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DOI
10.1016/j.msec.2020.111219

Publication date
2020

Document Version
Final published version

Published in
Materials Science and Engineering C

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

Aida Naghiloua,⁎, Lena Pöttschacherb, Flavia Millessa,c, Anda Mannc, Paul Suppera,c, Lorenz Semmlerd, Tamara Weissa,c, Ellen H.G. Backusd, Christine Radtkea,c,d

⁎ Corresponding author.
E-mail addresses: aida.naghilou@meduniwien.ac.at (A. Naghilou), lena.poettschacher@univie.ac.at (L. Pöttschacher), flavia.millesi@meduniwien.ac.at (F. Millesi), anda.mann@meduniwien.ac.at (A. Mann), paul.supper@meduniwien.ac.at (P. Supper), lorenz.semmler@meduniwien.ac.at (L. Semmler), tamara.weiss@meduniwien.ac.at (T. Weiss), ellen.backus@univie.ac.at (E.H.G. Backus), christine.radtker@meduniwien.ac.at (C. Radtke).

https://doi.org/10.1016/j.msec.2020.111219
Received 10 April 2020; Received in revised form 2 June 2020; Accepted 18 June 2020
Available online 20 June 2020

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ARTICLE INFO

Keywords:
Peripheral nerve reconstruction
Nerve regeneration
Nerve guidance conduit
Scanning electron microscopy
Nephila edulis
Avicularia avicularia

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 material properties for the integration of fibrous luminal fillings in nerve guidance conduits.

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 transaction 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].
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 shrunk 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].
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 \textit{N. edulis}, silk from three spiders, and for the experiments on \textit{A. avicularia}, silk from two spiders was 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 (\(\perp\)) or parallel (\(\parallel\)) 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 \textit{N. edulis} and 10 for \textit{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 [42,67,68]. 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 \(\beta\)-sheets, and 1684 and 1698 cm\(^{-1}\) for \(\beta\)-turns [40,42,46,69]. The peak at 1655 cm\(^{-1}\) could be attributed to either \(\alpha\)-helix or 3\_1-helix, both with a similar Raman shift [40,46]. Therefore, this peak will be denoted as helices. The amplitudes were kept as floating but positive. The area of each peak was calculated and its contribution to the overall area was evaluated.

Since the silk is used in an aqueous environment during the \textit{in vitro} experiments, in addition to the experiments in the native state, Raman spectroscopy was also performed on SPSI in deionized water. This can account for possible changes in the secondary structures after exposure to an aqueous environment. The fibers were placed under water for 30 min prior to the Raman measurements and the spectra were obtained while the fiber was still submerged under water. This guarantees similar experimental conditions in both material characterization and \textit{in vitro} studies.

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 \(\alpha\) (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% \(CO_2\). The derived cell suspension was seeded on culture dishes coated with 0.01% poly-l-lysine hydrobromide (PLL, SIGMA) and 4.8 \(\mu\)g/ml laminin (SIGMA). For culturing of rSCs, Minimum Essential Medium \(\alpha\) was supplemented with 5% FCS, 1% P/S, 1% Sodium Pyruvate Solution, 2.5% HEPES, 10 ng/ml recombinant Heregulin\(\beta\)-1 (PeproTech), 0.5% N-2 Supplement (GIBCO), 2 \(\mu\) M 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-pS) from six individual donors were used. For the process of seeding, a drop with a volume of 12.5 \(\mu\)l containing \(10^5\) rSCs was placed on the suspended SPSI on the frame and kept at 37 °C and 5% \(CO_2\) for 1 h. Afterwards, the silk and the silk frame were submerged in growth medium. Phase contrast imaging was performed with \(10 \times /0.25\) or \(20 \times /0.40\) objectives of a NIKON Eclipse Ti2R 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 (AF488Plus, 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 \(\mu\)g/ml 4’,6-Diamidino-2-Phenyldilone 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 \textit{N. edulis} as well as the connecting and attaching silk of \textit{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 \textit{N. edulis} show a relatively smooth and featureless morphology, with average diameters of (3.2 ± 0.2) \(\mu\)m for dragline and (4.6 ± 0.3) \(\mu\)m 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 $(6.7 \pm 0.4) \, \mu m$. Each of the fibers in the bundle has a diameter below 1 $\mu m$. 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.

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].
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 ⊥ (Fig. 5a) and || (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 β-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 ⊥ and || spectra are reflected in the results of the fit (Fig. 6 top and bottom rows, respectively). The β-sheet content is more perceivable in the ⊥ 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 β-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 || 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 β-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, β-sheets, and β-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 conformations (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 their adherence to each other and the agglomeration of rSCs as seen in Fig. 3. In contrast, the higher content of β-sheets in dragline, cocoon, and connecting silk ensured a straight and stiff fiber in the liquid, which served as a guiding structure for rSCs.

This direct comparison of the secondary protein structures and the in vitro experiments highlighted the importance of β-sheets conformation in the natural SPSI for a successful guidance of rSCs. Our findings enabled further understanding of the necessary material properties for the integration of fibrous luminal fillings in NGCs. Thus, the production of recombinant silk for the use in nerve regeneration should target the high content of β-sheets protein structure, which can be modulated by careful selection of proteins and manufacturing methods [59,60,65].

4. Conclusions

In this study, the material characteristics of dragline and cocoon silk from N. edulis and the connecting and attaching silk from A. avicularia were investigated and correlated to their ability to act as a guiding structure for rSCs during in vitro experiments. The results demonstrated that rSCs adhered well to all four SPSI fibers, regardless of the much smaller diameter of attaching silk and the bundled nature of connecting silk. During the in vitro experiments, well-distributed rSCs were found on dragline, cocoon, and connecting silks. The behavior of these silks showed prominent variations to the attaching fibers, which adhered to each other in culture, leading to the generation of cell agglomerations. Quantitative analysis of the secondary protein structures of the four SPSIs revealed a significant lower β-sheets conformation for the attaching fibers in comparison to dragline, cocoon, and connecting silks. 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.

Acknowledgments

The authors would like to thank Assistant Professor Dieter Baurecht, and Assistant Professor Sapun Parekh for fruitful discussions about...
Raman spectroscopy, as well as Dr. Stephan Puchegger and the faculty center for nanostructure research at the University of Vienna for support with scanning electron microscopy.

We are also grateful to Professor Bruno K. Podeszka and his team at the Center for Biomedical Research, Medical University of Vienna for sharing euthanized rats for tissue harvest.

We appreciate the constant support from Dr. Marion Gröger and her team at the Core Facility Imaging of Medical University of Vienna.

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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