Fabrication and Characterization of Dual-layer Multichannel Nerve Guidance Conduit

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Abstract: Nowadays, multichannel nerve guidance conduit (NGC) was designed by mimicking the architecture of nerve fascicles, and it was used to reduce dispersion of regenerating axons within the NGC lumen. In this paper, gelatin was used to prepare multichannel inner layer of NGC by freeze-drying, and poly(L-lactic acid-co-ε-caprolactone) (P(LLA-CL)) was used to fabricate nanofiber outer layer of NGC by electrospinning. The morphology of dual-layer multichannel NGC was observed by scanning electron microscopy (SEM). *In vitro* degradation experiment of the NGC demonstrated that the inner layer of NGC had the faster degradation rate than the outer layer of NGC. cell viability assay indicated that Schwann cells (SCs) showed better proliferation on dual-layer multichannel NGC than hollow NGC, because the multichannel structure introduced contact guidance for direct cell migration. Therefore, it was suggested that the dual-layer multichannel NGC had the potential for nerve tissue regeneration.

Key words: *poly(L-lactic acid-co-ε-caprolactone) (P(LLA-CL)); gelatin; electrospinning; multichannel; nerve tissue engineering*

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Introduction

Peripheral nerves injury is a common clinical problem, especially in trauma cases, and it causes an enormous socioeconomic burden[1-2]. The injured nerve is able to regenerate on its own and achieve functional recovery when the length of damaged gap is below 3-5 cm using autografts or synthetic conduits[3]. Initially, the simple hollow luminal nerve guidance conduit (NGC) is designed to guide Schwann cells (SCs) growth and axons regeneration, which is provided as a support matrix. However, previous research suggested that the hollow luminal NGC may lead to inappropriate target reinnervation by the dispersion of regenerating axons across the conduit, and this dispersion might cause misdirection of regenerating axons or polyinnervation of different targets by different axonal branches originating from the same motor neuron[4-6].

Multichannel NGC was fabricated and shown its function by mimicking the architecture of nerve fascicles, and it was used to reduce dispersion of regenerating axons within the NGC channel[7]. He *et al.* fabricated multichannel NGC using poly(lactide-co-glycolide) (PLGA), and *in vitro* study indicated that rat mesenchymal stem cells and SCs showed good attachment, spreading and proliferation on the PLGA NGC[8]. Yao *et al.* fabricated collagen NGC and characterized its degradation behavior and biocompatibility. However, the NGC which fabricated with natural materials (*e. g.*, gelatin, collagen and silk fibroin) shows better biodegradability and biocompatibility but poor mechanical properties; the NGC which fabricated with synthetic materials (*e. g.*, PLGA, poly(ε-caprolactone), and polylactic acid) shows good mechanical properties but poor biodegradability[4].

If the degradation of multichannel NGC is very slow which is fabricated with synthetic material, the axonal growth will be hampered. Therefore, in this study, gelatin as a natural material with good biodegradability, biocompatibility[9-11], was used to fabricate multichannel inner layer NGC via freeze-drying. Nanofiber outer layer of NGC was fabricated with poly(L-lactic acid-co-ε-caprolactone) (P(LLA-CL)) was used to fabricate by electrospinning[12-13], which provided mechanical properties for the NGC. The dual-layer multichannel NGC showed better biocompatibility, SCs proliferation and migration than hollow luminal NGC.

1 Experimental

1.1 Materials

P(LLA-CL) with an average molecular weight (M_w) of 300 kDa was supplied by Jinan Daigang Co., Ltd., (China). Hexafluoroisopropanol (HFIP) was obtained from Shanghai Darui Fine chemicals Co., Ltd., (China). 3-propanediamine, N'-(ethylcarbonimidoyl)-N, N-dimethyl-monohydrochloride (EDC), N-hydroxysuccinimide (NHS) and gelatin (Type B, from porcine skin) were purchased from Sigma Co., Ltd., (USA). The Dulbecco's modified eagle's medium (DMEM, Hyclone), fetal bovine serum (FBS, Gibico), trypsin (Hyclone) and 3-(4, 5-dimethylthiazol 2-yl)-2, 5-diphenyltetrazoliumbromide (MTT, Sigma) were purchased. 4', 6-Diamidino-2-phenylindole (DAPI) and Alexa Fluor 568 phalloidin was supplied by Life Technologies Co., Ltd., (USA). SCs were obtained from the Shanghai Institute of Biochemistry and Cell Biology (SIBCB, CAS, China).

1.2 Preparation of multichannel inner layer NGC

In this study, the multichannel inner layer NGC was prepared by mold. Cylindrical molds were constructed with ten wires spanning end caps to create channels for nerve guidance. NGC molds (l = 50 mm, Φ = 2 mm) were designed with polytetrafluoroethylene (PTFE). Channel diameter of channel mold was 250 μm, which was determined by the wire outer diameter. Figure 1(a) shows a schematic of a ten-channel mold.

Gelatin solution (25% (w/v)) was prepared by dissolving 4 g gelatin into 10 mL deionized water, and stirring 2 h at 60 ℃. At 60 ℃, the gelatin solution was injected into the PTFE mold quickly with a syringe. Then the mold containing gelatin solution was kept in the liquid nitrogen for 20 min. The mold with multichannel inner layer NGC was freeze-dried for 24 h. After which, the freeze-dried NGC was treated with a crosslinking solution of EDC (30 mmol/L) and NHS (10 mmol/L) in 95% ethanol solution overnight. After washing with deionized water, the NGC was freeze-dried for 24 h again. Eventually, molds and wires were removed from the inner layer

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NGC after freeze-drying.

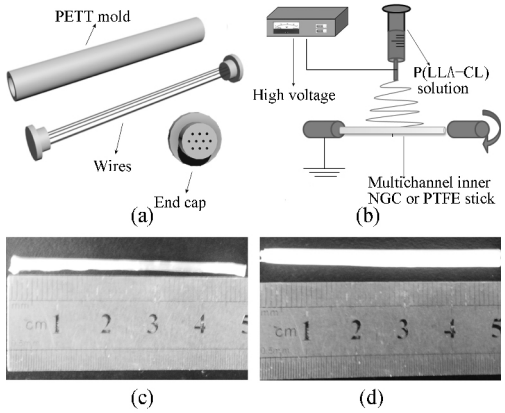

Fig. 1 Schematic of (a) multichannel NGC molds and (b) fabricate P(LLA-CL) out layer of dual-layer multichannel NGC with electrospinning; the digital photographs of (c) multichannel inner layer NGC and (d) dual-layer multichannel NGC

1.3 Preparation of outer layer NGC

P(LLA-CL) of 1 g was dissolved in 10 mL of HFIP to generate 10% (w/v) electrospinning solution. As illustrated in Fig. 1(b), the multichannel inner layer NGC was used as collector to collect P(LLA-CL) nanofibers with uniform rotation (the rotating speed was 50 r/min). The flow rate of P(LLA-CL) solution was set as 1.5 mL·h^{-1}, and high voltage was 12 kV. After electrospinning for 2 h, the dual-layer multichannel NGC was fabricated and stored in a vacuum oven. As a control, a PTFE stick (l = 50 mm, Φ = 2 mm) was set as a collector, and it had been removed after electrospinning to fabricated a hollow NGC.

1.4 Morphology of dual-layer multichannel NGC

The morphology of the dual-layer multichannel NGC was observed by scanning electron microscopy (SEM) (TM-3000, Japan) at an accelerated voltage of 15 kV. The multichannel inner layer of NGC and dual-layer NGC were observed by FUJI-Z200 digital camera. The diameter and length of NGC, and the diameter of channels were measured by the SEM images using the image visualization software Image J (National Institutes of Health, USA).

1.5 *In vitro* degradation of NGC

Electrospun hollow NGC and dual-layer multichannel NGC (l = 10 mm) were cut into rectangles for degradation testing *in vitro*. These samples were performed in 20 mL of phosphate-buffered solution at 37 ℃ for predetermined periods of time. After 3, 7, 14, 21, 28 and 35 d, the degraded samples were washed with deionized water then dried to constant weight in vacuum, and finally weighed, respectively. Weight loss (W) percentages were obtained using the following relationship:

$$W/\% = \frac{w_0 - w_d}{w_0} \times 100,$$

where w_0 is the initial weight and w_d is the dry weight after degradation. Each value was averaged from three specimens.

1.6 SCs proliferation and cell morphology observation

For the proliferation study, 1×10^4 SCs were seeded on the the NGCs (l = 10 mm) with tissue culture plate (TCP) as control. After culturing 1, 3 and 5 d, the amount of SCs was determined by MTT assay as previous study [14]. Noteworthy that, before we added the MTT assay into the plate, NGC samples were moved out from the post culturing plate, because only the SCs which growth on NGC should be counted.

After 5 d, SCs cultured on the NGC were fixed by 4% paraformaldehyde for 2 h at 4 ℃, and then the cells were stained with 25 μg·mL^{-1} phalloidin and 10 μg·mL^{-1} DAPI respectively. Before the staining, the cells on the samples were permeabilized by 0.1% Triton X-100 (Sigma, USA) for 10 min and washed 3 times with phosphate buffer solution (PBS). Confocal laser scanning microscopy (CLSM, Carl Zeiss, LSM 700, Germany) was used to visualize the morphology and distribution of cells on the scaffolds.

2 Results and Discussion

2.1 Morphology of dual-layer multichannel NGC

Freeze drying is a convenient method to fabricate 3D scaffolds in tissue engineering [15]. In this study, gelatin multichannel inner NGC was prepared by freeze-drying method using the mold as shown in Fig. 1(a). The outer layer of NGC was fabricated with electrospinning P(LLA-CL) using the multichannel inner NGC as collector (Fig. 1(b)). Figures 1(c) and 1(d) show the digital photographs of multichannel inner NGC and dual-layer multichannel NGC, respectively. It showed that the length of both multichannel inner NGC and dual-layer multichannel NGC was 50 mm, the diameter of NGCs were 2 mm and 2.5 mm, respectively. The parameters (such as length and diameter) of NGC can be controlled with different sizes of PTFE mold, and also the diameter and the number of channel in NGC can be controlled with wire.

SEM images of NGC were shown in Fig. 2. images of cross section (Fig. 2(a)) and longitudinal section (Fig. 2(b)) of multichannel inner NGC show the multichannel structure in NGC, and the diameter of channel is (230.43 ± 24.45) μm, which is determined by wires diameter. Figure 2(c) shows the nanofiber structure of P(LLA-CL) outer layer NGC, and the diameter of nanofibers is (967.90 ± 123.21) nm. As shown in Fig. 2(d), the inner layer and outer layer of dual-layer multichannel NGC are observed. It was found that a little irregular shrinkage of the gelatin inner layer of NGC occurred at the processing crosslink with EDC/NHS. This phenomenon was also documented in other crosslinking methods using EDC/NHS due to a well-known mechanism, NHS activate gelatin's COOH groups for further reaction with surrounding, nonprotonated NH_2 groups, and forming the peptide bonds [16-17].

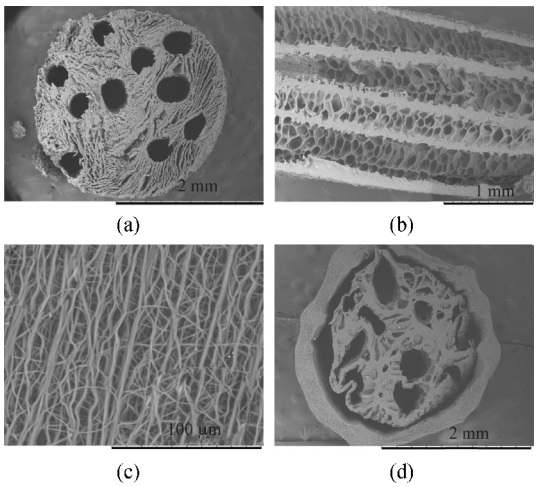

Fig. 2 SEM images of (a) cross section; (b) longitudinal section of gelatin multichannel inner layer NGC; (c) P(LLA-CL) nanofiber of outer layer NGC; (d) cross section of dual-layer multichannel NGC

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2.2 Degradation behavior of NGC

The degradation behavior of dual-layer multichannel NGC was investigated using PBS as solvent at 37 ℃, and the hollow P(LLA-CL) NGC was set as control. The degradation curves of NGCs were shown in Fig. 3, and it was found that the hollow NGC performed a slow degrade behavior, at 35 d, 20% weight loss of hollow NGC because of P(LLA-CL) degradation. However, the dual-layer multichannel NGC showed a faster degrade behavior in the initial two weeks degradation period, there was 58% weight loss due to the degradation of gelatin. After 35 d, 75% dual-layer multichannel NGC was degraded. The results indicated that the dual-layer multichannel NGC had a faster degradation behavior than hollow NGC, and the faster degradation of NGC was necessary for its application. On one hand, the slow degradation rate of the inner layer of NGC scaffold was not desirable, or else it would lead to the obstruction of axonal elongation, because the inner layer must be degraded to give space for axonal growth. On the other hand, appropriate degradation rate is also very important. If the inner layer of NGC scaffold shows a rapid degradation rate, it would result in that SCs could not proliferate on the scaffold before the degradation.

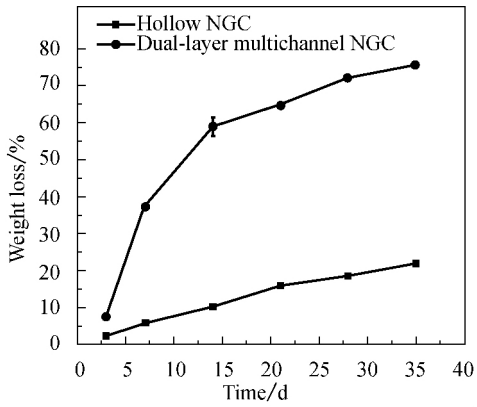

Fig. 3 *In vitro* degradation profiles of hollow NGC and dual-layer multichannel NGC

2.3 SCs proliferation and morphology of NGC *in vitro*

MTT assay was used to evaluate the SCs proliferation on NGC as shown in Fig. 4. In Fig. 4, * indicates significant difference of $p < 0.05$. During 5 d of culture, SCs go through a remarkable increase on NGCs, implying that both gelatin multichannel NGCs and P(LLA-CL) nanofiber can support the proliferation of SCs. Dual-layer multichannel NGC shows a better SCs proliferation than TCP group, which is indicated that the NGC is non-toxic for cell growth. In 3 d post-seeding, more SCs are detected on the P(LLA-CL) hollow NGC than dual-layer multichannel NGC and TCP group. It can be attributed to two aspects, on one hand, the nanofiber structure which formed by electrospun nanoscale fibers could offer the ECM-mimicking nano-sized structure with high surface area and porosity [18]; on the other hand, P(LLA-CL) materials have a good biocompatibility for cell growth [19]. Furthermore, 5 d later, the dual-layer multichannel NGC shows better SCs proliferation than hollow NGC, because the microscope structure of multichannel can provide more space foe cell spreading and migration with time.

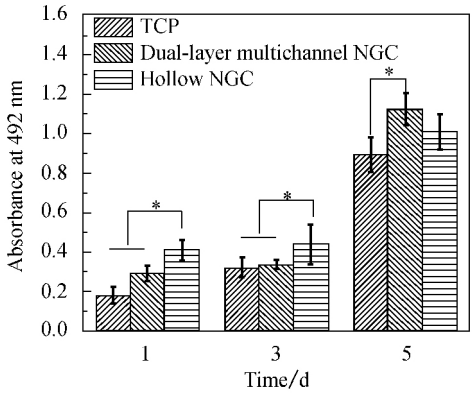

Fig. 4 Proliferation of SCs on TCP and NGCs

Meanwhile, the results can be verified by SCs morphology observation. SCs were visualized by phallodin and DAPI staining, respectively. In the confocal images (Fig. 5), the cytoplasm and nucleus of SCs were stained and performed white color. As shown in Figs. 5(a) and (b), SCs can be clearly observed spreading on the P(LLA-CL) nanofibers after 5 d culture. On dual-layer multichannel NGC (Figs. 5(c) and (d)), SCs were migrated into the channel and growth along the channel, which show the potential to guide axonal regeneration. Therefore, the SCs proliferation and morphology results indicated that dual-layer multichannel had good biocompatibility and application potential for peripheral nerves injury.

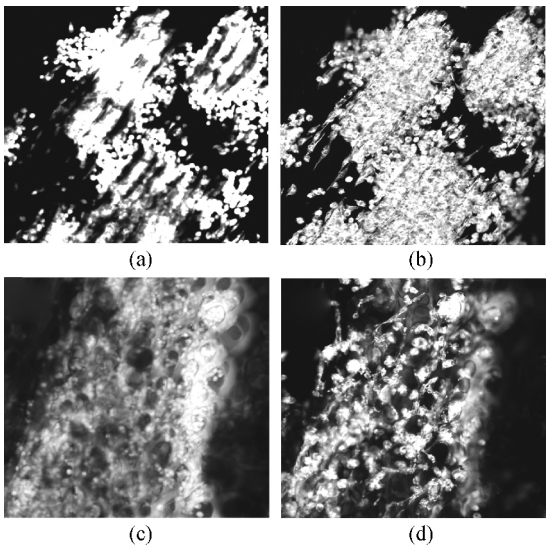

Fig. 5 CLSM micrographs for SCs cultured on (a) hollow NGC with DAPI staining; (b) hollow NGC with phallodin staining; (c) dual-layer multichannel NGC with DAPI staining; (d) dual-layer multichannel NGC with phallodin staining

3 Conclusions

Peripheral nerves injury is a common clinical problem, and multichannel NGC has the potential to reduce dispersion of axon regeneration within the channels. However, both the degradation and biocompatibility of NGC are very important for its application. Herein, a dual-layer multichannel NGC has been fabricated with freeze-drying and electrospinning methods. *In vitro* degradation experiments of NGC suggest the good

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degradability of dual-layer multichannel NGC. MTT assay and CLSM results indicated that the dual-layer multichannel NGC had good biocompatibility and guided SCs migration in the channels. 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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