injured extremity, entailing a considerable reduction of the patient’s quality of life [1]. However, the regeneration of minor injuries to the peripheral nervous system is inherently possible [2,3]. Schwann cells (SCs) have been identified as a crucial part of the regeneration process by helping to remove the degenerated axonal and myelin debris and also by forming a guiding structure (bands of Büngner) and enabling the parallel migration of regenerating axons [4–6].

The treatment of peripheral nerve injuries, where a straight transplantation is given and tension-free suturing is possible, involves the direct coaptation of the nerve endings [7]. In contrast, the repair of long-distance defects with a significant amount of lost nerve tissue still represents a major clinical challenge [7–9]. The current gold-standard procedure for nerve reconstruction above this length is an autologous nerve transplantation [1,10,11]. This method leads to donor site morbidity including sensory loss and possible neuroma formation, which may cause chronic neuropathic pain. Morphological mismatches, limited availability of donor nerves, and scar formation are also amongst the possible drawbacks of this method [8,12,13].

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ABSTRACT

The successful reconstruction of supercritical peripheral nerve injuries remains a major challenge in modern medicine. Progress in tissue engineering has enabled the development of nerve guidance conduits as an alternative to autologous nerve transplantation and the enrichment of conduits with fibrous materials or hydrogels has shown great potential in bridging nerve defects. The application of the dragline silk of spider genus Nephila as a filament for nerve guidance conduits has led to promising results. However, the use of spider silk has been phenomenological so far and the reasons for its success are still not identified. This renders a targeted tuning of synthetic fibrous luminal fillings such as recombinant silk out of reach.

In this work the existing research was extended and in addition to dragline, the cocoon silk of Nephila edulis, as well as the connecting and attaching silk of Avicularia avicularia were investigated. Scanning electron microscopy revealed a difference in size and morphology of the spider silks. However, in vitro experiments indicated that Schwann cells adhere to the four fibers, independent of these two attributes. Raman spectroscopy in native state and aqueous environment demonstrated similar secondary protein structures for dragline, cocoon, and connecting silk. In contrast, the attaching silk showed a significant lower conformation of β-sheets, crucial for the stiffness of the silk. This was in line with the in vitro experiments, where the flexible attaching silk fibers adhered to each other when placed in liquid. This resulted in their inability to guide Schwann cells, leading to the generation of cell agglomerations. This direct comparison demonstrated the crucial role of β-sheets confirmation for the guidance properties of natural spider silk, providing essential insights into the necessary material properties for the integration of fibrous luminal fillings in nerve guidance conduits.
Nerve guidance conduits (NGCs), hollow tubes used to bridge nerve defects, are a promising alternative to autologous nerve transplantation. These conduits can be made out of natural or synthetic materials [14–18]. A list of clinically approved sources for NGCs can be found in Refs [19, 20]. The performance of commercially available NGCs in bridging long nerve defects is not yet as effective as autologous nerve grafts [1,21,22]. This could be due to their lack of cellular support, neurotrophic factors, and endoneurial microstructures [14]. Indeed, conduits filled with materials such as fibers and hydrogels have been proven as superior in comparison to empty ones [21,23]. Overall, the necessity of a biodegradable and non-toxic material that does not induce an immunological response limits the options for a successful conduit implantation.

Recently, the use of natural spider silk (SPSI) for supporting nerve regeneration was investigated [1,19,24–29]. Spiders produce various types of silks, each suitable for a specific task such as catching prey and protecting their eggs [30]. The dragline silk from the major ampullate gland of adult female Nephila clavipes spiders was used to develop a NGC consisting of silk fibers aligned within a decellularized vein [31,32]. A 2.0 cm sciatic nerve defect in a rat model [31] and a critical size defect of 6.0 cm in the tibial nerve of sheep [24] could be successfully bridged without adverse effects.

SPSI has unique material properties that could be the reasons for its acceptance by SCs and its success in supporting nerve regeneration. It was in fact demonstrated on gel substrates, that SCs are highly sensitive to material characteristics such as stiffness and their morphology and motility can vary greatly based on this property [33,34].

The diameter of SPSI varies between species and between various silk types and is dependent on the health of the spider [35]. Scanning electron microscopy and atomic force microscopy have been used to investigate the size and the morphology of SPSI [36,37].

The secondary protein structure of SPSI can be studied with Raman spectroscopy [38–43]. The amide I region is used to quantify the contribution of various conformations such as unordered structures, helices, β-sheets, and also β-turns [38–40,42–46]. The properties of silk are highly dependent on its secondary protein structure and the stiffness of dragline silk in comparison to the more flexible silks such as flagelliform was related to the higher β-sheets content of dragline fibers [35,42,47]. Studies with Raman spectroscopy have revealed that the secondary protein structure of dragline silk of genus Nephila possesses a directionality, i.e. it is not symmetric in the long and the short axes of the fiber [40–42,48,49]. When exposed to liquids or high relative humidity, the dragline of genus Nephila showed supercontraction and shrank in the long axis [50]. The alteration of material properties after the exposure of dragline fibers to various liquids has also been investigated with Raman spectroscopy [39,48,49].

Although SPSI has peerless properties, it is still a natural material with limited availability. In an attempt to mimic the outstanding characteristics of natural SPSI with a synthetic material, recombinant silk has been the topic of intensive research in recent years [51–53]. In addition to higher obtainability, the possibility of tailoring the material properties is another advantage of recombinant silk over the natural fibers. The use of recombinant silk has provided a wide spectrum of applications including targeted drug delivery, tissue engineering, and implant coatings [54]. Recently, recombinant silk has also been used in regenerative medicine [55–58].

The choice of silk proteins, as well as the processing techniques and parameters affects the secondary protein structures and by that the characteristics of recombinant silk [59–61]. Computational methods can be employed to predict the correlation between the silk's sequence and its structure properties [62–64]. It was in fact demonstrated with Molecular Dynamics calculations that the length of poly-Alanine strands greatly affects the presence and the crystallinity of β-sheets [65]. In turn, the size of the crystals determines the fiber's mechanical properties as shown with Molecular Dynamics and Finite Element simulations [63]. The characterization of natural SPSI together with simulations and genetic synthesis of recombinant silk will enable a much brighter future for replacing the native fibers. With this iterative process, the necessary properties for a successful biomedical implementation are clarified and used as a target for the fabrication of synthetic materials by the correct choice of protein sequences and methods of production.

The spider genus Nephila has been in focus for both nerve regeneration and material characterization investigations. Much less attention has been paid to other spider species. Moreover, a systematic investigation relating the specific characteristics of SPSI to its guiding potential for cells is missing. In order to find a correlation between the material properties and the success of various SPSIs in NGCs, in addition to the well-studied dragline, the cocoon silk from Nephila edulis (N. edulis) as well as the connecting and the attaching silk from Avicularia avicularia (A. avicularia) were investigated. The silks were seeded with rat Schwann cells (rSCs) and cultured. In addition, the morphology and the secondary protein structures of the silks were studied with scanning electron microscopy and Raman spectroscopy, respectively.

2. Materials and methods

2.1. Spider silk harvesting

Spiders N. edulis and A. avicularia were kept in terraria with conditions optimum for each species. The terraria were sprayed with water regularly and the spiders were fed crickets (Acheta domestica). The two silk fibers from N. edulis used in this study are known as dragline (major ampullate silk) and cocoon (tubuliform, eggcase) [30], while the silk fibers from A. avicularia are recognized as connecting and attaching silk (also known as attachment silk) [66]. For harvesting the dragline silk of N. edulis and the connecting and attaching silk from A. avicularia, the spider was fixated under a gauze similar to procedures in Ref. [26]. The silk was directly reeled on 3D-printed silk frames (polyethylene), with outer dimensions of 21.3 and 23.5 mm and a height of 1.5 mm, fitting a 2-well chamber slide (ibidi). Cocoon silk from N. edulis was taken directly from the terraria and single strands were removed from the cocoon and fixed on the silk frame. All silks were taken with 15 full rotations on each axis of the frame and kept in closed containers at constant room temperatures and away from sunlight.

2.2. Scanning electron microscopy

The morphology and the size of SPSI were observed with scanning electron microscopy (Zeiss Supra 55). A 10 nm thin gold layer was coated on the SPSI with a Leica SCD050 sputter coater equipped with a quartz micro balance for monitoring the layer thickness. The micrographs were obtained by employing a secondary electron detector. For determining the diameters, five points were evaluated on two fibers from each silk type, leading to the average diameter from ten measurements.

2.3. Raman spectroscopy

Raman measurements were performed on single silk fibers in ambient conditions with a WITec alpha 300A micro-Raman device and a 50×/0.55 objective. A frequency doubled Nd:YAG laser at a
wavelength of 532 nm was used. The output powers of 5–10 mW for the native state and 15–20 mW for the measurements in the aqueous environment were employed. The resolution of the spectrometer was 2 cm⁻¹. The spectra were collected in a backscattering geometry. No degradation of the silk was observed under the chosen conditions.

For the experiments on the silk from N. edulis, silk from three spiders, and for the experiments on A. avicularia, silk from two spiders were harvested to avoid artefacts that may be related to an individual animal. In order to account for the anisotropy of the secondary protein structure of the silk, Raman measurements were performed in two orthogonal directions: the polarization of the incoming light was oriented perpendicular (⊥) or parallel (∥) to the long axis of the silk. At least 5 spectra were taken per spider, leading to a minimum of 15 spectra for each orientation and silk type for N. edulis and 10 for A. avicularia.

The spectra were corrected with a polynomial baseline function to account for the fluorescence background. For the quantitative evaluation of the secondary protein structures, the conformation sensitive amide I regions and the side chains (1550–1750 cm⁻¹) were analyzed [42, 46, 67]. The spectral decomposition was performed by fitting the spectra with Lorentzian distributions at constrained widths and resonance frequency parameters. The latter were centered at 1639 cm⁻¹ for unordered structures, 1655 cm⁻¹ for helices, 1669 cm⁻¹ for β-sheets, and 1684 and 1698 cm⁻¹ for β-turns [40, 42, 46, 69]. The peak at 1655 cm⁻¹ could be attributed to either α-helix or β-parallel (∥) to the long axis of the silk. At least 5 spectra were taken per spider, leading to a minimum of 15 spectra for each orientation and silk type for N. edulis and 10 for A. avicularia.

The normality of data’s distribution was verified by means of quantile-quantile plots and an unequal variance t-test was employed for the statistical evaluation [70–72].

2.4. Isolation and seeding of rat Schwann cells

Male and female adult Sprague Dawley rats (8–12 weeks of age) were used in this study. The animals were euthanized according to the Austrian’s Animal Testing Law (TVG 2012, §2, 1.c) and the Article 3 of the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes [73].

rSCs were isolated and cultured as described previously [74]. Briefly, sciatic nerves were excised, and the fascicles were pulled out of the epineurium. The collected isolated nerve fascicles were digested overnight in Minimum Essential Medium α (GIBCO) supplemented with 10% fetal calf serum (FCS, LINARIS), 1% Penicillin-Streptomycin (P/S, GIBCO), 1% Sodium Pyruvate Solution (GIBCO), 2.5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (HEPES, SIGMA), 0.125% (w/v) collagenase type IV (GIBCO), 1.25 U/ml Dispase II (Sigma-Aldrich), and 3 mM calcium chloride (Merck) at 37 °C and 5% CO₂. The derived cell suspension was seeded on culture dishes coated with 0.01% poly-l-lysine hydrobromide (PLL, SIGMA) and 4.8 μg/ml laminin (SIGMA). For culturing of rSCs, Minimum Essential Medium α was supplemented with 5% FCS, 1% P/S, 1% Sodium Pyruvate Solution, 2.5% HEPES, 10 ng/ml recombinant Heregulinβ-1 (PeproTech), 0.5% N-2 Supplement (GIBCO), 2 μM forskolin in Dimethyl sulfoxide (SIGMA), 10 ng/ml recombinant FGF basic (PeproTech), and 5 ng/ml PDGF-AA (PeproTech). Separation of rSCs from fibroblasts to a purity of over 95% was achieved during passaging using a two-step enrichment procedure [74,75]. rSCs cultures in passages 2–5 (p2-p5) from six individual donors were used. For the process of seeding, a drop with a volume of 12.5 μl containing 10⁵ rSCs was placed on the suspended SPSI on the frame and kept at 37 °C and 5% CO₂ for 1 h. Afterwards, the silk and the silk frame were submerged in growth medium. Phase contrast imaging was performed with 10×/0.25 or 20×/0.40 objectives of a NIKON Eclipse Ts2R optical microscope.

2.5. Immunofluorescence staining and confocal microscopy

After 14 days of cultivation, rSCs were washed with 1× Dulbecco’s phosphate-buffered saline (1× PBS) and fixed with 4.5% formaldehyde solution (SAV Liquid Production GmbH) for 20 min. For immunofluorescence staining, firstly the cells were permeabilized and blocked with 1× PBS containing 1% bovine serum albumin (BSA, SIGMA), 0.3% TritonX-100 (TX, SIGMA), and 3% goat serum (GS, DAKO) for 20 min. The primary antibody (S100, rabbit, DAKO, 1:200) was used in 1× PBS together with 1% BSA, 0.1% TX, and 1% GS overnight at 4 °C. After washing with 1× PBS, cells were incubated with the secondary antibody (AP488Plus, goat anti-rabbit, Invitrogen, 1:800) in 1× PBS with 1% BSA, 0.1% TX, and 1% GS for 1 h at room temperature. After washing, 50 μg/ml 4′,6-Diamidino-2-Phenylindole solution (DAPI, ThermoScientific) in 1× PBS was employed for 10 min for nuclei staining. Afterwards FluoromountG mounting medium (Invitrogen) was added and the stained rSCs cultures were kept at 4 °C. Images were obtained with a Leica SP8 confocal microscope, using a 20×/0.75 multi-immersion objective.

3. Results and discussion

3.1. Scanning electron microscopy

Scanning electron micrographs of dragline and cocoon silk of N. edulis as well as the connecting and attaching silk of A. avicularia are shown in Fig. 1. The morphology of the silk varies greatly between the two species. The dragline (Fig. 1a) and cocoon (Fig. 1b) from N. edulis show a relatively smooth and featureless morphology, with average diameters of (3.2 ± 0.2) μm for dragline and (4.6 ± 0.3) μm for cocoon.
In contrast, the connecting and the attaching silks of *A. avicularia*, show much larger variations between the two silk types. The connecting silk (Fig. 1c) consists of multiple fibers in the form of a bundle, which has been reported before [66], with an overall diameter of \((6.7 \pm 0.4) \, \mu\text{m}\). Each of the fibers in the bundle has a diameter below 1 \, \mu\text{m}. The attaching silk (Fig. 1d), on the other hand, is a single fiber with average diameter of \((477 \pm 60) \, \text{nm}\), which is an order of magnitude smaller than the other silks.

### 3.2. rSCs adhesion and behavior on spider silk

In order to investigate the adhesion and behavior of rSCs on SPSI, the four silk fibers were seeded with rSCs. Phase contrast optical micrographs (Fig. 2a, b) depict the morphology of rSCs in PLL/laminin coated dishes. The typical spindle-shaped morphology and the parallel alignment of rSCs are visible. Fig. 2c–f shows the adhesion and the morphology of rSCs 1 h after seeding onto the SPSI and submerging in growth medium. It is clear that there is a prominent adhesion between the cells and SPSI. Colonies of rSCs have adhered to the silk fibers. Although the attaching silk (Fig. 2f) has a much smaller diameter in comparison to dragline, cocoon, and connecting silk fibers (Fig. 2c–e, respectively), rSCs still adhered to it. No obvious morphological differences were observed between the cells on the four fibers.

For comparing the guiding properties of SPSIs, rSCs on silks were cultivated for 14 days. Afterwards, they were analyzed with immunofluorescence staining and confocal microscopy and compared to rSCs cultivated on PLL/laminin coated dishes (Fig. 3a, b). In all cases, the positive staining for SC marker S100 demonstrated the presence of rSCs. On the dragline, cocoon, and connecting silk (Fig. 3c–e, respectively) rSCs were found well distributed and arranged alongside the fibers. On the other hand, the attaching silk fibers have adhered together, and the single silk strands were barely perceivable (Fig. 3f). This has caused large amount of rSCs to form aggregates, which is in contrast to the distributed rSCs in PLL/laminin cultures and on the dragline, cocoon, and connecting silks. In the inset of Fig. 3f, the arrow indicates the presence of a single attaching silk, which is coupled to the rest of the fibers, fixed together.

The rSCs could take further actions such as myelination and wrapping around the SPSI. In fact, previous investigations demonstrated the ability of primary rSCs, but not the human SCs to myelinate axons when co-cultured with neurons [76,77]. However, other studies with human SCs seeded on a carbon fiber demonstrated the attachment and wrapping of the cells, with an expression of myelin basic protein as a fundamental constituent of wrapping [78]. In addition, transmission electron microscopy may be used to visualize the morphology of myelination [79]. Further investigations with rSCs on SPSI fibers could verify the presence or the lack of myelination.

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**Fig. 2.** rSCs cultures and adhesion of rSCs on spider silk. Phase contrast optical micrographs showing p3 rSCs after 3 days of cultivations in a) and b) PLL/laminin coated dishes, and rSCs 1 h after seeding on spider silk c) dragline, d) cocoon, e) connecting, and f) attaching silk.
3.3. Raman spectroscopy

In order to identify the secondary protein structures of SPSI and explain the difference in the response of the four silk fibers during the *in vitro* experiments, Raman spectroscopy was performed in native state and in aqueous environment for \( \perp \) and \( \parallel \) orientations. Average spectra for dragline and cocoon silk of *N. edulis*, as well as the attaching and connecting silk of *A. avicularia* in their native state are shown in Fig. 4a for \( \perp \) orientation. The small peaks at 643, 827, and 853 cm\(^{-1}\) and the peak around 1615 cm\(^{-1}\) are an indication of Tyr side chains, while the prominent peak at 905 cm\(^{-1}\) can be attributed to poly-Ala [38,43]. The peak at 1389 cm\(^{-1}\), which is present for the cocoon silk fibers might be related to contamination from the terrarium or the spider eggs.

Further qualitative analysis of the spectra shows that the amide I (1600–1700 cm\(^{-1}\)) and amide III (1200–1300 cm\(^{-1}\)) regions as well as the peak around 1092 cm\(^{-1}\) (characteristic for \( \beta \)-sheet conformation [38]) are similar in the dragline, cocoon, and connecting silk. The attaching silk on the other hand shows variations in all these regions. This contrast is more visible in Fig. 4b and c, where the superimposed amide I region of the four silk fibers in their natural state is depicted for \( \perp \) and \( \parallel \) orientations, respectively. The connecting silk of *A. avicularia* has a similar conformation as the dragline and cocoon silk fibers of *N. edulis*, whereas the attaching silk differs prominently with respect to the secondary protein structures. The spectral shape also varies between the \( \perp \) and \( \parallel \) orientations. The narrow bandwidth and the position of the peaks in the dragline, cocoon, and connecting silk, especially in the \( \perp \) orientation is a strong indication of the significant contribution of \( \beta \)-sheets [39,80].

Fig. 3. Behavior of rSCs on PLL/laminin and spider silk. Immunostaining images for rSCs on a) and b) PLL/laminin coated dishes as well as on c) dragline, d) cocoon, e) connecting, f) attaching silk with merged channels for S100 in green and DAPI in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
The Raman shift of the amide I region of SPSI in aqueous environment is represented in Fig. 4d and e. Small changes in the spectral shape of this region are visible. Similar results have been observed on dragline silk [39,48].

For a quantitative evaluation of the secondary protein structure, the spectra of the amide I region were decomposed as described in the Materials and methods section. Every spectrum was fitted separately. Examples are depicted in Fig. 5, where the single spectra of the native connecting silk of A. avicularia for \(\perp\) (Fig. 5a) and \(\parallel\) (Fig. 5b) orientations together with the spectral decomposition are shown. The fitted line agrees well with the experimental data points.

After the evaluation of each spectrum, the results of the decomposition were averaged and weighted with their confidence by Gaussian error propagation. The outcome of the fits is shown in Fig. 6 where the error bars are the variations between the fit of the spectra, based on the uncertainty of the measurement. The \(\beta\)-turns are added together and shown as one column as previously suggested in Ref. [40].

The different shapes of the amide I region between the \(\perp\) and \(\parallel\) spectra are reflected in the results of the fit (Fig. 6 top and bottom rows, respectively). The \(\beta\)-sheet content is more perceivable in the \(\perp\) orientation, suggesting that they are highly oriented parallel to the long axes of the fiber for dragline, cocoon, and connecting silk as the C=O groups of the \(\beta\)-sheets are mainly oriented perpendicular to the fiber axis [42]. The helices are also moderately oriented along the fiber axes and are more prominent in the \(\parallel\) orientation. These findings are in accordance with previous studies on dragline silk fibers [40–42]. In contrast, the attaching silk shows smaller variations between the spectra in the two orientations, indicating less directionality in respect to the secondary structures. A comparison between the four fibers also indicates a significantly higher \(\beta\)-sheets content for dragline, cocoon, and the connecting silk in comparison to the attaching silk from the fitted results (Supplementary material, Figs. S1–S3). The attaching silk also contains higher contents of helices.

The gray columns in Fig. 6 represent the conformation of the silks after exposure to water. The fibers become significantly more unordered, as this component of the secondary protein structures is increased. This is also the only conformation change that is consistent in all four fibers. Minimal changes could be detected for the helices, \(\beta\)-sheets, and \(\beta\)-turns between the native and aqueous state as indicated in Fig. 6.

Based on the in vitro experiments (Section 3.2), qualitative differences between the response of the silks were observed: while the
dragline, cocoon, and connecting silks were successful in guiding rSCs, the attaching fibers adhered together under liquid. Quantitative evaluation of the protein structures and a comparison between each of the dragline, cocoon, and connecting fibers with the attaching silk determined significant variations in the protein conformation (Supplementary data, Figs. S1–S3). The attaching silk becomes largely unordered in comparison to the other SPSIs when exposed to water. In addition, in both the native state and aqueous environment and in the ⊥ and ∥ orientations, the attaching silk shows a significant smaller β-sheets conformation. The lower content of β-sheets could give rise to the flexibility, lack of stiffness, and a high potential for elongation of attaching fibers [35,42,47]. This could cause them to move in culture, leading to the generation of cell agglomerations. Hence, the high content of β-sheets was found to be essential for natural SPSI fibers to guide rSCs and should be considered for the targeted fabrication of fibrous luminal fillings for NGCs.

CRediT authorship contribution statement

Aida Naghilou: Conceptualization, Methodology, Investigation, Resources, Software, Visualization, Formal analysis, Writing - original draft, Writing - review & editing, Project administration. Lena Pöttschacher: Methodology, Investigation, Visualization, Writing - review & editing. Flavia Millesi: Methodology, Investigation, Resources, Visualization, Writing - review & editing. Anda Mann: Methodology, Investigation, Writing - review & editing. Paul Supper: Resources, Writing - review & editing. Lorenz Semmler: Resources, Writing - review & editing. Tamara Weiss: Writing - review & editing. Ellen H.G. Backus: Conceptualization, Writing - review & editing, Supervision, Funding acquisition. Christine Radtke: Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2020.111219.

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