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Molecular Sequelae of Topographically Guided Peripheral Nerve Repair

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Abstract

Peripheral nerve injuries cause severe disability with decreased nerve function often followed by neuropathic pain that impacts the quality of life. Even though use of autografts is the current gold standard, nerve conduits fabricated from electrospun nanofibers have shown promise to successfully bridge critical length nerve gaps. However, in depth analysis of the role of topographical cues in the context of spatio-temporal progression of the regenerative sequence has not been elucidated. Here, we explored the influence of topographical cues (aligned, random, and smooth films) on the regenerative sequence and potential to successfully support nerve regeneration in critical size gaps. A number of key findings emerged at the cellular, cytokine and molecular levels from the study. Higher quantities of IL-1 α and TNF- α were detected in aligned fiber based scaffolds. Differential gene expression of BDNF, NGFR, ErbB2, and ErbB3 were observed suggesting a role for these genes in influencing Schwann cell migration, myelination, *etc.* that impact the regeneration in various topographies. Fibrin matrix stabilization and arrest of nerve-innervated muscle atrophy was also evident. Taken together, our data shed light on the cascade of events that favor regeneration in aligned topography and should stimulate research to further refine the strategy of nerve regeneration using topographical cues.

Keywords

Peripheral nerve regeneration; Topography; Extracellular matrix; Growth factors; Electrospun nanofibers; Schwann cell migration

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ELECTRONIC SUPPLEMENTARY MATERIAL

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INTRODUCTION

Peripheral nerve injuries lead to long term disability and decreased function in approximately 2.8% of patients.⁵ This is often followed by neuropathic pain which significantly impacts the quality of life of individuals suffering from peripheral nerve injuries. Even though there have been considerable advances made in microsurgical techniques, bridging of long peripheral nerve gaps remains a continuing clinical challenge. Autografts are the current standard for the treatment of long gaps, but several drawbacks of using autografts limit their use. The use of autografts can lead to donor site complications such as sensory defect, scar, pain as well as the need for multiple surgeries if the gaps are long.²⁵ Furthermore, due to modality mismatch, mismatch of dimensions between graft and host, functional recovery close to pre-injury levels is difficult to achieve. These limitations of autografts have led to efforts in finding alternatives to bridging peripheral nerve gaps.

Nerve conduits have been fabricated from biological materials such as veins,^{33,44} ECM proteins such as collagen¹⁸ as well as synthetic materials such as PCL,³⁷ PGA,²³ and PLGA.⁶ These guidance channels have had some success in approaching autograft performance over short gaps and in promoting nerve bridging by facilitating the formation of a provisional fibrin matrix which coalesce from the plasma entering the conduit space.⁵² Glial cells, Schwann cells (SC) and fibroblasts migrate along this provisional matrix laying down their own ECM. This enriched matrix guides axons along with SC facilitating the bridging of the nerve gap. Axons infiltrate the denervated distal stump and eventually make neuromuscular junctions with the target muscle.⁵ In larger gaps, the provisional matrix fails to coalesce and is not infiltrated by the supporting cells, further it loses structural integrity leading to failure in regeneration.⁴

Luminal fillers have been used to extend the bridging capacity of guidance channels.^{8,21,30} Several designs that replicate or replace the function of the initial provisional matrix have been explored to augment regeneration. Cellular fillers such as SC,²⁹ and genetically modified SC,^{20,24} structural fillers such as, micro channels,^{26,42} micro filaments,^{4,49,50} electrospun fibers,^{9,14,19} and neurotropic factors such as NGF,²⁸ BDNF,⁴⁵ NT-3⁴⁰ have been used individually and in combination to promote nerve bridging. In most studies, these techniques have been optimized *in vitro* for axonal growth and in non-critical gap *in vivo* models (gaps below 13 mm in rats). However, the extent to which these interventions interact and influence the molecular regenerative sequence of events is not completely understood.

We have previously shown that aligned electrospun nanofibers were able to successfully bridge the critical length nerve gap without the need of any exogenous factors using only 0.5% of the total volume of the guidance channel. Thus, aligned topographical cues were able to bridge a critical length nerve gap.^{9,19} Other studies have also shown the benefits of topographical cues in nerve regeneration.^{15,17,31,48} While a single film is known to be able to support cell migration and axonal bridging, the exact sequence of the regenerative mechanism still remains unknown. It is believed by several experts in the field of peripheral nervous system (PNS) repair that a thorough understanding of the mechanism(s) and the

way in which topography of scaffolds embedded within the nerve guidance channels guide the regenerative sequence is key to the design of next generation PNS repair scaffolds.

We explored the influence of topographical cues using three different types of scaffolds (aligned, random, and polymer smooth films) on the regenerative sequence in critical size gaps and assessed their potential to promote efficient nerve regeneration. While we have previously demonstrated the efficacy of topographical cues provided by electrospun fibers in bridging nerve gaps, the spatio-temporal sequence of events which contribute to that outcome have not been reported yet. In this study, we determined the influence of topographical cues using three different types of scaffolds (aligned, random, and smooth films) on the spatio-temporal progression of the regenerative sequence by assessing the role played by molecular markers such as growth factors, ECM proteins, and neuronal receptors in critical size gaps and assessed their potential to promote efficient nerve regeneration.

MATERIALS AND METHODS

Fabrication of Thin-Film Based Nerve Guidance Scaffolds

Fabrication of Aligned, Random, and Polymer Smooth Thin-Films—Polymer solutions (7%) were made by dissolving poly(acrylonitrile-co-methylacrylate) (PAN-MA) (Sigma, MW 8000) in *N,N*-dimethylformamide (DMF) at 60 °C. For electrospinning, the solution was pumped through a syringe at a rate of 1 mL/h at a voltage of 6–10 kV. The polymer stream was directed at an aluminum foil-covered metal drum rotating at 2400 rpm for 15 min in order to produce aligned fibers. A flat copper plate (McMaster Carr) was used as a collector to generate random fibers. A 2% solution of the same polymer prepared in DMF was casted on a glass coverslip to obtain smooth films with the same chemistry. The diameter of the fibers was characterized using scanning electron microscopy (S-800 SEM, Hitachi) and quantified using Image-Pro software (Media Cybernetics).

Assembly of Tubes for Implantation—Polysulfone nerve guidance channels (Koch Membrane Systems) were used as a carrier for the aligned fiber film scaffolding. Polysulfone tubes have a molecular weight cutoff of 50 kDa and allow for transfer of nutrients and gases while protecting the regenerating nerve against the immune cells. The semi permeable polysulfone tubing (inner diameter: 1.6 mm, outer diameter: 2.2 mm, molecular weight cutoff: 50 kDa) was first cut into tubes of 17 mm length, (to allow for a 15 mm gap plus 2 extra mm to allow for 1 mm of both the proximal and distal stump to be pulled into the scaffold during suturing.) The polysulfone tubing was halved lengthwise using a custom-machined aluminum template. Under a fabrication microscope, a 2.2 mm wide thin-film strip was fixed on one of the halved tubes, using medical grade UV light curing adhesive (1187-M-SV01, Dymax). To complete the scaffold, the other half of the tube was glued on top. Schematic diagrams of these tubes are shown in Fig. 1.

The scaffolds were sterilized by immersing in 70% ethanol for 30 min followed by washing in sterile diH₂O and overnight exposure to UV light. These scaffolds were stored in sterilized phosphate buffered saline (PBS) until surgical implantation.

In vivo Implantation

Adult Lewis male rats (250–300 g) were induced to anesthetic depth with inhaled isoflurane at 3–4%. Throughout surgery, the animals were maintained at 1.5–2% isoflurane. Microscissors were used to transect the tibial nerve branch, and the nerve stumps were pulled 1 mm into each end of the 17 mm (aligned, random, and polymer smooth film) guidance scaffolds (leaving a 15 mm gap) and fixed into place with a single 10–0 nylon suture (Ethicon). The muscles were reapposed with 4–0 vicryl sutures (Ethicon) and the skin incision was clamped shut with wound clips (Braintree Scientific). After the surgery, the rats were placed under a warm light until sternal, and then housed separately with access to food and water ad libitum at constant temperature (19–22 °C) and humidity (40–50%) on a 12:12 h light/dark cycle. To prevent toe chewing, a bitter solution (Grannick's Bitter Apple) was applied twice daily to the affected foot. Marcaine (0.25% w/v) was administered subcutaneously for post-surgical pain relief (0.3 mL/animal). Animals were maintained in facilities approved by the Institutional Animal Care and Use Committee (IACUC) at the Georgia Institute of Technology and in accordance with the current United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health regulations and standards.

Evaluation of Nerve Regeneration

Experimental Conditions—Nerve regeneration was evaluated at 5 days, 3 weeks, and 22 weeks post-implantation (Table 1). Aligned, random, and smooth thin-film based scaffolds were implanted for each time point. The 5 day (4 animals/condition) was chosen to capture the initial biochemical milieu within the scaffolds. During this time point, we were interested in the localization of fibrin throughout the guidance channel in response to the different topographical cues. Also, in order to determine the distance migrated by SC, it is important that two SC populations (proximal and distal) remain distinct. Previous work from our lab has shown that in a critical length gap, the 3 week time point was appropriate to quantify proximal and distal SC migration into the conduits. We implanted 4 animal/condition for the 3 week time point. The 22 week time point (8 animals/condition) was chosen to allow for functional muscular reinnervation as well as to assess mature cable formation.

Electrophysiology—In the 22 week groups, electrophysiological measurements were taken of nerve conduction velocity (NCV) through the regenerated nerves. NCV measurements are positively correlated with the size of myelinated axons and degree of myelination. Measurements were taken essentially as described previously.⁹ Briefly, the site of nerve injury was exposed, and two pairs of stainless steel bipolar hook electrodes were positioned on the nerve. One pair of electrodes was placed on the sciatic nerve, 10 mm proximal to the implanted guidance channel, and the other pair was placed 10 mm distal to the implanted guidance channel. A stimulator (Model S88, Grass Technologies) and stimulus isolation unit (Model SIU5B, Grass) was used to apply supramaximal square voltage pulses of 100 μ s duration at a rate of 1 Hz, through the distal pair of electrodes. The evoked compound nerve action potentials (CNAPs) were recorded upstream from the proximal pair of electrodes.

Nerve Conduction Velocity—The recorded signals were filtered and amplified (Model 1700, A-M Systems) and digitally sampled at 25 kHz. (Multichannel Systems DAQ card). The recordings were averaged, and the latency of the onset of the evoked CNAP was determined off-line. The distance between the stimulating and recording electrodes divided by this latency value was employed to calculate the conduction velocity of the CNAPs through the regenerated nerves.

Relative Gastrocnemius Muscle Weight Measurement—To evaluate the change in muscle weight due to the presence of different topographical thin-films, we ex-planted the medial gastrocnemius muscle from the operated and the contralateral side at the end of the 22-week study. Briefly, a longitudinal cut was made in lower leg, parallel to the Achilles tendon and gastrocnemius muscle. Insertions were made in the femoral area and at the heel through the Achilles tendon and the gastrocnemius muscle was explanted. The soleus muscle was excluded from the evaluation. Medial gastrocnemius muscles (GMW) were weighed and normalized against the contralateral muscle weight (CMW). The relative gastrocnemius muscle weight (RGMW) was calculated using the following equation:

$$\text{RGMW} = \frac{\text{GMW}}{\text{CMW}} \times 100\%$$

Muscle Atrophy—The medial gastrocnemius muscle from rats was explanted, fixed with 4% paraformaldehyde for 1 h, soaked in a 30% sucrose solution overnight, and then frozen in Tissue-Tek Optimal Cutting Temperature compound (Sakura Finetek USA Inc., Torrance, CA) at -80°C . Transverse sections of the muscle ($10\ \mu\text{m}$ thick) were obtained by cryosectioning the area of the muscle where the tendon of origin overlap. The muscle sections were then stained with Masson's trichrome stain using the following method. First, the sections were rinsed in distilled water and then submerged in Bouin's solution for 1 h at 56°C . Then the sections were thoroughly washed in tap water, to remove yellow coloration, followed by a rinse in distilled water. Next, the sections were placed in Weigert's hematoxylin for 90 s, followed by washing in running tap water for 5 min and then a rinse with distilled water. Then the sections were placed in Biebrich's scarlet-acid fuchsin for 5 min, followed by a rinse with distilled water. Next, the sections were placed in phosphomolybdic-phosphotungstic acid for 90 s. Then the sections were stained in aniline blue for 5 min, followed by rinsing with 1% acetic acid solution for 5 min. Finally, the sections were dehydrated first in 95% alcohol and then 100% alcohol, twice each, cleared with three changes of xylene, and mounted with Cytoseal 60 (Richard-Allan Scientific; Kalamazoo, MI). The sections were imaged using Axioskop 2 plus microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Image-Pro Plus 7.0 (Media Cybernetics; Bethesda, MD) was used to quantify the area stained blue (representing collagen), which in turn will allow estimation of the percent of sampled muscle area that stained blue.

Immunohistochemical Analysis—At the end of the prescribed regeneration times, guidance channels were explanted for histological analysis of nerve regeneration. Guidance channels from the 5 days, 3 weeks, and 22 weeks regeneration group were sectioned transversely at a thickness of $10\ \mu\text{m}$ and reacted with immunofluorescent markers to quantify

the different steps of regeneration. Explants were fixed in 4% paraformaldehyde in PBS (Sigma-Aldrich), washed, and stored in 30% sucrose in PBS for 24 h. Samples were embedded in O.C.T. gel (Tissue Tek) and frozen for cryosectioning (CM30505, Leica). Using techniques previously described,¹⁹ the sections were reacted for immunofluorescent demonstration of markers.

Extent of fibrin cable formation and macrophage infiltration within each scaffold were qualitatively determined using immunofluorescence. Fibrinogen antibody (Dako) staining was used to double stain for fibrin and Macrophages (ED-1, SeroTec) to evaluate the formation of provisional matrix and localization of macrophages that occurs during early regeneration (5 days). The fibrin cables in each scaffold type were characterized qualitatively in terms of whether a fibrin matrix forms and whether the matrix is continuous through the length of the nerve gap, and how it is affected by different topographies.

At 3 and 22 weeks, transverse cryosections were taken at regular intervals through the scaffold and immunostained. To stain the scaffolds for axons, SC, and fibroblasts, the following protocol was used: Sections were immunostained for markers of (1) regenerated axons (NF160, Sigma-Aldrich); (2) SC, (anti-S-100, Dako); (3) myelin (P0, Chemicon Intl.); (4) macrophages (ED-1, CD-68, Serotec); (5) fibroblasts: double stain of Vimentin (Sigma-Aldrich) and S-100 (to help differentiate non-specific staining of SC). Sections were double stained with the following secondary antibodies: Goat anti-rabbit IgG Alexa 488/594, and goat anti-mouse IgG1 Alexa 488/594 (Sigma-Aldrich), using the protocol as described previously.¹⁹ Briefly, sections were incubated for 1 h at room temperature in a blocking solution of 4% goat serum (Gibco) in PBS, incubated overnight at 4 °C in a mixture of primary antibody and blocking solution, washed and incubated for 1 h at room temperature in a solution of secondary antibody mixed in 0.5% triton in PBS. Slides were washed once more, then dried and cover slip mounted before evaluation.

Transverse cryosections were used to quantitatively evaluate the migration of SC and axonal growth into the scaffolds. At the 3 week time point, the distance that the migration front of each cell type penetrates into the scaffold was quantified. The migration fronts of SC were assessed using transverse sections taken at regular intervals through the scaffolds, with a resolution of at least 1 mm. This analysis was performed using ImagePro software. The penetration of the axonal front was quantified as well. In the 22 week study, regenerated cable area of SC and axons were quantified using Image Pro. The percentage of the cable area comprised of S100 positive SC as well as NF160 positive axons were quantified to evaluate how the presence of different topographical cues lead to a differential formation of the regeneration cable.

Trichrome Staining—Procedure similar to the one previously described to stain muscles was employed to stain for the presence of collagen in explanted nerve scaffolds. Collagen distribution throughout the scaffold in response to topographical cues was qualitatively determined.

Myelin Thickness, Axonal Diameter and Myelinated Axon Count—One millimeter long sections of regenerated nerve were removed from the center of each scaffold

in the 22 week study. These explants were resin embedded, cut into 1 μm thick slide mounted cross-sections, and stained with toluidine-blue. Images of regenerated nerve cross-sections were viewed under a Nikon Eclipse 80i light microscope (Japan), using a 100 $\times$ oil-immersion objective lens. A 40 $\times$ montage image was compiled for each nerve, using a Neurolucida® system (MBF Bioscience, Williston, VT), coupled to a mechanized stage and MicroFire™ camera (Optronics, Goleta, CA).

Myelinated axons were visually counted from these 40 $\times$ montages, using Image-Pro® Plus software (Media Cybernetics, Inc., Bethesda, MD). Myelin thickness and axonal diameters were quantified by sampling subsets of the entire axonal population. A 25 $\times$ 25 grid was overlaid above each image, using Adobe® Photoshop® CS3 (San Jose, CA), and a cell in the upper left-hand portion of the image containing myelinated axons was selected, and grid cells spaced evenly apart from the initially selected cell were chosen for analysis. Between 5 and 25% of the total myelinated axon population was analyzed from each nerve cross-section. Image-Pro® Plus software was used to measure myelin thickness and axonal diameter of myelinated axons. Axonal diameters were calculated using a circle-equivalent technique, in which the cross-sectional area of each axon was measured and then converted into an axonal diameter using a formula that assumes a circular cross-section.

Molecular Analysis of Explanted Nerve

Tissue Collection for Molecular Analysis—For tissue collection, animals were anesthetized using isoflurane and euthanized by cutting the heart at 5 days and 3 weeks post-surgery. Nerve conduits were explanted from each animal and the conduits were cleaned to remove tissue on the outside. All the samples were collected in 1.5 mL polypropylene tubes (RNase, DNase, and pyrogen free) and were placed in dry ice immediately after collection. Finally the nerve conduits were stored at -80°C for further protein and RNA extraction.

Cytokine Array for 5 Day Samples—Conduit from both the proximal and distal end (1 mm) were precisely cut using titanium blades to leave 15 mm conduit loaded with thin film based scaffolds. An empty scaffold was also used in this study as a control to test the recruitment and production of cytokines in response to topography. The conduits were further cut into three pieces of 5 mm each to evaluate the spatial distribution of cytokines. RIPA buffer (Sigma) (200 μL) was used to extract the tissue within each region of the conduit. The conduit was homogenized using Bullet Blender (Next Advance) at 4 $^{\circ}\text{C}$ followed by 10 min centrifugation at 10,000 rpm. The supernatants were stored at -80°C until ready for cytokine multiplex array analysis.

A 9-bead based immunoassay kit for rat cytokines (BIO RAD) was used to detect the concentration of IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, granulocyte-macrophage colony stimulating factor, interferon γ and TNF- α . Protocol provided by the manufacturer was used to quantify the amount of cytokines present in different areas of the conduits which were loaded with thin films as well as an empty tube. Multi wavelength fluorescence was determined with a luminometer (BIO RAD). Concentrations of cytokines that were outside the range of the standard curve were not reported as the concentrations of these samples were observed to be below the detection limit of the assay kit.

qRT-PCR for 3 Week Samples—To evaluate the effects of thin films of different topographies on nerve repair, we evaluated the gene expression of various growth factors and receptors that play a key role in the regenerative sequence. Similar protocol as described for the 5 day study was also used for the 3 week samples. Instead of cutting the conduits in thirds, the conduits were cut in half following the removal of 1 mm from both the proximal and distal end of the conduits. Total RNA was extracted from each half of the nerve conduit using Qiagen RNEasy Midi-Kit (Qiagen). Briefly, aligned, random, and smooth films based scaffolds, as well as empty tubes were explanted and cut in half. The tubes were homogenized using the Bullet Blender (Next Advance) and the lysis buffer provided in the RNEasy Midi-Kit. Samples were centrifuged and the supernatant was processed to extract the RNA.

Total RNA (5 μ g) was converted to cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems). PCR primers for BDNF, NT-3, FN, LN-1, ErbB2, ErbB3, NRG1, NGFR, and the housekeeping gene GAPDH were designed using Primer Express software (Applied Biosystems) and obtained from Integrated DNA Technologies. qRT-PCR and primer validation were performed on StepOnePlus real-time PCR machine (Applied Biosystems) using SYBR green mix. Fold differences were calculated using the comparative CT method and normalized to empty tubes.

Fiber Permeability

Constructs were created to test the ability of the thin films to allow for exchange of biomolecules. 5 mg/mL solutions of 500 and 70 kDa FITC-dextran (SIGMA) were added to the top chamber, while 1 $\times$ PBS was added to the bottom chamber to facilitate diffusion. Samples were collected from the bottom chamber and analyzed by using the spectrophotometer.

Statistical Analysis

ANOVA, combined with Tukey post hoc tests, was used to calculate the significance of differences between mean values. A *p* value <0.05 was considered statistically significant.

RESULTS

Histological Analysis of Regenerated Axons and Schwann Cell Migration (Regenerating Cable at 3 and 22 Weeks Post Implantation)

Axonal Growth and Schwann Cell Migration 3 Weeks Post Implantation—Three types of thin film conduits/scaffolds were examined for their potential in supporting nerve cell regeneration: (a) aligned fiber, (b) random fiber, and (c) polymer smooth film (Fig. 1a). We observed that fiber based scaffolds were permeable to proteins up to 500 kDa while smooth films were impermeable to both 70 and 500 kDa proteins (Figs. 1b and 1c). To monitor the extent of infiltration in scaffolds by neuronal cells during regeneration, the SCs were stained for S100 and axons for NF160. This staining also revealed the shape of the regenerating cable. Examination of the regenerating cable at the proximal end (first third) of the conduit showed that the regenerating cable was different in fiber based scaffolds vs. smooth film based scaffolds. Formation of a single cable was seen around the fiber-based

scaffolds while separate nerve cables were observed on either side of the smooth-film based scaffold. In the case of aligned and random fibers, neuronal cells were physically concentrated around the thin films whereas in the case of the smooth films the regenerating cable remained distinct and neither SCs nor axons were interacting with the smooth films. Further, in all the three conditions tested the regenerating cable showed the presence of S100-positive SCs and NF160 positive axons (Fig. 2). In the final third of the scaffold, the morphology of the regenerating cable was the same as the one observed at the proximal end. The migration of SCs and growth of axons from the proximal end of the scaffold were quantified (Figs. 3a and 3b). SC migration and axonal growth were significantly higher ($p < 0.01$) in aligned fiber based scaffolds compared to smooth films and random fiber based scaffolds. Also, the aligned fiber based scaffolds promoted the axonal growth up to 13 mm in a 15 mm nerve conduit whereas the smooth films and random fibers supported infiltration of axons only up to 11 and 9 mm, respectively (Fig. 3b).

Axonal Growth and Schwann Cell Migration 22 Weeks Post Implantation—

Long term influence of topographical guides on SC and axon maturation within the nerve conduit were qualitatively and quantitatively assessed 22 weeks post implantation. A distinct regenerating cable was observed 22 weeks post implantation in all the experimental groups. SC and axons closely associated with the fibers in the aligned fiber-based scaffolds while a layer of connective tissue was observed on the random fibers. In the smooth film based scaffolds two distinct cables were seen as previously observed in the 3 week study. Further, the regenerating cables surrounded the thin film scaffolds and were anchored to the thin-films extending from the edge of the scaffold creating an equal tissue distribution on either side of the thin-films. Representative images from the center of the scaffold are depicted in Fig. 4. In the 22 weeks study, a complete bridging of the nerve conduit was observed in all three experimental groups, as well as all three groups showed a bigger area of the cable at the proximal end compared to the distal thirds.

To delineate the organization of SC and axons within the regenerating cable, SC positive area was quantified within the transverse sections at 2, 4, 6, 11, 13, and 15 mm from the proximal end of the nerve conduit. Aligned fiber based scaffolds showed the highest SC area growth at 2 mm compared to smooth film-based scaffolds but not when compared to the random fibers, while at 4 mm, the aligned fiber based-scaffolds had significantly higher SC area compared to both smooth films and random fiber based scaffolds. At 6 mm into the conduit, a significantly larger area occupied by SC was observed in aligned fiber based scaffolds followed by the smooth films when compared to the random fibers based scaffolds. At 11, 13 and 15 mm into the conduits there was no statistical difference between the different experimental groups (Fig. 5a). Furthermore, to evaluate the effect of topography on the overall composition of the cable, the percentage of the regenerating cable tissue comprising of SCs were quantified. Significantly higher percentage of SCs were present in the regenerated cable of the aligned fiber at 2, 4, and 6 mm from the proximal end the conduit when compared to the smooth films. At conduit lengths of 11, 13, and 15 mm, no significant difference was observed between the different experimental groups. This data shows that aligned fibers based scaffolds influence the composition of SCs at several locations within the conduit compared to random and smooth film based scaffolds (Fig. 5b).

At 22 weeks, significantly higher area of NF160 positive axons were present in aligned fiber based scaffolds at 2 mm into the conduit compared to smooth film based scaffolds and at 4 mm into the conduit compared to random fiber based scaffolds. An increase in the growth of axons at the proximal end of the nerve conduit in the aligned fiber based group was observed. There was no significant difference from 6 mm onwards into the conduit between the different experimental conditions (Fig. 6a).

The area of the cable comprising of NF160 positive axons were also quantified. The percentage of axonal area within the aligned fiber based scaffolds was significantly higher compared to smooth films up to 11 mm into the conduit. Beyond 11 mm, there is no significant difference between the different experimental groups (Fig. 6b).

Quantification of Myelinated Axons

To investigate the effect of the different topographical guides on the maturation of the regenerated axons, toluidine blue stained myelinated axons were quantified in transverse sections from the center of the scaffolds. Aligned fiber-based scaffold showed a significantly higher number of myelinated axons compared to random fiber based scaffolds (Fig. 7a). No significant differences were observed in the total number of myelinated axons between the aligned and smooth film scaffolds.

Myelinated Sheath Thickness and Axonal Diameter

The scaffolds were further investigated to elucidate the axonal width and myelin thickness as measures of the level of growth of axon within the conduits (Figs. 7b and 7c). There was no significant difference in myelin thickness between the different experimental groups suggesting that axons were myelinated equally in response to topographical cues provided by aligned, random and smooth thin films (Fig. 7b). Axon maturity was also quantified within the conduit by evaluating the axonal width. A significantly higher percentage of fibers that were $>2\ \mu\text{m}$ in diameter were observed in aligned fiber based scaffolds compared to random fiber based scaffolds (Fig. 7c). These observations show that at 22 weeks post implantation, topographical cues influence the regenerative capacity of peripheral nerves and lead to axonal maturation compared to non-oriented fiber based scaffolds.

Muscle Weight and Muscle Fiber Diameter

Functional evaluation of axonal reinnervation into the muscle was indirectly tested by performing muscle weight analysis. The innervated muscle weight ratio was significantly higher in aligned fiber based scaffolds compared to random and smooth films based scaffolds (Fig. 8d). Furthermore, trichrome staining of sectioned muscle tissue (Figs. 8a–8c) showed that the amount of atrophy measured by the shrinking of the bundle of muscle fibers was significantly higher in random fibers and smooth films compared to aligned fiber based scaffolds (Fig. 8e). This data suggests that muscle innervation was enhanced by aligned topography.

Compound Action Potential Velocity

To further understand the functional implications of the presence of different topographical cues within the nerve guide, the ability of the regenerated nerve cable to transmit electrical

signals was measured. Compound action potential was measured and the conduction velocity in response to various topographies was calculated. The compound action potential velocity was not significantly different between all the three experimental groups (Fig. 9), suggesting that all the conditions supported the growth of functionally active axons.

Fibrin Organization 5 Days Post Injury

Presence of the fibrin matrix was observed in all conditions throughout the length of the conduit. A denser fibrin matrix was observed in the smooth film based scaffolds. A more disrupted provisional matrix was observed in random fiber based scaffolds compared to smooth film based scaffold. Fibrin matrix was interconnected in the aligned fiber scaffolds but not as dense as in the smooth film. A representative image from the center of the scaffold is shown in Fig. 10).

ECM Organization at 3 Weeks and 22 Weeks Post Injury

Cryosectioned samples were stained for laminin at 22 weeks and for collagen at 3 weeks as well as 22 weeks post-surgery using trichrome staining. In all thin film based scaffolds (aligned, random, and smooth film) positive laminin signal was detected within the area where SC were present and could be seen to have localized within the fiber based thin films, in contrast laminin was significantly less in smooth films within the cross section (Fig. 11). Tissue sections were analyzed using trichrome staining to evaluate the presence of collagen within the regenerating cable at 3 and 22 weeks post injury. Collagen fibers (blue stain) surrounded the regenerating cable comprised of SC, axons and other supporting cells creating an epineurial like coating (Supplementary Fig. 1). A higher density of collagen fibers were observed encapsulating the random fiber based scaffolds compared to aligned fiber based scaffolds and smooth films. The maturation of the nerve cable could also be observed by the presence of increased red (cell cytoplasm) staining in 22-week samples when compared to the 3-week samples. Tri-chrome staining also confirms our previous observation of two distinct cables on each halves of the smooth film based scaffold in the 3 week study. A cross section from the proximal third of the scaffold is shown in the Supplementary Fig. 1. Similar observations were made throughout the scaffold length.

Macrophage Response to Topography in a Long Gap

Localization of macrophages within the nerve conduits was also evaluated at all three time points (5 days, 3 weeks, and 22 weeks). Qualitative analysis of macrophage density within the conduit suggests that macrophage presence within the conduit 5 days post-surgery was minimal in all three conditions. Macrophages were mainly localized at the thin films and near the periphery of the fibrin cable 5 days post-surgery. At 3 weeks, a higher density of macrophages were observed throughout the regenerating cable in all three conditions. Finally at 22 weeks post-surgery, aligned fiber based scaffolds contained the least macrophages in the vicinity of the thin film compared to random fibers and smooth films (data only shown for cross sections near the distal end). A higher density of macrophages were seen encapsulating the random fiber based scaffolds throughout the length of the nerve gap compared to the other conditions. Localization of macrophages close to the distal end of the scaffold for each time-point is shown in Supplementary Fig. 2.

Cytokine Profile Analysis

To gain mechanistic insights into the role of topography in augmenting nerve bridging within the conduit, presence of cytokines at various locations (proximal, middle, and distal) within the nerve conduits were analyzed. We observed that IL-1 α , IL-1 β , IL-6, and TNF- α were present in detectable amounts at all three locations within the aligned, random, smooth film conduits as well as in the empty tube. Concentrations of IL-2, IL-4, IL-10, GM-CSF, and IFN- γ could not be assessed because their levels were below the detection limit of the cytokine array kit, suggesting that these cytokines were not present at the same levels as the former cytokines. IL-1 α was observed to be significantly higher in conduits loaded with thin films in comparison to empty tubes in the proximal region of the nerve. However, levels of IL-1 α were not significantly different between the various thin film topographies. In the distal 5 mm segment of the conduits, IL-1 α amounts were significantly higher in aligned fiber based scaffolds when compared to all the other conditions. IL-1 β and IL-6 levels were not significantly different between all the conditions as well as at all locations within the nerve conduits; whereas the concentration of TNF- α was significantly higher in aligned fiber based scaffolds when compared to the empty conduits in all three locations within the conduits. Further, at the proximal and distal ends, both random fibers and smooth film loaded conduits contained higher concentration of TNF- α when compared to empty tubes (Fig. 12).

qRT-PCR—qRT-PCR was performed to evaluate the differential expression of regeneration specific markers in response to the various topographies. The two halves (proximal and distal) of the nerve conduits were evaluated to examine the phenotype of cells migrating from both the proximal and distal stumps. Empty tube implantation were used as controls to normalize the expression levels of the various markers.

The markers assessed can be classified into two categories—(a) growth factors and ECM proteins and (b) neuronal receptors. At the proximal half of the conduit, the expression of BDNF was up regulated in the aligned films although only marginally by 0.8 fold. There was no significant change in the smooth film whereas in the random fiber scaffolds there was significant down regulation by 4 fold. At the distal end, the up regulation of BDNF was maintained but the increase was significantly higher by >3 fold in the aligned fibers. There was no change in BDNF levels in the smooth film but a modest 1.5 fold increase in expression was observed in the random fiber at the distal end unlike at the proximal end where there was a significant down regulation of BDNF. Next, the expression of fibronectin, laminin-1, NRG-1 at both ends and in all three types of conduits did not show a significant change.

The expression of the neuronal receptors such as ErbB2 and ErbB3 exhibited down regulation by >3 fold and 5 fold respectively at the proximal end in the aligned fibers whereas there was no significant change in the random and the smooth film conduits. At the distal end however, there was no change in both the ErbB2 and ErbB3 in the aligned fiber. A very marginal increase of 0.7 fold was seen in the random fiber. No significant changes in the levels of both ErbB2 and ErbB3 could be seen in the smooth film scaffolds. The expression of NGFR again showed only a very marginal increase of 0.7 and 0.5 fold in the

aligned fibers and the random fibers respectively with no change in the smooth films at the proximal end. However at the distal end there was a significant up regulation in NGFR expression by >4 fold in the aligned fiber and a 3 fold increase in the random fiber, while a down regulation was observed in the smooth film scaffolds (Supplementary Figs. 3 and 4). Analyzing samples for NT-3 was excluded as the CT values were high (>35 cycles). This suggested that significant expression of NT-3 does not occur in response to all conditions 3 weeks post injury in both the proximal and distal part of the conduits.

DISCUSSION

While it is known that synthetic filler materials can enhance nerve growth within guidance channels^{8,39,42} and augment bridging of non-critical peripheral nerve gaps, it is not clear if they can bridge critically sized gaps. Previous work from our group has shown that minimal topographical cues provided by electrospun thin-film scaffolds have led to bridging of nerve gaps in critically sized defects.^{9,19} These earlier studies showed that the presence of cues from oriented electrospun nanofibers as well as the organization of thin films within the nerve conduits influences regenerative events. These scaffolds also support and optimize the function of glial cells and help guide axons to bridge the nerve gap. While these studies have provided us insights into the role of electrospun fibers in eliciting the endogenous repair, it remains to be determined if it is the presence of topographical cues or the orientation of thin films within the scaffold that leads to successful bridging of the critical size nerve gaps. Furthermore, there is very little information on the temporal progression of the regenerative sequence in response to different topographical cues in long gaps. Thus, in this study we explored the influence of topographical cues on the regenerative sequence in critical size gaps. Additionally, detailed exploration of the cellular and molecular events in a spatio-temporal fashion was carried out. The insights gained here will help develop rational designs to augment nerve regeneration using thin film synthetic fillers.

To better design scaffolds and understand the mechanistic interaction between thin-film enhanced nerve guidance channels and improved regeneration, we evaluated the molecular and cellular response to thin-film containing nerve guidance channels starting from 5 days post guidance channel implantation across critically sized defects. PAN-MA, a non-degradable co-polymer was used to fabricate scaffolds with different topographical cues. The purpose of using a non-degradable polymer was to evaluate the effects of just the topography of the thin films without the presence of other factors such as degradation byproducts generated from biodegradable materials. We did not observe any chronic influence of the PAN-MA material on the functionality of the regeneration cable and the major influence was attributed to the varying topographical cues. In future studies, it would be interesting to see whether the mechanical properties of the polymer would positively or negatively impact the regeneration events.

We investigated SC migration and neurite outgrowth in response to the presence or absence of topographical cues. The short term migration of SC and axons were analyzed while the composition of the cable comprised of SC and axons were elucidated at a longer time point. We observed that at the 3 weeks' time point, aligned topographical cues enhance the migration of SC and infiltration of axons within the conduit compared to random and

smooth film scaffolds. This suggests that aligned topographical cues lead to the acceleration of regeneration in a critical gap model. Histological evaluation of the regeneration at 3 weeks also showed that the localization of SC and axons was greatly influenced by the topography, suggesting that the differential interaction with the thin films could have led to the difference in migration that we have observed. Several studies have emphasized the importance of the speed of regeneration and not just the quality of regeneration.^{2,22} Therefore, acceleration of the initial regeneration process might be important in enhancing outcomes in long peripheral nerve gaps.

Analysis of the temporal progression of SC recruitment and axonal regeneration suggest that different thin film topographies lead to a very distinct regeneration. Histological analysis *via* immunofluorescence of SC, axons, fibroblasts, laminin, and fibrin as well as Tri-chrome staining suggest that aligned, random, and smooth film based scaffolds cause a differential cable formation and influence the regenerative sequence. As suggested by fibrin staining at 5 days post injury, all the thin film based scaffolds were able to sustain some form of the provisional matrix throughout the length of the conduit. While previously it has only been hypothesized that the thin films play a role in either replacing or aiding in the function of the provisional matrix, this is the first instance where it has been demonstrated that thin films promote the stabilization of the fibrin matrix. We also observed that the provisional matrix has a similar shape to that of the mature cable leading us to conclude that the long-term maturation of the nerve cable is dependent on the ability of the thin films to organize the fibrin matrix. The fibrin matrix had more disruption in the random film based scaffolds when compared to aligned fibers and smooth film based scaffolds. This could have caused the slow migration of SC and neurite outgrowth that we observed in the *in vivo* studies. Earlier studies have shown that varying the crosslinking and density of fibrin gels can affect neurite outgrowth and SC migration.^{1,32,36} While the aligned and smooth film based scaffolds both exhibit a contiguous fibrin cable, a difference in the density of the fibrin was observed in cross-sections at different locations within the conduit. These differences could be one of the major reasons why we see a difference in initial migration of SC and neurite outgrowth between aligned and smooth film based scaffolds. However, this inference was made based on the histological analysis obtained in our study and previous studies involving fibrin matrix characterization. This aspect warrants a more thorough investigation because crosslinking as well as the density of the provisional matrix plays a pivotal role in influencing the regenerative sequence in nerve conduits.

In contrast to smooth film based scaffolds, both random and aligned fiber based scaffolds promote the formation of a consolidated axon/SC cable surrounded by collagen bands of epineurial-like tissue. Both fiber based scaffolds led to a unified cable surrounding the thin films whereas segregated cables on either side of the smooth thin films based scaffolds were observed. One factor contributing to this difference in organization of the regenerating cable could be the permeability of the thin films. In fact, FITC-dextran based permeability studies revealed that fiber based topographies were permeable to biomolecules of up to 500 kDa. This property of the fiber based scaffolds could have aided in promoting an efficient organization of the regenerating nerve, while smooth films were impermeable to similar sized biomolecules. Thus, in addition to the topographical cues, the porosity of the regeneration guides may also play a role in the regeneration cascade.

Evaluation of the percentage of both SC and area of the axons within the cross sections suggested that aligned fibers promoted significantly higher percentage of regenerating axons and SC compared to the other conditions. We also observed that aligned fiber based scaffolds support the highest number of myelinated axons compared to the other conditions. Previous studies have shown that myelin thickness directly affects conduction velocity.¹⁰ Our observations indicate that the myelin thickness was similar for all conditions. This suggests that while topography affects the number of axon infiltration in the conduit, it does not affect the myelination of the axons. Myelin thickness for each condition was similar to the normal nerve.¹² Thus, we can speculate that the 22 week time point might have been too long to observe any differences in myelin thickness due to the presence of topographical cues. Evaluation of the axonal diameter showed that the highest numbers of axons with width greater than 2 μm were observed in aligned fiber based scaffolds. While in this study we did not measure the strength of the action potential, we can hypothesize based on previous studies that highest number of larger diameter axons would lead to an increased action potential in the aligned case compared to other conditions.⁴¹

Studies involving the measurements of the compound action potential velocity (ability of the regenerated cable to carry electrophysiological signals), revealed that all thin film based scaffolds were able to sustain the signal through the tube. Nerve conduction velocity was slightly higher but not significantly different in aligned fiber based films in comparison to the other conditions. Our previous studies showed that aligned fiber based scaffolds promoted the highest number of myelinated axons and axon diameter which could together lead to a higher nerve conduction velocity. Further, comparing to a previous study from our lab, the velocity that we achieved was similar to the gold standard autographs in a similar gap.¹² Thus, this data suggests that all thin film based scaffolds are able to generate a functional cable. Since measurements were performed 5 mm past the distal end, it is not clear if all conditions would support such nerve conduction velocities closer to the innervation site.

Several studies have emphasized that, the final goal of all therapies for PNS injuries is accelerating nerve growth and achieving functional recovery to the rein-nervated muscle. In this context, we monitored muscle atrophy because muscle atrophy will increase if axon growth is slow in any of the conditions tested. The RGMW and quantitative data of the individual muscle fiber area we obtained, suggests that aligned topo-graphical cues were able to accelerate axonal growth and lead to the arrest of muscle atrophy compared to other conditions. Thus our results strongly suggest that aligned topographical cues enhance the endogenous regenerative sequence to accelerate nerve growth.

The impact of topography on the regenerating microenvironment (recruitment and production of cytokines) were evaluated at 5 days and also changes in the regeneration specific genes were assessed at 3 weeks post implantation. Several molecular and cellular events occur directly following nerve injury that lead to Wallerian degeneration as well as prime the proximal and distal end of the severed nerve for repair. Following nerve injury, non-immune cells and macrophages work together to remove degenerated myelin.²⁷ Previous work has shown that IL-1 α and TNF- α , both proinflammatory cytokines that aid in the recruitment of macrophages and clearance of myelin debris are first produced by SC

directly after injury.^{34,43} Our study demonstrates that IL-1 α and TNF- α production is influenced by the nature of topography. We observed that higher quantities of IL-1 α and TNF- α were detected in aligned fiber based scaffolds when compared to other conditions. This suggests that aligned topographical cues augment the proinflammatory phenotype of SC. Saino *et al.*,³⁵ have also shown that topographical cues from fibers and smooth films are capable of inducing macrophage activation and secretion of proinflammatory cytokines. Furthermore, in our study all thin film based scaffolds (aligned, random and smooth film) increased the production of TNF- α at the proximal end of the scaffold as compared to empty tube control. This phenomenon could further explain why thin film based scaffolds are able to support nerve bridging while it fails in an empty tube. TNF- α has also been shown to cause IL-1 β production in SC *via* autocrine mechanism.³⁴ Even though we did not observe any differences between any of the conditions, our data confirms this phenomenon as we observed detectable quantities of IL-1 β throughout the conduit at day 5. We believe that this is more likely caused by the effect of TNF- α on SC in the conduits.

It has also been shown that Wallerian degeneration is a biphasic process, wherein during the first phase, M2 phenotype macrophages are recruited due to the actions of proinflammatory cytokines such as TNF- α , IL-1 α , IL-1 β , IL-6, and GM-CSF.^{13,27,34} These macrophages have been implicated in the nerve repair process. Second phase is predominantly anti-inflammatory where IL-1 β and TNF- α levels are reduced and the production of IL-6 and IL-10 are increased. Hence, lack of detection of IL-10 in our studies are consistent with this because at day 5 we are still in the first phase of Wallerian degeneration. Overall, this data suggests that the presence of topographical cues alters the cytokine production after nerve injury. Topographical cues also augment the proinflammatory phenotype of glial cells which helps clear the inhibitory environment and enhance the overall nerve regeneration. We can thus conclude that some of the long term gains we have observed in earlier studies with the aligned topographical cues are largely due to their ability to modulate the proinflammatory phenotype of SC compared to other topographies. Since we limited our study to 5 days, we were not able to observe the effects of topography on the second phase of Wallerian degeneration which is usually observed between 7 days and 3 weeks post injury.

Studies on the expression of the different markers such as the growth factors, ECM proteins and neuronal receptors by qRT-PCR revealed differential regulation in expression in the three types as well as at the two halves of each of the conduits used in this study. BDNF is known to be a regulator of myelination. In our studies, a very modest increase in BDNF expression was observed at the proximal end, while a significant increase of >3 fold was seen at the distal end of the aligned fibers. BDNF has been shown to be involved in the myelination process and neuronal regeneration process.⁵¹ Further, a significant down regulation of BDNF was observed at the proximal end of the random fibers with no changes in the smooth films reflecting upon the process of myelination being influenced by the different topographical cues. No significant changes in the expression of fibronectin and laminin and NRG-1 were observed at both ends and in all the three types of scaffolds. Previous studies have shown that IL-1 β and TNF- α lead to NGF synthesis.³⁸ Also, studies by Anton *et al.*,³ have shown that NGF and NGFR together stimulate SC migration. In our study, we observed that both IL-1 β and TNF- α were present in all conditions after injury while NGFR was differentially regulated. NGFR mRNA synthesis was moderately up regulated in scaffolds

with fiber based topographies at the proximal end, while a significant increase of 5 fold was observed at the distal end in the aligned based scaffolds and a 3 fold increase in the random based scaffolds, but it was down regulated in the smooth films scaffolds. This suggests that NGFR expression is modulated by surface topography. Thus the ability of aligned topographical cues to increase TNF- α production leading to increased NGF release when combined with topography induced up regulation of NGFR could explain the increase in the short term SC migration and neurite outgrowth at 3 weeks. Migration data from 3 weeks also indicated that smooth film also enhance SC migration compared to random fibers but RT-PCR analysis suggests that they relatively down regulate NGFR production. Therefore we believe that smooth film induced SC migration might be due to alternative pathways. Earlier studies have suggested that increased NGFR expression might lead to increased myelination¹¹ and that SC with a myelinatory phenotype do not migrate very well.^{46,47} The lower expression of NGFR observed in smooth film scaffolds could keep the infiltrating SC in a more migratory phenotype and lead to migration of glial cells as well which we did observe in our histologically analysis. The changes in fibronectin, laminin and NRG-1 in all the conditions were in the range of 2–2.5 fold, so were considered not significant. However, we cannot rule out if marginal changes in these biomolecules could suffice to initiate changes leading to the regeneration sequence.

NT-3 is shown to increase SC migration.^{16,46} NT-3 was not expressed in any of the conditions in our study based on real-time PCR analysis. Since topographical cues are not able to influence NT-3 expression, future therapies could incorporate exogenous NT-3 to synergistically work with aligned topographies to enhance regeneration.

We observed down regulation of ErbB2 and ErbB3, which are receptors involved in SC proliferation⁷ in the aligned fibers compared to empty tube controls at the proximal side of the scaffold. Previous work by others has speculated that ErbB2 and ErbB3 have the ability to keep SC in a non-myelinating phenotype and their down regulation could initiate the onset of SC transition into myelinating stage.⁴⁶ In our studies, the highest down regulation of ErbB2 and ErbB3 were observed in aligned fiber based scaffolds suggesting that migrating SC on aligned topographical cues could be maturing faster and transitioning into their myelinatory phenotype compared to other conditions. This confirms our earlier observations by histological assessment wherein aligned fibers demonstrated enhanced maturation of the nerve cable.

Our results show that topographical cues affect the normal regeneration process significantly both spatially and temporally. We also observed that thin films transiently create a pro-inflammatory environment by influencing the phenotype of SC and macrophages to speed up the removal of inhibitory debris from around the injury sites. This ability of the fiber-based scaffolds can be utilized in spinal cord repair as well, where an inhibitory glial scar prevents regeneration. Along with their ability to direct growth, fiber based thin films can be used to modulate the local microenvironment to influence repair. Also, the ability of the different topographies to alter the organization of the regeneration cable can be applied to design devices such as neural interfaces which require precise interaction both spatially and temporally.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

(a) Schematic diagram of film-based scaffolds used to bridge long peripheral nerve gaps. (b) Schematic of the permeability assay. (c) Assessment of fiber permeability. Smooth film based scaffolds were impermeable to FITC-dextran molecules of 70 and 500 kDa.

FIGURE 2. Schwann cell cable formation at 3 weeks in the proximal section of the film-based scaffolds. (a) Schematic diagram of the location in the tube that corresponds to the stained sections shown by a green circle. (b, c) Aligned fibers based scaffold stained with NF160 (Axons) and S100 (Schwann cells) respectively. (d, e) Random fiber based scaffold stained for NF160 and S100 respectively. (f, g) Smooth film based scaffold stained for NF160 and S100 respectively. Scale bar 5 400 μm .

FIGURE 3.

Schwann cell migration and axonal growth from the proximal end of the nerve graft. (a) Distance moved by SC. (b) Axonal growth in the 15 mm conduit. ** $p < 0.01$ aligned vs. random and smooth film condition. *** $p < 0.01$ for smooth film vs. random. X-axis represents the distance within the nerve conduit.

FIGURE 4. Schwann cell cable formation at 22 weeks. Sections were taken from the middle of the film-based scaffolds. (a) Schematic diagram of the location in the tube that corresponds to the stained sections shown by a green circle. (b, c) Aligned fiber based scaffold stained with NF160 (axons) and S100 (Schwann cells). (d, e) Random fiber based scaffold stained for NF160 and S100. (f, g) Smooth film based scaffold stained for NF160 and S100. Scale bar 5 400 μm . Yellow dotted lines indicate thin film localization within the nerve conduit.

FIGURE 5.

Quantification of SC in the regenerating cable after 22 weeks of implantation. (a) Total area that was positively stained for S100 (Schwann cells) at different locations in a nerve conduit. ** $p < 0.01$ and * $p < 0.05$. (b) Percentage of the total cable area positive for S100 (Schwann cells) at different locations in a nerve conduit. * $p < 0.05$.

FIGURE 6.

Quantification of axons in the regenerating cable after 22 weeks of implantation. (a) Total area that was positively stained for NF160 (axons) at different locations in a nerve conduit. ** $p < 0.01$ and * $p < 0.05$. (b) Percentage of the total cable area positive for NF160 (axons) at different locations in a nerve conduit. * $p < 0.05$.

FIGURE 7.

Toluidine blue staining of cross sections from the middle of the scaffold 22 weeks post injury was performed for quantification of myelinated axons. (a) Graph shows the number of myelinated axons vary based on the topographical guides used in the study. Aligned fibers exhibit a significant increase in the number of myelinated axons compared to random fibers, * $p < 0.05$. (b) Quantification of myelin thickness. The three different conditions did not show a significant difference with respect to the myelin thickness. (c) Quantification of myelinated fiber diameter. At 22 weeks post-implantation, the aligned fibers promoted the formation of axons with the largest diameter.

FIGURE 8.

Characterization of gastrocnemius muscle fiber. Cross-sectional view of the scaffold from medial gastrocnemius muscle. (a) Aligned. (b) Random. (c) Smooth film. (d) Quantification of RGMW ratio. * $p < 0.05$. The innervated muscle weight ratio was significantly high in the aligned fibers. (e) Muscle fiber width. Pixel average diameter of muscle fibers is represented on the Y axis. * $p < 0.05$.

FIGURE 9.

Determination of compound action potential velocity. Nerves were stimulated distal to the injury site and the signal was recorded proximal to the site of injury. The compound action potential is not different in all the three conditions studied.

FIGURE 10.

Fibrinogen staining 5 days post-surgery near the proximal end of the conduit. (a) Aligned. (b) Random. (c) Smooth film. Scale bar = 100 μm . Broken lines indicate the region of the conduit in each of the images.

FIGURE 11.

Laminin (green) and fibroblast (red) localization midway in the nerve conduits 22 weeks post-surgery. (a) Aligned, (c) random, (e) smooth film: 4 $\times$ images on upright scope. Scale bar 5 400 μm . (b, d, f) Enlarged 20 $\times$ images of areas indicated in (a), (c), and (e), respectively. Scale bar 5 100 μm .

FIGURE 12.

Cytokine array analysis 5 days after injury. In the left panel the changes in the cytokines such as IL-1 alpha and IL-6 in aligned, random, smooth film as well as in empty tubes is assessed at three different locations (proximal, middle, and distal) of the scaffolds. Similarly on the right panel, graphs representing differential expression of IL-1 beta, TNF alpha in aligned, random, smooth film as well as in empty tubes at three different locations (proximal, middle, distal) of the scaffolds are shown. * $p < 0.05$.

TABLE 1.

Experimental scheme and endpoint analysis of the study.

Studies	Conditions	No. of animals	Outcome measures
5 Days	Aligned	4 per condition	Histology
	Random		Fibrinogen distribution
	Smooth		
3 Weeks	Aligned	4 per condition	Histology
	Random		Schwann cell/axon migration
	Smooth		Fibronectin/laminin distribution
22 Weeks	Aligned	8 per condition	Histology
	Random		Cable area
	Smooth		Schwann cell/axon distribution
			Myelin thickness
			Axonal diameter
			Functional
			Muscle weight/fibrosis
			Electrophysiology—conduction velocity