Published in final edited form as:

Biomaterials. 2008 July ; 29(21): 3117–3127.

The role of aligned polymer fiber-based constructs in the bridging long peripheral nerve gaps

Young-tae Kim¹, Valerie K. Haftel², Satish Kumar³, and Ravi V. Bellamkonda^{1*}

¹Neurological Biomaterials and Therapeutics, Laboratory for Neuroengineering, Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30332

²Department of Biology, Morehouse College, Atlanta, GA 30314

³School of Polymer, Textile and Fiber Engineering, Georgia Institute of Technology, Atlanta, GA 30332

Abstract

Peripheral nerve regeneration across long nerve gaps is clinically challenging. Autografts, the standard of therapy, are limited by availability and other complications. Here, using rigorous anatomical and functional measures, we report that aligned polymer fiber-based constructs present topographical cues that facilitate the regeneration of peripheral nerves across long nerve gaps. Significantly, aligned but not randomly oriented fibers elicit regeneration, establishing that topographical cues can influence endogenous nerve repair mechanisms in the absence of exogenous growth promoting proteins. Axons regenerated across a 17mm nerve gap, reinnervated muscles, and reformed neuromuscular junctions. Electrophysiological and behavioral analyses revealed that aligned, but not randomly oriented constructs facilitated both sensory and motor nerve regeneration, significantly improved functional outcomes. Additionally, a quantitative comparison of DRG outgrowth *in vitro* and nerve regeneration *in vivo* on aligned and randomly oriented fiber films clearly demonstrated the significant role of sub-micron scale topographical cues in stimulating endogenous nerve repair mechanisms.

INTRODUCTION

Severe traumatic injury or invasive surgical procedures on a peripheral nerve can result in a gap between two nerve stumps. The clinical “gold standard” for bridging peripheral nerve gaps is the use of autografts (typically, the sensory sural nerve). However, the use of autografts is limited by the following issues: 1) limited availability of nerves to use in the autograft [1], 2) secondary surgery, 3) lack of coaptation between the injured nerve and the nerve graft due to size/length/modality mismatch [2], and 4) functional loss, such as numbness at the donor sites [3]. Moreover, complications at the donor site such as hyperesthesia or formation of painful neuromas also have to be addressed [4,5]. Therefore, it is imperative that alternative approaches that are ready-to-use, pre-customized for reducing the mismatch, and suitable for both sensory and motor nerve regeneration are developed.

*Corresponding author: Ravi V. Bellamkonda, Ph.D., Professor of Biomedical Engineering, Wallace H. Coulter Dept. of Biomedical Engineering, 313 Ferst Drive, Room 3108, Georgia Institute of Technology and Emory University, Atlanta, GA 30332, E-mail: ravi@gatech.edu, Phone: 1-404-385-5038, Fax: 1-404-385-5044.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Polymeric tubular nerve constructs have been used clinically for repairing peripheral nerve injury [6]. These nerve constructs, made of either non-porous silicone or porous natural/synthetic polymers, bridge the injured nerve stumps when the gaps are <10–12mm in rats by enabling the formation of a fibrin cable that provides a substrate for the migration of Schwann cells. The migrated Schwann cells from both nerve stumps reorganize to create longitudinally oriented bands of Bungner, which serve as guiding substrates and a source of neurotrophic factors to foster axonal regrowth [7,8]. However, polymeric conduits alone are limited in their ability to enable regeneration across long nerve gaps (>15mm in rats). Failure of nerve regeneration across long gaps seems to be the result of a lack of the formation of an initial fibrin cable, which is necessary for Schwann cell migration into the constructs and the formation of the bands of Bungner which are aligned columns of Schwann cells and laminin [9].

In this study, we used an electrospinning process to create 10–20µm thin polymer films made of either aligned or randomly oriented sub-micron scale polymeric fibers (~400–600nm in diameter). To examine the effects of sub-micron topography on neurite outgrowth/Schwann cell migration, dorsal root ganglia (DRG) were seeded on the films *in vitro*, and the extent of neurite outgrowth/Schwann cell migration was quantified. These two dimensional (2D) fiber films were stacked in a 3D configuration to build implantable polymeric constructs with inherent topographical cues what were either aligned or randomly oriented. Polymer fiber-based constructs were implanted across 17mm tibial nerve gaps in adult rats to determine if topographical cues were sufficient to stimulate endogenous regeneration of nerves even in the absence of biochemical cues such as neurotrophic factors or extracellular matrix proteins. Saline filled polymeric constructs and autografts were used as controls. Our results indicate that robust regeneration across the tibial nerve gap occurred in the aligned fiber and autograft implants, but not in the randomly oriented or saline-filled constructs. These results clearly illustrate the significance of using fibers to create sub-micron scale, oriented topographical features in stimulating endogenous repair mechanisms, presumably by enhancing the efficiency of Schwann cell migration and axon outgrowth across the nerve gap. These conclusions are strengthened by a quantitative analysis of regeneration that included evaluation of reinnervation of distal targets (i.e., muscle) by immunohistological, histomorphometrical, electrophysiological, and behavioral analysis.

METHODS

Fabrication of aligned and random fiber films

Uniaxially aligned fiber films were fabricated by electrospinning poly(acrylonitrile-co-methylacrylate, random copolymer, 4 mole percent of methylacrylate) (PAN-MA) on a high-speed rotating metal drum [10]. Briefly, an 18% PAN-MA (w/v) solution was prepared by dissolving PAN-MA in the organic solvent, N, N-Dimethyl Formamide (DMF, Acros) at 60°C. The polymer solution was loaded into a 10ml syringe and delivered at a constant flow rate (1ml/hour) through a metal needle (19 gauge) connected to a high-voltage power supply. Upon applying high voltage (15–18kV), a fine polymer solution jet was ejected from the needle and deposited on thick aluminum foil wrapped around a custom designed high-speed rotating metal drum (Fig. 1E). The foil carrying the electrospun aligned fiber film (10–20µm thick) was then removed and stored at 60°C overnight to remove residual DMF. The alignment and morphology of the fibers was examined using scanning electron microscopy (S-800 SEM, Hitachi) and quantified with Image-Pro software (MediaCybernetics). Randomly oriented fiber films were fabricated as an experimental control. The fabrication procedure for these control films was exactly the same as for the aligned fiber films, except that electrospun fibers were collected on a flat metal surface, instead of on the high-speed rotating metal drum.

DRG neuron outgrowth on fiber films

The fiber films were peeled off from the aluminum foil and placed on tissue culture plates. To secure the fiber films on the bottom of the plates, a medical grade UV light curing adhesive (Dymax) was applied to the edges of the film. For sterilization, the fiber films were exposed to UV light overnight, soaked in 70% ethanol for 20 minutes, and washed three times with sterilized deionized water. Dorsal root ganglion (DRG) neurons were isolated from postnatal day 1 (P1) rat pups. Individual ganglia were removed from the spinal column, nerve roots were stripped, and the ganglia were placed on the fiber film. To aid the attachment of ganglia on the film, the culture medium was removed and then 1–2 drops of medium was reapplied on the film before seeding the DRG neurons. After four hours of incubation at 37°C, culture medium (DMEM/F12 with 10% fetal bovine serum at 37°C) with nerve growth factor (NGF, 50ng/ml, Roche Applied Science) was slowly added to the plate. The culture medium with NGF was replaced every two days. No proteins (e.g., laminin or collagen) or poly-L-lysine solution was applied to the film for a surface coating.

Immunohistochemical staining

One week after seeding the DRGs, the DRGs were fixed for 30 minutes in a 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C and washed three times with PBS. For transverse cross-sections, fixed DRGs on the film were cryosectioned into 10µm thick slices. The DRGs were double-immunostained overnight at 4°C with the following primary antibody solutions: 1) neurofilament 160kDa (NF160, 1:500, mouse IgG1, Sigma) to identify the neurons and S-100 (1:250, rabbit IgG, DakoCytomation) to identify the Schwann cells, 2) NF160 and laminin (1:500, rabbit IgG, Sigma) to identify the endogenously deposited laminin protein on the film, and 3) S-100b (1:250, mouse IgG1, Sigma) to identify the Schwann cells and laminin. Secondary antibodies were diluted 1:220 in 0.5% triton in PBS and included goat anti-mouse IgG1 Alexa 594 (Invitrogen) for NF160 and S-100b, and goat anti-rabbit IgG Alexa 488 for S-100 and laminin. The immunostained neurons, Schwann cells, and laminin protein were visualized with a fluorescence microscope (Axioskop 2, Zeiss) and captured with a digital camera (Olympus).

For the quantitative comparison of neurite outgrowth orientation and neurite outgrowth *in vitro* on either aligned or random fiber film, at least 25 of the longest NF160⁺ neurites per DRG were selected. The extent of neurite outgrowth was measured by the distance between the DRG edge and the end tip of each neurite. For the extent of Schwann cell migration, at least the ten furthest migrated S-100⁺ Schwann cells per DRG were selected, and a distance between the DRG and the selected Schwann cells was measured and averaged.

Polymeric constructs containing fiber films

Either aligned or random fiber films were cut into 17mm × 1mm pieces and peeled off from the aluminum foil. A total of 10–12 films were then stacked within two halves of a longitudinally split polysulfone nerve conduit (Koch Membrane Systems, 50,000 molecular weight cutoff - 19mm long and 1.5mm inner diameter) (Supplementary Figs. 2A and B). The longitudinal half-cut was closed and sealed with UV light curing adhesive. For sterilization, the nerve constructs containing either aligned or random fiber films were exposed to UV light overnight, soaked in 70% ethanol for 30 minutes, washed three times with sterilized deionized water, and placed in the sterilized PBS until implantation into transected rat tibial nerve. Saline filled polymeric constructs (19mm long and 1.5mm inner diameter) were fabricated as an experimental control.

Implantation of fiber constructs or autografts

All procedures were conducted according to Institutional Animal Care and Use Committee (IACUC) approved protocols. The implants were classified into four categories: 1) saline filled polymeric constructs (n=12); 2) aligned polymer fiber-based constructs (n=12); 3) random polymer fiber-based constructs (n=12); and 4) 17mm nerve autografts, which were resected from the rat tibial nerve being bridged (n=12). Adult male rats (250–300g, Fischer 344 rats, Harlan) were induced to anesthetic depth with inhaled isoflurane at 5%. Throughout surgery, the animals were maintained at 2–2.2% isoflurane. Before surgery, the surgical area was shaved and the incision site cleansed with a chlorohexaderm scrub. After skin incision along the femoral axis, the thigh muscles were separated with a blunt-tip scissors and the tibial nerve was dissected. The nerve was then transected using microscissors, and the polymeric constructs or autografts were sutured in place. The proximal and distal nerve stumps were secured in either 19mm long polymeric constructs or 17mm autografts, using a 10-0 Nylon monofilament suture (Ethicon). The 19mm polymeric constructs enabled a 17mm nerve gap when used as guidance channels, due to the 1mm overlap needed on each end for suturing in the nerves. The muscles were closed with a 4-0 suture and the incised skin was closed with wound clips. The animals were monitored regularly and self-mutilation was prevented by applying bitter apple solution to their toes. At 7 days post-surgery, the wound clips were removed.

Behavioral assessment: grid walking test

After baseline testing (prior to surgery), the behavior of the animals was measured at sixteen weeks after implantation. The grid walk test was used to evaluate small deficits in fine motor control. We slightly modified the standard protocol in our experiment as described in Kunkel-Bagden, *et al.* [11]. Briefly, the animals were trained to walk on a metal wire grid (50cm × 30cm, 2cm = distance between wires in the grid). The number of affected hindlimb slips below the plain of the wire grid over a span 2 minutes was counted and averaged over three trials per animal.

Electrophysiology of regenerated nerve

To test the restoration of functionality of the regenerated nerve through the implants (i.e., polymeric construct or autograft), the compound action potential (CAP) across the nerve gaps was measured. Sixteen weeks after implantation, the animals were anesthetized with a mixture of ketamine (65mg/kg), xylazine (7.5mg/kg), and acepromazine (0.5mg/kg), and the sciatic nerve, posterior tibial nerve, and triceps surae muscles were exposed in the affected hindlimb as described in Figure 6B. A laminectomy was performed to expose the L4–L6 dorsal and ventral roots. The nerve was stimulated proximal to the site of the implants via a platform electrode, (1Hz, 100μs duration, variable amplitude). During stimulation, mixed (i.e., regenerated sensory and motor nerves) compound action potentials were recorded at the posterior tibial nerve in each of the following groups: 1) normal controls (unoperated rats, n=3); 2) autograft treated animals (n=3); 3) aligned fiber-based polymeric construct treated animals (n=3); and 4) random fiber-based polymeric construct treated animals (n=3). Recordings were then taken at various stimulus intensities.

To determine whether the implants were suitable for both sensory or motor nerve regeneration, CAPs were recorded extra-axonally from the tibial nerve distal to the site of implantation following extracellular stimulation (1Hz, 100μs duration, variable amplitude) of lumbar dorsal roots (for sensory neurons) or ventral roots (for motor neurons) (Fig. 6B). The stimulus intensity was varied. Recordings were amplified at a gain of 10,000 and bandpass filtered with cutoffs at 10Hz and 3kHz (Grass Inst.). The samples were acquired using LabVIEW software and stored for offline averaging and analysis with custom designed MATLAB software. For conduction latency measurement, a time latency from the application of an electrical pulse at

the stimulation site to the detection of the resulting CAP at the recording site was measured from offline averaged CAPs.

Tissue preparation and immunohistochemical analysis

One day after behavior assessment (i.e., 16 weeks after implantation), the animals were perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. Three different types of polymeric constructs (i.e., saline filled, aligned, or random fiber-based) and autografts were then explanted (Supplementary Fig. 2C), and post-fixed overnight in the paraformaldehyde solution. The gastrocnemius muscles from both the transected and intact sides of the animals were collected, weighed, and then also post-fixed overnight in the paraformaldehyde solution. The fixed explants and muscles were soaked in 30% sucrose (w/v, Sigma) in PBS at 4°C for cryosectioning.

Fiber polymeric constructs and autografts (longitudinal sections)—Longitudinal 18µm thick sections of the constructs were obtained with a cryostat (six animals/each category). Serial sections were collected from each specimen and every fourth section was processed for a particular antigen pair using immunohistochemical methods. Serial sections were incubated overnight at 4°C with the following primary antibody solutions: NF160, S-100, laminin, and rat endothelial cell antibody (RECA-1, 1:500, mouse IgG1, Serotec) to identify blood vessels. Secondary antibodies were diluted 1:220 in 0.5% triton in PBS and included goat anti-mouse IgG1 Alexa 594 for NF160 and RECA-1, goat anti-rabbit IgG Alexa 488 for S-100, and goat anti-rabbit IgG Alexa 594 for laminin. Sections treated only with secondary antibody but with no primary antibody were used to determine non-specific binding. Tissue sections were mounted on glass microscope slides with mounting medium and allowed to cure for 1 hour prior to analysis.

Fiber polymeric constructs and autografts (transverse cross-section)—Coronal 10µm thick sections were cryosectioned (six animals/each category). Sections were collected from each specimen at three different sites: 5mm and 12mm from the proximal nerve stump, and 3mm into distal nerve stump. Sections were processed for a particular antigen pair using immunohistochemical methods with the following primary antibody solutions: NF160, S-100, laminin, and protein zero (P0, 1:100, chicken IgY, Chemicon) to identify glycoprotein expressed in the compact myelin of the peripheral nerve. Secondary antibodies were diluted 1:220 in 0.5% triton in PBS and included goat anti-mouse IgG1 Alexa 594 for NF160, goat anti-rabbit IgG Alexa 488 for S-100 and laminin, and goat anti-chicken IgG Alexa 488 for P0.

Explanted gastrocnemius muscles—Longitudinal 25µm thick sections of the gastrocnemius muscle were cryosectioned (seven animals/each category). Sections were collected from each specimen and processed for the following markers: NF160 for axons, S-100 for terminal Schwann cells, and alpha-Bungarotoxin-tetramethylrhodamine (α -Bung, 1:220, Sigma), which was used to identify the distribution of nicotinic acetylcholine receptors at neuromuscular junctions. SV2 antibody (1:25, synaptic vesicle protein 2, Developmental Studies Hybridoma Bank, Iowa City, IA) was used to immunostain synaptic vesicles at neuromuscular junction.

Quantitative analysis of peripheral nerve regeneration

Nerve regeneration through the implants (n = 6 for each category and n = 2 for normal control) was quantified by the following two parameters: 1) number of NF160⁺ axons/cross-section at three different sites: 5mm and 12mm from the proximal nerve stump, and 3mm into the distal nerve stump and 2) NF160⁺ axon diameter distribution using a fluorescent microscope equipped with a digital camera and Image-Pro software.

Reconstruction of neuromuscular junctions at the affected gastrocnemius muscle ($n = 7$ for each category and $n = 2$ for normal control) was quantified by the following two parameters: 1) percentage of NF160⁺ axons innervated with α -Bung⁺ neuromuscular junctions. For this analysis, 15 sample areas (i.e., NF160 and α -Bung double immunostained region in longitudinally sectioned muscles, Figs. 5A, D, G, J, and M) per animal were captured. The number of co-localization of NF160⁺ axons and α -Bung⁺ NMJs was counted and divided by the total number of α -Bung⁺ NMJs to determine the percentage of axons innervated with NMJs; 2) percentage of SV2⁺ NMJs. For this analysis, 12 sample areas (i.e., SV2 and α -Bung double immunostained region in longitudinally sectioned muscles, Figs. 5C, F, and I) per animal were captured. The number of co-localization of SV2⁺ synaptic vesicles and α -Bung⁺ NMJs was counted and divided by the total number of α -Bung⁺ NMJs to determine the percentage of SV⁺ NMJs.

Statistical analysis

An ANOVA, Tukey post hoc test, was used to calculate the significance of differences between mean values. A p value less than 0.05 was considered statistically significant.

RESULTS

Fabrication and morphological characterization of fiber films

Either uniaxially aligned or random fiber films were fabricated by an electrospinning process (see Methods section) (Fig. 1E). The diameter of the individual fibers ranged between 400 and 600nm (Figs. 1F and H) and the thickness of the films was approximately 10–20 μ m (Supplementary Fig. 1D). This thickness was chosen to enable manual handling (e.g., peeling off from the aluminum foil), which was required for stacking them into the polymeric constructs. Quantification of individual fiber alignment within the films was performed with SEM pictures and Image-Pro software. This quantification revealed that approximately 72% of all fibers fell within ± 20 degrees of their long axis (Fig. 1G). However, the alignment of random fibers was evenly distributed through all angle bins (Fig. 1I).

DRG neurite outgrowth and Schwann cell migration

The topography of sub-micron scale fiber films determined the orientation and extent of DRG neurite outgrowth and Schwann cell migration *in vitro* (Fig. 1). Quantitative comparison of outgrowth of DRGs on the aligned fiber film demonstrates that the majority of neurite outgrowth and Schwann cell migration from the DRGs extended unidirectionally, parallel to the aligned fibers (Figs. 1A–D). However, the orientation of neurite outgrowth and Schwann cell migration on the random fiber films was randomly distributed (Figs. 1J, K, and L). While Schwann cell migration always led growth cones in all culture conditions, this lead was significantly greater in aligned fiber film cultures as measured by quantifying the average distance between the NF160⁺ neurite front and the S100⁺ Schwann cell migratory front (Fig. 1M). No difference in initial DRG adhesion (see Methods section), was observed between the films.

Double immunohistochemical analysis of transverse cross-sectioned DRGs on the aligned fiber films demonstrated that DRGs adhered to the fiber films (Supplementary Fig. 1D), and that migrating S-100⁺ Schwann cells on the films form laminin⁺ circular pocket structures (Supplementary Figs. 1A, B, and C). NF160⁺ axons were observed in these structures (Supplementary Fig. 1G).

Characterization of nerve regeneration *in vivo*

Immunohistochemical analysis (longitudinal sections stained with NF160 for regenerated axons and S-100 for Schwann cells) revealed that the aligned polymer fiber-based constructs (aligned construct) facilitated the nerve regeneration of transected tibial nerves across 17mm nerve gaps, and that host derived Schwann cells infiltrated into the constructs from both proximal and distal nerve stumps (Fig. 2A, 2B, Fig. 3A, and 3B). The transected NF160⁺ axons entered into the proximal end of the construct, regenerated through entire length of the construct along the stacked aligned fiber films (Supplementary Fig. 3A and Fig. 3E), and moved into the distal nerve stump (Figs. 2A and 2.3). This successful nerve regeneration via the constructs was observed in all aligned construct treated animals (n=12). Interestingly, the regenerating axons always co-localized with the migrated S-100⁺ Schwann cells through the entire construct and no NF160⁺ axons were observed without co-localization with the Schwann cells. However, migrated Schwann cells in the absence of axons were observed (Figs. 2.1–3). The regenerated axons and the migrated Schwann cells regrew along the aligned fiber film surface, suggesting that the aligned fiber films guide the direction of regenerating axons and the migrating Schwann cells after injury, akin to the *in vitro* observations of DRG neurite extension (Fig. 1).

Unlike the nerve regeneration observed through the aligned constructs, significantly lower numbers of NF160⁺ axons were observed in all random polymer fiber-based constructs (random construct, n=12) (Figs. 2C, D, and 2.4–5). The transected NF160⁺ axons entered into the proximal end of the construct, but significantly lower numbers of axons regrew through entire length of the construct and entered into the distal nerve stump (Fig. 2.5). The majority of these axons regrew through the outer surface of the cable structure that was formed between proximal and distal nerve stump. Besides a few NF160⁺ axons, the numbers of S-100⁺ Schwann cells, migrating into the construct from both nerve stumps, were significantly lower than in the aligned construct. As in the case of aligned constructs, no NF160⁺ axons were observed without co-localization with the Schwann cells. However, migrated Schwann cells in the absence of axons were observed (Figs. 2.4–5).

In autograft treated animals, the transected NF160⁺ axons regenerated through the entire length of the graft and moved into the distal nerve stump (Figs. 2G, H, and 2.8). This successful nerve regeneration was observed in all autograft treated animals (n=12). Unlike with the nerve regeneration through aligned constructs, which have both axon/Schwann cell rich areas and axon/Schwann cell depleted areas through the constructs, regenerated axons within the autograft treated animals were tightly packed in with the aligned Schwann cells, suggesting that the autografts allowed less infiltration of non-neuronal cells such as fibroblasts or macrophages as compared to the aligned constructs.

No regenerative cable structure was observed in more than 85% of the saline filled polymeric construct treated animals (n=12, Figs. 2E, F, and 2.6–7). Even though a cable structure between proximal and distal nerve stumps was observed in ~15% of the animals, it was thin and stained positive for Collagen type 1, but not for NF160 or S-100 (data not shown). These results demonstrate no Schwann cell or axonal infiltration, confirming the stringency of the animal model used in this study. In the ~85% of the cases in which the nerve construct was empty, NF160⁺ axons were observed only at the proximal nerve stump, along with Schwann cells (Fig. 2.6), but not in the distal nerve stump (Fig. 2.7). This observation suggests that the saline filled polymeric constructs fail to facilitate formation of the initial fibrin cable between proximal and distal nerve stump due to the greater length of the nerve gap (17mm gap).

Schwann cell migration and laminin deposition

Double immunostaining for extracellular matrix protein laminin and NF160⁺ regenerated axons (either longitudinal or transverse cross-sectioned) revealed that the transected axons

regenerated through laminin⁺ circular pocket structures *in vivo* (Fig. 3). In the longitudinal sections, regenerated axons were always observed with both S-100⁺ migrated Schwann cells and endogenously deposited laminin protein, resembling bands of Bungner cellular structures (Figs. 3A–E). In the transverse cross-sections, it was clearly observed that axons regrew through laminin⁺ circular pocket structures (Figs. 3F and G). These structures are also strongly positive for S-100, which marks Schwann cells (data not shown). Figure 3G shows axons of several different diameters, which are encircled by pocket structures. In some cases, laminin⁺ pocket structures without NF160⁺ axons were observed (arrows in inset in Fig. 3G). Additionally, many small and large blood vessels were observed among these structures (Supplementary Fig. 3B and Fig. 3G). These pocket structures were also observed on the DRG that were seeded on the aligned fiber films *in vitro* (Supplementary Fig. 1).

Transverse cross-sections and histomorphometric analysis

Immunohistochemical and histomorphometric analysis of cross-sections of the implants revealed that the pattern of nerve regeneration through polymer fiber-based constructs (i.e., aligned or random constructs) was different from that of both autografts and normal nerves (Figs. 4A–H). However, there were significant similarities between the regenerated axons through the implants (i.e., aligned, random, and autograft) as compared to normal nerves in terms of diameter of axons (Figs. 4K–U). In all implants except the saline filled polymeric constructs, the regenerated NF160⁺ axons always co-localized with S-100⁺ Schwann cells, which ensheathed the axons with myelin through entire implant (Figs. 4N–U). Unlike the autografts and normal nerves, however, axon/Schwann cell depleted areas are observed in random and aligned polymer fiber-based constructs (Figs. 4A–H, and J). The axon/Schwann cell depleted areas are filled with non-neuronal cells (presumably fibroblasts, macrophages, or pro-inflammatory cells, indicated by arrows in 4E and F). Despite the different nerve regeneration pattern through the aligned constructs, the regenerated axons entered into the distal nerve stumps and regrew through the distal nerve stump (Fig. 4I).

Histomorphometric analysis revealed that significantly more sprouting axons were observed at the proximal portion of the aligned constructs as compared to autografts and normal nerves (1.7 times and 2.3 times as compared to autograft and normal nerve, respectively) (Fig. 4K). However, the number of axons was significantly reduced at the distal portion of the constructs, and was lower than in the normal nerve at distal nerve stump. The number of axons regenerated through the random constructs was significantly lower at all locations in the construct as compared to other conditions. Despite the differences in number of regenerated axons between the implants, the distribution of axon diameters is similar between the implants. Unlike in normal nerves, the majority of regenerated axons through the implants are smaller than 4 μm (Figs. 4L, M, and N–Q). Additionally, NF160⁺ axons, P0⁺ myelin sheaths, and surrounding S-100⁺ Schwann cells were distinct in the normal nerves (i.e., axons were tightly ensheathed by myelin and encircled by Schwann cells). However, these distinct structures were disrupted in all implants (Figs. 4N–U).

Characterization of reinnervated muscle

The regenerated axons that crossed over the 17mm nerve gaps through either the aligned constructs or the autografts continuously regrew through the distal nerve stump, reinnervated the affected gastrocnemius muscle, and reestablished SV2⁺ (a marker for synaptic vesicles) neuromuscular junctions (Fig. 5). The NF160⁺ regenerated axons (approximately 45% for aligned constructs, 4% for random constructs, and 55% of autografts, Fig. 5N; the number of co-localized NF160⁺ axons and α-Bung⁺ NMJs divided by the total number of α-Bung⁺ NMJs, see Methods section) at the muscle terminated at the end plates (confirmed by staining for alpha-Bungarotoxin-tetramethylrhodamine⁺ nicotinic acetylcholine receptors), resulting in new neuromuscular junctions (Figs. 5A, D, G, J, and M). Terminal Schwann cells, known to

play a crucial role in terminal sprouting [12], were also observed at the reformed neuromuscular junctions (Figs. 5B, E, and H, indicated by arrows in insets), and some of the axons extended beyond the end plate in the aligned construct treated animals (Fig. 5D). Additionally, there were SV2⁺ neuromuscular junctions (approximately 31% for aligned construct and 40% for autograft, Fig. 5O; the number of co-localization of SV2⁺ synaptic vesicles and α -Bung⁺ NMJs divided by the total number of α -Bung⁺ NMJs, see Methods section) at the affected muscle, suggesting that the regenerated axons in the aligned constructs and autografts not only reinnervated the end plates (resulting in neuromuscular junctions), but also that the neuromuscular junctions were functional.

The gastrocnemius muscle ratio (affected muscle/contralateral intact muscle) was significantly higher in the autograft treated animals as compared to other experimental conditions. However, this muscle ratio in the aligned construct treated animals was significantly higher than in random construct and saline filled polymeric construct treated animals (Fig. 5L).

Characterization of regenerated nerve modality

Electrophysiological and behavioral analysis revealed that the aligned constructs enabled both sensory and motor nerve regeneration and significantly improved functional recovery as compared to either random constructs or saline filled polymeric constructs. Figure 6A (and supplementary figure 4) display representative compound action potentials (CAP) of normal controls, autograft, aligned construct, and random construct treated animals. Recordings were taken from mixed (Fig. 6A), sensory, and motor nerve (supplementary figure 4) components of the regenerated nerve in response to tibial nerve, dorsal root, and ventral root stimulation respectively (Fig. 6B, see Methods section). In all CAP testing (i.e., mixed, sensory, and motor nerve), significantly higher stimulation levels were required to recruit regenerated axons in autograft and aligned construct treated animals as compared to normal animals. However, these regenerated axons were functional (e.g., able to propagate action potentials and to produce observable muscle contraction) and it is important to note that aligned constructs performed comparably to autografts in all CAP measurements. In the random construct treated animals, no CAPs were detected.

Recovery of mixed, sensory, and motoneuron conduction after bridging with aligned constructs or autografts was determined by measuring the time latency from the application of an electrical pulse at the stimulus site to the detection of the resulting CAP at the recording site (Fig. 6C, see Methods section). Conduction latency in all CAPs was significantly higher across autografts and aligned constructs than across normal controls, demonstrating that regenerated axons propagate action potentials more slowly than in intact nerve. These results also suggest a larger composition of smaller diameter axons, a finding that agrees with the measured distribution of axon caliber and the extent of myelination in both autograft and aligned construct treated animals (Fig. 4). Comparing autografts and aligned constructs, conduction latency across mixed and sensory nerves was significantly shorter through autografts. However, in motor nerves there was no significant difference in conduction latencies between autograft and aligned construct.

Grid walking measurements demonstrated significantly fewer foot slips in autograft and aligned construct treated animals than in random construct or in saline filled polymeric construct treated animals (Fig. 6D). These results suggest that both autograft and aligned construct treatment reduce the functional deficits after peripheral nerve injury.

DISCUSSION

The results of this study demonstrate that aligned polymer fiber-based constructs (aligned constructs), but not randomly oriented polymer fiber-based constructs (random constructs),

significantly enhance peripheral nerve regeneration in a challenging, 17mm nerve gaps in rats, as evaluated by anatomical as well as functional measures. The significance of these results is that sub-micron scale topographical cues alone, with no exogenous neurotrophic or extracellular matrix proteins, enabled significant regeneration. Immunohistochemical analysis clearly shows that transected tibial nerve axons entered into the aligned construct along with migrated Schwann cells, regrew through the entire construct, and moved into the distal nerve stump. The regenerated axons are myelinated, co-localized with migrated Schwann cells, and encircled by endogenously deposited laminin⁺ pocket structures. The regenerated axons continuously regrew, reinnervated target tissues (e.g., gastrocnemius muscle), and reformed synaptic vesicle-rich neuromuscular junctions along with terminal Schwann cells. Electrophysiological and behavioral analysis also demonstrate that aligned constructs facilitated both sensory and motor nerve regeneration, significantly improving functional deficit after peripheral nerve injury.

Topographical cues involving grooves and fibers have been previously demonstrated to cause Schwann cell alignment and subsequent neurite extension *in vitro* [13–15], and some studies have used magnetically aligned collagen filaments [16], microbraided polymer fibers [3], or micron-scale filaments to promote nerve regeneration [17,18]. In this study, the quantitative comparison of both DRG outgrowth *in vitro* and nerve regeneration *in vivo* on two different polymer surface topographies (i.e., aligned and randomly oriented fiber films, but with identical material and fabrication process) clearly provides key insights into the importance of the structural cues (i.e., alignment) for promoting nerve regeneration. The extent of neurite outgrowth and Schwann cell migration *in vitro* was significantly higher on the aligned fiber films than on the random fiber films (Fig. 1M). Also, the number of regenerated axons per cross-section *in vivo* was significantly higher in the aligned construct treated animals than in the random construct treated animals, through the length of the constructs (Fig. 4K). Since the alignment of the fiber films was the only difference between these two experimental conditions, these results clearly demonstrate the significance of topographical cues in influencing endogenous repair mechanisms for promoting nerve regeneration across challenging gaps.

The aligned constructs, consisting of a stack of aligned sub-micron fiber films, were designed to 1) maximize the topographic directional cues for neurite outgrowth and Schwann cell migration in 3D configuration by stacking multiple films while minimizing the cross-sectional space occupied by the polymer and 2) facilitate the recreation of bands of Bungner-like cellular structures by providing topographical cues for migrating Schwann cells, resulting in the maximization of the innate regenerative capabilities of the peripheral nervous system after injury. The importance of providing a 3D substrate for neurite outgrowth has been demonstrated in many studies. To achieve this goal, nerve constructs have been filled with neurite outgrowth permissive gels, including either extracellular matrix (e.g., collagen and fibrin gel) or hydrogels (e.g., agarose gel) [19–21] or with polymer-based microfilaments [17]. One important design criterion for 3D constructs is to maximize directional topographic cues while minimizing cross-sectional area occupied by the polymer (i.e., dead space where nerve cannot grow). Due to our design of stacking thin polymer films inside the polymeric conduit only 10% of the total cross-sectional area of polymer conduit was occupied by the polymer films.

After peripheral nerve injury, Schwann cells proliferate, reorganize, and align to form bands of Bungner, oriented columns of extracellular matrix protein laminin and Schwann cells [7]. These bands are an essential key element of axon regeneration for the following reasons: they serve as a guiding substrate to direct axons to the target organs and provide regenerating axons with neurotrophic factors, including nerve growth factor, brain-derived nerve growth factor, and ciliary neurotrophic factor. They also provide cell adhesion molecules/basement membrane components such as laminin, fibronectin, proteoglycan, and collagen [8,22]. In this

study, we observed these bands of Bungner-like cellular structure along with the aligned fiber films: Schwann cells migrated from both proximal and distal nerve stumps and reorganized along the oriented fiber films, with laminin deposition (Fig. 3). We believe that these bands of Bungner-like cellular structures within the aligned constructs provides topographical cues and directed axonal regeneration across long nerve gaps, without requiring the addition of exogenous neurotrophic or ECM proteins.

Only ~40–50% of patients regain useful function when treated with autografts, the current clinical gold standard [23]. These imperfect results might be explained by a recent study that examined the effects of motor versus sensory nerve grafts on peripheral nerve regeneration. The study showed that while robust nerve regeneration was observed through motor and mixed nerve grafts, poor nerve regeneration was seen through sensory nerve grafts. These results suggest that motor or mixed nerve grafts might improve clinical outcomes as compared to the current autograft approach that employs a sensory nerve [2]. However, there are few available motor or mixed nerves suitable for use as autografts in the human body. Therefore, it is important to develop an alternative clinical approach suitable for repairing both sensory and motor nerve injuries. In this study, we chose a mixed tibial nerve for use as an autograft, so that our aligned constructs were compared against the ‘ideal’ autograft, not the less functional, clinically used sensory grafts. To determine whether aligned constructs are suitable for both sensory and motor nerve regeneration, electrophysiological analysis was used to measure compound action potentials (CAP) across the regenerated nerve segments. The results revealed that aligned constructs promote both sensory and motor nerve regeneration (supplementary figure 4); CAPs measured at the lumbar dorsal root (for sensory nerves) and ventral root (for motor nerves) revealed that both sensory and motor neurons regenerated through the aligned construct and were functional (i.e., able to propagate an action potential across the construct and to produce observable muscle contraction). However, the conduction latency was significantly higher than normal nerve in all CAP measurements, and higher stimulation levels were required to elicit CAPs, as compared to normal nerve (presumably due to significantly higher numbers of smaller diameter (<4µm) regenerated axons with a thinner myelination than in normal nerve).

Our results clearly demonstrate that with the use of a synthetic polymer-based aligned construct, a 17mm peripheral nerve gap in a rodent model can be reliably bridged without the exogenous delivery of regenerative agents such as neurotrophic factors (e.g., NGF), ECM molecules (e.g., laminin), or cell transplantation (e.g., pre-seeded Schwann cells). This feature suggests several potential advantages of aligned constructs over autografts and conventional tubular nerve conduits for promoting long nerve gap regeneration as follows: 1) unlimited availability, and ready-to-use: the aligned construct are relatively easy to fabricate, handle, store, replace, and sterilize, compared to autografts which require a secondary surgery and cause donor site morbidity. Also, since the aligned construct does not require exogenous proteins or cells, its use avoids the complications associated with the addition of exogenous proteins or cells; 2) pre-customization and off-the-shelf: the aligned construct can be pre-customized (e.g., diameter or length) for different types of nerve injury. This potential customization may significantly minimize the lack of coaptation between the injured nerve and the nerve graft due to size/length/modality mismatch; and 3) suitable for both sensory and motor nerve repair: the aligned construct promotes both sensory and motor nerve regeneration. This is important, because the nerves mainly used for current autografts are sural nerves, which are sensory and thus not ideal as described above.

The presence of non-degradable PAN-MA amidst the regenerated axons/migrated Schwann cells and polysulfone-based nerve conduit resulted in no adverse effects on nerve regeneration, as indicated by immunohistological and functional evaluation. However, the next generation of aligned constructs could employ biodegradable fiber films and conduits, and we anticipate

that degradable fiber films would enable regeneration, provided that their degradation does not interfere with the stability of the bands of Bungner-like cellular structures during the initial regeneration period. In this sense, our results are generalizable to a wide-range of polymers with appropriate and efficient topographical cue presentation, including non-degradable polymers and our findings with regarding to aligned polymer fibers facilitating and augmenting endogenous Schwann cell migration mediated regeneration are significant and important.

CONCLUSION

Topographical cues can influence endogenous peripheral nerve repair mechanisms even in the absence of exogenous growth promoting proteins. Aligned, but not randomly oriented, polymer fiber constructs successfully promoted regeneration of axons across a 17mm nerve gap, reinnervating muscles, and reforming new neuromuscular junctions, as verified through histological, electrophysiological, and behavioral analysis. Thus, aligned polymer fiber-based constructs are potentially a significant alternative to the current clinical standard of using autografts for the repair of long peripheral nerve gaps.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Funding for this research project was provided by NIH NS44429 (RVB) and GTEC (NSF EEC-9731643). The authors thank Isaac Clements, Dr. Robert H. Lee, Dr. Mahesh Dodla, George McConnell, Tong Wang, and Hong Shen for technical assistance toward this work.

REFERENCES

1. IJkema-Paassen J, Jansen K, Gramsbergen A, Meek MF. Transection of peripheral nerves, bridging strategies and effect evaluation. *Biomaterials* 2004;25:1583–1592. [PubMed: 14697860]
2. Nichols CM, Brenner MJ, Fox IK, Tung TH, Hunter DA, Rickman SR, et al. Effects of motor versus sensory nerve grafts on peripheral nerve regeneration. *Exp Neurol* 2004;190:347–355. [PubMed: 15530874]
3. Bini TB, Gao S, Xu X, Wang S, Ramakrishna S, Leong KW. Peripheral nerve regeneration by microbraided poly(L-lactide-co-glycolide) biodegradable polymer fibers. *J Biomed Mater Res A* 2004;68:286–295. [PubMed: 14704970]
4. Itoh S, Takakuda K, Kawabata S, Aso Y, Kasai K, Itoh H, et al. Evaluation of cross-linking procedures of collagen tubes used in peripheral nerve repair. *Biomaterials* 2002;23:4475–4481. [PubMed: 12322967]
5. Matsuyama T, Mackay M, Midha R. Peripheral nerve repair and grafting techniques: a review. *Neurol Med Chir (Tokyo)* 2000;40:187–199. [PubMed: 10853317]
6. Taras JS, Nanavati V, Steelman P. Nerve conduits. *J Hand Ther* 2005;18:191–197. [PubMed: 15891977]
7. Bungner O. Über die Degenerations- und Regenerationsvorgänge am Nerven nach Verletzungen. *Beitr Pathol Anat* 1891;10:610–616.
8. Ide C. Peripheral nerve regeneration. *Neurosci Res* 1996;25:101–121. [PubMed: 8829147]
9. Lundborg G, Dahlin LB, Danielsen N, Gelberman RH, Longo FM, Powell HC, et al. Nerve regeneration in silicone chambers: influence of gap length and of distal stump components. *Exp Neurol* 1982;76:361–375. [PubMed: 7095058]
10. Wang T, Kumar S. Electrospinning of polyacrylonitrile nanofibers. *J Appl Polym Sci* 2006;102:1023–1029.
11. Kunkel-Bagden E, Dai HN, Bregman BS. Methods to assess the development and recovery of locomotor function after spinal cord injury in rats. *Exp Neurol* 1993;119:153–164. [PubMed: 8432357]

12. Love FM, Son YJ, Thompson WJ. Activity alters muscle reinnervation and terminal sprouting by reducing the number of Schwann cell pathways that grow to link synaptic sites. *J Neurobiol* 2003;54:566–576. [PubMed: 12555269]
13. Rangappa N, Romero A, Nelson KD, Eberhart RC, Smith GM. Laminin-coated poly(L-lactide) filaments induce robust neurite growth while providing directional orientation. *J Biomed Mater Res* 2000;51:625–634. [PubMed: 10880110]
14. Rutkowski GE, Miller CA, Jeftinija S, Mallapragada SK. Synergistic effects of micropatterned biodegradable conduits and Schwann cells on sciatic nerve regeneration. *J Neural Eng* 2004;1:151–157. [PubMed: 15876634]
15. Wen X, Tresco PA. Effect of filament diameter and extracellular matrix molecule precoating on neurite outgrowth and Schwann cell behavior on multifilament entubulation bridging device in vitro. *J Biomed Mater Res A*. 2005
16. Dubey N, Letourneau PC, Tranquillo RT. Guided neurite elongation and schwann cell invasion into magnetically aligned collagen in simulated peripheral nerve regeneration. *Exp Neurol* 1999;158:338–350. [PubMed: 10415141]
17. Cai J, Peng X, Nelson KD, Eberhart R, Smith GM. Permeable guidance channels containing microfilament scaffolds enhance axon growth and maturation. *J Biomed Mater Res A* 2005;75:374–386. [PubMed: 16088902]
18. Ngo TT, Waggoner PJ, Romero AA, Nelson KD, Eberhart RC, Smith GM. Poly(L-Lactide) microfilaments enhance peripheral nerve regeneration across extended nerve lesions. *J Neurosci Res* 2003;72:227–238. [PubMed: 12671998]
19. Evans GR, Brandt K, Katz S, Chauvin P, Otto L, Bogle M, et al. Bioactive poly(L-lactic acid) conduits seeded with Schwann cells for peripheral nerve regeneration. *Biomaterials* 2002;23:841–848. [PubMed: 11774850]
20. Nakayama K, Takakuda K, Koyama Y, Itoh S, Wang W, Shirahama N, et al. Regeneration of peripheral nerves by bioabsorbable polymer tubes with fibrin gel. *J Nanosci Nanotechnol* 2007;7:730–733. [PubMed: 17450824]
21. Yu X, Bellamkonda RV. Tissue-engineered scaffolds are effective alternatives to autografts for bridging peripheral nerve gaps. *Tissue Eng* 2003;9:421–430. [PubMed: 12857410]
22. Bunge RP. Expanding roles for the Schwann cell: ensheathment, myelination, trophism and regeneration. *Curr Opin Neurobiol* 1993;3:805–809. [PubMed: 8260833]
23. Lee SK, Wolfe SW. Peripheral nerve injury and repair. *J Am Acad Orthop Surg* 2000;8:243–252. [PubMed: 10951113]

Figure 1.

DRGs on aligned and random fiber film *in vitro*. A–D) Double immunostained DRG on the aligned fiber film: (A) representative montage of NF160 (a marker for axons) immunostained DRG neurons on the film and (B) montage of S-100 (a marker for Schwann cells) immunostained Schwann cells on the film. (C) Magnified NF160 (red, from box in A) and S-100 (green, from box in B) overlapped image. (D) Double immunostained aligned axons (NF160, red) and endogenously deposited laminin protein (Laminin, green). E–I) Fabrication of the fiber films and distribution of alignment of the films. E) Schematic of aligned fiber film fabrication by electrospinning process. Random fiber film was deposited on a flat metal target instead of on a high-speed rotating metal drum. F and H) Representative SEM image of the aligned fibers (F, magnified fibers below) and the random fibers (H). Scale bar = 1 μ m and 30 μ m, respectively. G and I) Distribution of fiber alignment in aligned (G) and random fiber (I). J and K) Double immunostained DRG on the random fiber film: (J) representative montage of NF160⁺ neurons and (K) S-100⁺ Schwann cells. Scale bar = 500 μ m. L) shows the quantitative comparison of orientation of neurite outgrowth on the aligned and random fiber film. Direction of arrows indicates the orientation of neurite outgrowth, and length of arrows indicates the rate of occurrence (percentage) (n=25 per DRG). M) shows the quantitative comparison of the extent of neurite outgrowth and Schwann cells migration on the films. The distance between the longest neurite outgrowth (n=25 per DRG)/the furthest migrated Schwann cells (n=10 per DRG) and DRG was measured and averaged. *P<0.05. Error bar = s.e.m.

Figure 2.

Immunohistochemical analysis of nerve regeneration through implants *in vivo* (longitudinal section, 17mm nerve gap). A, B, and 1–3) Representative nerve regeneration through aligned fiber-based polymeric construct (aligned construct); C, D, and 4–5) nerve regeneration through random fiber-based polymeric construct (random construct); E, F, and 6–7) nerve regeneration through saline filled polymeric construct; and G, H, and 8) nerve regeneration through autograft. A and B) Double immunostained nerve regeneration (A, NF160) and Schwann cells infiltration from both proximal and distal nerve stump (B, S-100). Scale bar = 1mm. Numbered three images are magnified and NF160/S-100 overlapped images from boxes in A. Scale bar = 200μm. C and D) Double immunostained nerve regeneration (C) and Schwann cells (D). Two numbered images are from boxes in C. E and F) Double immunostained nerve (E) and Schwann cells (F). Numbered images are magnified and NF160/S-100 overlapped image from boxes in E. G and H) Double immunostained nerve regeneration (G) and Schwann cells (H). Numbered image is magnified and NF160/S-100 overlapped images from box in H. Abbreviation: g = autograft. Scale bar = 800μm.

Figure 3.

Nerve regeneration through aligned construct and presence of both migrated Schwann cells and endogenously deposited laminin protein. A and B) Representative double immunostained nerve regeneration (A) and migrated Schwann cells from both proximal and distal nerve stump (B). Abbreviation: m = polysulfone nerve conduit. C–E) Magnified and double immunostained endogenously deposited laminin (green, C) and regenerating axons (red, D) from box in A. E) Triple overlapped images: laminin (green), axon (red), and aligned fiber films (blue). Arrows indicate fiber films. Scale bar = 200 μ m. F) Representative NF160 (a marker for axons) immunostained aligned construct (transverse cross-section, 5mm from proximal nerve stump). Scale bar = 500 μ m. G) Magnified and double immunostained regenerated axons (red) encircled by laminin⁺ pocket structures (green) from box in F. Arrow heads indicate blood vessels and inset shows magnified axons and laminin⁺ pocket structures. Arrows indicate laminin⁺ pocket structures without re-grown axon.

Figure 4.

Quantitative immunohistochemical analysis of nerve regeneration through implants (transverse cross-section). A, E, I, and J) aligned construct; B and F) random construct; C and G) autograft; D) saline filled polymeric construct; and H) normal tibial nerve. Scale bar = 500μm. A and E) Double immunostained (NF160/S-100) cross-section (5mm from proximal nerve stump). B and F) Double immunostained (NF160/S-100) cross-section (5mm from proximal nerve stump). C and G) Double immunostained (NF160/S-100) cross-section (5mm from proximal nerve stump). I) NF160 immunostained axons at 10mm into distal nerve stump. Inset is magnified and NF160/S-100 immunostained images. Scale bar = 200μm. J) Regenerated axons (red, immunostaining) and aligned fiber films (white lines, bright field) overlapped image (5mm from proximal nerve stump). K) Quantitative comparison of average number of axons/cross-section between the implants and normal nerves at three different sites: 5mm and 12mm from proximal nerve stump, and 3mm into distal nerve stump. Error bar is s.e.m. L and M) Comparison of axon diameter distribution: 5mm from proximal nerve stump (L) and 3mm into distal nerve stump (M). N–Q) Magnified NF160 (red)/S-100 (green) double immunostained cross-section of normal nerve (N), aligned construct (O), autograft (P), and random construct (Q). Insets are magnified images. Scale bar = 50μm. R–U) Magnified NF160 (red)/P0 (protein zero, green) double immunostained cross-section of normal nerve (R), aligned construct (S), autograft (T), and random construct (U). Insets are magnified images.

Figure 5.

Reconstruction of functional neuromuscular junctions. A–C) normal control; D–F) aligned construct; G–I) autograft; J) random construct; and M) saline filled polymeric construct. Scale bar = 100µm. A, D, G, J, and M) Double immunostained reinnervated axons (NF160, green) and nicotinic acetylcholine receptors (alpha-Bungarotoxin-tetramethylrhodamine, red) at neuromuscular junctions. Insets are magnified images. B, E, and H) Double immunostained terminal Schwann cells (S-100, green) and nicotinic acetylcholine receptors (red). Arrows indicate terminal Schwann cells at the neuromuscular junctions. Insets are magnified images. C, F, and I) Double immunostained synaptic vesicles (SV2, green) and nicotinic acetylcholine receptors (red) at neuromuscular junctions. Insets are magnified images. K) Schematic of a

neuromuscular junction. Abbreviation: SV = synaptic vesicle, NAR = nicotinic acetylcholine receptors, and TSC = terminal Schwann cells. L) Comparison of relative gastrocnemius muscle weight ratio (affected muscle/contralateral intact muscle) between the implants. * $P < 0.05$ between aligned and others. % $P < 0.05$ between random and others (autograft and saline). & $P < 0.05$ between autograft and saline. Error bar = s.e.m. N) Comparison of percentage of NF160⁺ axons (green) innervated with alpha-Bungarotoxin-tetramethylrhodamine⁺ neuromuscular junctions (red) between the implants and normal controls. See the detail in Methods section. O) Comparison of percentage of co-localization of SV2⁺ synaptic vesicles and alpha-Bungarotoxin-tetramethylrhodamine⁺ neuromuscular junctions (red) between the implants and normal controls. See the detail in Methods section. Error bar is s.e.m. # $P < 0.05$ between normal control and others. * $P < 0.05$ between aligned and others. % $P < 0.05$ between random and others (autograft and saline). & $P < 0.05$ between autograft and saline.

Figure 6. Electrophysiology and behavioral outcomes. A) Comparison of compound action potentials (CAP) recorded from the animals following electrical stimulation of mixed nerve. See the detail in Methods section. S represents stimulus voltage. B) Schematic of CAP measurements. C) Comparison of conduction latency measured from mixed, sensory, and motoneurons in normal control, autograft, and aligned construct treated animals. [#] $P < 0.05$ between normal control and others, and ^{*} $P < 0.05$ between autograft and aligned construct. Error bar is s.e.m. D) Quantitative comparison of the number of foot slips between conditions. [#] $P < 0.05$ between normal and others. ^{*} $P < 0.05$ between autograft and others. [%] $P < 0.05$ between aligned and others (random and saline filled polymeric construct).