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Article

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**Three-dimensional nanofiber hybrid scaffold directs and enhances axonal regeneration
after spinal cord injury**

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Abstract

Spinal cord injuries (SCI) are followed by a complex series of events that contribute to the failure of regeneration. To date, there is no robust treatment that can restore the injury-induced loss of function. Since damaged spinal axons do not spontaneously regenerate in their native inhibitory microenvironment, a combined application of biomaterials and neurotrophic factors that induce nerve regeneration emerges as an attractive treatment for

SCI. In this study, we report the novel use of a three-dimensional (3D) hybrid scaffold to provide contact guidance for regrowth of axons *in vivo*. The scaffold comprises of 3D aligned sparsely distributed poly (ϵ -caprolactone-*co*-ethyl ethylene phosphate) nanofibers that are supported and dispersed within a collagen hydrogel. Neurotrophin-3 was incorporated into the scaffold as an additional biochemical signal. To evaluate the efficacy of the scaffold in supporting nerve regeneration after SCI, the construct was implanted into an incision injury, which was created at level C5 in the rat spinal cord. After 3 months of implantation, scaffolds with NT-3 incorporation showed the highest average neurite length ($391.9 \pm 12.9 \mu\text{m}$, $p \leq 0.001$) as compared to all the other experimental groups. In addition, these regenerated axons formed along the direction of the aligned nanofibers, regardless of their orientation. Moreover, the presence of the hybrid scaffolds did not affect tissue scarring and inflammatory reaction. Taken together, these findings demonstrate that our scaffold design can serve as a potential platform to support axonal regeneration following SCI.

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Key words: sustained release, electrospinning, NT-3, collagen, neural tissue engineering, neurite ingrowth

INTRODUCTION

Spinal cord injuries (SCI) often result in the extensive loss of nerve connections. As damaged spinal axons do not spontaneously regenerate in their native inhibitory microenvironment, SCI lead to permanent functional impairment below the injury sites. Unfortunately, to date, there has not been any robust treatment that can promote tissue regeneration and restore the injury-induced loss of function to prevent life-long disability¹⁻².

One of the current strategies for SCI repair is the introduction of biomaterial scaffolds to bridge the injury gap and support neo-tissue formation. Currently, hydrogels³⁻¹³ and self-assembled peptide nanofibers¹⁴⁻¹⁵ are often used as they mimic the mechanical properties of the spinal cord. However, the isotropic architecture of these materials cannot direct cellular and neuronal regeneration in the rostral-to-caudal direction^{9, 14, 16-20}, resulting in relatively few axons ultimately forming reconnections with the host tissue^{9, 16, 19-20}. Moreover, due to the fast degradation rate of these materials, the problem of cyst formation remains poorly addressed^{15, 21}.

To provide physical contact guiding cues, scaffolds with topography, such as microchannels, microfilaments, and conduits made out of electrospun fiber meshes, have been used. In general, guidance effect was seen for axonal regeneration^{9, 20, 22}. However, these regenerated axons did not re-penetrate the host spinal cord beyond the lesion site partially due to the formation of thick fibrous scars around the scaffolds^{9, 20}. In addition, there was limited cell penetration into the walls of the scaffolds^{9, 20, 22}, ultimately resulting in regenerated tissue structures that are far from the physiological architecture of the spinal cord.

Given the limitations of previous scaffold designs, we hypothesized that a combination of hydrogel and aligned nanofibers would be favorable to SCI treatment. The hydrogel gel component provides a three-dimensional structure that mimics the mechanical environment of the spinal cord to facilitate cell penetration. More importantly, its three dimensional isotropic architecture could be exploited to disperse aligned nanofibers throughout the hydrogel matrix to guide nerve regeneration along the longitudinal axis of the spinal cord. On the other hand, aligned electrospun fibers have been shown to promote axonal regeneration and improve functional recovery after injuries in the peripheral nervous system²³⁻²⁵. In addition, as compared to the microchannels and microfibers that were used previously, nanofibers more closely mimic the size scale and architecture of the natural extracellular matrix. Correspondingly, these constructs are expected to improve host-implant integration with reduced scar tissue formation^{22, 26}.

In this study, we demonstrate the effects of a three-dimensional hydrogel-nanofiber hybrid scaffold on axonal regrowth, host-implant integration, and blood vessel formation after SCI. Specifically, the scaffold was fabricated with sparsely distributed aligned poly (ϵ -caprolactone-*co*-ethyl ethylene phosphate) (PCLEEP) nanofibers that were dispersed throughout a collagen hydrogel. This scaffold design is robust and easy to handle *in vivo*, thus allowing us to explore the effects of fiber orientation on axonal regrowth. Furthermore, neurotrophin-3 (NT-3) could be easily incorporated into the scaffold as an additional biochemical signal for nerve regeneration. NT-3 promotes neuronal survival, axonal sprouting, and regeneration²⁷⁻²⁹. It also facilitates the proliferation and differentiation of oligodendrocyte precursor cells (OPCs)^{27, 30-31}. Thus, a combined treatment that comprises of scaffold topography and neurotrophic factor delivery would be promising in enhancing nerve regeneration after SCI.

EXPERIMENTAL PROCEDURES

Scaffold fabrication

a. Aligned PCLEEP fibers

The PCLEEP copolymer (Mw = 40,509, Mn = 17,288) was synthesized by previously reported methods³²⁻³³. PCLEEP (22 % wt.%) was dissolved in 2,2,2-Trifluoroethanol (TFE, Sigma-Aldrich). One-hour prior to electrospinning, Tris-EDTA buffer (TE buffer; pH 8.0, 1st BASE) was added to the polymer solution at a 1:10 (TE: TFE) volume ratio to form the spinning solution. Sparsely distributed aligned PCLEEP fibers were fabricated using the two-pole air gap electrospinning technique³⁴. In brief, the spinning solution was loaded into a 1 ml syringe that was capped with a 21 gauge blunt-tipped needle. This solution was then charged at + 10 kV and released at a flow rate of 1.9 ml/h. The PCLEEP fibers were deposited within a 5.0 cm air gap area that was between two stationary collector poles (- 5.0 kV). Each set of fiber was obtained after 1.0 minute of spinning. These fiber bunches were then sterilized under UV light for 30 minutes.

b. Scaffolds

Rat-tail Collagen type I (10.08 mg/ml, Corning, LOT 4041009) was used to fabricate hydrogel according to the manufacturer's protocol (BD Biosciences). Briefly, 10x phosphate buffered saline (PBS; pH 7.4, Life Technologies), collagen type I, 1.0 N NaOH, and de-ionized (DI) water were added into a sterile microtube and mixed properly to get a final collagen concentration of 6.0 mg/ml. The collagen mixture was kept on ice until used. 50 µl of the mixture was then added into a sterilized cylinder mold of 6.0 mm in length and 3.0 mm in inner diameter to obtain isotropic hydrogel scaffolds. To obtain scaffolds with aligned nanofibers, the mold was pre-set with 4 sets of fibers in the core region prior to the addition

of the collagen mixture. For NT-3-incorporation, 1.0 μl of 10x PBS and 9.0 μl of DI water in the collagen mixture was substituted by 10 μl of 0.1 % bovine serum albumin (BSA, Sigma-Aldrich) containing 5.0 μg of human NT-3 (PeproTech). The mixture was then added into the PCLEEP fiber-embedded mold. Hydrogel formation took place at 37 $^{\circ}\text{C}$ in the incubator for 45 minutes. After gelation, the scaffolds were rinsed with 1x and 0.1x PBS sequentially. Thereafter, the scaffolds were lyophilized and kept at - 20 $^{\circ}\text{C}$ until used.

Scaffold characterization

a. Fiber diameter

The PCLEEP aligned fibers and scaffolds were sputter-coated with platinum (JEOL, JFC-1600) at 10 mA for 120 s and 140 s, respectively. Thereafter, these samples were observed under the scanning electron microscope (SEM; JEOL, JSM-6390 LA) at an accelerating voltage of 10 kV. The fiber diameters were measured using ImageJ software (<http://imagej.nih.gov/ij/>) on SEM images. At least 100 fibers were measured.

b. Fiber distribution

Scaffolds were fixed with 10 % formalin for 45 minutes at room temperature. Thereafter, the scaffolds were rinsed with PBS and then placed at - 80 $^{\circ}\text{C}$ overnight. These scaffolds were then cross-sectioned in liquid nitrogen. Subsequently, they were sputter-coated with platinum (JEOL, JFC-1600) at 10 mA for 150 s and observed under the SEM at an accelerating voltage of 10 kV. The ratio of fiber area to scaffold area was measured using ImageJ software. Three scaffolds were used for this measurement.

For fiber density, 3 different cross-sectioned areas (50 μm x 50 μm) of a scaffold were imaged and the number of fibers in each area was counted using ImageJ software. Three scaffolds were used for this measurement.

Animals

Adult female Sprague Dawley rats (6 - 8 weeks, 200 - 250 g) were obtained from In Vivos Pte Ltd (Singapore). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC, NTU).

Animals were randomly divided into five groups. Specifically, the experimental groups comprised of scaffolds with aligned fibers that were implanted parallel (denoted as Parallel or NT-3 Parallel, where NT-3 was incorporated) or at an angle (Angled) to the longitudinal axis of the spinal cord. The control groups comprised of plain collagen hydrogels without aligned nanofibers (Gel) and SCI animals without any treatment (SCI). Table 1 summarizes the experimental groups and test conditions.

Time point	Group name	Description	Sample size
4 weeks	Parallel	Plain nanofiber-hydrogel scaffolds, with nanofibers aligned parallel to spinal cord longitudinal axis	3
12 weeks	Parallel	Plain nanofiber-hydrogel scaffolds, with nanofibers aligned parallel to spinal cord longitudinal axis	8
	Angled	Plain nanofiber-hydrogel scaffolds, with nanofibers aligned at an angle to spinal cord longitudinal axis	8

	NT-3 Parallel	NT-3 incorporated nanofiber-hydrogel scaffolds, with nanofibers aligned parallel to spinal cord longitudinal axis	4
	Gel	Plain collagen hydrogel scaffolds	6
	SCI	Injured, untreated animals	6

Table 1. Experimental groups and test conditions.

Implantation Surgeries

Rats were injected with buprenorphine subcutaneously (0.05 mg/kg) 30 minutes before surgery. Right before surgery, they were anesthetized using a combination of ketamine (73 mg/kg) and xylazine (7.3 mg/kg). Thereafter, the surgical field was shaved and cleaned with Betadine. The skin was cut open and the muscle was cut, peeled back to the sides, and held in place. The C5 vertebra was located and its right side was broken open to gain access to the C5 spinal cord. A 1/3 incision was then made on the right side (Fig. 1A) using a pair of iridectomy scissors. Thereafter, scaffolds (1.0 mm in length) were implanted into the incision site such that the fibers were either parallel to the long axis of the spinal cord (Parallel and NT-3 Parallel groups, Fig. 1B, C), or at an angle (Angled group, Fig. 1D, E). For Gel group, a plain collagen hydrogel (1.0 mm in length) was implanted into the site of injury (Fig. 1F, G). For SCI group, the incision was left untreated. Thereafter, in all the groups, a layer of fat was used to cover the injury, the muscle was then sutured, and the skin was closed with auto clips. All animals were injected with buprenorphine subcutaneously (0.05 mg/kg) twice a day for 3 days post-operation. Subsequently, they were fed with acetaminophen (200 mg/kg) three times a day and meloxicam (5.0 mg/ml, 1.0 ml in 500 ml drinking water) continuously for the next 7 days. If forepaw biting was noticed, the rats were fed with acetaminophen (200 mg/kg)

three times a day for another 3 days and injected with Baytril (5.0 %, 0.1 ml/kg) subcutaneously once a day until the symptom ceased.

Immunohistochemistry and Histology

At 4 weeks and 3 months post-operation, animals were perfused with 0.9 % saline followed by 4.0 % ice-cold paraformaldehyde (Millipore). After perfusion, spinal cords were post-fixed for 2 h at 4 °C and then sequentially transferred to 15 % sucrose and 30 % sucrose after 24 h and 48 h, respectively. All samples were stored at 4 °C until sectioned. Spinal cord samples of 1.5 cm containing the lesion site were sectioned horizontally on a cryostat set at 20 µm thickness. Immunohistochemistry and histology were performed to evaluate axonal regrowth, scar formation, host-implant reaction, and blood vessel reformation.

a. Immunohistochemistry

For immunohistochemistry, all the steps were done at room temperature. Spinal cord sections were treated with 0.3 % Triton X-100 (Sigma-Aldrich) for 5 minutes and then incubated in 10 % normal goat serum (NGS, Sigma-Aldrich) blocking solution for 1 h at room temperature. Thereafter, the sections were incubated with primary antibodies diluted in 5.0 % NGS overnight followed by secondary antibodies diluted in PBS for 1.5 h. The following primary antibodies were used: rabbit anti-neurofilament 200 (NF, Sigma-Aldrich, 1:700 dilution), mouse anti-glial fibrillary acidic protein (GFAP, clone GA5, Millipore, 1:500 dilution), mouse anti-CD11b/c (anti-Ox42, Abcam, 1:200 dilution), mouse anti-macrophages/monocytes (clone ED-1, Millipore, 1: 200 dilution), rabbit anti-laminin (LN, SigmaAldrich, 1:200 dilution), mouse anti-Rat RECA-1 (clone HIS52, AbD Serotec, 1:800 dilution). Secondary antibodies were Alexa Fluor 488-conjugated Goat Anti-Mouse/Rabbit IgG (Invitrogen, 1:700 dilution) and Alexa Fluor 555-conjugated Goat Anti-Mouse/Rabbit

IgG (Invitrogen, 1:1000 dilution). Nuclear staining was performed by incubating the sections with DAPI (1:1000, Life Technologies) for 10 minutes after the secondary antibodies. Thereafter, all samples were examined using an Olympus IX71 inverted microscope and a Zeiss LSM710 META confocal microscope.

b. Histology

Masson's trichrome staining was performed at room temperature following the standard protocol (Sigma-Aldrich). All samples were then examined using an Olympus IX71 inverted microscope.

Quantification of cystic cavity size

Only samples that exhibited cystic cavity were evaluated ($n = 5$) (Table 2). The length and width of the cystic cavity were measured using the cellSens Dimension 1.11 software (Olympus). Each cavity had one measurement for length and 2 - 11 measurements for width depending on its extent.

Measurement	Sample size (animals)				
	SCI	Gel	Parallel	Angled	NT-3 Parallel
Cystic cavity	$n = 5$	-	-	-	-
Neurite ingrowth	$n = 6$	$n = 6$	$n = 3$ (4 weeks) $n = 8$	$n = 8$	$n = 4$
Glial scar	$n = 6$	$n = 6$	$n = 7$	$n = 8$	$n = 4$

Table 2. Sample sizes in each experiment. All animals were examined at 12 weeks post injury, unless otherwise stated.

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Quantification of axonal length and glial scar thickness

a. Axonal length

Axonal regrowth was examined at 4 and 12 weeks post injury. NF signal was traced as individual neurites and measured using the ImageJ software. Specifically, for the analysis of the average axonal ingrowth length, all NF signals in two to three tissue sections of each animal were measured. While for the longest neurite length, only the longest NF signal of each animal was taken into account. The sample pool is stated in Table 2.

b. Glial scar thickness

For quantification of glial scar formation, the GFAP-positive area in the grey matter surrounding the injury sites or the scaffolds at 12 weeks post injury was measured. The sample pool is stated in Table 2. Briefly, a horizontal plane cutting through the middle of the injury site or the scaffold was defined on each tissue section. The side facing the spinal cord was then divided by nine lines, which were 20° apart from each other (Supp Fig. 1). The lengths of these lines that passed through the glial scar area were measured using the ImageJ software.

Qualitative analysis of laminin expression

Laminin expression at 12 weeks post injury was analyzed qualitatively. The grades were given from “0” indicating the absence of LN signal or alignment of LN-positive structures to “++++” indicating the strongest LN signal or the highest alignment of LN-positive structures.

Evaluation of functional recovery

Two behavioral tests, the cylinder test and the grid walk test, were performed. The extent of recovery was described by the recovery index where a higher value indicated better recovery. For the cylinder test, after placing each rat into a cylinder (13 cm in diameter, and 24 cm in height), the rat was monitored until its left paw has touched the wall of the cylinder for 50 times. During this time period, the number of times the right paw touched the wall in an open configuration was recorded (Supp Fig. 3A). The recovery index at each designated time point was then obtained using the right paw values normalized to the value at day 3 post-injury. For those day 3 results that happen to be zero, a value of one was used instead.

For the grid walk test, each rat was allowed to walk freely on a grid (32 x 50 cm) elevated 60 cm above the ground for 3 minutes (Supp Fig. 3C). The struts of the grid were 0.2 cm in thickness and formed 3.5 x 3.5 cm squares. The right paw normal step was computed as the percentage of the number of times the right paw did not miss the grid over the total steps. The recovery index at each designated time point was then obtained using the right paw normal step normalized to the value at day 3 post-injury. For those day 3 results that happen to be zero, a value of one was used instead.

Statistical Analysis

All data are presented as mean $\pm$ standard error of the mean (SEM). For the analysis of average NF ingrowth length, the differences between experimental groups were examined using the Kruskal Wallis test followed by the Mann-Whitney U test. All other experimental results were verified to have equal variances and were analyzed using one-way ANOVA followed by Turkey post hoc test.

RESULTS

Characterization of the aligned PCLEEP fibers and scaffolds

Fig. 2A and B show the morphology and appearance of electrospun PCLEEP fibers. Bead-free and uniformly aligned nanofibers with an average diameter of 818 ± 11 nm were obtained. Regardless of the presence of aligned PCLEEP fibers, all collagen hydrogels appeared similar macroscopically with three-dimensional interconnected macroporous structures (Fig. 2C-E, G, H). Aligned PCLEEP fibers were distributed uniformly in the middle of the scaffold with a density of $1.37 \times 10^5 \pm 755$ fibers/mm² (Fig. 2G - J). The fibers occupied 46.3 ± 3.9 % of the scaffold cross-sectional area. The presence of collagen hydrogel within the scaffold supported and maintained fiber orientation and alignment without inducing their fusion (Fig. 2H, J).

Host-implant integration of the scaffolds

As indicated in Fig. 1C and E, the orientation and alignment of the nanofibers were maintained inside the rat spinal cords at 12 weeks post-implantation. Moreover, this scaffold design allowed one to easily introduce different fiber orientations *in vivo* (Fig. 1B - E).

As indicated in Fig. 3, all scaffolds remained intact after 12 weeks *in vivo*. In addition, there was no difference in collagen density within the scaffolds between 4 and 12 weeks post-injury (data not shown). The Gel group exhibited highly compacted collagen hydrogel with limited cell penetration (Fig. 3D). On the other hand, the Parallel, Angled, and NT-3 Parallel groups showed less compact collagen with good cell penetration throughout the scaffolds (Fig. 3A - C).

As shown in Fig. 3A - D, fibrous scars were rarely detected in the scaffold-treated groups and they did not exceed 15 μm in thickness. Moreover, fibrotic scars surrounding the scaffolds were more extensive in the Gel group than in all the fiber-containing groups.

For the SCI group, the outer white matter (WM) was always densely filled with cells. Due to the contraction of the scar tissue around the lesion, the injury site in the SCI group appeared smaller as compared to the other groups with scaffold implantation. However, this healing process was only partial and resulted in cystic cavity formation. We observed that 5 out of 6 animals developed a cystic cavity in the gray matter (GM) (Fig. 3E). For these five animals, the average cavity length was $440.1 \pm 74.9 \mu\text{m}$ and the average cavity width was $91.5 \pm 9.9 \mu\text{m}$. In contrast, the presence of scaffolds prevented the contraction process and bridged the two ends of the host tissue. As a result, cavity formation was not observed in all the scaffold-treated groups (Fig. 3A - D).

Neurite ingrowth

For the Parallel group, neurites extended along the aligned PCLEEP nanofibers at 4 weeks post-injury (Fig. 4A, B). The average axonal ingrowth length was $268.7 \pm 13.0 \mu\text{m}$ while the longest neurite reached $856.0 \pm 92.2 \mu\text{m}$ in length (Fig. 4K, L). At 12 weeks post-injury, more neurofilaments were observed inside the scaffolds as compared to 4 weeks (Fig. 4C, D). Furthermore, the average axonal ingrowth length was also significantly longer ($326.1 \pm 15.6 \mu\text{m}$) (Fig. 4K).

Similarly, neurite extension was also observed along the aligned PCLEEP nanofibers in the Angled group (Fig. 4E, F). In addition, the angled orientation of the fibers decreased the average neurite length significantly as compared to the parallel fibers ($326.1 \pm 15.6 \mu\text{m}$

vs. $271.7 \pm 13.0 \mu\text{m}$ for Parallel vs. Angled, Fig. 4K). However, fiber orientation did not significantly affect the longest attainable neurite length ($731.4 \pm 125.3 \mu\text{m}$ vs. $788.6 \pm 78.2 \mu\text{m}$ for Parallel vs. Angled, Fig. 4G, H).

The NT-3 Parallel group (Fig. 4G, H) showed the highest average neurite length as compared to all the other experimental groups ($391.9 \pm 12.9 \mu\text{m}$) (Fig. 4K). However, the longest attainable neurite length was not significantly different between all groups that received hybrid nanofiber-hydrogel scaffolds (Fig. 4L, NT-3 Parallel vs. Parallel and Angled).

In general, at 12 weeks post injury, the longest neurite lengths of the Parallel, Angled, and NT-3 Parallel groups were significantly longer as compared to the Gel group (Fig. 4L). Furthermore, no orientation of regrown neurites was observed in the Gel group (Fig. 4I). The SCI group also exhibited regrown neurites inside the injury site. However, similar to the Gel group, their growth was highly disorganized, losing the distinct rostro-caudal alignment within the spinal cord (Fig. 4J). Moreover, when comparing the length of the longest neurites in all groups, the SCI and Gel groups showed the lowest recorded lengths (Fig. 4L).

Glial scar

As illustrated in Fig. 5, there was no significant difference in glial scar thickness among all experimental groups at 12 weeks post injury.

Inflammatory response

Inflammatory reaction towards the scaffolds was evaluated by the appearance of microglia and macrophages at the injured sites.

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5 In general, microglial cells reacted well towards the hybrid scaffolds as they remained
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7 in a quiescent state with a highly ramified morphology within host-tissues that were
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9 surrounding the implants (Fig. 6A - D). The cells stayed activated, displaying cellular
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11 hypertrophy, only within the implant, where the aligned PCLEEP fibers remained present at
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13 12 weeks post-injury (Fig. 6A - C). The Gel group showed restricted microglial penetration
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15 (Fig. 6D). Meanwhile, there was almost no microglia in the lesion cavities of the SCI group
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17 (Fig. 6E).
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23 Contrary to the microglial reaction, no clearly distinguishable macrophages could be
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25 found along the aligned PCLEEP fibers in all the fiber-containing groups (Fig. 7A - C). The
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27 fluorescence signals within the implants, as seen in the low magnification images (Fig. 7A -
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29 C), were mainly attributed to the auto-fluorescence of the scaffolds. In addition, macrophages
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31 were only found at the outer border of the scaffold but not inside in the Gel group (Fig. 7D).
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33 Moreover, the amount of macrophages around all the scaffolds was limited and not
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35 significantly different from the SCI group (Fig. 7).
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40 **Laminin expression and blood vessel formation**

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43 In all animals that received the hybrid scaffolds, laminin expression was found
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45 extensively along the aligned PCLEEP fibers regardless of the fiber orientation (Fig. 8A - C).
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47 Moreover, the amount of laminin inside the scaffolds of the NT-3 Parallel group was the
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49 highest as compared to the other hybrid scaffold groups (Fig. 8C, F).
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55 Similarly, newly regenerated blood vessels, as indicated by RECA-1⁺-tube like
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57 structures, were found along the aligned PCLEEP fibers in all the fiber-containing groups
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(Supp Fig. 2A, B). On the other hand, limited blood vessel formation with no orientation was found inside the isotropic hydrogels in the Gel group (Supp Fig. 2C). Thus, the compaction of the collagen hydrogel seemed to restrict cell penetration and blood vessel formation. Meanwhile, the SCI group displayed typical hypertrophied blood vessels at the lesion site (Supp Fig. 2D). However, these newly formed blood vessels were tortuous and disorganized.

Functional recovery

Both of the cylinder (Supp Fig. 3B) and the grid-walk tests (Supp Fig. 3D) revealed that the scaffold-implanted groups had faster functional recovery as compared to the SCI group. Importantly, the NT-3 Parallel group appeared to give the best functional improvement, especially in the cylinder test. On the other hand, the fiber-containing groups (Parallel, Angled, and Nt-3 Parallel groups) clearly showed improved functional recovery in the grid-walk test as compared to the Gel and SCI groups, suggesting the supporting role of the aligned nanofibers. However, there was no significant difference among these groups.

DISCUSSION

Despite the intrinsic regeneration capacity of neurons, restoration of neuronal functions following SCI is still generally poor due to the complex inhibitory microenvironment that exists after injuries. Hence, methods that provide support for spinal nerve regrowth without causing host rejection are attractive.

In this study, we hypothesized that by implanting a hybrid scaffold with three-dimensionally distributed aligned nanofibers into the injured spinal cord, contact guiding cues can be provided to direct neurite regrowth along the longitudinal axis, thereby facilitating tissue regeneration. In our previous work, a sheet of aligned nanofibers was rolled into a

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3 spiral conduit and implanted into the spinal cord. Cell penetration was only observed
4 throughout the spaces in between the layers of conduit, but not within the nanofiber sheet²².
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6 This poor cell penetration was attributed to the small pore sizes within the nanofiber mesh.
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8 Hence, in this study, in order to enhance cell penetration, we introduced the use of three-
9 dimensionally, sparsely distributed aligned nanofibers. Correspondingly, robust cell
10 penetration was observed throughout the scaffold (Fig. 3A - C, Fig. 6A - C, and Fig. 7A - C)
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12 without compromising contact guidance effect.
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21 PCLEEP is a biodegradable copolymer of (ϵ -caprolactone) and (ethyl ethylene
22 phosphate (EEP)). Our previous study showed that after three months of incubation in distilled
23 water at 37 °C, the mass loss of PCLEEP fibers was less than 8 %³⁵. *In vivo*, the presence of
24 PCLEEP fibers was still detected at 3 months post-implantation into the rat's sciatic nerve²⁵.
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26 Importantly, the incorporation of EEP into the poly (ϵ -caprolactone) backbone enhanced the
27 hydrophilicity of the resulting polymer, making it an ideal material for sustained drug and
28 protein delivery^{25, 35-38}. Incorporated with glial cell-derived neurotrophic factor, aligned
29 PCLEEP fibers enhanced peripheral nerve regeneration across a 15-mm critical defect gap in
30 the rat model at 3 months post injury²⁵. Together with the results from this study, PCLEEP
31 fibers are useful in enhancing nerve regeneration in the peripheral and central nervous
32 systems.
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47 Compared to the conventional way of obtaining meshes of fibers by using a rotating
48 target, the air-gap electrospinning provided sparsely distributed aligned fibers. In addition,
49 electrospun nanofibers are at least 1 - 3 orders of magnitude smaller in diameter than other
50 micron-sized features that have been explored for contact guidance in SCI treatment³⁹⁻⁴¹.
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52 This reduction in size of fibers clearly hinders their handling process. Therefore, the
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3 implantation of sparsely distributed aligned nanofibers into the injured spinal cord has not
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5 been achieved prior to this study. Here, the scaffold is fabricated by suspending the aligned
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7 nanofibers within a collagen hydrogel. This scaffold design allows us to implant aligned
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9 nanofibers *in vivo* without compromising fiber alignment. Thus, the effects of fiber
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11 orientation on axonal regrowth can be studied. Importantly, the fact that regenerated neurites
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13 extended along the fibers regardless of their orientation clearly confirmed the significant role
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15 of aligned nanofibers as topographical signals that directed the alignment of regenerated
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17 axons *in vivo*. The current fiber density was chosen through an initial optimization process.
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19 With a lower fiber density, the impact of physical guidance on axonal regrowth was
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21 insignificant; while cell and neurite penetrations were largely hindered with higher fiber
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23 densities.
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30 An ideal scaffold for SCI treatment should bridge the injury gap and integrate well
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32 into host tissues. However, most of the scaffolds developed to date have yet fulfilled these
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34 requirements since they are unable to prevent the formation of lesion cavities^{14, 42}. These
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36 cystic cavities formed after SCI is one of the factors that impede axonal ingrowth and nerve
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38 reconnection⁴³⁻⁴⁷. We observed that 5 out of 6 animals in the SCI group developed cystic
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40 cavity (Fig. 3). In contrast, cystic cavity formation was absent in all animals that received
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42 scaffold implantation at 12 weeks post-injury (Fig. 3). These results suggest that the collagen-
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44 based scaffolds integrated well into adjacent tissues.
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50 Besides cystic cavity formation, SCI also leads to the formation of scar tissues around
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52 the lesion. These scar tissues, consisting of glial and fibrotic scars, act as physical barriers
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54 that prevent regenerated axons from entering/exiting the lesion, thereby inhibiting neuronal
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56 reconnections⁴⁸⁻⁵². As indicated in Fig. 5, there was no significant difference in glial scar
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3 thickness among all the experimental groups. These results demonstrated that the scaffolds
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5 did not enhance the extent of astrocytic activation. On the other hand, there were thicker
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7 fibrotic capsules in the Gel group than in all the fiber-containing groups (Fig. 3). One
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9 possible reason may be the presence of nanofibers, which prevented the hydrogel from
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11 collapsing or becoming compacted after implantation. Consequently, robust cell penetration
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13 and enhanced host-implant integration were observed in the nanofiber-hydrogel scaffolds as
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15 compared to the isotropic hydrogel constructs.
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21 Good host-implant integration was also observed in terms of inflammatory response.
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23 Activated microglia and macrophages are mainly responsible for the inflammatory reactions
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25 after SCI^{50, 53}. Hence, Ox42 and ED-1 were used as markers for microglia and macrophages,
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27 respectively. Quiescent microglia was seen within the host tissue surrounding our implants.
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29 These cells remained activated only within the implants in all experimental groups that
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31 contained PCLEEP aligned nanofibers. This observation is similar with the implantation of
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33 the RADA16-I scaffolds for SCI treatment in rats¹⁵. The presence of the nanofibers within
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35 the injury is likely the reason for the prolonged activation of microglial cells within the
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37 implants. In contrast to microglial infiltration, the amount of macrophages found inside and
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39 around the nanofiber-hydrogel scaffolds was similar with the SCI group at 12 weeks post-
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41 injury. Comparatively, Nomura *et al.* reported a massive aggregation of macrophages inside
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43 chitosan scaffolds and near-by spinal cord tissues even after 14 weeks post-implantation⁵⁴.
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45 Similarly, a large number of macrophages was also found surrounding the RADA16-I
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47 scaffolds¹⁵. Taken together, these results indicate that our scaffolds were well accepted by
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49 the host tissue and did not induce extensive chronic inflammatory reaction in comparison
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51 with other scaffold models.
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Laminin, a major component of the extracellular matrix in the CNS⁵⁵⁻⁵⁶, has been widely known to promote neurite outgrowth as well as to direct axon development⁵⁶⁻⁵⁹. As such, the extensive presence of laminin within our scaffolds at 3 months post-injury indicates a favorable microenvironment for axonal regeneration. Moreover, the existence of laminin-positive structures in the injured area is also an indicator of blood vessel reformation⁶⁰⁻⁶¹. Indeed, the NT-3 Parallel group possessed greater amounts of both laminin expression and aligned blood vessels within the scaffold as compared to the Gel group (Fig. 8C, D, F and Supp Fig. 2B, C). This result is in agreement with Cristofaro *et al.*'s study, which showed that NT-3 promoted neovascularization in both rat mesenteric angiogenesis assay and mouse limb ischemia⁶². Revascularization of the injured spinal cord is a critical tissue repair process as newly formed blood vessels provide trophic and metabolic support, which is essential for wound healing⁶³. Under normal physiological conditions, blood vessels create a favorable environment for axonal growth⁶⁴⁻⁶⁵. Thus, axons can be frequently found in close association with the walls of newly formed blood vessels⁶⁴. Correspondingly, our hybrid scaffold, especially with the inclusion of NT-3, provided a beneficial local habitat for axonal regrowth and tissue regeneration.

Although a general trend of enhanced neurite length was observed in the presence of NT-3, there was no significant effect on the longest attainable neurite length as compared to the other hybrid scaffold groups. This minor improved neurite regeneration outcome suggests that either a higher loading dose of NT-3 or the synergistic effect of NT-3 and other factors, is required. The release of NT-3 from our scaffold has been evaluated (data not shown) to reveal that after an initial burst release at day 1, a steady release of NT-3 was obtained for at least 1 month with a cumulative release of 48.3 ± 13.2 ng/mg of scaffold. On the other hand, a combination of NT-3 and BDNF might be considered to achieve the synergistic effect. This

combination was shown to generate the longest neurite elongation from hippocampal progenitor HiB5 cells, as compared to the over-expression of either NT-3 or BDNF alone⁶⁶. Therefore, future works should expand along these lines. Similarly, the incorporation of factors that minimize neurite inhibition, such as antibodies against myelin inhibitor⁶⁷⁻⁷⁰ or enzymes against chondroitin sulfate proteoglycans⁷¹⁻⁷⁴, may also be explored for additional synergistic effects to further promote nerve regeneration and functional recovery. The versatility of our scaffold fabrication process will allow the easy extension to incorporate multiple factors within the hydrogel and nanofibers^{38, 75}.

In contrast, the SCI group displayed typical hypertrophied and disorganized blood vessels at the lesion site (Supp Fig. 2D), which were in sync with the lowest score for average axonal ingrowth length (Fig. 4). These results are consistent with the literature showing that after SCI, newly formed blood vessels do not guide regenerated axons into and beyond the injury site because of their tortuous form⁶⁴⁻⁶⁵. Similarly, nerve regeneration also remained sub-optimal with limited neurite extension in the Gel group, which also demonstrated correspondingly limited and disorganized revascularization (Fig. 4 and Supp Fig. 2C). These results are in agreement with the study by Joosten *et al.*, which demonstrated that nerves did not penetrate a solid collagen implant⁷⁶.

The incision SCI model was chosen in this study as it is repeatable, thus allowing us to carry out preliminary morphometric evaluation and focus on critical factors affecting nerve regeneration, such as neurite extension, cell penetration, revascularization, host responses, and host-implant integration. Correspondingly, our behavioral tests, the cylinder and the grid-walk tests (Supp Fig. 3), only revealed that the scaffold-implanted groups had faster functional recovery as compared to the SCI group. In addition, aligned nanofibers possessed

supporting role in enhancing functional recovery. However, there was no significant difference among the groups. It is possible that this model was not sufficiently severe to result in significant differences in rat locomotion among different groups. We believe that this may be due to the spontaneous recovery processes following incomplete injuries in the CNS ⁷⁷⁻⁷⁸. Specifically, after partial SCI, a new intraspinal axonal circuit may form spontaneously, bypassing the lesion and contributes to the recovery of function ⁷⁹⁻⁸⁰. Furthermore, it is known that intensive locomotor training can improve functional recovery after an incomplete SCI, especially when it is initiated immediately post-injury ⁸¹⁻⁸². As such, the exercise we introduced while doing the grid walk test assessment might possibly enhance functional recovery, thus masking our results. Hence, future studies should involve a complete transection SCI model to examine the effectiveness of the hybrid scaffolds in promoting functional recovery.

CONCLUSIONS

In this study, we introduced the three-dimensional hybrid scaffold as a potential platform for SCI treatment. *In vivo*, aligned nanofiber topography guided neurite extensions and neovascularization into the lesion site in an organized and aligned manner. In addition, the scaffold provided good host-implant integration, as it did not trigger additional inflammatory response and scar tissue formation. These findings clearly demonstrate the advantages of our scaffold design for SCI repair in terms of axonal regeneration guidance and host-implant integration.

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Supporting Information

Supplemental Figure 1: Illustration of glial scar measurement.

Supplemental Figure 2: Immunofluorescent staining of endothelial cells and laminin at 12 weeks post-injury.

Supplemental Figure 3: Motor performance at the cylinder test and the grid walk test.

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Three-dimensional nanofiber hybrid scaffold directs and enhances axonal regeneration
after spinal cord injury

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GM

Neurofilament

500 μm

Figure 1. Injury model.

(A) 1/3 incision was performed at C5. For the scaffold-treated groups, 1mm scaffold was implanted into the injury site at the moment of the surgery. (B, D, F) Schematic representation of a horizontal section containing an implant with parallel fibers (B), angled fibers (D), and isotropic hydrogel (F). (C, E, G) Bright-field images of the scaffold with aligned PCLEEP fibers (C), angled fibers (E), and isotropic hydrogel (G). Rostral, left; caudal, right. WM, white matter; GM, grey matter. Scale bar 500 μm.

Figure 2. SEM images of the aligned PCLEEP nanofibers and the scaffolds.

(A, B) Aligned electrospun PCLEEP nanofibers. (C, D) Longitudinal section of the isotropic hydrogel scaffold. (E, F) Side view of the isotropic hydrogel scaffold. (G, H) Longitudinal section of the scaffold containing aligned PCLEEP nanofibers. (I, J) Cross section of the scaffold containing aligned PCLEEP fibers. (B, D, F, H, and J) The high magnifications of the insets in (A, C, E, G, and I, respectively).

Figure 3. Masson's Trichrome staining of spinal cord sections at 12 weeks post injury. (A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel groups. (D) The Gel group. (E) The SCI group. Rostral, right; caudal, left. Arrowheads: fibrous scar. Scale bar: 500 μm (A - E); 100 μm (the insets).

Figure 4. Immunofluorescent staining of neurite ingrowth (NF⁺, red) in spinal cord sections.

(A, B) The Parallel group at 4 weeks post-injury. (C, D) The Parallel group, (E, F) The Angled group, (G, H) The NT-3 Parallel group, (I) The Gel group, and (J) The SCI group at 12 weeks post-injury. (A, C, E, G, I, J) Fluorescent images of NF⁺ staining. (B, D, F, H) Merged images of the fluorescent images (NF⁺ staining) and bright-field images (fibers). (K) Quantification of the average neurite ingrowth length and (L) the longest neurites of different experimental groups. Rostral, left; caudal, right. Scale bar: 500 μm (A – J); 100 μm (the insets). NS non-significant; * $p \leq 0.05$. ** $p \leq 0.01$; *** $p \leq 0.001$. Data are shown as mean $\pm$ SEM.

Figure 5. GFAP⁺ scar formation around the scaffolds and injury sites at 12 weeks post-injury.

(A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel group. (D) The Gel group. (E) The SCI group. (F) Quantification of the scar thickness around the injury site. Rostral, left; caudal, right. Scale bar: 500 μm (A – E); 100 μm (the insets). Data are shown as mean ± SEM.

Figure 6. Immunofluorescent staining of microglia (Ox-42⁺, red) in spinal cord sections at 12 weeks post-injury.

(A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel group. (D) The Gel group. (E) The SCI group. Rostral, left; caudal, right. Scale bar: 500 μm (A - E); 100 μm (the insets).

Figure 7. Immunofluorescent staining of macrophages (ED1⁺, red) in spinal cord sections at 12 weeks post-injury.
(A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel group. (D) The Gel group. (E) The SCI group. Rostral, left; caudal, right. Scale bar: 500 μm (A - E); 100 μm (the insets).

Figure 8. Immunofluorescent staining of laminin (LN⁺, green) at 12 weeks post-injury. (A) The Parallel group. (B) The Angled group. (C) The NT-3 Parallel group. (D) The Gel group. (E) The SCI group. (F) Qualitative analysis of the LN⁺ structures inside the scaffolds/ injury site. Rostral, left; caudal, right. 0, absence of the orientation/no signal; +, the lowest score; +++++, the highest score. Scale bar: 500 μ m (A - E); 100 μ m (the insets).