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Auditory Neurons on the Silk Road – spider silk for bridging the nerve-electrode-gap

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Abstract: The cochlear implant (CI) restores hearing to patients with severe to profound sensorineural hearing loss by stimulating the spiral ganglion neurons (SGN). Due to the inner ear anatomy, a fluid-filled gap remains between the SGN and the electrode array. This gap impedes focused stimulation and thus optimized performance with the CI. Regenerating neurites may bridge this fluid-filled gap for a direct nerve-electrode-link but require a supportive matrix. Spider silk might be a suitable candidate for this support. Therefore, silk fibers were tested for biocompatibility with inner ear neuronal tissue, suitability as neurite outgrowth matrix and applicability to the CI-electrode array.

Dragline silk from female *Trichonephila* spiders was woven around a frame and autoclaved. Spiral ganglia of young rats were prepared, cut and placed in a drop of medium on the parallel silk fibers for pre-cultivation to support adherence before remaining medium was added. After 5d of cultivation, cells were fixed and immunocytologically stained. For CI-application, silk was wrapped around a silicone dummy and a one-sided spacer. Opposite the spacer, alginate-hydrogel was applied for silk-fixation and -shielding. After gelation, silk loops were cut in the middle to form protruding threads.

Cells showed a high degree of migration and neurite regeneration along the silk fibers and in some cases the outgrown neurites directly contacted the silk. It was possible to fix the silk to the dummy as protruding fibers.

The tested spider silk is compatible with the inner ear tissue in culture, supports cell growth and seems to be an attractive material for neurite contacting. Application to the CI as protruding fibers is possible, making it a promising candidate for structural support to bridge the nerve-electrode-gap.

Keywords: spiral ganglion neurons, *Trichonephila* spider, neurite outgrowth matrix, cochlear implant, alginate coating

1 Introduction

The cochlear implant (CI) is a very successful neuroprothesis that restores hearing of patients suffering from severe hearing loss or deafness [1]. Its electrode array is surgically placed in the perilymph-filled scala tympani, from where it electrically stimulates the spiral ganglion neurons (SGN) located more centrally in the bony modiolus of the cochlea. This leads to a fluid-filled gap between the SGN and the electrode of about 2 mm [2], which impedes focused stimulation and thus an optimized performance with the CI [3]. Neurite regeneration induced by neurotrophic factor treatment may overcome this obstacle, but most likely requires a supportive and guiding matrix for the outgrowing neurites in the fluid-filled gap [4]. Spider silk fibers extending from the electrode contacts to the modiolus could provide such support. Natural spider silk is known for its biocompatibility [5] and support of neurite outgrowth and has successfully been used as longitudinal guiding fibers in nerve guidance conduits to bridge long-distance peripheral nerve defects by regenerating neurites [6,7,8,9]. In case of the CI, the fibers would need to be fixed to the electrode, should protrude modiolar from the contacts, and must not impede electrical stimulation by covering the contacts or impairing the insertion behavior. A one-sided, thin hydrogel coating with alginate could meet these requirements. Applied on the opposite side of the contacts to attach silk-loops to the implant, the alginate may: shield the silk in this area against cell attachment to prevent encapsulation, smoothen the implantation [10], act as a neurotrophic factor delivery matrix to induce neurite outgrowth [11].

To assess the suitability of natural spider silk as matrix for SGN neurite outgrowth, we cultured inner ear neuronal tissue on silk fibers and tested the application of spider silk to a CI-electrode dummy.

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2 Methods

2.1 Inner ear tissue culture

To test compatibility with neuronal inner ear tissue and influence on neurite outgrowth, spiral ganglion explants (SGE) were cultured on spider silk. Dragline silk from female *Trichonephila edulis* spiders was woven around a metal frame as described before [6] with finally 70-80 stretched, aligned silk fibers on each side of the frame. Silk covered frames (N= 3) were autoclaved to secure sterility and placed in a 24-well plate. In preparation for tissue seeding, a 50 µl-drop of cell culture medium (see below) was placed and spread on the silk fibers of each frame.

Inner ear tissue was harvested from four day old rats. Animals were rapidly decapitated, the temporal bones removed and collected in ice-cold PBS (phosphate-buffered saline; Gibco, Thermo Fisher Scientific, Waltham, USA). By using fine forceps and under microscopic control, cochleae were exposed, spiral ganglia dissected and divided in three pieces (apical, medial, basal; 3 SGE / frame, in total n=9 SGE). These SGE were placed crosswise on the aligned, stretched silk fibers (Fig. 1) in the drop of cell culture medium.

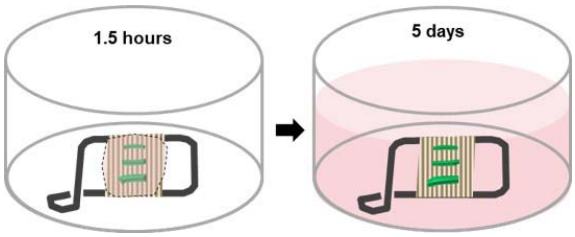

Figure 1: Scheme of SGE cultivation on spider silk: Pre-cultivation (left) in a 50 µl-drop of medium (pink, circled by dashed line) to support tissue adhesion and final cultivation (right) in 500 µl medium (pink). SGE (green) were placed crosswise on the aligned silk fibers (light yellow) on the metal frame (grey) in the 24-well plate.

Cells were pre-cultivated for 1.5 hours to support adhesion to the silk fibers, before wells were supplied with additional 450 µl of prewarmed cell culture medium (in total 500µl/well) consisting of Panserin 401 (PAN BIOTECH, Passau, Germany) complemented with penicillin, glucose, insulin, N2-supplement (for detailed composition manufacturer specification see [11]) and 10 % serum (fetal calf serum, Bio&Sell GmbH, Feucht, Germany). Cultivation was performed for five days without medium change in the incubator (37 °C, 5 % CO_2, humidified atmosphere; Binder, Tuttlingen, Germany). Afterwards, medium was removed, cells washed once with PBS and fixed with 4 % paraformaldehyde for 13 minutes followed by three PBS-washes. Subsequently, cells were stained for neurofilament and DAPI to label neurons and cell nuclei as described in table 1. Frames with stained cells were stored in PBS at 4°C and cell and neurite growth were examined by inverse fluorescence microscopy in floating condition (Fig. 2).

Table 1: Immunocytochemical staining procedure for neurons and cell nuclei. (AB: antibody, h: hour, min: minute, RT: room temperature)

step	solution	performance
permeabilisation	0.5 % Triton X in PBS	15 min, RT
washing	PBS	3x, RT
blocking	10% normal horse serum and 0.1% Triton X in PBS	1h, RT
primary AB	anti-neurofilament chicken (ab 4680, Abcam) (1:10,000) in blocking solution	1h, RT or overnight, 4°C
washing	PBS	3x, RT
secondary AB	goat anti chicken (ab 96947, Abcam, DyLight 488) (1:200) in blocking solution + DAPI (1:100)	1h, RT

2.2 Attachment of silk fibers to the electrode

The use of spider silk fibers as guiding structure for regenerating neurites between CI-electrode and SGN requires the application and fixation of the silk as protruding threads on silicone electrodes. For a first proof of concept, the silk was harvested and autoclaved as described above, manually wrapped around a CI-dummy and fixed with a one-sided alginate hydrogel coating (dummy production and coating procedure previously described in [10,12]).

Molded silicone CI-electrode-dummies (Sylgard 184; Dow, Barry, UK) were UV-radiated for decontamination and pre-coated with poly-L-lysine (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) to allow alginate adhesion. A 2 mm wide spacer (thin plastic foil) was connected to one side of the dummies to allow the spider silk to form loose loops, while the silk lay directly on the dummy surface on the other side. After moistening, a single thread of spider silk was detached from the spool and manually wrapped around the dummy with the spacer until several loops were applied. For fixing the silk to the silicone dummy on the opposite side of the spacer, alginate sol (*Lessonia negrescens* and *trabeculata* 1:1, 0.65 % in saline; Alginatec®, Riedenheim, Germany) was applied with a pipette. Alginate was then gelled for 10 minutes in a bath of barium-crosslinking solution (20 mM $BaCl_2$, 115 mM NaCl,

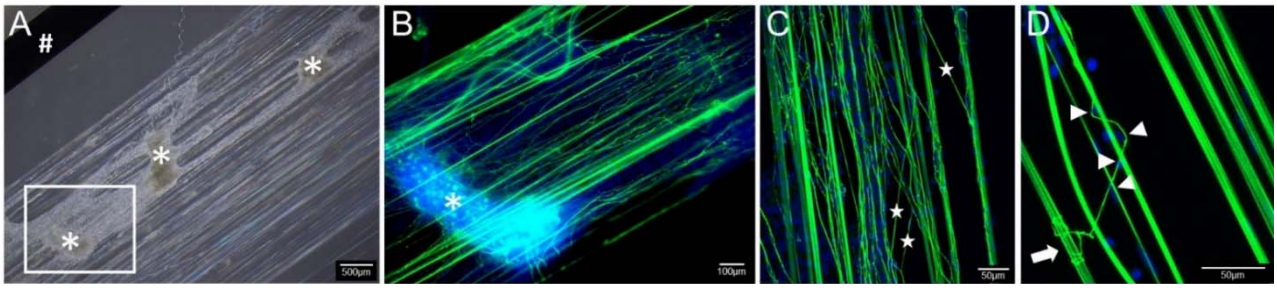

Figure 2: Cell and neurite growth on aligned spider silk fibers woven on a frame. The phase contrast image (A) depicts cell growth and migration, the fluorescence images (B, C, D) show the intense neurite regeneration along the silk fibers after five days of cultivation. C and D are close-ups of the regenerated neurites along the spider silk fibers. The fibers (autofluorescence, thick green lines) provide the neurofilament-negative cells (blue, DAPI-labeling) and the regenerating neurites (neurofilament-labeling, thin green lines) a growth matrix and direction in the medium. Some neurites bridge longer gaps between fibers and project to another one (C, stars). D focuses on a neurite wrapping around a silk fiber first accompanied by neurofilament-negative cells (arrow heads) then projecting to another fiber where it ends (arrow). Initially seeded SGE are market with asterisk (A, B), the frame with hashtag, B shows the magnified SGE indicated in A by the white rectangle. Scale bars: 500 µm (A), 100 µm (B), 50 µm (C, D)

(both Sigma-Aldrich), 5 mM L-Histidin (GENAXXON bioscience, Ulm, Germany) in 100 ml ddH_2O, pH 7.0) followed by washing in saline (B. Braun Melsungen AG). Silk loops were cut open with microscissors in the middle of the spacer to form protruding fibers. The silk and hydrogel coated dummies were subsequently transferred to a petri dish filled with saline for microscopy and imaging.

3 Results

Placement of the SGE on the longitudinally woven spider silk fibers in the drop of medium was easy to implement and the pre-cultivation for 1.5 hours supported a sufficient tissue attachment. No SGE (n=9) was detached by final medium addition, fixation, or during ICC and microscopy of the floating frames was possible. As exemplarily depicted in Fig. 2, cells as well as neurites migrated and grew along the parallel fibers of spider silk during cultivation. The SGN regenerated long and branched neurites on the silk fibers, which sometimes projected from one fiber to the other (Fig. 2C, stars). Interestingly, in some cases, the neurites appeared to make direct contact with the silk fibers and spiral around them (Fig. 2D, arrow).

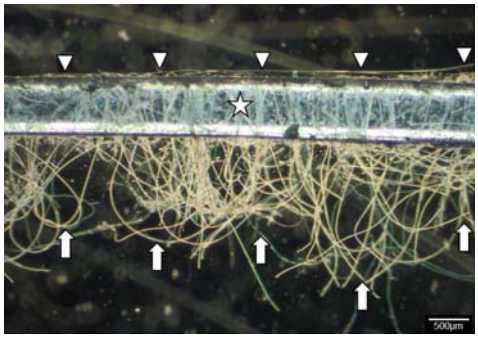

Figure 3: Silicone dummy functionalized with spider silk fibers and hydrogel-coating. Spider silk (light yellow fibers) is attached to the dummy (light blue, star) and covered by a thin layer of alginate hydrogel (arrow heads) on one side and ends in protruding fibers (arrows) on the other side.

Manual wrapping of spider silk fibers around the electrode-dummy was possible and the attached spacer allowed formation of loose loops on one side of the dummy. The applied and crosslinked alginate formed a thin hydrogel layer on the opposite side of the spacer and by this attached the silk to the dummy and shielded it. By cutting the loops in the middle of the spacer, the attached silk formed the intended protruding fibers of about 2 mm length on the uncoated side of the dummy (Fig. 3).

4 Discussion

In this study we prove for the first time the compatibility of natural spider silk with inner ear neuronal tissue and present a procedure for application of the silk to a silicone CI-electrode dummy in combination with an alginate hydrogel coating.

A remarkable growth and migration of cells of the spiral ganglion and regeneration of neurites along the stretched silk fibers were observed. Particularly noteworthy is the direct contacting and growth of the neurites along the fibers. This indicates an excellent compatibility of the spider silk with the spiral ganglion tissue and suitability as matrix to support regenerating neurites even in a liquid environment. These findings are in accordance with previous studies showing high suitability of natural spider silk as cell and neurite growth and guiding matrix for human model neurons (NT2) [6] and primary dorsal root ganglia neurons [7] *in vitro* or in nerve conduits for peripheral nerve regeneration *in vivo* [8,9] and

patients [13]. In contrast to peripheral nerve regeneration where several cm long defects have to be bridged by the neurons of a relatively compact nerve, in case of the CI a relatively short distance of about 2 mm [2] has to be overcome by regenerating neurites of the several thousand neurons of the spiral ganglion over the whole length of the electrode array (about 2.6 cm [14]). An additional challenge in case of the CI is that a fluid-filled gap has to be bridged for a direct nerve-electrode-link. This most likely requires a matrix that extends from the laterally positioned electrode to the modiolar neurons to support and guide regenerating neurites to the electrode contacts. The matrix needs to be flexible, robust, and not disruptive during implantation and must not hinder electrical stimulation or support cell adhesion, potentially increasing fibrotic encapsulation of the electrode. In the case of residual hearing, a low impact on the cochlear traveling wave by the growth matrix is favorable as well. Also suitability for drug delivery to induce neurite regeneration would be beneficial. The here presented combination of spider silk fibers on one side and thin hydrogel coating on the other side of the electrode-dummy may fulfil these requirements. The spider silk provides flexible, protruding fibers on the dummy and supported neurite outgrowth of SGN in the cell tests. For silk fixation to the electrode, there are several materials one could think of like fibrin glue. However, alginate combines the advantages of being suitable for electrode-coating, factor release, support of smooth insertion of the implant, and being stable in artificial perilymph for more than one year [10,11,15]. In this first proof of concept the alginate coating worked well and attached and covered the spider silk as intended.

In conclusion, we demonstrated that natural spider silk is a favorable material for peripheral auditory neurite regeneration and it can be applied as flexible, protruding fibers with a one-sided smooth alginate hydrogel coating to a silicone CI-dummy making it a promising combination to bridge the nerve-electrode gap in the CI-implanted inner ear. Further studies have to prove the attractiveness of the protruding spider silk fibers for neurite regeneration, a more precise application of the fibers at the electrode contacts, a better oriented direction of the fibers off the CI, and particularly its robustness and impact during implantation.

Author Statement

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