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Title

Biofunctional scaffolds with high packing density of aligned electrospun fibers support neural regeneration

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Abstract

Neurons of the central nervous system do not regenerate spontaneously after injury. As such, biofunctional tissue scaffolds have been explored to provide a growth-promoting environment to enhance neural regeneration. In this regard, aligned electrospun fibers have proven invaluable for regeneration by offering guidance for axons to cross the injury site. However, a high fiber density could potentially limit axonal ingrowth into the scaffold. Here, we explore which fiber density provides the optimal environment for neurons to regenerate. By changing fiber electrospinning time, we generated scaffolds with different fiber densities and implanted these in a rat model of spinal cord injury (SCI). We found that neurons were able to grow efficiently into scaffolds with high fiber density, even if the gaps between fiber bundles were very small ($< 1 \mu\text{m}$). Scaffolds with high fiber density showed good host-implant integration. Cell infiltration was not affected by fiber density. Efficient blood vessel ingrowth likely requires larger gaps between fibers or faster degrading fibers. We conclude that scaffolds with high fiber densities, and thus a large number of small gaps in between fiber bundles, provide the preferred environment for nerve regeneration after SCI.

Keywords

Spinal cord injury, Tissue Engineering, Electrospinning, Nerve regeneration, Aligned fibers

1. Introduction

Spinal cord injury (SCI) leads to the loss of vital neuronal connections between the brain and the rest of the body. Although neurons of the central nervous system do not regenerate spontaneously, recent research has provided evidence that, given the right environment, neurons can regenerate and re-establish connections, which may lead to functional recovery (1). Finding the ideal conditions to support and direct neural regeneration is currently a major goal in spinal cord injury research.

Fibrous tissue scaffolds hold great potential to provide a regeneration-promoting environment and have shown promising results in several tissue engineering areas including skin, musculoskeletal, cardiovascular, bone, cartilage, tendon and ligament (2–8). Electrospinning is one of the most popular techniques to generate fibrous scaffolds for tissue regeneration (9–11). Because of the versatility of the technique, fibers can be designed to provide the ideal conditions for nerve regeneration. A lot of work has been done to optimize fiber properties like diameter, alignment, porosity, mechanical strength and biocompatibility (9). Investigating fibers with a wide range of diameters from nano- to micrometer scale has led to the conclusion that neurites grow most efficiently on electrospun fibers with diameters of a few microns (1–2 μm)(2,12). In addition, multiple *in vitro* and *in vivo* studies have demonstrated that highly aligned electrospun fibers improve neurite outgrowth more than randomly oriented fibers by guiding neurites along the length of the fibers (2,13–16). Other fiber characteristics, like surface topography or coating with extracellular matrix proteins such

as laminin, also significantly influence neurite extension, neurite direction and branching (17,18). In addition, scaffolds can be designed to locally release biochemical signals like growth factors, drugs and genes to stimulate growth or control inflammation (16,19–21).

One important and possibly limiting factor in the design of fibrous scaffolds is the fiber density, that is, the number of fibers in a given area of the scaffold. Previous studies have suggested that a low fiber density would benefit regeneration, because large gaps in the scaffold improve cellular infiltration and tissue ingrowth (9,22–26). Indeed, for non-fibrous scaffolds, several studies have indicated that channel or pore sizes around 100 μm seem to be suitable for the regeneration of neurons (9). Because of this idea, considerable efforts have been made to increase the gaps between electrospun fibers (27,28). However, when gaps were too large, axons lost directionality (25). In addition, it was noted that neurites prefer growing near the surface of large channels, as opposed to in the center (24).

Fibrous scaffolds offer the advantage of a high ratio of surface area versus volume, which provides more opportunities for cell attachment and improves directional growth (9). To investigate the effect of fiber density on neural regeneration, if the fiber density is too high, neurons may not find sufficient space to grow. On the other hand, if the fiber density is too low, neurons may not find sufficient guidance cues for attachment and directional growth. While the effect of fiber properties like diameter and alignment has been thoroughly investigated, the specific influence of fiber density on axonal growth remains unclear.

Based on the literature and our preliminary observations, we anticipated that a lower fiber density would allow more axonal ingrowth into the scaffold, because neurons would have more space to grow (22–25). To put this theory to the test, we designed scaffolds with different fiber densities and quantified axonal ingrowth in a rat model of SCI. Surprisingly, we found that a higher fiber density supported more axonal ingrowth. Detailed analysis of individual gaps in between fiber bundles confirmed that axonal ingrowth was more efficient when gaps between the fiber bundles were small ($< 1\ \mu\text{m}$). Furthermore, our results suggest that axons preferred growing individually in narrow gaps ($< 1\ \mu\text{m}$), rather than as axon bundles when the space in between fiber bundles was larger ($> 1\ \mu\text{m}$).

2. Materials and methods

2.1. Materials

Normal goat serum (NGS, G9023), Triton X-100 (X100), Masson's trichrome staining kit (HT15), 2, 2, 2-trifluoroethanol (TFE, $\geq 99.0\%$, T63002) and heparin sodium (H0200000) were purchased from Sigma-Aldrich. Bovine serum albumin (BSA, A1000801) and phosphate buffered saline (PBS; pH7.4, 10010049) were obtained from Life Technologies. Paraformaldehyde (1.04005) was purchased from Millipore and diluted in PBS. Rat-tail Collagen type I (354249) was purchased from Corning. Neurotrophin-3 (NT-3, 450-03-100) was purchased from PeproTech.

The following primary antibodies were used: rabbit anti-neurofilament 200 (NF, N4142, Sigma-Aldrich, 1:700 dilution), mouse anti-glial fibrillary acidic protein (GFAP, clone GA5, MAB360, Millipore, 1:500 dilution), mouse anti-Rat Endothelial Cell Antigen-1 (RECA-1, MCA970R, SeroTec, 1:100 dilution). Secondary antibodies were Alexa Fluor 488-conjugated Goat Anti-Mouse/Rabbit IgG (A11001/A11034, Invitrogen, 1:700 dilution) and Alexa Fluor 555-conjugated Goat Anti-Mouse/Rabbit IgG (A-21422/A21429, Invitrogen, 1:1000 dilution). Nuclear staining was performed with DAPI (1:1000, 62248, Life Technologies).

2.2. Fiber-hydrogel scaffold fabrication

Fibers. Poly (caprolactone-co-ethyl ethylene phosphate) (PCLEEP) copolymer ($M_w = 59,102$, $M_n = 25,542$) was a gift from Dr. Yucai Wang's lab. It was synthesized as reported previously (29). To prepare the polymer solution for electrospinning, PCLEEP was dissolved in TFE at 33% w/w and allowed to settle overnight prior to use to ensure homogeneity. A two-pole air-gap electrospinning technique was used to fabricate aligned PCLEEP fibers. Briefly, the electrospinning solution was loaded into a 3 ml syringe that was subsequently capped with a 21-gauge blunt-tipped needle. A +8 kV charge was applied to this needle tip and the two-pole air-gap collector was charged at -4 kV. The electrospinning solution was released at a flow rate of 1.5 ml/h by a syringe pump. PCLEEP fibers were then deposited within the 5.0 cm air gap area between the stationary poles. To vary the fiber density of the scaffolds, the electrospinning time per fiber layer was varied. Low fiber density scaffolds were fabricated by

electrospinning each layer of fibers for 3 min while high fiber density scaffolds were made with fiber layers that were electrospun for 6 min 30 s. Each layer of fibers was subsequently stacked into a stack of 7 layers. These fiber layers were sterilized under UV light for 30 min prior to stacking and rolling them into a bundle of fibers. A sterilized cylindrical mould (8.0 mm in length and 3.5 mm in inner diameter) was used to set each fiber bundle in the core region before the addition of collagen matrix (Supplementary Figure 1A).

Collagen matrix. Rat-tail type 1 collagen was used to fabricate the hydrogel matrix according to the manufacturer's protocol. Briefly, 10x PBS, 1.0 N NaOH, deionised (DI) water and collagen type 1 were added into a sterile 600 μ l microtube in the order listed. All components were gently mixed to obtain a final collagen concentration of 3.0 mg/ml. To promote infiltration of neurofilaments, growth factors were incorporated into this matrix. Specifically, NT-3 was reconstituted in 0.1% BSA and 400 μ g/ μ l heparin at 1:1 v/v to arrive at a stock concentration of 2 μ g/ μ l. Thereafter, 4 μ l of NT-3 stock solution was used to substitute 4 μ l of DI water in the 250 μ l collagen mixture per 8 mm mould. Therefore, a total of 8 μ g of NT-3 was added into this collagen mixture. Subsequently, a total of 2 μ g of NT-3 was used per animal. This collagen mixture was kept on ice until dispensed into the mould that contained the electrospun fiber bundle in its core region. Hydrogel formation occurred at room temperature for 30 min before placing into -20 °C for 4 h prior to overnight lyophilization. Freeze dried scaffolds were subsequently cut into 2.0 mm length under sterilized conditions before implantation in each animal.

2.3. Fiber-hydrogel scaffold characterization

The morphology of the aligned PCLEEP fiber layers and fiber-hydrogel scaffolds were evaluated by scanning electron microscopy (SEM) (JOEL, JSM-6390LA, Japan) under an accelerating voltage of 10 kV after sputter-coating with platinum at 10 mA for 100 s and 120 s, respectively. Both the fiber diameter and alignment of fiber were quantified by measuring 100 fibers per fiber layer. Three fiber layers were measured for both 3 min and 6.5 min spinning time. Fiber-hydrogel scaffolds that were 8.0 mm in length were cut into 2.0 mm long scaffolds to arrive at 3 different sectioning interfaces before these cross-sectional areas were examined under the SEM. To evaluate fiber density, at each 2.0 mm sectioning interface, 2 different fields of view were captured and the number of fibers in each field of view was counted (Supplementary figure 1B). Three different fiber bundles were made for both 3 min and 6.5 min spinning time and 6 different fields of view were quantified for each fiber bundle. All measurements were done with ImageJ software (NIH, USA).

2.4. Animal Care and Surgical Procedures

Eight adult female Sprague–Dawley rats (7–8 weeks, 190–250 g) were obtained from In Vivos Pte Ltd. (Singapore). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanyang Technological University, Singapore. Animals were randomly divided into two groups to receive scaffolds with 3.0 min or 6.5 min

electrospun fibers. Animals were anesthetized using intraperitoneal injection of ketamine (73 mg/kg) and xylazine (7.3 mg/kg). The surgical field was shaved and disinfected with Betadine. The skin was cut open, the muscle was cut, peeled back to the sides, and held in place. A laminectomy was performed to gain access to the T9-T10 spinal cord. A 2 mm complete transection was then made using a pair of iridectomy scissors. Thereafter, scaffolds (2.0 mm in length) were implanted into the incision site. PCL film was used to cover the injury, the muscle was sutured, and the skin was closed with auto clips. All animals were subcutaneously injected once a day with Meloxicam (1 mg/kg) for 3 days and injected with Baytril (5.0%, 0.1 mL/kg) for 10 days after surgery. Bladders were manually expressed at least twice daily until bladder function recovered.

2.5. Tissue preparation and sectioning

At 2 weeks post-injury, animals were transcardially perfused with 0.9% saline followed by 4.0% paraformaldehyde. After perfusion, spinal cords were post-fixed for 2 h at 4 °C and sequentially transferred to 15% sucrose and 30% sucrose after 24 h and 48 h, respectively. All samples were stored at 4 °C until sectioned. Spinal cord samples of 1.5 cm containing the lesion site were sectioned horizontally into 15 µm sections.

2.6. Immunohistochemistry

Immunohistochemistry was performed to evaluate axonal regrowth and host-implant reaction. All steps were done at room temperature. Spinal cord sections were treated with 0.3% Triton X-100 for 5 min and then incubated in 10% NGS blocking solution for 1 h at room temperature. Thereafter, the sections were incubated with primary antibodies diluted in 5.0% NGS overnight followed by secondary antibodies diluted in PBS for 1.5 h. Nuclear staining was performed by incubating the sections with DAPI for 10 min after the secondary antibodies. Samples were examined using a Leica DMI8 inverted microscope or Leica SP2 AOBS confocal microscope (Leica, Milton Keynes, UK). Analyses of the images were performed using Fiji/ImageJ. For all quantifications, at least three sections per animal were analyzed.

2.7. Masson's trichrome staining

Slides were post-fixed with 4% PFA for 5 min and rinsed with PBS before trichrome staining. Slides were stained in Working Weigert's Iron Hematoxylin Solution for 10 min, in Biebrich Scarlet-Acid Fuchsin for 8 min, in Phosphomolybdic Acid Solution for 5 min and in Aniline Blue Solution for 2 min. Samples were rinsed with DI water in between each step. Samples were placed in acetic acid, 1%, for 2 minutes. Finally, samples were dehydrated in 70% and then in 100% ethanol.

2.8. Quantification of cystic cavity size

Cystic cavities were identified on GFAP- and DAPI-stained tissue sections as cell-free areas in the lesion sites surrounding the scaffold. The size of the cavities was measured on images of GFAP-stained sections after thresholding, using the magic wand tool in FIJI. The sum of cavity areas on rostral and caudal sides was calculated per tissue section. Three tissue sections per animal were analyzed.

2.9. Quantification of axonal ingrowth

Axonal ingrowth into the scaffold was quantified based on immunostaining for neurofilament 200 (NF). The scaffold area was identified on brightfield images and the NF-positive area inside the scaffold area was measured after thresholding in FIJI. The percentage NF ingrowth into the scaffold was calculated as NF-positive area divided by the total scaffold area. Five tissue sections per animal were analyzed.

2.10. Quantification of glial and fibrotic scarring

For quantification of glial scar formation, the GFAP-positive proportional area in the scar areas surrounding the scaffold was measured. A region of interest (ROI) was defined within 500 μm from the glial scar border (30). The glial scar border was identified as a dense lining of GFAP-positive astrocytes in the lesion site rostral and caudal to the scaffold. A freehand line was drawn along the glial scar border, the line was then translated 500 μm in

the rostral or caudal direction (away from the scaffold), and the ROI was defined as the area between these two lines (Figure 6B). Cystic cavities were excluded from the area. The GFAP-positive area in the ROI was measured after thresholding in FIJI. The GFAP-positive proportional area was calculated as GFAP-positive area in the ROI divided by the total ROI area. Both rostral and caudal scars were quantified.

The size of the fibrotic scar was measured on Masson's trichrome stained tissue sections. The area of the collagen-rich (blue) scar tissue adjacent to the scaffold was outlined on both the rostral and the caudal side and measured using FIJI (Figure 6E).

2.11. Quantification of gap distance between fiber bundles

All quantification was done with FIJI. The gap distance between fiber bundles was quantified by first converting all polarized images of fibers into 8-bit images (Supplementary Figure 2A,B). Subsequently, background signals of these images were subtracted (rolling = 20) before applying a Gaussian blur filter (sigma= 5.0). A threshold was then applied to these images (Algorithm: Default) before using plugin (Analyze Particle; pixel size = 500 – infinity) to identify all the gaps between the bundles of fibers. All these identified gaps were then fitted into an ellipse (elliptical gap) and the minors of these ellipses were measured to obtain the gap distance between fiber bundles (Supplementary Figure 2C). The number of gaps per mm² was quantified as number of gaps per image / image size.

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To ensure the accuracy of the ellipse fitting, gap distances between fiber bundles were measured manually and compared with the minors of elliptical gaps. Five gaps of six polarized images of fibers from different animals were randomly chosen for manual quantification. The height of each gap was measured at three different locations that were spaced out in this gap. These three heights were then averaged to obtain the manually quantified gap distance of that particular gap. A comparison between the manual quantification and the minors of the elliptical gaps can be found in Supplementary Figure 2D.

2.12. Quantification of cells in each elliptical gap

All quantification was done using FIJI. DAPI images were first separated into red, green and blue channels. All subsequent image processing was done on the blue channel. These images underwent a subtraction of background signals (rolling = 20), application of Gaussian blur filter (sigma = 1.0) before the final application of a threshold (Algorithm: Default).

The elliptical gaps (as identified in subsection 2.11) were converted into a mask and then overlaid onto the corresponding DAPI images. Within each elliptical gap, the number of cells was counted with the aid of the plugin (Analyze Particle; pixel size = 150 – 300).

2.13. Quantification of NF-positive area per gap and likelihood of NF ingrowth

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All quantification was done using FIJI. NF immunofluorescence images were first separated into red, green and blue channels. All subsequent image processing was done on the red channel. These images underwent a subtraction of background signals (rolling = 50), application of Gaussian blur filter (sigma = 1.0) before the final application of a threshold (Algorithm: Default). All elliptical gaps (as identified in subsection 2.11) were converted into a mask and then overlaid onto the corresponding NF images. Within each elliptical gap, every NF-positive pixel was tabulated with the aid of the plugin (Analyze Particle; pixel size = 0-infinity) and summation of all these NF-positive pixels would reflect the NF-positive area within each elliptical gap. The likelihood of NF Ingrowth was measured as (Number of gaps with at least one NF-positive pixel) / (Total number of gaps) * 100%.

2.14. Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad Software, Inc.). All results were presented as mean $\pm$ standard deviation. The Brown-Forsythe test was used to test for equal variances while the Shapiro–Wilk test was used to test for normality. If the variances of the groups were equal and normally distributed, One-way ANOVA and Tukey's Post-hoc tests were used to compare the means of more than two samples. Where only 2 samples were compared, the Student's t-test was used. Where variances were not equal, the non-parametric tests, Kruskal Wallis and Mann-Whitney U-tests, were used. Outliers were identified using Dixon's test for a single outlier.

3. Results

3.1. Reducing electrospinning time per fiber layer reduced the fiber density, without affecting fiber diameter and fiber alignment

To change the density of PCLEEP fibers in the collagen hydrogel scaffolds, we altered the electrospinning time per fiber layer. Figures 1A,B show the morphology and appearance of electrospun PCLEEP fibers. Reducing the electrospinning time from 6.5 min to 3.0 min significantly reduced the fiber density (Figure 1C, $p < 0.001$). Electrospinning times between 3.0 min and 6.5 min did not generate significantly different fiber densities. On the other hand, further reducing the electrospinning time below 3.0 min yielded scaffolds that collapsed easily when handled and these would not be suitable for subsequent *in vivo* implantation. Electrospinning times longer than 6.5 min were not tested, as we presumed that the fibers would be too dense to allow axonal ingrowth. Thus, to test the effect of fiber density on axonal ingrowth, scaffolds with 3.0 min and 6.5 min electrospun fibers were used for *in vivo* implantation. Changing the electrospinning time per fiber layer did not affect fiber diameter or fiber alignment (Figures 1D,E).

3.2. The higher fiber density enhanced axonal ingrowth into the scaffold

To evaluate the effect of fiber density on *in vivo* axonal ingrowth, scaffolds were implanted in a 2 mm T9-10 complete spinal cord transection injury in rats. We hypothesized

that reducing the fiber density would provide more space for neurites to grow into the scaffold, which could enhance recovery after SCI. However, as shown in Figure 2, reducing the electrospinning time, and hence the fiber density, did not improve axonal ingrowth at 2 weeks post-implantation. On the contrary, the higher fiber density scaffolds showed 2.8 times more ingrowth as compared to the low fiber density group (Figure 2D).

3.3. Higher fiber spinning time resulted in a larger number of narrow gaps

To gain insight into the effect of fiber density on axonal ingrowth, scaffolds were further characterized by measuring the width of the gaps between small bundles of fibers ('gap distance') and assessing the influence of gap distance on NF ingrowth. The gap distance between the fiber bundles was measured using ellipse approximation in ImageJ (Supplementary Figure 2). As expected, a higher fiber spinning time resulted in a smaller average gap distance ($p = 0.0418$; Figure 3A). However, the fibers were not uniformly distributed in the scaffold. The gap distance distribution in Figure 3B shows that the 6.5 min spinning time resulted in a higher number of gaps with a gap distance smaller than $0.5\ \mu\text{m}$. The number of gaps larger than $2\ \mu\text{m}$ was very small and comparable between the two groups. The average number of gaps per mm^2 was also higher for the 6.5 min electrospun fibers (Figure 3C). The overall width of the scaffolds was similar between 3.0 min and 6.5 min electrospun fiber scaffolds, indicating that the lower fiber density did not cause the scaffolds to collapse after *in vivo* implantation (Figure 3D).

3.4. Axons prefer growing into small gaps

To investigate which gap distance provides the optimal environment for axonal ingrowth, we analysed the effect of gap distance on the presence of NF-positive axons in the scaffold. The likelihood of NF ingrowth (percentage of gaps with NF-positive signal) was slightly higher for the gaps in the 6.5 min spinning time fibers but the difference was not significant (Figure 4A; $p = 0.686$). To understand which gap size supports the most efficient NF ingrowth, the percentage NF-positive area per gap was plotted against the gap distance for all gaps (Figure 4B). If the percentage NF-positive area per gap represents the efficiency of the gap to support axonal ingrowth, Figure 4B clearly shows that smaller gaps ($< 1 \mu\text{m}$) provide the optimal environment for axonal regeneration, independent of fiber spinning time. To confirm this relationship between gap distance and axonal ingrowth, a simple linear regression was calculated for each animal (lines in Figure 4B). The slope of the regression line was negative for 7 out of 8 animals, which supported the observation that the smaller gap distances were correlated with higher NF ingrowth.

3.5. High fiber density limited blood vessel ingrowth but did not affect cell infiltration

To test the effect of fiber density on blood vessel ingrowth at 2 weeks after scaffold implantation, the number of blood vessels was quantified on RECA-1 immunostained scaffold sections (Figure 5A). RECA-1 positive cells clearly formed tubes with a lumen, which shows

that they not only infiltrated the scaffold, but also formed blood vessel-like structures (Supplementary Figure 4). The scaffolds with the higher fiber density contained a slightly smaller number of blood vessels, but the effect was not significant ($p=0.40$; Figure 5B).

Cell infiltration into the gaps between the fibers was analysed based on DAPI staining (Figure 5C). Although the longer electrospinning time resulted in smaller gaps, cell infiltration was similar in both groups (Figure 5C, D).

3.6. The higher fiber density scaffolds showed reduced cavity formation, glial and fibrotic scarring

Scarring and cavity formation were analyzed to evaluate the effect of fiber density on host-implant integration. Two weeks after implantation of the scaffold, a clear GFAP-positive glial scar was formed at the injury site (Figure 6A,B). The extent of the glial scar was measured based on the expression of the reactive astrocyte marker GFAP (Figure 6B; (30)). Scaffolds with a higher fiber density showed a significant reduction in glial scarring at the rostral side of the scaffold (Figure 6B, $p = 0.0304$). Between the glial scar and the scaffold, a fibrotic scar composed of collagen was observed (Figure 6D,E). A trend of smaller fibrotic scars was seen for the 6.5 min spinning time (Figure 6F; $p = 0.398$ for rostral and $p = 0.469$ for caudal side). GFAP-positive cells were readily observed in the fibrotic scar area (Figure 6A). However, at 2 weeks after implantation, we did not observe GFAP-positive cells within the scaffold.

To test the effect of the fiber density on the formation of cavities around the scaffold, the size of cystic cavities in the scar area was measured. A trend of smaller cavities was seen for the 6.5 min spinning time scaffolds as compared to the 3.0 min spinning time. (Figure 6G-I; $p = 0.724$ for rostral and $p = 0.701$ for caudal side).

4. Discussion

In recent years, biomaterial scaffolds containing aligned fibers have become a promising approach to improve neural regeneration after spinal cord injury (31). In particular, aligned fibers enhanced regeneration by guiding neurite growth along the fibers (2,14–16). Electrospinning is a popular method for generating polymeric fibers because of advantages like the ability to control the fiber diameter, alignment and porosity.

Previous research has focussed on optimizing scaffold characteristics like scaffold materials, fiber diameter, porosity, mechanical properties, degradation rate and biocompatibility. In this paper, we investigated the influence of fiber density on axon regeneration *in vivo*. In our previous work, we used aligned nanofiber scaffolds with a fiber density of $1.37 \times 10^5 \pm 755$ fibers/mm² and found that higher fiber densities hindered cell infiltration (14). Further optimization of the electrospinning process led to the upper limit of 6.5 min spinning time we used in this study. We hypothesized that reducing the fiber density would enhance axonal ingrowth into fiber-hydrogel scaffolds by providing more space for axons to grow. However, we found the opposite effect. In our experiments, the higher fiber

density showed more axonal ingrowth. Although this effect did not show high statistical significance (likely due to our small samples sizes), our results converge on the idea that axons prefer growing into scaffolds with a higher fiber density and thus smaller gaps between the fibers.

The likelihood of axonal ingrowth in the gaps between the fiber bundles was slightly higher in the high fiber density scaffolds (6.5 min fiber spinning time) as compared to the lower fiber density scaffolds (3.0 min spinning time) (Figure 4A). Combined with a higher number of gaps in these scaffolds, (Figure 3C), the high fiber density substrates showed a higher overall axonal ingrowth (Figure 2D).

One of the animals in the 3.0 min group was excluded from the axonal ingrowth analysis (Figure 2D) because it was an outlier ($p = 0.023$; Dixon's test for a single outlier). We believe that the outlier may have been caused by compression of the scaffold during implantation. Although all scaffolds were the same width before implantation, after sacrifice, the scaffold width in this animal was smaller than in the other animals in this group. Likewise, the average gap size was also smaller. Compression of the scaffold could have altered the fiber density, which could have affected NF ingrowth. Therefore, we believe this data point was unreliable and since it was an outlier statistically, it was removed.

The fibers were not uniformly distributed in the scaffold. Because of this nonuniform distribution of the fibers and gaps, we chose to analyse the effect of individual gap distances on neurite ingrowth. We found that axonal ingrowth was most efficient if the gaps between

the fibers were narrow (Figure 4B). This shows that the fibers in these scaffolds are not just aggregating together to form big bundles with large gaps in between. Instead, they do form small gaps where axons can grow.

A simple model illustrating the relationship between gap distance and % NF-positive area per gap is shown in Supplementary Figure 3. If the gap distance increases, but the number of axons in the gap remains the same, then the % NF-positive area decreases (curve 1, negative slope). In this case, a large number of small gaps would be most effective to support axonal ingrowth in the scaffold. If the gap distance increases, and the number of axons increases to the same degree, the % NF-positive area in the gap remains constant (curve 2, flat slope). This would imply that all gap sizes are equally efficient in supporting axon ingrowth. If the gap size increases, and the % NF-positive area increases to a greater degree, then larger gaps support proportionally more axonal ingrowth, thus scaffolds with larger gaps would be more beneficial (curve 3, upslope). This would suggest that axons prefer to grow in bundles, rather than individually. Based on the regression analysis in Figure 4B, our results match situation 1. Thus, optimal axonal growth would be obtained by providing a large number of small gaps.

Axons in the spinal cord show a broad range of diameters, depending on the species studied, location in the spinal cord, and the thickness of the myelin sheath (32). The smallest unmyelinated axons in the spinal cord have diameters smaller than 0.1 μm , while the largest myelinated axons can measure up to 20 μm in diameter. However, in rats as well as in

humans, large diameter axons represent only a small fraction of all axons in the spinal cord and the majority of axons are smaller than 1 μm in diameter (32). This means that the majority of axons would fit nicely in the small gaps ($< 1 \mu\text{m}$) between the fibers in our scaffolds. Because the distribution of fibers in the scaffold is not homogeneous, larger axons could still find space to grow in areas where the gaps between the fiber bundles are larger.

Besides fiber density, the amount of topography could also be an important factor for axonal regeneration. Indeed, a higher fiber density implies more topography. Previous studies have demonstrated the effects of topography on axonal growth (17,18,33). In this study, we focussed on the effect of the amount of space between the fibers, even though the amount of topography may have played an important role in axon guidance and regeneration.

In addition to enhancing axonal ingrowth, scaffolds with the higher fiber density also tended to reduce glial and fibrotic scarring and cavity formation at the lesion site. Although these results were not significant, they show a promising and consistent trend. Further studies with larger sample size could provide more insight to the effect of fiber density on axonal ingrowth and host-implant integration.

To support neural regeneration in the long term, scaffolds should allow the ingrowth of blood vessels for the supply of oxygen and removal of waste products. Since majority of the gaps between the fibers in our scaffolds were smaller than 1 μm and the smallest capillaries have a diameter of 5-10 μm (34), the high fiber density may have restricted blood vessel ingrowth into the scaffold. Indeed, we observed a lower number of blood vessels in

scaffolds with the higher fiber density (Figure 5B). Using biodegradable fiber materials could help to create more space for blood vessel formation after axons have grown into the scaffold. The fiber material used in this study, PCLEEP, is a biodegradable polymer. Our previous studies have shown that PCLEEP was still present without significant changes even at 3 months after *in vivo* implantation in a rat model for peripheral nerve-injury treatment (33). However, in the presence of protein-encapsulation, the electrospun fibers completely degraded within 3 months *in vivo*. Further studies should investigate the fiber degradation rate in more detail. This may be done by measuring molecular weight changes of PCLEEP polymer by gel permeation chromatography, after retrieving implants from *in vivo* samples. To balance between the requirement of higher fiber density for robust axonal ingrowth and lower fiber density for more extensive blood vessel penetration, optimization of fiber degradation rate using a faster degrading material may be beneficial and should be explored in future studies.

Several previous studies have attempted to increase the open space in the scaffold in order to improve axonal ingrowth (23–25). These studies concluded that scaffolds containing microchannels with diameters around 100 μm were most suitable for the regeneration of neurons (9). In marked contrast, our results indicate that neurons prefer to grow in very narrow gaps of 1 μm diameter or less. Since the measurement of gap distance in our study was based on 2D images of a 3D scaffold, the actual space available for axons may have been larger than represented. Nevertheless, further research is necessary to compare the effect of large channels versus fiber-hydrogel scaffolds on neural regeneration. We speculate that the

high fiber density scaffolds in our study provide more opportunities for the axons to interact with the fibers and thereby enhanced axon guidance and growth.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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Figure legends

Figure 1. Reducing the electrospinning time per fiber layer reduced the fiber density of fiber-hydrogel scaffolds without affecting fiber diameter and fiber alignment. (A) Cross-sectional SEM images of fiber-hydrogel scaffold. High magnification field of view on the right taken from the center of scaffold (red box). Scale bars in main images are 1 mm, scale bars in high magnification images are 100 μm . (B) SEM images of fiber layers. Scale bars in images on the left are 10 μm , scale bars in images on the right are 20 μm . (C) Quantification of fiber density. The number of fibers per mm^2 was 2177 ± 172 and 5279 ± 531 for 3.0 min and 6.5 min spinning time, respectively. *** $p < 0.001$; Student's t-test. (D) Quantification of fiber

diameter ($p = 0.235$; Mann-Whitney). (E) Quantification of fiber alignment ($p = 0.0653$; Mann-Whitney). Ns, not significant.

Figure 2. Effect of fiber spinning time on axonal ingrowth into the scaffold. (A)

Brightfield images showing scaffold structure 2 weeks after implantation in the rat spinal cord. Scale bars, 1 mm. (B) Immunofluorescent staining of axonal ingrowth (NF, neurofilament) into scaffolds at 2 weeks post-SCI. Scale bars, 500 μm . Dotted line, tissue-scaffold interface. (C) NF-staining merged with brightfield images show axons growing in between the fibers. Scale bars, 100 μm . (D) Quantification of NF ingrowth into the scaffold. Average NF ingrowth into the scaffold was $1.26 \pm 0.22\%$ for 3.0 min spinning time and $3.52 \pm 1.40\%$ for 6.5 min spinning time ($p = 0.0506$; Welch's t-test; 3 animals in the 3.0 min group and 4 animals in the 6.5 min group, 3 to 5 sections per animal).

Figure 3. Effect of fiber electrospinning time on gap distance between the fibers.

Effect of fiber electrospinning time on gap distance between the fibers. (A) The longer electrospinning time resulted in a smaller average gap distance between the fibers. The average gap distance was $0.775 \pm 0.163\ \mu\text{m}$ for 3.0 min and $0.571 \pm 0.025\ \mu\text{m}$ for 6.5 min electrospun fibers ($*p = 0.0481$; Student's t-test. $n = 4$ per group with at least 100 gaps per n analysed). (B) Gap distance distribution for 3.0 min and 6.5 min spinning time groups. The 6.5 min spinning time resulted in a higher number of gaps smaller than 0.5 μm . (C) Effect of fiber

electrospinning time on the number of gaps per mm² ($p = 0.258$). (D) Effect of fiber electrospinning time on scaffold width ($p = 0.855$).

Figure 4. Influence of gap distance on axonal ingrowth. (A) Effect of fiber electrospinning time on likelihood of NF ingrowth. Likelihood of NF ingrowth was defined as (Number of gaps with NF+ signal) / (Total number of gaps) * 100%; Likelihood of NF ingrowth was $16.2 \pm 3.8 \%$ for 3.0 min spinning time versus $18.7 \pm 6.9 \%$ for 6.5 min spinning time. $p = 0.686$; Mann-Whitney test; $n = 4$ per group with at least 100 gaps per n analyzed. (B) Influence of gap distance on the NF-positive area per gap, normalized to gap area. Each dot represents one gap. Different colors represent different animals. For both groups, narrower gaps show a higher proportion of NF-positive area. Colored lines show linear regression for each animal.

Figure 5. Influence of gap size on blood vessel ingrowth and cell infiltration into the scaffold. (A) Immunofluorescent images of blood vessels (RECA-1, green) growing into the scaffold at 2 weeks post-SCI. Examples of RECA-1-positive blood vessels in the scaffold are indicated with white arrows. Dotted line, tissue-scaffold interface. Scale bars, 100 μm . (B) Effect of electrospinning time on the number of blood vessels in the scaffold ($p=0.400$; Student's t-test). (C) DAPI staining merged with brightfield images showing cell infiltration into the scaffold. Scale bars, 50 μm . (D) Effect of electrospinning time on cell infiltration. Although a shorter electrospinning time resulted in larger gaps, the average number of cells

per gap was not significantly different between groups ($p > 0.999$. Mann-Whitney test. $n = 4$ per group with at least 100 gaps per n analyzed).

Figure 6. Effect of fiber density on host-implant integration. (A) GFAP immunofluorescent staining of spinal cord sections at 2 weeks post injury show glial scar formation at the lesion site. Blue dotted line, tissue-scaffold interface. (B) Method for quantification of glial scarring. A line was drawn along the dense, continuous layer of GFAP+ astrocytes ('glial scar border') in FIJI. This line was translated 500 μm away from the scaffold (yellow arrow) and the area between the 2 lines was defined as glial scar ROI (white dashed lines). Glial scarring was quantified by measuring the percentage of GFAP+ area inside the ROI. Blue dashed line, tissue-scaffold interface. (C) Quantification of glial scar formation (* $p = 0.0304$ for rostral and $p = 0.945$ for caudal side). (D) Masson's trichrome staining shows fibrotic scarring. Orange dotted line, tissue-scaffold interface. (E) The fibrotic scar area (yellow dotted line) was defined as the collagen-rich (blue) area between the scaffold and glial scar region. (F) Quantification of fibrotic scarring ($p = 0.398$ for rostral and $p = 0.469$ for caudal side). (G) DAPI merged with GFAP immunofluorescent staining revealing the cell-free areas in the scar. Orange dotted line, tissue-scaffold interface. (H) Cystic cavity areas were measured as the sum of cell-free areas in the glial and fibrotic scar areas. (I) Quantification of cystic cavity formation ($p = 0.724$ for rostral and $p = 0.701$ for caudal side). Student's t-test with 4 animals per group, 3 sections per animal. Scale bars, 500 μm .

A
3.0 min **B** **C** **D** **6.5 min**

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A

B

C

D

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