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RESEARCH ARTICLE

A novel bioactive nerve conduit for the repair of peripheral nerve injury

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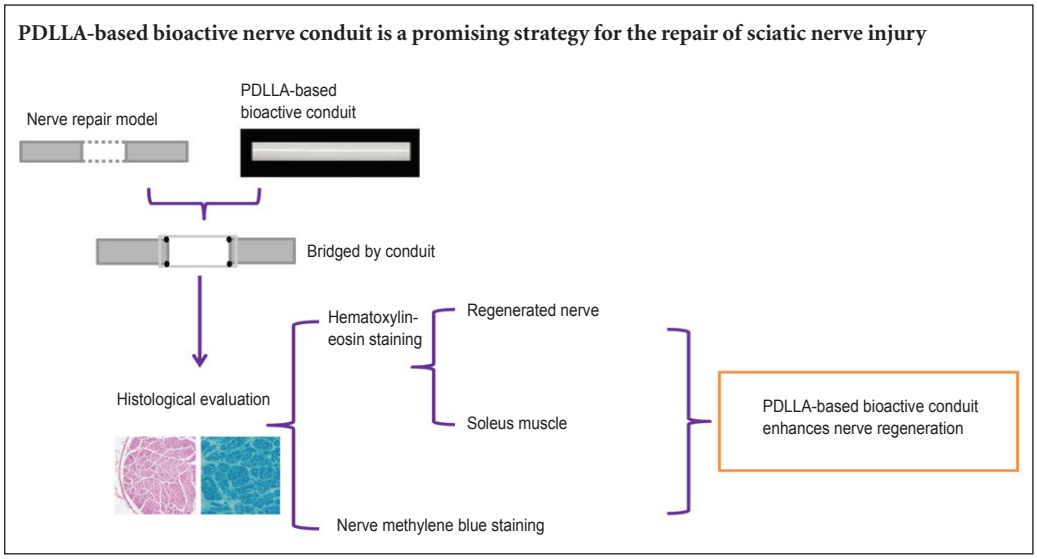
Graphical Abstract



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Abstract

The use of a nerve conduit provides an opportunity to regulate cytokines, growth factors and neurotrophins in peripheral nerve regeneration and avoid autograft defects. We constructed a poly-D-L-lactide (PDLLA)-based nerve conduit that was modified using poly{(lactic acid)-co-[(glycolic acid)-alt-(L-lysine)]} and β-tricalcium phosphate. The effectiveness of this bioactive PDLLA-based nerve conduit was compared to that of PDLLA-only conduit in the nerve regeneration following a 10-mm sciatic nerve injury in rats. We observed the nerve morphology in the early period of regeneration, 35 days post injury, using hematoxylin-eosin and methylene blue staining. Compared with the PDLLA conduit, the nerve fibers in the PDLLA-based bioactive nerve conduit were thicker and more regular in size. Muscle fibers in the soleus muscle had greater diameters in the PDLLA bioactive group than in the PDLLA only group. The PDLLA-based bioactive nerve conduit is a promising strategy for repair after sciatic nerve injury.

Key Words: *nerve regeneration; polylactic acid; poly{(lactic acid)-co-[(glycolic acid)-alt-(L-lysine)]}; β-tricalcium phosphate; nerve conduit; nerve fiber; neural regeneration*

Introduction

The two detached ends of an injured peripheral nerve cannot rejoin successfully in the absence of an external aid. A nerve-like device is necessary for the repair of the injured nerve to reduce the loss of muscle function and sensory disorder (Evans et al., 1999; Li et al., 2014). The application of a nerve conduit is considered as an effective method that avoids the shortfalls associated with an autograft. It can also provide an opportunity to regulate the responses to the cytokines and neurotrophins during the peripheral nerve regeneration (Mohammad et al., 2000; Kehoe et al., 2012; Azizi et al., 2015). To resolve these issues, composite materials with excellent biodegradability and biocompatibility are employed to imitate the structure and function of natural

nerves (Mligiliche et al., 1999; Chen et al., 2005; Luis et al., 2007; Subramanian et al., 2009; Das et al., 2013). Poly-D-L-lactide (PDLLA) was employed in this study as the nerve conduit due to its excellent biodegradability and biocompatibility. However, the degradation products of PDLLA can lower the local pH which is harmful to the surrounding cells and tissues. The addition of β-tricalcium phosphate (β-TCP) is beneficial not only because of its good biocompatibility, biodegradability and non-toxicity, but also because its basic degradation products restore the local pH to its normal value. The peptide Gly-Arg-Gly-Asp-Gly (RGD) has been shown to enhance Schwann cell attachment and elongation *in vitro* (Yan et al., 2012; de Luca et al., 2013), and thereby facilitate axon growth in the early stage *in vivo* (Liu et al., 2009).

Most previous studies focused on the long-term (over 3 months) nerve regeneration after nerve conduit implantation (Den Dunnen et al., 1993; Toba et al., 2002; Bian et al., 2009; Xu et al., 2011, 2014; Yan et al., 2012), but there have been fewer reports on the morphology of regenerated nerves in the early stages of nerve regeneration (Yang et al., 2001; Jaminet et al., 2013; Kawasaki et al., 2013; Schrems-Hoesl et al., 2013; Seo et al., 2013; Qiu et al., 2014; Li et al., 2015).

In this study, both RGD and β-TCP were first used to modify a PDLLA conduit to offer a bioactive microenvironment for nerve regeneration using a biomimetic method. We planned to analyze the biological performance of the nerve conduit in the repair of a 10-mm deletion of the sciatic nerve in rats by observing the changes at an early stage (35 days) during nerve regeneration.

Materials and Methods

Preparation of PDLLA-based bioactive nerve conduit

A polymer RGD peptide (GL Biochem, Shanghai, China) modification of poly{(lactic acid)-co-[(glycolic acid)-alt-(L-lysine)]} (PRGD) was fabricated by the following steps. Firstly, (3S)-3-[4-(benzyloxycarbonylamino) butyl] morpholine-2,5-dione (BMD) was synthesized by bromoacetyl bromide and Nε-(benzyloxycarbonyl)-L-lysine. Secondly, poly(lactic acid)-co-[(glycolic acid)-alt-(Nε-benzyloxycarbonyl-L-lysine)] was obtained by copolymerization of D, L-lactide and BMD. Then, poly{(lactic acid)-co-[(glycolic acid)-alt-(L-lysine)]} (PLGL) was synthesized by catalytic hydrogenation. Finally, PLGL was modified with RGD peptide. PRGD (0.05 g) and PDLLA (0.9 g) (molecular weight 250,000, synthesized in the State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Wuhan University of Technology in China) were dissolved in ethyl acetate at a concentration of 0.05 g/L β-TCP (0.05 g) (synthesized in the State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Wuhan University of Technology) was then added to the ethyl acetate solution and mixed thoroughly. The PRGD, PDLLA and β-TCP were used to prepare PDLLA-based bioactive composite using a solvent volatilization method (Zhang et al., 2015). The PDLLA-based composite and PDLLA membranes were fabricated and then rolled to form the PDLLA-based composite or PDLLA bioactive nerve conduits. The product was 14 mm long, 2 mm in diameter and 0.2 mm thick. The nerve conduits scheduled for bridging sciatic nerve defects were sterilized with ultraviolet light for 60 minutes. The material characterizations and *in vitro* evaluations of these conduits have been reported in our previous study (Zhang et al., 2015).

Ethics statement and animals

The study protocol was approved by the Animal Care and Use Committees of Wuhan University of Technology, China and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (No.85-23, revised 1996). Precautions were taken to minimize the number of animals used and their suffering.

Adult male Wistar rats, weighing 200–250 g, 8 weeks old, specific pathogen-free level, were purchased from the Centers for Disease Control and Prevention of Hubei Province (China) (license No. SCXK(E)2015-0018). The rats were randomly divided into three groups each with 10 rats: PDLLA conduit group; PDLLA-based bioactive conduit group and normal nerve group.

Sciatic nerve injury model establishment and nerve conduit repair

The rats were anesthetized with 50 mg/kg pentobarbital sodium by intraperitoneal injection. The right sciatic nerve was exposed after a skin incision was made and the muscles around the nerve tissues were separated using blunt dissection. Subsequently, the right sciatic nerve was severed into proximal and distal segments at the center of the right thigh. Both the proximal and distal stumps were sutured with 9-0 nylon sutures to a depth of 1 mm into the conduits, leaving a 10-mm gap between the stumps, bridged by the nerve conduits (**Figure 1**). The muscle and skin layers were re-approximated using 6-0 nylon sutures. In the normal nerve group, no surgery was carried out on the sciatic nerve.

Hematoxylin-eosin staining

At 35 days after conduit implantation, the rats were anesthetized again with 50 mg/kg pentobarbital sodium to expose the right sciatic nerve. The conduits were opened and samples of the regenerated nerves, or normal nerves, and small pieces of the soleus muscle in all groups were collected. All the rats were sacrificed by cervical dislocation after all the samples had been collected. The soleus muscle and nerve specimens were fixed in a solution containing 1% paraformaldehyde, 1.25% glutaraldehyde and 0.1 M cacodylate buffered to pH 6.5–7.0, then dehydrated and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin and observed using an inverted microscope (IX71, Olympus, Tokyo, Japan). The muscle fiber size in each group was analyzed by selecting 200 muscle fibers in 10 random areas. The average diameter of the muscle fibers was analyzed using an image analysis system (Image-Pro Plus, Media Cybernetics, San Francisco, CA, USA).

Toluidine blue staining

To evaluate the physiological status of axons and myelin sheath regeneration at 35 days after conduit implantation, the regenerated nerve specimens were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffered saline for 2 hours and postfixed in 1% osmium tetroxide for 1.5 hours. Then they were dehydrated in a graded ethanol series, and embedded in paraffin. The specimens were cut into 1-μm-thick cross-sections with an ultramicrotome (MT-XL, RMC Inc., New York, NY, USA), stained with toluidine blue, and observed by inverted microscopy. The fiber sizes in all groups were analyzed by selecting 200 nerve fibers in 10 random areas. The average diameter of the regenerated nerve fibers was analyzed using the image analysis system (Image-Pro Plus, Media Cybernetics).

Statistical analysis

Data are expressed as the mean ± SD. Experimental data were processed using the statistical software SPSS 10.0 (SPSS Inc., Chicago, IL, USA), and analyzed with one-way analysis of variance followed by a Bonferroni *post-hoc* test. *P* values less than 0.05 were considered statistically significant.

Results

The biocompatibility of the PDLLA-based bioactive conduits

All the rats used in this study appeared to be well by their daily behavior. Macroscopically, axonal sprouts were found at both the distal and proximal ends, and these were successfully connected by the 35th day. The conduit was well integrated with the sciatic nerve and had not yet degraded.

Regenerated nerve morphology after rat sciatic nerve repair with PDLLA-based bioactive conduits

To observe the morphology of the nerve in the early stage of regeneration, hematoxylin-eosin staining was done 35 days post surgery. These hematoxylin-eosin images illustrate that **Figure 2C** appears to have denser packing of nerve fibers than either **Figure 2A** or **B**. Also, the fiber bundles in **Figure 2C** are more regular than those in **Figure 2B**, which in turn are more regular than those in **Figure 2A**. **Figure 2B** shows less in-growth of irregular connective tissues from the sciatic nerve sheath than that in **Figure 2A**. The regenerated nerve fibers of sciatic nerves in the rats (**Figure 2D**, **E**) were smaller in diameter and less uniform in morphology than those in the normal nerve group (**Figure 2F**). Those in the PDLLA conduit group were thinnest and most irregular with the most in-growth of connective tissues (**Figure 2D**). The sections in the two conduit groups showed more activated Schwann cells and blood vessels than those in the normal nerve group. These findings showed that the axons were actively supported and that the neurotrophic substances were delivered to the lesion for the nerve regeneration.

Results of toluidine blue staining showed that nerve fibers were densely packed in all groups (**Figure 3A–C**), the myelinated fibers in the PDLLA-based bioactive conduit group had more compact and uniform structures than those in the PDLLA conduit group, but less than those in the normal nerve group. The average fiber diameter analysis showed that the mean fiber diameter in the PDLLA-based bioactive conduit group was significantly smaller than that in the normal nerve group, but 1.42 times larger than that in the PDLLA conduit group (**Figure 3D**).

Morphology of the soleus muscles after rat sciatic nerve repair with PDLLA-based bioactive conduits

To evaluate the nerve function recovery, the soleus muscles in all groups were subjected to hematoxylin-eosin staining. Compared with the normal nerve group (**Figure 4C**), the soleus muscles following surgery had degenerated in the PDLLA conduit and PDLLA-based bioactive conduit groups, and presented smaller fiber diameters in the same area. The muscle atrophy and connective tissues in the PDLLA-based bioactive conduit group (**Figure 4B**) had re-grown, showing a better morphology, while the muscles in the PDLLA conduit group (**Figure 4A**) were still in a poor condition. The mean diameter of muscle fibers in the PDLLA-based bioactive conduit group was 1.34-times larger than that in the PDLLA conduit group, yet still smaller than that in the normal nerve group (**Figure 4D**).

Discussion

The RGD peptide has been proven to enhance Schwann cell attachment and elongation *in vitro* (Yan et al., 2012; de Luca et al., 2013), and RGD *in vivo* facilitates the axonal regeneration in the early period after sciatic nerve injury in rats (Liu et al., 2009). The presence of RGD-coated conduits in the early phase of peripheral nerve regeneration provides a permissive surface for activated Schwann cells around the lesion to secrete vital trophic factors to support axon regeneration (Rafiuddin Ahmed and Jayakumar, 2003; Liu et al., 2009). The other supplement, β-TCP, upregulates the mRNA expression of cytoskeletal protein and has been proved to be nontoxic (Qiu et al., 2014).

In this study, we aimed to observe the morphology of regenerated nerves bridged by the PDLLA-based bioactive nerve conduit in the early period of nerve regeneration. The

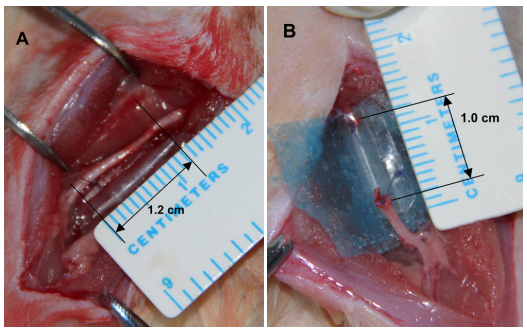

Figure 1 Sciatic nerve defects bridged by poly-D-L-lactide (PDLLA)-based bioactive conduits.
(A) A sciatic nerve defect before bridging by PDLLA-based bioactive conduit; (B) sciatic nerve after bridging by PDLLA-based bioactive conduit.

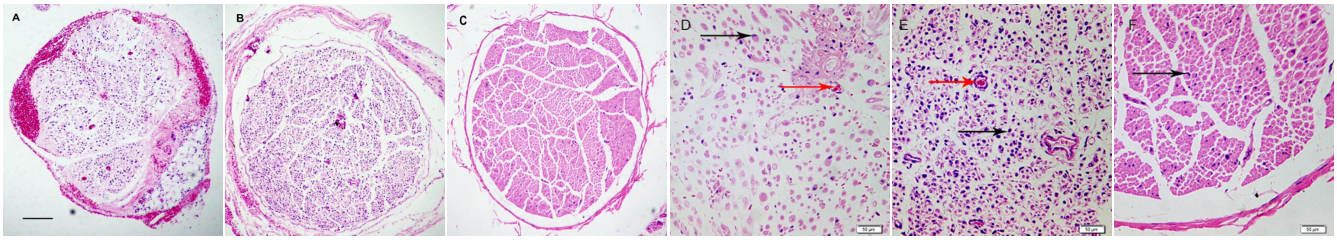

Figure 2 Morphology of regenerated sciatic nerves in rats at 35 days after sciatic nerve injury (hematoxylin-eosin staining).
PDLLA conduit group (A, D). PDLLA-based bioactive conduit group (B, E). Normal nerve group (C, F). In D–F, black arrows indicate the nuclei of Schwann cells and the red arrows indicate blood vessels. Scale bars: 200 μm in A–C; 50 μm in D–F. PDLLA: Poly-D-L-lactide.

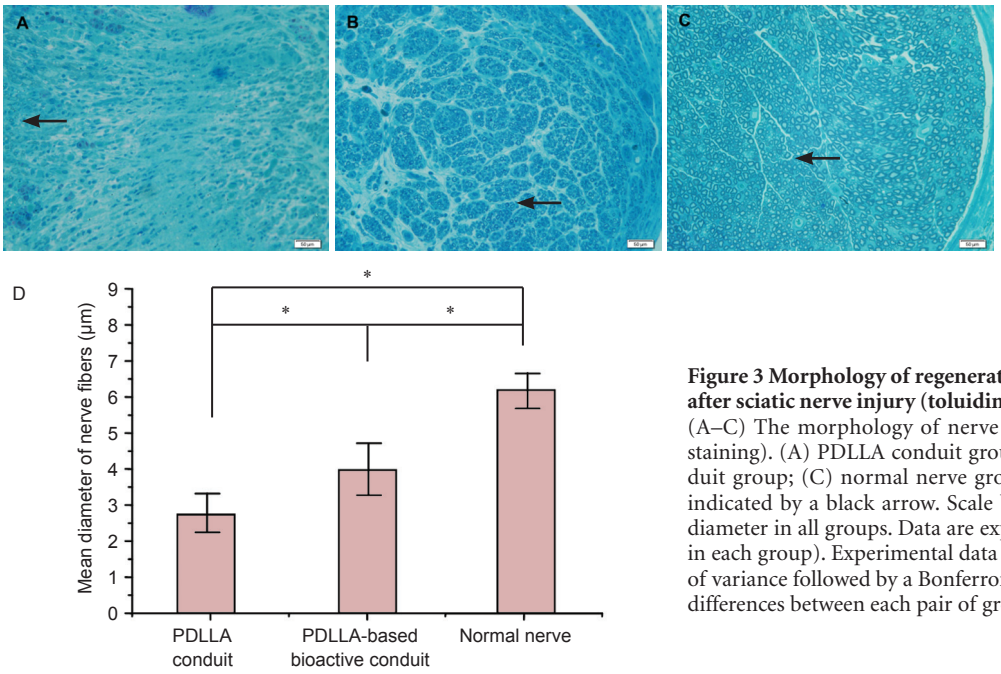

Figure 3 Morphology of regenerated sciatic nerves in rats at 35 days after sciatic nerve injury (toluidine blue staining).
(A–C) The morphology of nerve fibers in all groups (toluidine blue staining). (A) PDLLA conduit group; (B) PDLLA-based bioactive conduit group; (C) normal nerve group. In A–D, the myelinated fiber is indicated by a black arrow. Scale bars: 50 μm. (D) Size of nerve fiber diameter in all groups. Data are expressed as the mean ± SD (n = 5 rats in each group). Experimental data were analyzed with one-way analysis of variance followed by a Bonferroni *post-hoc* test. *P < 0.05, significant differences between each pair of groups. PDLLA: Poly-D-L-lactide.

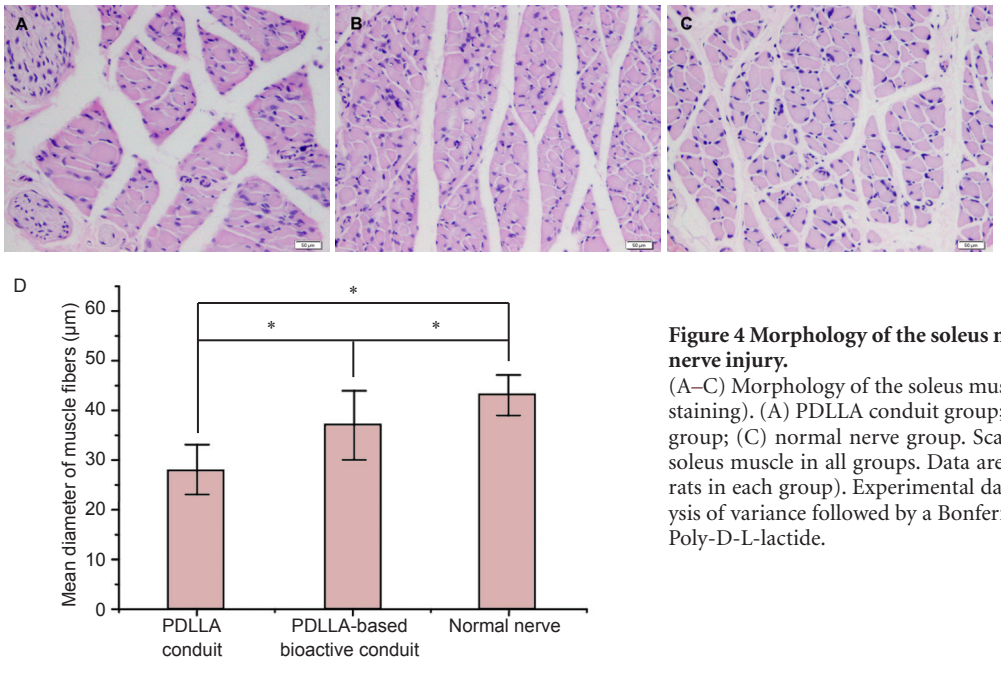

Figure 4 Morphology of the soleus muscle in rats at 35 days after sciatic nerve injury.
(A–C) Morphology of the soleus muscle in all groups (hematoxylin-eoxin staining). (A) PDLLA conduit group; (B) PDLLA-based bioactive conduit group; (C) normal nerve group. Scale bars: 50 μm. (D) Diameter of rat soleus muscle in all groups. Data are expressed as the mean ± SD (n = 5 rats in each group). Experimental data were analyzed with one-way analysis of variance followed by a Bonferroni *post-hoc* test. *P < 0.05. PDLLA: Poly-D-L-lactide.

nerve morphology of nerve fibers at different stages would directly demonstrate the maturation of axons, which is also a marker for assessing the efficiency of indirect conduit bridging. Many of the fibers with small diameters could be non-conducting and degenerating rather than regenerating. As the nerve fibers regenerate distally and reach the appropriate target organs, the fiber diameter increases and the myelin sheath grows (Weiss, 1945; Schröder, 1972). If sprouting axons are incapable of establishing a suitable connection with the target organ, they are deprived of vital growth factors and degenerate.

The morphology and size analysis of nerve fibers have demonstrated that the PDLLA-based bioactive nerve conduits promote early-stage peripheral nerve regeneration by enhancing the nerve regeneration rate and significantly increase the myelinated fiber and soleus muscle fiber density compared with PDLLA conduit controls. At 35 days after sciatic nerve surgery, the fibroblasts and macrophages concentrated around the periphery of the newly formed nerve tissues (Brown et al., 1991; Zhou and Snider, 2006). Schwann cells and endothelial cells moved into the lesion and secreted the necessary cytokines and neurotrophins to enhance synthesis of new nerve tissue and axon elongation (Markus et al., 2002; Leibinger et al., 2009; Liu et al., 2011). The injury detected by the neuronal body switched the axons from the normal state to growth mode with an associated gene expression and protein synthesis (Kretz et al., 2005; Agthong et al., 2006; Miao et al., 2006; Luo et al., 2007; Trenchi et al., 2009; Yamazaki et al., 2009; Liu et al., 2011). Concurrently, cell-adhesion molecules, myelin proteins, and extracellular matrix proteins around the lesion supported the growth cone sprouting and axons remodeling (Skene et al., 1986; Fernandes et al., 1999; Janke and Bulinski, 2011). The nerve fiber morphology would be different in this early stage during nerve regeneration because of the different cytokines and gene regulation. Therefore, we concluded that the PDLLA-based bioactive nerve conduit might promote axon growth and soleus muscle recovery in the early stage of nerve regeneration.

Our evidence indicated that a PDLLA-based conduit modified with bioactive compounds enhanced regeneration of the injured nerve during the first 35 days. However, the regenerating nerve morphology should be explored at other time points. Similarly, research at the molecular level is necessary to explore how the bioactive conduit affects the changes in the cytokines and neurotrophins.

In this study, we developed a novel PDLLA-based bioactive nerve repair conduit, which we used in *in vivo* trials for the repair of rat sciatic nerve injury with a 10 mm gap. We evaluated the outcomes of nerve regeneration by observing the nerve morphology with histological staining in the early period. Our results demonstrated that compared with the PDLLA conduit group, the nerve recovery in the PDLLA-based bioactive conduit group showed larger diameter nerve fibers in more ordered array. Soleus muscle fibers were also larger. This enhanced nerve conduit offers new opportunities for research in the field of nerve regeneration.

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Conflicts of interest: *None declared.*
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