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Nerve Conduit Constructed by Electrospun P(LLA)CL Nanofiber and PLLA Nanofiber Yarn

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Injuries of peripheral nerve occur commonly in various people of different ages and backgrounds. Generally, surgical repairing such as suturing the transected nerve stumps and transplanting autologous nerve graft is the only choice. However, tissue engineering provides an alternative strategy for regeneration of neural context. Functional nerve conduits with three dimensional (3D) support and guidance structure are badly in need. Herein, uniform PLLA nanofiber yarn constructed by unidirectionally aligned nanofiber was fabricated via dual spinneret system. Which was subsequently incorporated into a hollow poly(L-lactide-co-caprolactone) (P(LLA-CL)) tube to form a nerve conduit with inner aligned texture. The biocompatibility of poly(L-lactic acid) (PLLA) yarn was assessed by in vitro experiments. Schwann cells (SCs) presented better proliferation rate and spread morphology on PLLA yarn than that of PLLA film. Confocal images indicated the axon spread along the length of yarn. SCs were also cultured in the conduit. The data indicated that SCs proliferated well in the conduit and distributed dispersedly throughout the entire lumen. These results demonstrated the potential of PLLA nanofiber yarn conduit in nerve regeneration.

Introduction

Peripheral nerve injury is a common global problem occurs to people of different backgrounds, which often leads to the loss of sensory and motor function. Various methods were invented to repair the nerve injury. Surgically, short nerve lesion can be appropriately repaired by end to end coaptation. However, for the long distance nerve defection, end to end anastomosis is no longer an option for it causes detrimental tension along the nerves and retards healing [1]. In these cases, a graft is needed to bridge the nerve gap and provide better regenerative outcomes. Currently, autologous nerve grafts are considered as the "gold standard" for gap injuries greater than 5-10 mm [2]. However, some drawbacks restrict its application, such as limited donor resource, sacrifice of the donor, extra incision, and possible size dismatch. Customized grafts for specific nerve injuries are desired for better cure with little side effect and additional sacrifice.

The progress of tissue engineering scaffold provides a promising alternative for nerve repair. Nerve conduits can act as a bridge between adjacent ends, providing directional guidance and biological support during nerve regeneration. To better adjust the interaction of cells, tissue, and the conduit, scaffolds with nanoscale topology are extensively introduced to the manufacture of nerve scaffolds. Diverse fabrication methods have been used to prepare nanostructured scaffolds, such as phase separation [3, 4], self-assembly [5], as well as electrospinning [6-8]. The most commonly used designs include the hollow tubes (Fig. 1 (a)), multiple channels conduits (Fig. 1 (b)), tube filled with internal matrix with longitudinal oriented channels or pore (Fig. 1 (c)) [9-12], as well as lumen filled with aligned polymer fibers as longitudinal guidance (Fig. 1 (d), (e)) [8, 13-21].

Electrospinning is mostly reported due to its easy handleability, cost efficiency, quality controllability, and availability for various natural and synthetic materials. Electrospun nanofiber scaffolds can mimic the basic nanoscale structure of natural extracellular matrix. Parallel fibers have demonstrate the ability to guide the spreading and migration of Schwann cells (SCs)[8, 22-24]. Additionally, aligned fibers may induce the differentiation and maturation of neural stem cells and SCs [25, 26]. Dorsal root ganglia (DRG) cultured on the

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parallel nanofiber tended to generate long and unidirectionally ordered neurites. The predetermined aligned nanofiber could cause SC alignment and subsequent neurite extension in vitro. Studies also have shown the unidirectional electrospun nanofiber, but not the randomly oriented nanofiber, could guide nerve regeneration across long nerve gaps [19]. However, the traditional electrospinning process always manufactured 2D film with densely compacted structure which inhibited cell infiltration and immigration into the scaffold [27]. Andreas Kriebel et al have tried v-shaped collector to collect aligned nanofibers with 3D structure which was subsequently incorporated into collagen matrix [21]. While the guiding function of parallel nanofiber was struck for axons also adhere to the surrounding hydrogel. Several types of nerve conduits have been designed to incorporate aligned electrospun nanofiber in the internal of the tube. The most commonly used method is to fabricate tubes with axially aligned nanofibers constructing the inner surface of the tube[23, 28, 29]. Jingwei Xie et al. fabricated double layered nerve conduits with the aligned PCL nanofiber as the inner surface while the random fiber served as the supporting wall [23]. In vivo results indicated that the bilayer conduits could effectively improve nerve fiber sprouting and motor recovery. This approach could facilitate cell spreading and migration but did not provide effective support for cell growth in the space of the lumen. Eric M Jeffries et al. reported 3D multichannel nerve conduit incorporated with parallel electrospun fibers [18]. This guide had thin walls and high channel numbers to maximize surface area and facilitated cell spreading and migration. However, the manufacturing process was labor intensive and varied by operator, which limited the effective conduit length and reproducibility. Another commonly used method is to insert a bundle of aligned nanofibers in the nerve guide[15, 17, 19-21]. Highly aligned nanofiber film was cut into thin strips and incorporated in a hollow nerve conduit by Young-tae Kim et al [19]. The presence of aligned nanofiber film could maximize the topographic directional cues for neurite outgrowth and SCs migration in 3D configuration. However, the width of the stripes may also lead to unevenness of tissue regeneration in the sectional direction. 3D scaffold consisting parallel fibers were fabricated via modulating the collector by Andreas Kriebel et al [21] and Balendu S. Jha et al [15]. Both studies demonstrate the parallel fibers could direct axonal regeneration and SCs migration along a defined axis. Nevertheless, the scaffolds constructed by parallel fibers were quite soft and difficult to handle. Moreover the densely packed nanofibers may inhibit cell infiltration[21, 27].

In this study, a novel approach is introduced to manufacture long distance nerve conduits with aligned electrospun nanofiber as the filler and randomly electrospun nanofiber as the shell. Our method is based on the fabrication of nanofiber yarn by a dual spinneret electrospinning system. Yarn constructed by nanofiber inherits various features of nanofiber, but also possessed unique properties such as easy post-processing. Nanofiber yarn can be manufactured by textile and related methods into a fabric and other predetermined structure. Various techniques were introduced to fabricated nanofiber yarn. Ko et al. firstly electrospun continuous nanoscale composite yarn with a complex setup with orientation, twisting, and take up components[30]. Smit et al. drawn the electrospun nanofiber web from the water bath and collected continuous yarns[31]. Teo et al. used the water vertex while water flowing from the hole of a basin to generate continuous nanoscale yarn[32]. A grounded tip was applied to induce self-bundling nanofiber yarn by Wang et al[33]. Recently, nanofiber yarn was fabricated by the oppositely charged dual nozzle system[34-36]. In which, the nanofiber yarn could be twisted and collected at the same time.

Herein, PLLA nanofiber yarn was fabricated use the dual nozzle electrospinning system. The random nanofibers electrospun from P(LLA-CL) solution possess excellent mechanical properties for the nerve conduits and provide the conduit tear-resistant during surgical procedure. Whereas the highly aligned nanofiber in the nanofiber yarn made of PLLA serve as the guidance for axons spreading and cell migration. Characterization of the PLLA nanofiber yarn and nerve conduit was conducted, while SCs was cultured on the PLLA nanofiber yarn and in the nerve conduits to study the biological performance.

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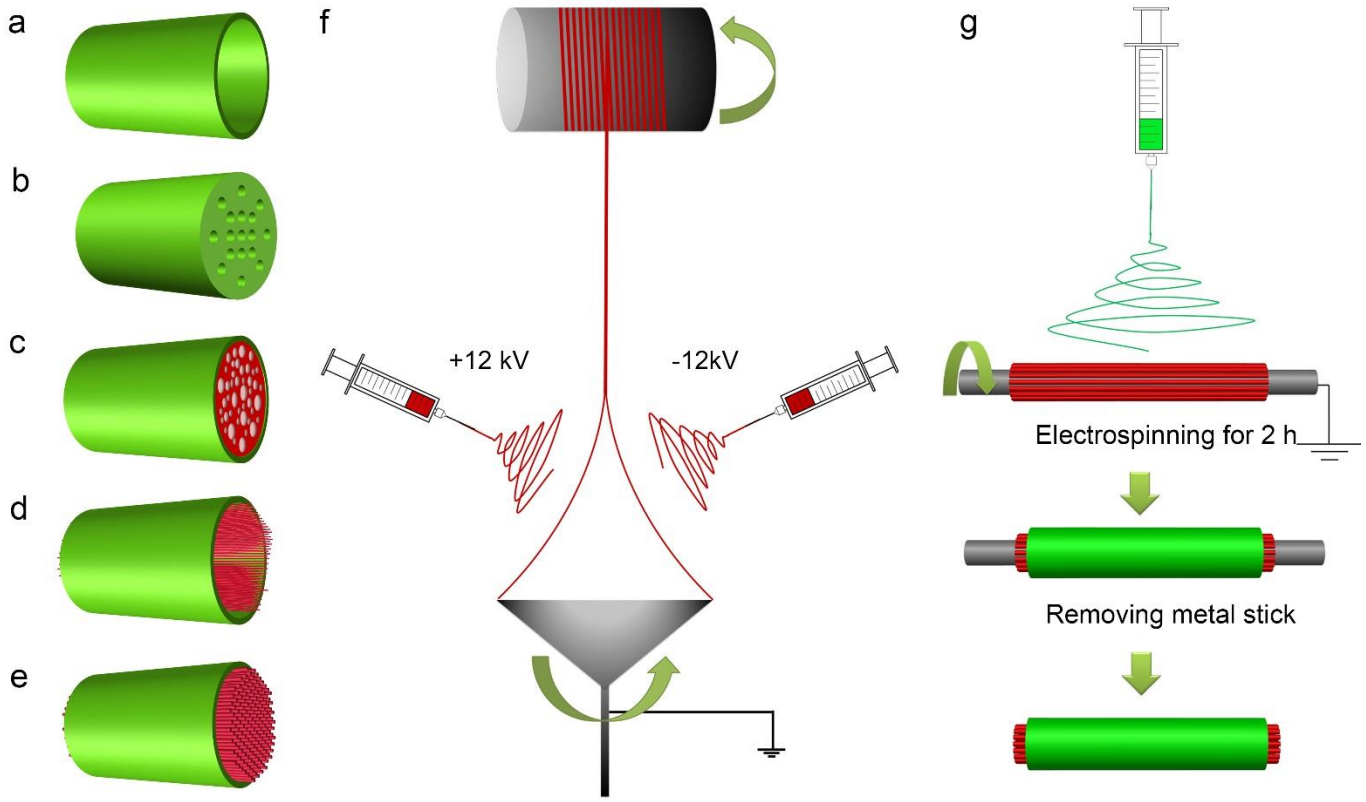

Fig. 1 Schematic of various conduits: (a) hollow lumen conduit, (b) multichannel conduit, (c) sponge-containing conduit, (d) fiber-containing conduit, and (e) nanofiber yarn-containing conduit. (f) The mechanism of nanofiber yarn fabrication. (g) Schematic of incorporating nanofiber yarn into the conduit.

2. Materials and experiments

2.1 Materials

PLLA with an average molecular weight (Mw) of 500 kDa was purchased from Daigang Biomateriasl Inc. (Jinan, China). P(LLA-CL) (Mw=300 kDa, LA:CL=50:50) was supplied by Nara Medical University, Japan. Hexafluoroisopropanol (HFIP) was obtained from Shanghai Darui Finechemical Co., Ltd. (China). The Dulbecco's Modied Eagle's Medium (DMEM, Hyclone), fetal bovine serum (FBS, Gibico), trypsin (Hyclone) 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT, Sigma) was purchased from Yuanzhi Biotechnology Co., Ltd (Shanghai, China). 4', 6-Diamidino-2-phenylindole (DAPI) and Alexa Fluor® 568 phalloidin was supplied by Life Technologies Co., Ltd. (USA).

2.2 Preparation of PLLA nanofiber yarn

0.75 g PLLA was dissolved in 10 mL HFIP to generate 7.5% w/v PLLA solution. The nanofiber yarn was fabricated by a dual spinneret electrospinning system as described by Usman Ali et al [36]. As illustrated in Fig. 1 (f),

the setup consists of two spinneret, a plastic funnel (diameter=10 mm) with conductive edge which was grounded, a yarn winder (diameter 8 mm), and two high voltage DC power supplies (Gamma High Voltage Research, USA). During electrospinning, the PLLA solution was added in the two oppositely positioned syringes and squeezed out through the metal needles of 20 gauge. The flow rate of PLLA solution was set at 1.0 mL/h. Two needles were separately charged with positive (+ 12 kV) and negative (-12 kV) high voltage. Electrospun nanofibers from two nozzles were deposited on the rotary funnel and covered the funnel end with a nanofiber layer. A cone shaped nanofiber layer could form on the funnel edge by initial inducing. After drawing by the winder and twisting by the rotary funnel, a continuous nanofiber yarn was obtained. As a control, PLLA nanofiber film was prepared by a single electrospinning nozzle and collected on aluminum foil. The voltage, spinning rate, and collecting distance was set as +12 kV, 1.0 mL/h, and 15 cm, respectively.

2.3Characterization

The surface of nanofiber yarn was sputter-coated with gold and subsequently observed by a Digital Vacuum Scanning Electron Microscope (SEM, TM 3000, Hitachi, Japan) at the accelerating voltage of 15 kV. Diameter of fibers in the nanofiber yarn was measured on the SEM images by the image visualization software Image J (National Institutes of Health, USA). 100 fibers were randomly selected for each sample.

2.4 In vitro experiments

SCswere maintained and expanded in DMEM culture medium, incubated in humidified atmosphere with 5% CO2 at 37 °C. The culture medium was refreshed every other day. For in vitro biocompatibility assessment, the PLLA nanofiber yarn was wound on a square glass slip (a side of 10 mm) till the entire surface was covered by the yarn. The PLLA film was cut into round pieces with a diameter of 15 mm. The samples were fixed in the 24-well culture plates by stainless steel rings with an inner diameter of 10 mm. Subsequently, the plates were sterilized by alcohol steam in sealed desiccator for 48 h. Scaffolds in the plate were rinsed with phosphate-buffered saline solution (PBS) for 3 times and washed with culture medium for 1 time.

2.5 Adhesion and proliferation of SCs

For the assessment of SCs adhesion on the PLLA nanofiber yarn, a total number of 4×10^4 cells were seeded on the scaffolds in 24-well plate to compare the cell adhesion PLLA nanofiber yarn and film with tissue culture plates (TCPs) s as control. 40 min, 60 min, 120 min and 240 min after seeding, the culture medium was removed and the specimens were rinsed with PBS 3 times to remove the unattached and dead cells. Then, the amount of the attached cells was determined by standard MTT assay. Briefly, the specimens were incubated in 360 µL FBS-free DMEM culture medium and 40 µL 5 mg mL^{-1}MTT solution for 4 h. Thereafter, the culture media were pipetted out and 400 µL dimethylsulfoxide (DMSO) was added. Afterwards, the plate was incubated in a shaker at 37 °C for 30 min. While the crystal was thoroughly dissolved, 100 µL of the solution was transferred to a 96-well plate and tested by a microtiter plate reader (Multiskan MK3, Thermo, USA), at the absorbance of 492 nm.

For proliferation study, 1×10^4 cells were seeded on the scaffolds with TCP as control. The amount of the cells on each specimen was determined by the standard MTT assay. 1, 3, and 7 days post seeding, the culture medium was removed and unattached cells were washed away with PBS for three times, MTT assay was conducted as described above to determine the amount of viable cells on the scaffolds. For each group 6 specimens were tested.

2.6 Cell morphology observation

A total number of 1.0×10^4cells were seeded on the nanofiber yarn and film in 24-well plates. After culturing for 1, 3, 7 days, cells cultured on the scaffolds were fixed by 4% paraformaldehyde for 2 h min at 4 °C, dehydrated with gradient ethanol solution (30%, 50%, 70%, 90%, 95%, and 100%) and followed by freeze drying at -60 °C for 12 h. Afterwards, the samples were sputter coated with gold and observed by SEM at the accelerating voltage of 15 kV.

Confocal laser scanning microscopy (CLSM, Carl Zeiss, LSM 700, Germany) was used to visualize the morphology and distribution of cells on the scaffolds. After 1, 3, 7 days of culture, the specimens with cells were rinsed twice with PBS and then fixed with 4% paraformaldehyde for 2 h min at 4 °C. Subsequently, the cells on the scaffolds were permeabilized by 0.1% Triton X-100 (Sigma, USA) for 10 min. After rinsing 3 times with PBS, the cytoskeletons and nuclei of cells were stained with 25 µg/mL rhodamine-conjugated phalloidin and 10 µg/mL DAPI for 30 min and 5 min, respectively. Subsequently, the cells were visualized using CLSM.

2.7 Fabrication of nerve conduit

The schematic of fabrication of nerve conduit was illustrated in Fig. 1 g. Briefly, 1.2 g P(LLA-CL) was dissolved in 10 mL HFIP to generate 12% w/v P(LLA-CL) solution, which was subsequently applied in single spinneret electrospinning. The applied voltage, electrospinning distance and flow rate were controlled at 12 kV, 15 cm, and 1.5 mL/h, respectively. The as-prepared PLLA nanofiber yarns (red in Fig. 1 (g), effective length of 6 cm) were parallelly fixed around the metal stick (grey in Fig. 1 (g), diameter of 2.5 mm) along the axis of the

stick. The stick was fixed on a motor with a rotating rate of 5 rpm and grounded to collect the electrospun P(LLA-CL) nanofiber (green in Fig. 1 (g)). The electrospinning lasted for two hours. Finally, the metal stick was removed and the conduit was well prepared. The nerve conduit was incubated in vacuum oven for 48 h to remove the residual solvent.

2.8 In vitro cell culture

To test the biocompatibility of the nerve conduit, the as-prepared conduit was cut into short sections with a length of 9 mm. SCs was co-cultured with the conduit sections. The ethanol steam sterilized conduit section was placed in wells of 24-well plate. After washed with PBS and culture medium, 500 µL of cell solution of 4×10^5 cells/mL was pipetted into the end of each section of nerve conduit. Afterwards, the conduit sections were let lied in the culture plate and incubated on a shaker (60 rpm) in the incubator. The culture medium was refreshed every other day. After 1, 3, 7 days of culture, the conduit section combined with cells was transferred into a new plate. MTT assay was conducted as described above to determine the amount of viable cells in the conduit.

2.9 Cell proliferation and migration in the nerve conduit

To observe the distribution of cells in the conduit, conduits combined with SCs was fixed after 7 days of culture with 4% paraformaldehyde for 2 h min at 4 °C. The samples were embedded in sample freezing medium and plunge frozen at -80 °C. The frozen samples were sliced into thin slices with a thickness of 30 µm at -40 °C. Cross sections in axial direction were obtained. Slices were collected on glass slides and stained with DAPI, followed by observed with CLSM.

2.10 Statistical analysis

All the data were obtained at least in triplicate and all values were presented as the mean and standard deviation (SD). Statistical analysis was performed by the one-way analysis of variance using Origin 8.0 (OriginLab Inc., USA). The statistical difference between two sets of data was considered when * $p < 0.05$ and ** $p<0.01$.

Results and discussion

3.1 Electrospun nanofiber yarn

Dual spinner etelectrospinning was conducted to fabricated PLLA nanofiber yarn with highly aligned nanofiber. The schematic of yarn fabrication is illustrated in Fig. 1 (f) and the SEM images of nanofiber yarn were shown in Fig 2. It can be seen that PLLA nanofibers in the surface of the nanofiber yarn were unidirectionally oriented along the axis of the yarn body within a tiny twisting angle α (8.37±1.69°, Fig. 2 (a), (b)). Ali Usman et al reported that increasing the rotating speed of the metal funnel could rise the twisting angle and meanwhile strengthen the mechanical property [36]. Herein, we chose a low funnel rotating speed of 300 rpm to minimize the twisting angle and got nanofibers aligned to the axis of the yarn. The cross-section of the nanofiber yarn was illustrated in Fig. 2 (c). Two or three fibers combined into a bundle with grooves forming between adjacent fibers. Porosity could still be observed in the yarn, which could increase the specific surface area and thus facilitate the transport of nutrition and degradation. The average diameter of the PLLA nanofibers in the yarn was 598.2 ±215.1 nm (Fig. 2 (d)). The average diameter of the PLLA yarn was 49.7±14.6 µm.

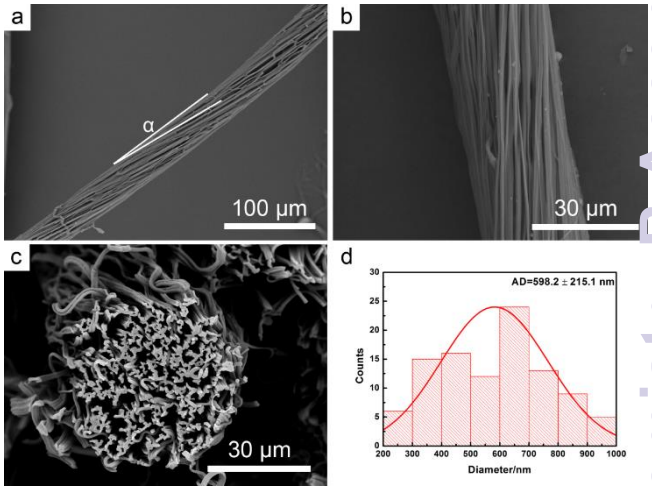

Fig. 2 SEM images of the nanofiber yarn. Morphology of the surface of PLLA nanofiber yarn (a), (b). Cross section of the nanofiber yarn (c). Diameter distribution of PLLA nanofiber in the nanofiber yarn (d).

3.2 SC adhesion and proliferation

Cell adhesion on PLLA nanofiber yarn and film was assessed by determining the amount of viable SCs attached to each scaffold with MTT assay. According to our previous research, most SCs could attach to scaffolds in about 4 hours. Thus the time period for cell adhesion was set as 240 min. As illustrated in Fig 3 (a), cells attach to the PLLA nanofiber film kept the least in 240-min culture period. After 240 min of incubation, SCs on the nanofiber yarn are significantly more than that of TCP. MTT analysis shows that nanofiber yarn had better cell adhesion capacity than nanofiber film, which might further benefit the spreading and proliferation of cells on the scaffold. Nanofiber is

believed that the nanoscale topology and high specific surface area could mimic the natural extracellular matrix (ECM) and facilitate cell growth and tissue regeneration. However, nanofibrous structure also reduces the pore size of the scaffold, inhibiting cells on the very surface and hindering cell migration into the scaffold. The nanofiber yarn can provide fluctuant surface of microscope, which increases the effective surface for cell adhering. Thus, more cells can attach to the scaffold in the initial several hours, enhancing the adhesion of SCs.

Longer period culturing was conducted to study the proliferation of SCs on the nanofiber yarn. MTT assay was conducted after 1, 3, 7 days of culture to determine the amount of viable cells. As shown in Fig. 3 (b), during 7 days of culture, SCs go through remarkable increase on three groups of substrates, implying that the PLLA nanofiber scaffolds can support the proliferation of SCs. 3 days post-seeding, more SCs are detected on the PLLA nanofiber yarn than film. The difference is enlarged over time. 7 days later, amount of SCs on the nanofiber yarn even surpasses that on TCP. It can be obviously concluded that the PLLA nanofiber yarn could significantly enhance SCs proliferation. As mentioned above, the microscope structure of nanofiber yarn can provide more space for cell spreading and migration. As shown in Fig. 4 (f), SCs covers the whole surface of the yarn, including the upper side, lateral sides, and even the underside. However, restricted by the small pores between the nanofibers, the film possesses no extra space for cell growth except the upper surface. In addition, the micro-structure of the yarn also facilitates the transport of nutrition and metabolic waste, which also contributes to the cell proliferation.

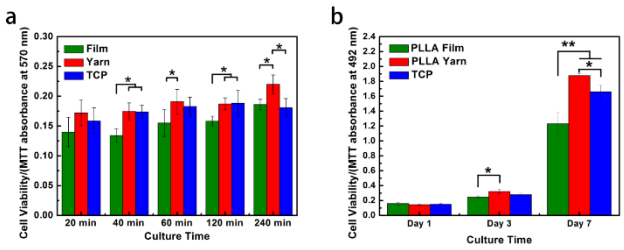

Fig. 3 Analysis of MTT assay for SCs adhesion (a) and proliferation (b) on PLLA film, PLLA nanofiber yarn and TCP. * indicates statistically difference for $p<0.05$; ** indicates statistical difference for $p<0.01$.

3.3 Cell morphology

The structure of scaffolds is the key issue for cell colonization in tissue engineering. To study the interaction between cells and different scaffolds, the morphology of SCs on PLLA nanofiber yarn and film was observed via SEM and CLSM images after 1, 3, 7 days of culture. Fig. 4 and Fig. 5 illustrates the SEM images and confocal microscopy of SCs, respectively. For the confocal observation, the SCs were visualized by staining the F-actin and nuclei into red and blue, respectively.

After 1 day of culture, polarized SCs are observed on the nanofiber yarn with the long axis oriented in the direction of yarn (Fig. 4 (d), (d')), while on the film, randomly oriented cells of spindle or polygonal shapes are observed (Fig. 4 (a), (a')).This phenomenon can be confirmed by the confocal images in Fig. 5 (a), (a'), (d), (d'). Longer axons of SCs can be clearly observed along the nanofiber yarn (Fig. 5 (d)). These two different phenotypes of SCs evidently observed on two scaffolds become more obviously different in later culturing period. After 3 days, more cells can be found on both scaffolds (Fig. 4 (b), (e), Fig. 5 (b), (e)). Part of SCs cultured on the film become spread-out, while the rest cells are still randomly aligned across multiple fibers and elongated along the fiber axes (Fig. 4 (b'), Fig. 5 (b')). In contrast, more SCs with long axons stretched along the nanofiber yarn are observed (Fig. 4 (e), (e'), Fig. 5 (e), (e')). The parallel red filaments indicates that the aligned nanofiber induces cell extending undirectionally. 7 days after cell seeding, rounded shaped SCs form a densely compacted layer and occupied the whole surface of the film with no evident axons observed (Fig. 4 (c), (c'), Fig. 5 (c), (c')). However, the trend on nanofiber yarn keeps unchanged as the number of SCs expands over time. More aligned SCs surround the nanofiber yarn with only part of the surface taken up, leaving sufficient space for further cell migration and proliferation.

Previous studies have indicated that parallel nanofibers determined the spreading and migration, as well as the neurite outgrowth of nerve cells [8, 19]. However, the crossed fibers inhibited the further axonal extension of SCs, which may be detrimental in the growth of efficient and directed axons. Additionally, aligned fibers also induced the differentiation and maturation of neural stem cells and SCs [25, 37]. Highly aligned nanofiber scaffolds possessed the potential in nerve regeneration for the cure of peripheral nerve injuries. Herein, nanofiber yarn constructed by nanofibers highly aligned along the axis of the yarn was fabricated via dual needle electrospinning system. SCs were cocultured with the yarn as well as film to assess axon out growth and SCs behavior. SCs culture on the

showed a polarized structure along the axis of yarn, and the trend did not change over time during culture period. However, SCs on the film was randomly oriented with spread-out phenotype. As the time prolonged, SCs occupied the finite surface of the film and transformed into rounded shape. Without more space for cell spreading, the proliferation of SCs was inhibited and the outgrowth of axons was hindered. In contrary, the microscope structure constructed by the nanofiber yarn enlarged the effective space for cell migration and proliferation. Moreover, SCs on the nanofiber yarn could colonize in a three-dimensional space, which may be favorable for long time implantation and leave enough time for the regeneration of new nerve tissue in vivo.

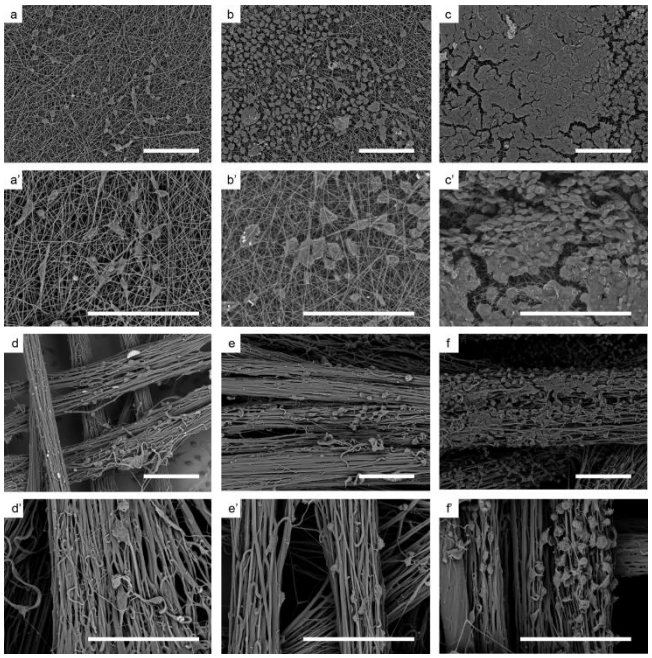

Fig. 4 SEM images of SCs on PLLA film and PLLA nanofiber yarn after cultured on PLLA film for 1 day(a), (a'), 3 days (b), (b'), 7 days (c), (c'), and PLLA nanofiber yarn for 1 day (d), (d'), 3 days (e), (e'), 7 days (f), (f'). (x) and (x') represent different magnification of 500× and 2000×. (Double sided arrows indicated the yarn axis. Scale bar: 100 μm.)

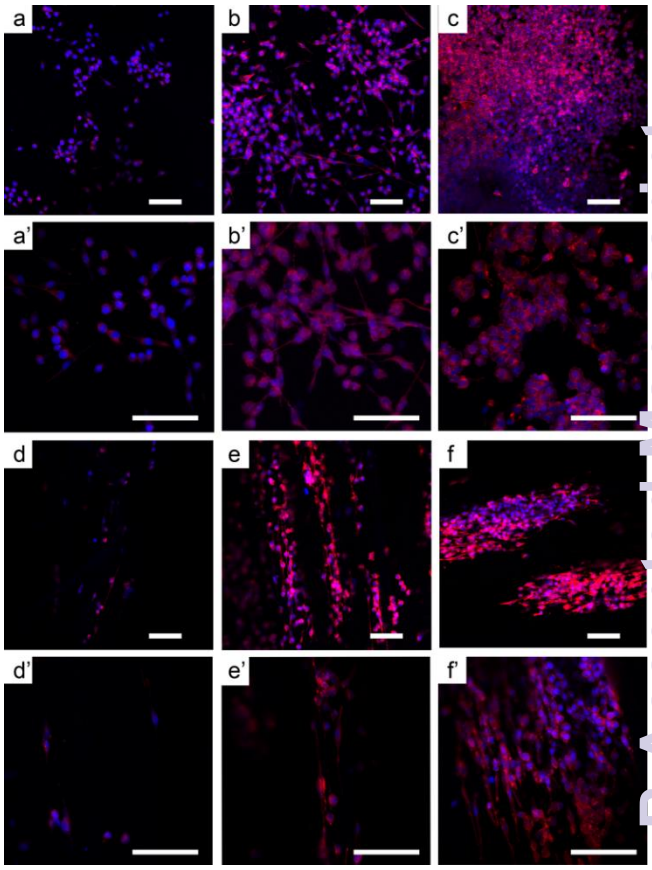

Fig. 5 CLSM microscopy images of SCs on PLLA film and PLLA nanofiber yarn after cultured on PLLA film for 1 day(a), (a'), 3 days (b), (b'), 7 days (c), (c'), and PLLA nanofiber yarn for 1 day (d), (d'), 3 days (e), (e'), 7 days (f), (f'). (x) and (x') represent different magnification of 500× and 2000×. (Double sided arrows indicated the yarn axis. Scale bar: 100μm.)

3.4 Fabrication of nerve conduit with PLLA nanofiber yarn

As manually inserting the nanofiber yarn into a hollow tube may cause additional curves and entanglements of yarns during operation, resulting in disordered structure in the lumen. The entangled yarn would mislead the spread and migration of cells in the couduit, consuming more time for the enclosure of the nerve defection. To generate a uniform arrangement, the nanofiber yarn constructed by highly aligned nanofiber was incorporated into a nanofibrous conduit of P(LLA-CL) nanofiber as illustrated in Fig. 1 (g). PLLA yarns of two hundred are straightened and parallelly adhered around a metal stick as shown in Fig. 6 (a). After electrospinning P(LLA-CL) for 2 h, the surface of the yarn is covered with a thin layer of P(LLA-CL) nanofiber (Fig. 6 (b)), which can act as a barrier to limit the penetration of interstitial cells into the guide, as well as facilitate necessary suture while applied in clinic. After

the removal of internal metal stick, a novel P(LLA-CL) nerve conduit filled with PLLA nanofiber yarn is obtained. The conduit was immersed into liquid nitrogen and then cut into short sections for further characterization. The radial cross section of the conduit was observed by SEM and shown in Fig. 6 (c) and (d). It can be seen that the yarns are parallelly inserted in the lumen of the conduit with less curve and entanglement. The inserted yarns can provide proper support and the highly aligned nanofiber in the yarn can generate topological guidance for cell migration and neurites outgrowth across the nerve bridge. The SEM image of the P(LLA-CL) layer is shown in Fig. 6 (e). The average diameter of the P(LLA-CL) nanofiber is 899.4±266.3 nm.

In addition, porosity in the lumen of conduit is a key factor for nerve cell spreading, colonization, proliferation, as well as new nerve tissue ingrowth. Simply filling conduits with aligned fibers may block the channels and hinder the infiltration and migration of cells [18, 21]. For this conduit, the porosity between yarns left enough space for further cell migration. The percentage of open region was determined by the density of nanofiber yarns. For a given inner diameter of conduit, the porosity increases while decrease the number of nanofiber yarns inserted in. Thus the porosity was controllable for a specific demand.

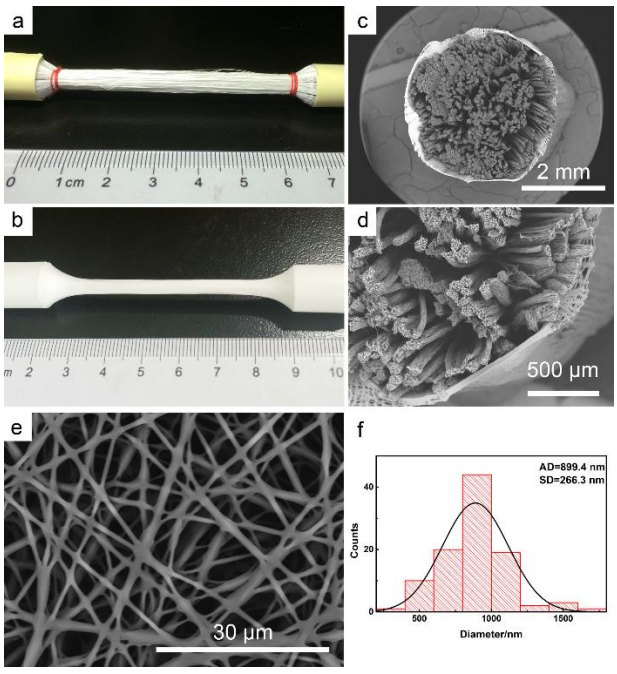

Fig. 6 Photo of the metal stick surrounded by PLLA nanofiber yarn (a). Photo of nerve conduit after electrospun P(LLA-CL) (b). SEM images of the cross section of nerve conduit 30×(c), 100×(d). SEM image (e) and diameter distribution (f) of the P(LLA-CL) layer.

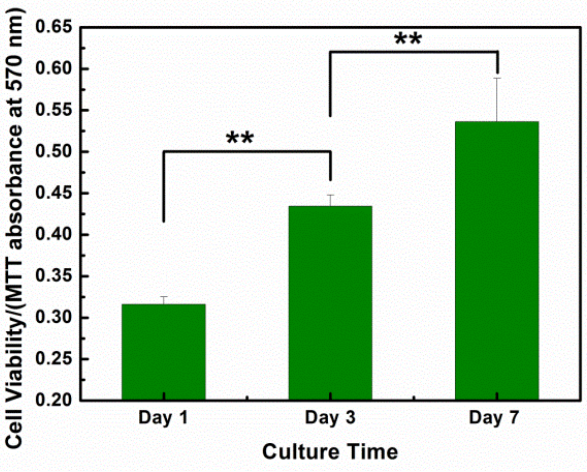

Fig. 7 MTT assay of SCs cultured in the nerve conduit.

3.5 SCs proliferation and migration in the conduit

SCs were seeded in the nerve conduit sections with a length of 9 mm by pipette a certain amount of SCs into the lumen from one end of the conduit. The substrates seeded with cells were placed in a shaker. The nutrition and metabolic waste could only be transferred through the P(LLA-CL) wall or the two ends of the conduit. As illustrated in Fig.7, the MTT assay indicates that the amount of SCs in the conduits go through slightly increase during the 7 days of culture, indicating the good biocompatibility of the nerve conduit. Obviously, the increasing rate is much slower compared with those cultured on PLLA nanofiber yarn (Fig. 3 (b)). This can be attribute d to the methods of viability determination. Part of the formazan form during MTT incubation may not be dissolved by DMSO owing to the barrier of P(LLA-CL) wall, resulting a relatively lower absorbance.

Fig. 8 illustrates the morphology of SCs growing in the conduit. The cultivated SCs show no visible difference in appearance compared with those cultured on the nanofiber yarn. On a single yarn, the amount of SCs increases over culturing time. On the conduit wall of P(LLA-CL), randomly oriented SCs are observed in Fig. 8 b.

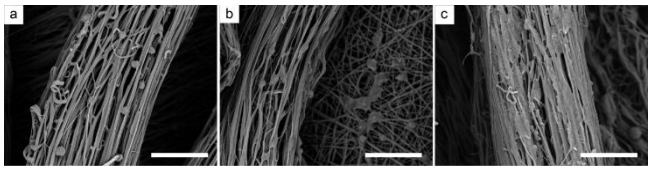

Fig. 8 SEM images of SCs cultured in the conduit sections for 1 (a), 3 (b), and 7 days (c). (Scale bar: 50μm)

To determine the distribution of the SCs in the conduit, cells were labeled by DAPI to generate blue fluorescence and visualized by CLSM. Fig. 9 demonstrates the confocal microscopy photos of the longitudinal section of nerve conduit combined with SCs after 7 days of culture. SCs can be observed from the wall (Fig. 9(a)) to the center of the conduit lumen (Fig. 9 (a)). Moreover, across the longitudinal axis of the conduit, the amount of SCs shows little difference, indicating that SCs had migrated through the entire lumen. According to the SEM images showed in Fig. 8 (c), after 7 days of culture, SCs have already covered most surface of the nanofiber yarn, which made it evident that the filling nanofiber yarn can positively promote the spreading and migration of SCs in the conduit.

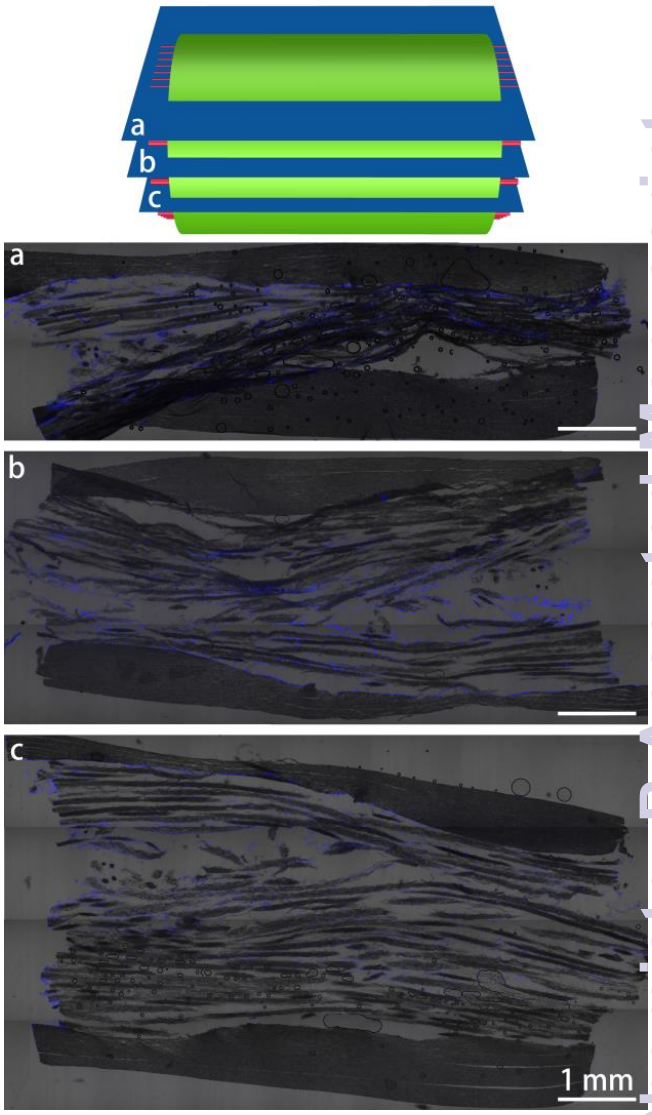

Fig. 9 Longitudinal cross section of the nerve conduit after coculturing with SCs for 7 days. (a), (b), and (c) presented different levels of the conduit shown in the schematic diagram.

Topological cues could significantly affect the behavior of SCs including elongation, migration, alignment, as well as subsequent axon extension. Previous study indicated the size scale matters the alignment and outgrowth of axons, which became significantly improved while the fiber diameter was lowered down from hundreds micrometer to hundreds nanometer[38, 39]. Electrospun fiber with a diameter ranging from tens nanometer to several micrometer has drawn considerable attention in the fabrication of nerve tissue engineering scaffolds. Herein, PLLA nanofiber yarn with highly aligned nanofiber was fabricated via dual spinnerets electrospinning system. Parallel nanofiber twisted into a bundle maintained the guidance cues as

the 2D film in addition of microscale structure and feasibility for further processing. In vitro experiments demonstrated that the PLLA nanofiber yarn scaffold could promote SCs proliferation for its 3D structure and high effective surface. The proliferating SCs could express various ECM cell adhesion molecules and plentiful growth promoting factors, enhancing the further outgrowth of axons[40]. The unidirectional nanofiber in the surface also accelerated the elongation and orientation of SCs and increased the length of axons.

After injury of peripheral nerve, SCs proliferate, reorganize, and align to form bands of Bungner [19]. Nerve conduit is needed to bridge the lesion, providing a guiding framework for the proliferation of neurons and promote the related cells to generate inductive factors for axonal outgrowth. Nanofiber filaments, bundles, or 3D scaffolds with parallel nanofibers were inserted in hollow tubes for enhanced cell alignment and migration. However, the manual operation always brought in entanglement of nanofibers and collapse of the parallel structure, which would impede the growth of regenerated nerve. Moreover, the structure of the filaments, bundles, and 3D nanofibers was quite hard to qualitatively control. In this study, we incorporated the uniform PLLA nanofiber yarn consist of longitudinally aligned nanofiber into a P(LLA-CL) tube to fabricate a novel nerve conduit for peripheral nerve regeneration. The PLLA nanofiber yarn distributed in a 3D configuration in the conduit, providing support for cell adhesion and migration through the entire lumen, while the aligned PLLA nanofiber guided the SCs growth in a predetermined direction. In addition, the structure of the conduit including conduit diameter and open area could be adjusted to realize a specific design for clinical demands.

In the present study, synthetic PLLA nanofiber yarn could enhance the alignment, elongation of SCs and outgrowth of axons. However, for further research, in vivo experiment needs to be conducted to assess the actual function of this novel conduit in nerve regeneration. Natural materials of better biocompatibility or the combination of natural and synthetic materials could also be employed to construct aligned yarn and conduit. Thus, quite a number of materials and their combination could be fabricated into nanofiber yarn to study the ability in generating new nerve. Moreover, surface modified or drug loaded nanofiber might be processed in the same method to lead to better results in the repair of peripheral nerve injuries.

Conclusion

In peripheral nerve injuries, nerve conduit bridges between the broken stumps provides proper configuration to facilitate support cell distribution and the growth of injured nerve tissues in a predetermined direction. Herein, a novel conduit was fabricated with PLLA nanofiber yarn as the inner filler and P(LLA-CL) nanofiber layer as the surrounding shell. In vitro experiments indicated the good biocompatibility and guiding capacity for spreading, migration, and alignment of SCs. SCs cultured in the conduit section migrated through the entire space in the conduit. Based on the present data, it was believed that the conduit possessed the ability for peripheral nerve repair, which would be experimentally evaluated in further studies.

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Abstract

PLLA was electrospun into nanofiber yarn, which was then covered by P(LLACL) nanofiber tube to form a nerve conduit. PLLA nanofiber yarn as the inner filler and P(LLA-CL) nanofiber film as the surrounding shell. In vitro experiments indicated the good biocompatibility and guiding capacity for spreading, migration, and alignment of SCs.

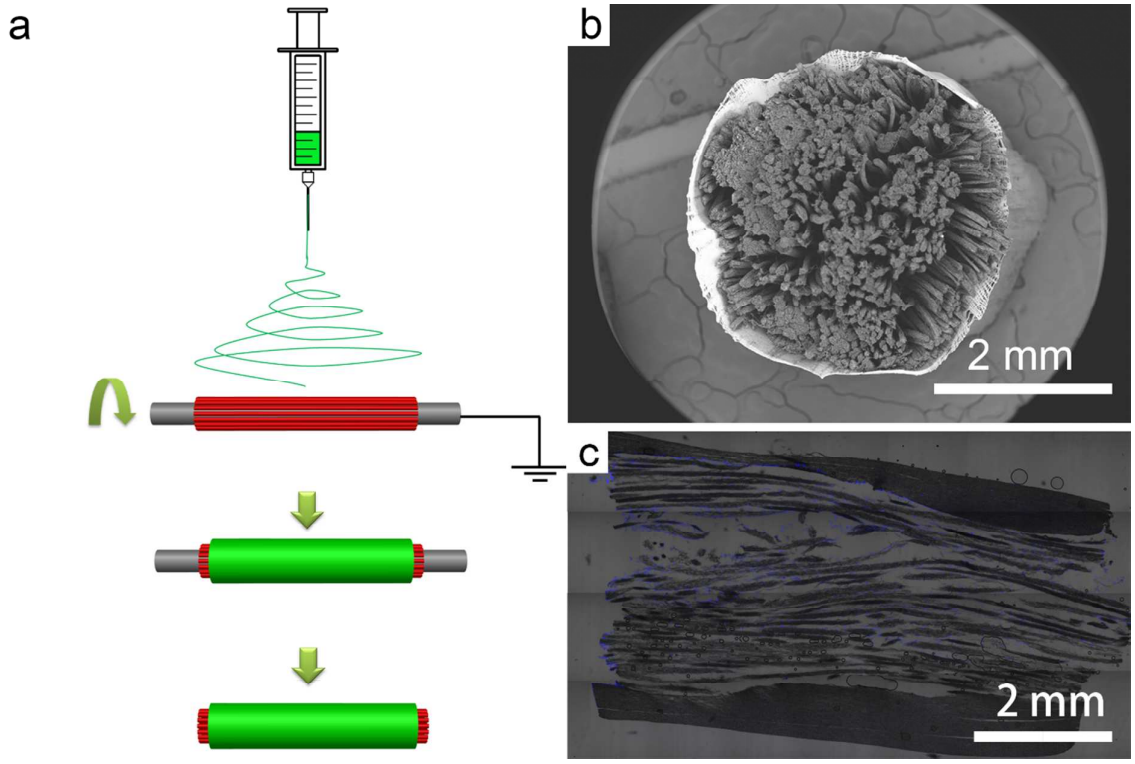

Fig (a) Schematic of incorporating nanofiber yarn into the conduit. (b) SEM image of the crosssection of nerve conduit after electrospun P(LLA-CL). (c) Longitudinal cross section of the nerve conduit after coculturing with SCs for 7 days.