J Mater Sci: Mater Med (2016) 27:188
DOI 10.1007/s10856-016-5783-5

Original Research

Covalent binding of placental derived proteins to silk fibroin improves schwann cell adhesion and proliferation

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Received: 3 April 2016 / Accepted: 20 September 2016 / Published online: 5 November 2016

Abstract Schwann cells play a key role in peripheral nerve regeneration. Failure in sufficient formation of Büngner bands due to impaired Schwann cell proliferation has significant effects on the functional outcome after regeneration. Therefore, the growth substrate for Schwann cells should be considered with highest priority in any peripheral nerve tissue engineering approach. Due to its excellent biocompatibility silk fibroin has most recently attracted considerable interest as a biomaterial for use as conduit material in peripheral nerve regeneration. In this study we established a protocol to covalently bind collagen and laminin, which have been isolated from human placenta, to silk fibroin utilizing carbodiimide chemistry. Altered adhesion, viability and proliferation of Schwann cells were evaluated. A cell adhesion assay revealed that the functionalization with both, laminin or collagen, significantly improved Schwann cell adhesion to silk fibroin. Moreover laminin drastically accelerated adhesion. Schwann cell proliferation and viability assessed with BrdU and MTT assay, respectively, were significantly increased in the laminin-functionalized groups. The results suggest beneficial effects of laminin on both, cell adhesion as well as proliferative behaviour of Schwann cells. To conclude, the covalent tailoring of silk fibroin drastically enhances its properties as a cell substratum for Schwann cells, which might help to overcome current hurdles bridging long distance gaps in peripheral nerve injuries with the use of silk-based nerve guidance conduits.

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1 Introduction

Peripheral nerve lesions occur with a prevalence of approximately 5 % in trauma associated injuries [1]. Even though autologous nerve grafts are the clinical gold standard, the functional regeneration is often not satisfactory and autografts show several severe disadvantages such as limited availability and potential donor site morbidity [2–5]. Alternatives are sought to facilitate nerve regeneration, such as artificial nerve guidance tubes, luminal fillers containing neurotrophic substances and/or Schwann cells. Some of these approaches are currently used in clinical nerve repair, although there is an ongoing debate concerning their appropriate use, effectiveness and observed side-effects [6, 7]. One of the major reasons for the unsatisfactory outcome after repair of long distance gaps is the limited proliferative capacity of Schwann cells [8] and their selective adhesion to surfaces [9]. Schwann cells play a key role in peripheral nerve regeneration: they participate in the removal of myelin and axonal remnants, start proliferating and align to build the so called bands of Büngner [10]. After the axon has elongated along these bands of Büngner, the Schwann cells start to remyelinate the newly formed axon to complete the regenerative process. Failure to form sufficient bands of Büngner due to impaired Schwann cell proliferation has significant effects on the functional outcome after regeneration [11]. Therefore, from a biomaterials

point of view, the growth substrate for Schwann cells should be considered with highest priority.

Over the last decades, silk fibroin in its pure form or modified, has been shown to be a promising biomaterial in several areas of tissue engineering [12–15]. In the recent past silk fibroin has attracted considerable interest as a biomaterial for use as a conduit in peripheral nerve regeneration due to its excellent biocompatibility [14, 16–18]. In previous studies our group demonstrated that carbodiimide chemistry can be utilized to covalently bind the plant lectin wheat germ agglutinin to silk fibroin leasing to a prompt and stable cell adhesion to silk fibroin in a short time frame (<30 min) [19, 20]. In this study we used this method to tailor silk fibroin based nerve guidance conduits with laminin and collagen which are known to interact with integrins on the surface of Schwann cells, supporting adhesion and proliferation of Schwann cells [21].

We hypothesize that covalent binding of laminin and collagen molecules to silk fibroin improves cell adhesion and might have beneficial effects on Schwann cells cultured on silk fibroin. Therefore we investigated the influence of covalently bound laminin and collagen molecules on the adhesion time, viability and proliferation of Schwann cells.

2 Materials and methods

Unless indicated otherwise, all reagents were purchased from Sigma Aldrich and of analytical grade.

2.1 Schwann cell isolation and culture

All animals were euthanized according to established protocols, which were approved by the City Government of Vienna, Austria in accordance with the Austrian Law and Guide for the Care and Use of Laboratory Animals as defined by the National Institute of Health. Animals and treatment/control groups were randomly chosen and analysed without pre- or post selection of the respective nerves or cultures.

Sciatic nerves of adult male Sprague Dawley rats were dissected and kept in phosphate buffered saline (PBS; PAA, Austria) pre-chilled on ice until further use, but not longer than one hour. Schwann cells were isolated from sciatic nerve tissues according to a method adapted from Kaekhaw et al. [22]. Briefly, the epineurium was removed and nerves were strained and minced. Nerve fragments were incubated with 0.05 % collagenase for 1 h at 37 °C subsequently filtered through a 40 µm cell strainer and centrifuged at 400 × g for 6 min. After washing the cell pellet in Dulbecco's Modified Eagle Medium (DMEM; PAA, Austria) containing 10 % fetal calf serum (FCS; PAA, Austria), the pellet was resuspended in DMEM-D-valine (PAA, Austria), supplemented with 10 % FCS, 2 mM L-Glutamine (PAA, Austria), 1 % antibiotics (PAA, Austria), N_2 supplement (Invitrogen, Germany), 10 µg/mL bovine pituitary extract, 5 µM forskolin. Cell suspension was seeded on 6-well plates (PAA, Austria) coated with poly-L-lysine and laminin. Purity of Schwann cell cultures was assessed with flow cytometry (BD FACS Canto II) and Schwann cell markers anti-P75 NGFR (goat polyclonal, Santa Cruz Biotechnology, USA), anti-S100b (rabbit polyclonal; Dako) and anti-P0 (rabbit polyclonal, Santa Cruz Biotechnology, USA) conjugated with APC (Lynx Rapid Conjugation Kit, ABD Serotec, Kidlington, UK).

2.2 Silk fibres and silk mats

White raw *Bombyx mori* silkworm fibres of 20/22 den and 250T/m were purchased from Testex AG, (Zürich, Switzerland). As described in a previous study [16] these fibres have been used as starting material to textile engineer tubular silk conduits. In the here presented study these conduits were functionalized with laminin and collagen to enhance their substratum properties for the growth of Schwann cells. For easier analysis such as visualization the conduits have been cut open resulting in braided silk mats (see Fig. 5).

2.3 Degumming of silk fibres and mats

As sericin is thought to potentially elicit immune reactions [23], silk fibres were degummed. Sericin was removed from raw *Bombyx mori* silkworm fibres or silk mats by boiling twice in 0.2 M boric acid in a 0.05 M sodium tetraborate buffer at pH 9.0 for 45 min each time. Then silk fibres or constructs were thoroughly rinsed in ddH_2O at least 3 times. The so-degummed silk fibres and mats have been air-dried over night and stored at RT for later use.

2.4 Preparation of silk films

After degumming of silk fibres silk films were prepared as described by Teuschl et al. [19]. Two gram of fibres were dissolved by boiling them in 50 mL of calcium chloride, ethanol and ddH_2O at a molar ratio of 1:2:8 for 45 min. The solution was then filtered (0.22 µm, Rotilab, Germany), dialyzed against ddH_2O using a Slide-a-Lyzer dialysis cassette (Thermofisher Scientific, US) with 3–12 mL capacity and a molecular weight cut off of 3.500 Da. Therefore, 12 mL of the silk-solution was injected into the cassette with a 20 mL syringe and an 18-gauge needle. The 12 mL of silk-solution were dialyzed 6 times against one liter of ddH_2O within 48 h. The resulting aqueous silk solution was lyophilized using an Alpha 1–2 LDPlus dry freezer (Christ L-1 alpha 1–4 Freeze-dryer, SciQuip, NewTown, UK). Silk films were prepared on tissue culture plastic after

reconstitution of the lyophilized silk powder with hexafluoroisopropanol at a concentration of 25 mg/mL. Then aliquots of 15 or 90 µL were used to cast single wells of 96-well or 24-well plates, respectively. Well plates were air-dried for at least 2 h and the silk was stabilized by induction of beta-sheets by immersion with methanol/H_2O (ratio 9/1 v/v) for at least 15 min. Then the solution was removed and the plates dried overnight at room temperature. The so-prepared coated well-plates have been stored at 4 °C for later use.

2.5 Coating of silk films with laminin and collagen

Placental collagen (mixture of collagen I and III) and laminin (routinely isolated from placental tissue at the Ludwig Boltzmann Institute for experimental and clinical Traumatology) were resuspended in HEPES/NaOH pH 7.4 to a concentration of 1 mg/ml and a blend of collagen/laminin to a concentration of 1 mg/ml each overnight on a roller mixer. In parallel plates were immersed in 100 % methanol for an hour and allowed to air-dry. After hydration for at least 1 h in 20 mM HEPES/NaOH (pH 7.0) buffer, the carboxy groups were activated by adding 5 mg/mL 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 7 mg/mL *N*-hydroxysuccinimide (NHS) solution in 20 mM HEPES/NaOH (pH 7.0) for 2 h and subsequently rinsed in 20 mM HEPES buffer pH 7.0. Incubation with laminin or collagen was performed at 4 °C over night. Finally films were rinsed with 6 M urea to reduce unspecific binding, and washed with HEPES buffer pH 7.4. For all assays the following control groups were used: uncoated TCPS (ctrl), poly-L-lysine/laminin coated TCPS, fibroin coated TCPS. To assses effect of the EDC treatment itself fibroin coated TCPS functionalized with glycine (to saturate the activated groups) was used.

2.6 Cell adhesion assay

To evaluate alterations in Schwann cell adhesion to the silk films after coating with placental proteins, an attachment assay was performed. Briefly, Schwann cells were detached from cell culture plastic with Accutase. Accutase activity was stopped with cell culture medium and cells were counted using a cell counter (BioRad T20). 5×10^3 cells were pipetted onto the silk films as well as control wells of a 96 well plate (triplicates per Schwann cell culture; a triplicate of 1 donor equals 1 n, total $n = 12$), and tube containing cell suspension was inverted after every triplicate. The plates were incubated for 20 min, 2 and 24 h under standard cell culture conditions (37 °C, 5 % CO_2 and 80 % humidity). Subsequently supernatant fluid containing non-adhered cells was collected. Residual cells were collected by a washing step with PBS. Cells were counted and ratio of viable to non-viable cells was assessed using a cell counter and trypan blue dye exclusion.

2.7 Cell viability

Cell viability of Schwann cells on the coated silk fibres was determined using MTT assay. Schwann cells, seeded at a density of 4×10^3 cells/cm^2 (triplicates/Schwann cell culture; a triplicate of 1 donor equals 1 n, total $n = 12$), incubated with culture medium containing 650 µg/ml MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide for 1 h at standard cell culture conditions (37 °C, 5 % CO_2 and 80 % humidity) (30 h after seeding). Medium was discarded and MTT formazan precipitate was dissolved in 100 µl DMSO by shaking in dark for 20 min. Light absorbance at 540 nm was measured immediately and optical density (OD) values were corrected for an unspecific background on an automatic microplate reader (Tecan Sunrise; Tecan Switzerland).

2.8 Proliferation

Proliferation of Schwann cells on the coated silk fibres was evaluated using a 5- bromo-2-deoxyuridine uptake assay (BrdU; Cell Proliferation ELISA assay Kit; Roche Diagnostics, Switzerland), according to manufacturer's instructions. Briefly, coated and control 96 well plates were seeded with Schwann cells at a density of 4×10^3 cells/cm^2 (triplicates/Schwann cell culture; a triplicate of 1 donor equals 1 n, total $n = 12$). Cells were left to adhere for 24 and 72 h. Subsequently, medium was changed to Schwann cell medium containing 100 µM BrdU and cells were incubated for 24 h at standard cell culture conditions (37 °C and 5 % CO_2). The culture plates were fixated with FixDenat® solution and incubated with anti-BrdU POD antibody solution for 60 min at room temperature. After washing the plate with PBS twice, substrate solution (tetramethyl benzidine) was added for 20 min. The reaction was stopped with 1 M H_2SO_4 and absorption was measured at 450 nm with 690 nm as reference wavelength on an automatic microplate reader. Results were normalized on cell count after 30 h attachment period.

2.9 Visualization of cells on silk fibres

Schwann cells were seeded on functionalized fibroin mats (woven) (described by Teuschl et al. [16]) and fibroin fibres at an initial cell seeding density of 4×10^3 cells/cm^2. Forty eight hours after cell seeding, cell adherence on the fibres was visualized using a live/dead staining (Calcein AM/Propidium iodide; Thermo Fisher, US) according to manufacturer's instructions. Briefly, cell-fibre constructs were incubated with Calcein AM (3 µM in DMSO

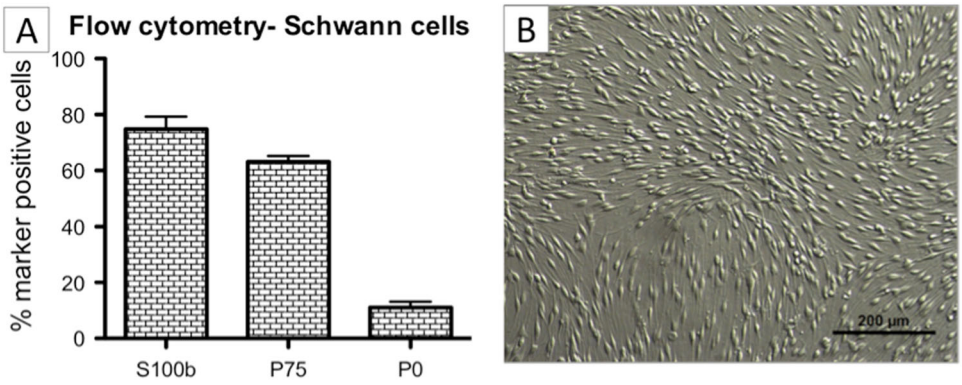

Fig. 1 **a** Flow cytometric analysis of Schwann cells for Schwann cell specific markers S100b, P75 and P0; **b** light microscopy image depicting typical Schwann cell morphology

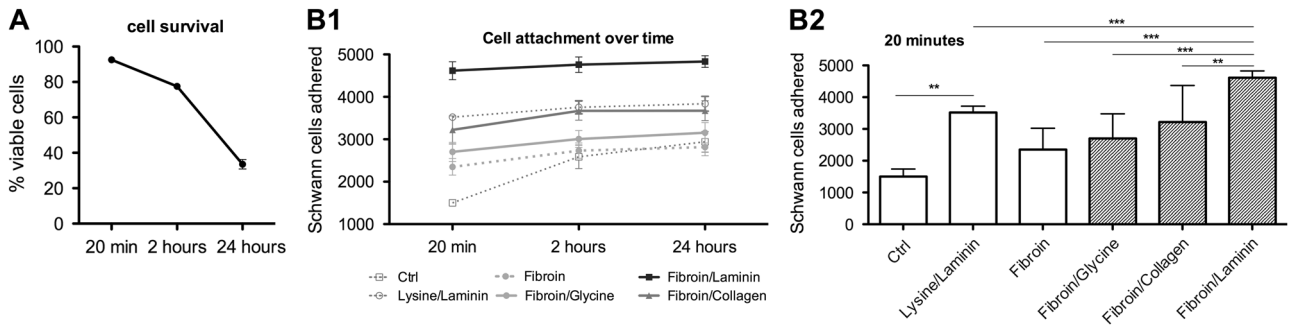

Fig. 2 Schwann cell adhesion to silk fibroin films; **a** percentage of viable cells assessed with trypan-blue dye exclusion after 20 min, 2 and 24 h of all groups; **b** Schwann cells adhered after 20 min (B2), 2 and 24 h after seeding on uncoated control (ctrl-dotted light grey), standard Schwann cell coating (lysine/laminin- dotted dark grey) and fibroin films alone or functionalized with glycine, collagen or laminin; statistical significance was tested with 1- way ANOVA and Tukey range test; data is presented as Mean±SD; $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$; $n = 12$

(Dimethylsulfoxid) and propidium iodide (2.5 µM in water) in cell culture medium for 30 min. Subsequently cell-fibre constructs were washed twice with PBS and kept in PBS until examination using a Leica DMI6000B microscope (Leica, Solms, Germany).

2.10 Statistical analysis

Statistical difference was analysed using one-way ANOVA (analysis of variance) followed by Tukey range test for significant differences between the means. Significance was considered for $P < 0.05$. For statistical calculations GraphPad Prism 5 for Mac OS X, Version 5.0b (GraphPad Software, Inc., La Jolla, CA, USA) was used. All data in this study is shown as mean ± standard deviation (SD).

3 Results

3.1 Improved immediate Schwann cell adhesion to modified silk films

Prior to assays Schwann cell purity and proliferative status were assessed using flow cytometry and common Schwann cell specific markers. As seen in Fig. 1a, Schwann cells expressed the Schwann cell marker S100b and the proliferation-associated marker P75, while myelin marker P0 is only mildly expressed. Figure 1b depicts Schwann cells revealing typical bipolar, spindle shaped Schwann cell morphology.

Schwann cell adhesion was evaluated in a cell attachment assay. 20 min, 2 and 24 h after cells were pipetted onto silk films, residual non-adhered cells were counted and viability assessed with trypan blue dye exclusion. Tissue culture plastic (ctrl) and the coating commonly used for Schwann cells (lysine/laminin) acted as negative and positive control, respectively. Cell viability of the non-adhered Schwann cells decreased significantly over time (Fig. 2a), independently of the growth substrate. While after 20 min around 95 % of the non-adhered cells were negative for trypan blue, the amount of viable cells decreased to 35 % after 24 h (Fig. 2b). Figure 2b depicts that in all three time points significantly more cells adhered to the standard lysine/laminin coating than to the untreated control (mean cells adhered: 3520 to 1507 (20 min), 3754 to 2582 (2 h) and 3837 to 2941 (24 h) allowing a comparison of the treatment groups. Schwann cells adhered faster and overall better to tailored fibroin, independent of protein type, than

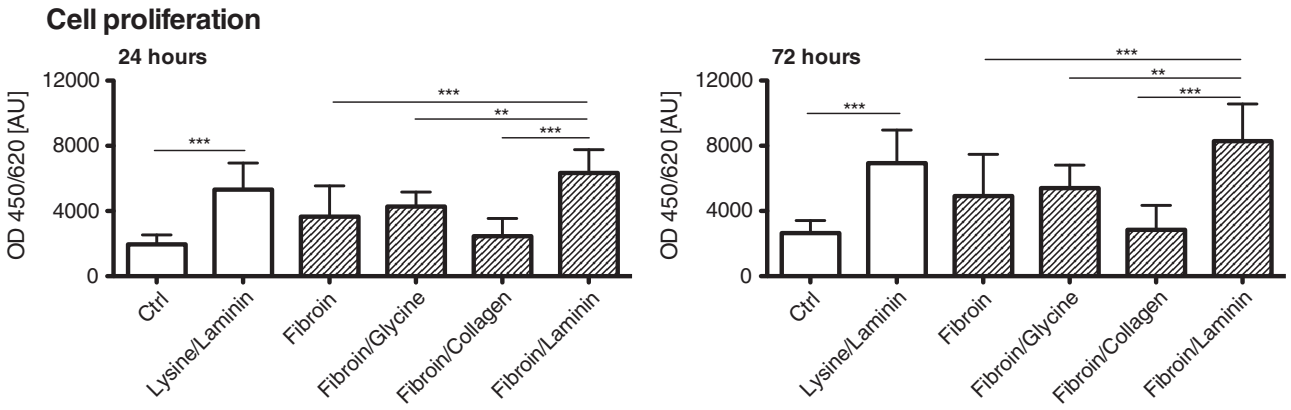

Fig. 3 Improved Schwann cell proliferation on silk fibroin films modified with laminin; BrdU assay of Schwann cells seeded on uncoated tissue culture plastic (ctrl), standard Schwann cell coating (lysine/laminin) and fibroin films alone or functionalized with glycine, collagen or laminin; OD values were normalized on BrdU start point cell counts per group; statistical significance was tested with 1- way ANOVA and Tukey range test; data is presented as Mean ± SD; $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$; $n = 12$

to uncoated control. However, a significant improvement of attachment, also compared to the standard coating group, could be observed in the fibroin group coated with laminin (mean cells adhered: 4616 (20 min), 4758 (2 h), 4833 (24 h)).

3.2 Schwann cell proliferation on modified silk films

Proliferation behaviour on silk fibroin films was assessed with a BrdU incorporation assay (Fig. 3). Cells were seeded on the films and left to adhere for 24 and 72 h. Tissue culture plastic (ctrl) and the standard coating for Schwann cells (lysine/laminin) acted as negative and positive control, respectively. Schwann cells proliferated at both time points significantly more on lysine/laminin films than on the uncoated control (Fig. 3; mean OD 5309 to 1940 (24 h) and 6930 to 2649 (72 h)). Proliferation on fibroin films and fibroin/glycine films was improved compared to the uncoated control, but lower than in the lysine/laminin group (mean OD: 3640 (24 h) and 4914 (72 h) for fibroin film and 4262 (24 h) and 5405 (72 h) on fibroin/glycine films). Modification of the fibroin film with collagen negatively affected proliferation (mean OD: 2441 (24 h) and 2847 (72 h), while modification with laminin led to a significant improvement (mean OD: 6329 (24 h) and 8285 (72 h), comparable to the positive control lysine/laminin. Between the early time point and later time point proliferation increased in all groups between 25–30 %.

3.3 Schwann cell viability on modified fibroin films

Influence on Schwann cell viability was evaluated with an MTT assay (Fig. 4). Cells were seeded on the films and left to adhere for 24 and 72 h. Uncoated control (ctrl) and the standard coating for Schwann cells (lysine/laminin) acted as negative and positive control respectively. As seen in Fig. 4, no significant differences in cell viability could be observed after 24 h, with a slight tendency towards a decreased viability in the uncoated control group (ctrl) and the fibroin/collagen group. However, after 72 h Schwann cell viability was significantly higher in the lysine/laminin and fibroin/laminin group. OD values normalized on start point cell count revealed an increase between the early time point (24 h) and the later time point (72 h) of approximately 60 % in the lysine/laminin and fibroin/laminin group, 45 % in the fibroin/glycine group, and in the other groups OD values increased only 25–30 % (Fig. 4).

3.4 Improved adhesion to laminin-modified silk fibre mats and silk fibres

To investigate efficacy of the coating on single fibres and woven mats in terms of Schwann cell attachment, cells were visualized using a live/dead stain (Fig. 5). Cells were seeded on the mats and fibres and left to adhere for 48 h. After the staining procedure, silk mats and fibres were positioned with the surface containing the cells facing the light beam of the microscope. Calcein AM/PI staining shows a high number of viable cells adhered to the laminin-coated fibroin mats and fibres, while only a few cells could be detected on the uncoated and collagen-coated fibres and mats.

4 Discussion

In this study, we hypothesized that covalent binding of laminin and collagen molecules to silk fibroin could accelerate cell adhesion and as a result has beneficial effects on Schwann cell viability and proliferation compared to unmodified silk fibroin.

A well-known method was used to isolate Schwann cells by first dissociating the nerve and then cultivating the cells

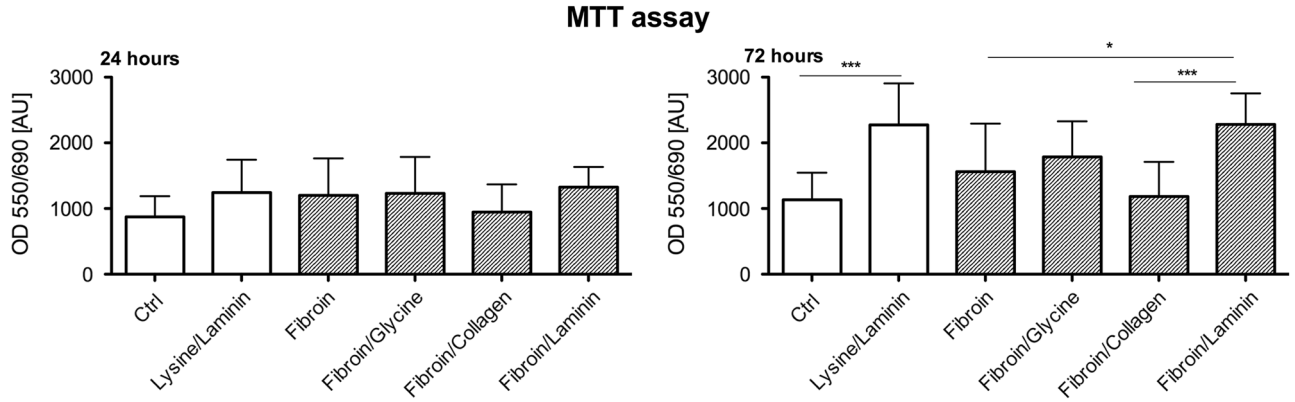

Fig. 4 Increased Schwann cell viability on fibroin films modified with laminin; MTT assay of Schwann cells seeded on uncoated tissue culture plastic (ctrl), standard Schwann cell coating (lysine/laminin) and fibroin films alone or functionalized with glycine, collagen or laminin; OD values were normalized on BrdU start point cell counts per group; statistical significance was tested with 1- way ANOVA and Tukey range test; data is presented as Mean ± SD; $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$; $n = 12$

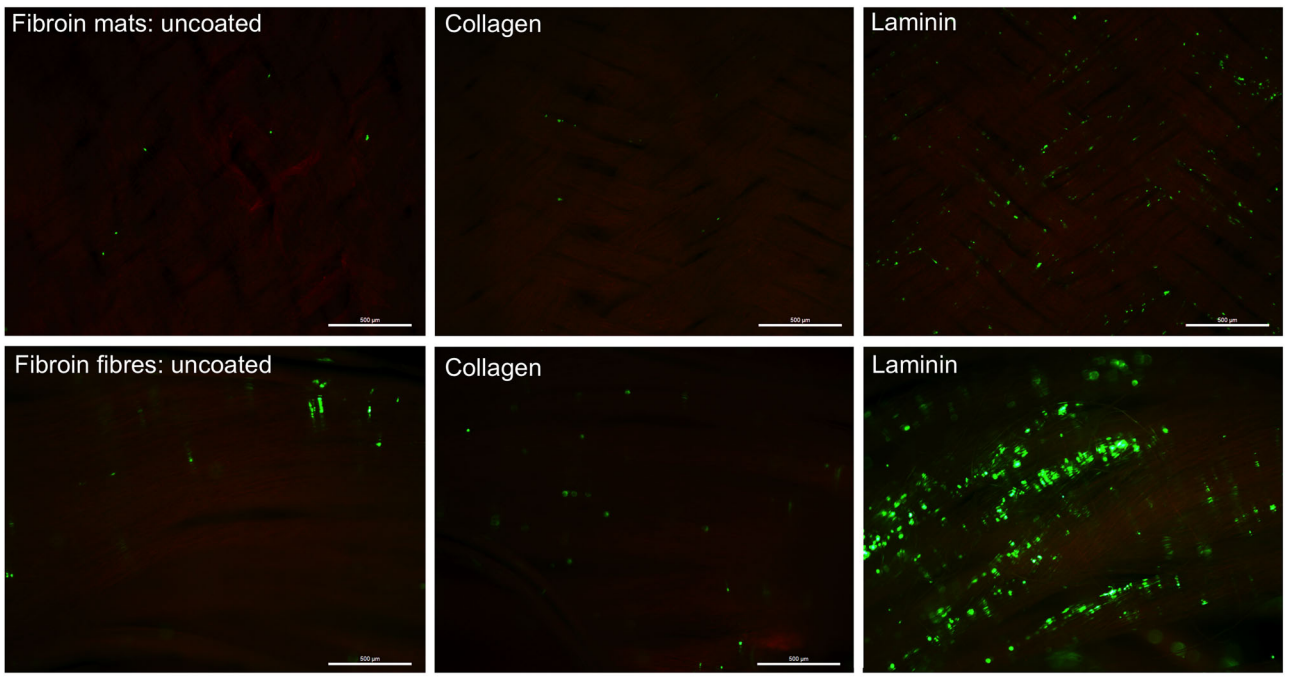

Fig. 5 Representative fluorescence micrographs of Schwann cells visualized using Calcein AM/propidium iodide staining; upper panel: Schwann cells seeded on woven silk fibroin mats coated with collagen or laminin; lower panel: Schwann cells seeded on silk fibroin fibres coated with collagen or laminin

in a selective medium introduced by Kaekhaw et al. 2012 [22]. Purity of the Schwann cells used was proven with S100b, P75 and P0 antibody staining, evaluated with flow cytometry. Furthermore cells showed the typical Schwann cell bipolar, spindle-like morphology.

Vleggeert et al. [24] showed that Schwann cells can adhere to a number of materials, contradicting the common opinion that attachment is limited to exclusive materials coated with ECM molecules and therefore an important consideration for Schwann cultures [24, 25]. In our study we demonstrated that—seeded in a relatively low concentration—Schwann cells adhered to a certain degree to all substrates, even to the negative control (uncoated tissue culture polystyrene), but there were significant differences between the substrates providing ECM molecules and the uncoated controls at all three time points evaluated. Covalent binding of laminin to fibroin significantly improved cell adhesion, also compared to the standard coating used for Schwann cells (lysine/laminin). Moreover, it accelerated attachment: more than 90 % of the seeded cells adhered

within the first 20 min. As seen in Fig. 2, time is a crucial factor. Percentage of viable cells within the non-adhered cells decreased significantly after 2 and 24 h. This effect might not only result in high variations of cell numbers, but also in a selection of potential sub-populations within the Schwann cells and therefore a faster and more efficient cell seeding results in a lower initial cell number needed and a reduction of donor material necessary.

It is known that integrins on the surface of Schwann cells interact with laminin and collagen, resulting in increased adhesion and proliferation [21]. We observed a positive effect of both, laminin and collagen, on Schwann cell adhesion compared to the control groups (Fig. 2). However, only the laminin-coated groups showed an increase in proliferation, while proliferation on the collagen-coated fibroin films was comparable to tissue culture plastic and even lower than fibroin alone (Fig. 3). Laminin has been shown to be a crucial factor in Schwann cell morphogenesis, required for Schwann cells to form their bipolar shape and extensions [26]. Cell metabolism assessed by MTT assay revealed no differences between the groups after 24 h, but a significant increase in laminin coated groups after 72 h. This elevation in metabolic activity may be reflected in the increased proliferation. It has been shown that a number of neural cells respond with enhanced viability and a later onset of apoptosis to adhesion on laminin [27–29]. This reported increase in viability is in accordance with our findings, in further in vitro and in vivo studies long term effects (e.g., on adhesion and apoptosis) have to be evaluated.

Summarizing, collagen and laminin covalently bound to silk fibroin increased cell adhesion, while only laminin also improved proliferation and cell viability. The modification of biomaterials, in this study of silk fibroin, might help to exploit the full neuroregenerative capacity of applied Schwann cells by (1) increasing the number of adhered Schwann cells and (2) elevating their proliferation and viability. These findings could find application in tissue engineering approaches for peripheral nerve regeneration, e.g., to improve in vitro Schwann cell adhesion on fibroin guiding structures (fibres, electrospun fibroin sheets) and direct axonal outgrowth, which might help to overcome current hurdles bridging long distance gaps in peripheral nerve injuries. However, as laminin-mediated cell adhesion is not prone to Schwann cells alone (e.g., keratinocytes [30] or myoblasts [31]) the findings of functionalizing the versatile biomaterial silk fibroin with the cell adhesion promoting factor laminin, could be translated into other regenerative fields, such as skin or muscle tissue engineering. In future studies we will use the here described laminin-functionalization to refine our established silk-based nerve guidance conduits facilitating nerve regeneration.

Acknowledgments The financial support by the City of Vienna Competence Team reacTissue Project (MA27#12-06) as well as the financial support by the Lorenz Böhler Fond are gratefully acknowledged.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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