At present, reconnecting the transected nerve in clinic is still mainly reliant on surgery suture. This is a procedure that requires thorough training and is also time consuming. Here, an octa-poly(ethylene glycol) (PEG)-based adhesive for fast reconnecting of the transected peripheral nerve is reported. To enhance the therapeutic efficacy, a succinyl unit is applied to endow the controllably dissolvable property of the adhesive, and lithium is loaded in the adhesive to improve the axonal regeneration. Present data reveal that this adhesive possesses good cytocompatibility and can significantly shorten the reconnecting time of the transected nerve ends compared to that required for suture surgery. Histology, electrophysiological, and behavior assessments indicate that the adhesive reconnected nerves exhibit a low grade of fibrosis, inflammation response, and myoatrophy as well as robust axonal regeneration and functional recovery. Together, these results indicate that this octa-PEG adhesive can act as an alternative to traditional nerve suture in peripheral nerve injury.

1. Introduction

Tens of millions of people are currently affected by some form of peripheral nerve injuries (PNI), of which most patients are working-age people, raising serious socioeconomic implications.[1] Generally, PNI results from traumatic, non-traumatic, and iatrogenic causes, which will cause painful neuropathies and neuromas, poor sensation, and paralysis for patients due to the loss of function in damaged motor and sensory axons. Peripheral nerve surgery has proved to be a life-changing surgery as it not only improves the patients’ ability to perform every day function but also to enhance professional activities which affects the physical and psychological well-being of the patients.[2]

Nowadays, suture surgery is indispensable for peripheral nerve transection whether with short-distance gap or long-distance nerve gap.[3] For short-distance gap, the transected nerve can be sutured and reconnected end-to-end. For long-distance gap, the treatments rely mainly on the application of donor tissues in clinic. In such a case, after implanting the donor tissues, suture is needed to connect the donor tissues and the original nerve.[4] On the other hand, it is difficult to suture tissues which are tiny and brittle, so skillful surgeons are needed in these operations.[5] There are also concerns that suture will cause second tissue...
damage and later a potential inflammatory response, which might hinder the healing process in the sutured nerves. Adhesives are thought able to revolutionize the wound closure. They are inherently non-invasive and can be easily applied without special request. Moreover, drugs or cells can be readily loaded in the adhesives for a better wound healing outcome. There are now some commercialized adhesives which can be classified into the natural and the synthetic. Fibroin glue is the most widely used natural adhesive, but it has long been blamed for the risk of viral and disease transmission as well as for allergic problems. Cyanoacrylates are widely used in the synthetic adhesives. However, they are extremely limited in applications of the inner of the human body, especially in the sensitive nerve system because of their exothermal polymerization, long retention time and potential toxic degradation products.

Mussel-inspired adhesives have attracted massive attention. Dopamine has been used in these applications to fabricate precursors, which were then crosslinked with an oxidant to form strong bonding with tissues. On the one hand, to obtain the greatest adhesive strength, a strong oxidant sodium peroxide or hydrogen peroxide system is used, which might cause lesion to tissues. On the other hand, neurological effect of dopamine on the nerve system is always a concern. And, while people are assiduously searching for methods to produce a robust anchor, they seldom consider how to remove the adhesives when they are no longer needed, especially when the adhesives are applied on nerves, a notably vulnerable part of the body.

We previously reported the synthesis of a controllably dissolvable adhesive with succinyl units using Tetra-poly(ethylene glycol)(PEG)-NH\textsubscript{2} and Tetra-PEG-succinimidyl succinate (Tetra-PEG-SS). The appealing properties of a sealant system is that the use of succinyl units endowed the system with almost the same adhesive strength as that of fibrin glue, a controllably dissolvable property as well as good biocompatibility, which will not induce a long-term inflammatory response. It was reported that PEG was able to connect the distal and proximal ends of an injured nerve at the cellular level through axonal fusion to avoid Wallerian degeneration of the injured distal nerve and promote peripheral nerve regeneration.

Previous study demonstrated that lithium has anti-apoptotic, anti-inflammatory properties and a neuroprotective effect, which have been explored for use as a treatment in the central nervous system against trauma such as spinal cord injury and chronic neurodegenerative diseases. Lin et al. proved that the synergistic lithium chloride polycaprolactone enhances peripheral nerve regeneration. Chen et al. showed that the oral administration of adult mice with lithium after sciatic nerve crush accelerated the myelin debris clearance, restored the myelin structure and promoted the functional recovery of the sciatic nerve.

In the present study, we used octa-arm poly(ethylene glycol) succinimidyl succinate (octa-PEG-SS) and octa-arm poly(ethylene glycol) amine (octa-PEG-NH\textsubscript{2}) to construct hydrogel adhesives (OSS) with succinyl units to act as an adhesive for the suture of peripheral nerve transection (Scheme 1). First, we hypothesized that OSS would gel fast enough to avoid nerve fracture after injury, offer sufficient adhesive strength for wound closure, and can be controllably eliminated for future therapeutic purposes. We then hypothesized that through combination with LiCl, LiCl loaded OSS could accelerate the nerve regeneration, which has the potential to act as an alternative to the traditional suture technique.

2. Results and Discussion

The octa-PEG-SS can be easily synthesized in two steps according to our previous literature (Figure 1A). All the peaks in the \textsuperscript{1}H and \textsuperscript{13}C NMR spectrum (Figure 1B,C) can be justly attributed to the structure in Figure 1A and the ratio of integration of peak d to the sum of that of a, b, and c nearly equals to 1–4, demonstrating the successful synthesis of octa-PEG-SS. The adhesive must gel fast enough to avoid the shrinking of the nerve transect. To achieve a speedy gelation, we used octa-PEG, which has...
Figure 1. Preparation and characterization of OSS. A) Synthetic route of the octa-PEG-SS and its B) $^1$H NMR and C) $^{13}$C NMR spectra of octa-PEG-SS in CDCl$_3$. D) Rheological analysis of OSS with the PEG concentration to be 12 wt%. E) Gelation times of OSS and OSSL with the different PEG concentrations (6, 8, 12, and 16 wt%). F) Adhesive strength of PEG with the PEG concentration to be 12 wt% compared with that of fibrin glue. G) Pictures showing that two pig skins adhered by the hydrogel (1 cm x 1 cm) can hold the weight of 400 g. ($n = 3$)

double the density of the functional group as that of the Tetra-PEG with same molecular weight. First, the gelation of PEG was confirmed by the rheology tests (Figure 1D). The elastic modulus ($G'$) is larger than the viscous modulus ($G''$), proving that the hydrogel formed after mixing the two components. Then, the inversion method was used to the gelation time of OSS, which showed that OSS could gel in 4.2, 2.0, 1.4, and 1.2 s with the concentration to be 6, 8, 12, and 16 wt%, faster than that of Tetra-arm based PEG hydrogel at the same condition.\[^{18}\] It was also observed that after adding LiCl (OSSL), the gelation time has no significant difference with that of OSS, proving that LiCl will not influence the gelation of the adhesives. Wanting to use as little materials as possible in the sensitive nerve part, we chose the PEG concentration of 12 wt% in the experiments that followed. Because of the reaction between the active ester and amine groups from tissues, the hydrogel can adhere to tissues.\[^{25}\] It was proved that the average adhesive strength (15.7 ± 3.8 kPa) of the hydrogel was nearly the same as that of the commercialized fibrin glue (13.2 ± 3.5 kPa).
which is the gold standard adhesive in the market.\textsuperscript{[16]} The adhesive properties were further evaluated by loading weight using the hydrogel-adhered porcine skin and the binding area was 1.5 cm × 1.5 cm. After being adhered, the porcine skins could stick together even when the loaded weight increased to 400 g (Figure 1G).

Adhesives are more convenient than suture in most cases. However, it might cause bigger problem in cases where we have to remove the adhesives for exposure of the tissues, because unlike sutures, the adhesives usually adhere to the tissues tightly.\textsuperscript{[26]} Inspired by controllably dissolvable properties of succinyl units, we hypothesized that the adhesive system can be easily removed.\textsuperscript{[18]} The dissolution experiments using 5 wt% cysteamine solution demonstrated that a block of 500 µL hydrogel could be dissolved in less than 50 min (Figure 2A). While 20 µL of hydrogel on the transected nerve could be dissolved by cysteamine solution in 40 min (Figure 2B). Recognizing that this is a relatively long time in clinic, we used the swab, which has been used to clean wounds in hospital, to accelerate the dissolution. It turned out that the hydrogel in the injury site could be wiped out by the cysteamine solution-soaked swap in less than 2 min (Figure 2C). This controllably dissolvable property made OSS advantageous in brittle nerve system applications when the removal of adhesives is needed.

Lithium has a positive influence on nervous system regeneration.\textsuperscript{[27]} Here, we loaded LiCl into the adhesive system (OSSL) and the release assessment was studied by photometrical spectroscopy as described in previous literature.\textsuperscript{[28]} When the OSSL was immersed in water in vitro, the assessment indicates that the lithium could be continuously released from the OSSL for 14 days with the total release amount of 85% (Figure 2D). To further detect the slow release capability of OSSL in vivo, OSSL was immersed in water in vitro, the assessment indicates that the lithium could be continuously released from the OSSL for 14 days with the total release amount of 85% (Figure 2D). To further detect the slow release capability of OSSL in vivo, OSSL was immersed in water in vitro, the assessment indicates that the lithium could be continuously released from the OSSL for 14 days with the total release amount of 85% (Figure 2D). To further detect the slow release capability of OSSL in vivo, OSSL was immersed in water in vitro, the assessment indicates that the lithium could be continuously released from the OSSL for 14 days with the total release amount of 85% (Figure 2D). To further detect the slow release capability of OSSL in vivo, OSS and OSSL were used to adhere the transected nerve within 2 s too. Meanwhile ≈5–10 min was needed to finish a successful microsurgery of nerve end to end suture even by a skilled operator. These findings illustrated the significant advantage of the adhesives in reducing time for the nerve reconnection (Figure 4A). When the nerve was re-exposed at 8 weeks post-surgery, no breakage in the reconnected nerve was found in all groups (Figure 4A). After the nerve injury, the invasion of fibroblasts and macrophages from the surrounding tissues might lead to fibrosis and inflammation in the lesion area. Both of fibrosis and inflammation are considered as adverse factors that impede nerve regeneration. Our previous study proved that succinyl unit-based PEG hydrogel can prevent fibroblast invasion and induce little inflammation in a liver wound model.\textsuperscript{[19]} Herein, Masson staining and ED1 immunohistochemistry were performed to illustrate the fibrosis and inflammation in the injured nerves.\textsuperscript{[34,35]} As shown by Masson staining, the sutured nerve has remarkable signs of collagen fibers (blue stained components in the section) at the link points (Figure 4B). In contrast, the nerves in the OSSL group showed few of blue signals. Notably, the nerves in the OSS group also showed blue signals similar to those of the suture group but the HIS groups had extremely strong blue signals. Further, ED1 immunohistochemistry revealed that densities of macrophages in the lesion areas were similar between the suture group and OSSL group (Figure 4C,F). Compared with the previous two groups, the macrophage density of the OSS group and the HIS group increased significantly, with the largest being in the HIS group. These patterns were the same at 1 week and 8 weeks post-surgery. These data indicate that the adhesives could significantly shorten the surgery time to reconnect the transected peripheral nerves compared with that of microsurgery suturing. Moreover, using adhesives to repair the nerve does not rely on a surgery microscope and well-trained operators. We believe that these advantages are of great importance for acute neurotrauma repair, especially for the treatment out of hospital. On the other hand, we found the HIS reconnected nerves exhibited conspicuous fibrosis and inflammation, which were indexed by high levels of collagen fibers and macrophages in the lesion area. OSS and OSSL treatments, meanwhile, significantly decreased the fibrosis and inflammation. In particular, levels of fibrosis and inflammation in the OSSL group were almost the same as those in the suture group. Overall, the present findings demonstrated that the adhesive of OSSL can reconnect the transected nerves with low fibrosis and inflammatory responses.

After nerve injury, axons in the distal trunks must degrade and then the axons in the proximal trunk will extend into the distal trunk in the cases where the nerve was reconnected. One of biggest challenges of the peripheral nerve regeneration is that the slow axonal outgrowth, so it is the rate of the axonal regeneration that attracts the attention of all researchers in the field of nerve injury. Existing literature and our previous studies demonstrated that GAP43 was a reliable marker for regenerating axons.\textsuperscript{[36,37]} GAP43 is specifically expressed in developing or regenerating axons. Immunofluorescence illustrated that the GAP43-positive axons extending from the proximal site of the injury site to the distal trunk were obviously longer and denser in the OSSL group than in the OSS group and HIS group (Figure 5A). To further quantify the regenerating axons, the numbers of axons crossing these
Figure 2. Dissolution and release studies. A) Dissolution of the hydrogel in 5 wt% cysteamine solution. B) Dissolution of the hydrogel in the injured nerve with cysteamine solution. C) Wiping the hydrogel in the injured nerve using a swab soaked with cysteamine solution. D) In vitro release profiles of OSSL in water. E) In vivo release profile of OSSL after being subcutaneously implanted. (n = 5)
Figure 3. Cell viability studies. A) Fluorescent images showing Live/Dead staining result of cells cultured for 24 h with extraction solution of OSS and OSSL, compared with those cultured in normal medium (control) and 30% DMSO. B) Quantification of the ratio of the life cells in the Live/Dead assay of each group. *p < 0.05, ns means no significance. (n = 6)

Peripheral nerve injury will result in atrophy and dysfunction of myofibers and neuromuscular junctions. When the injured nerve recovers, the muscle will be re-innervated and myoatrophy can be attenuated. As a result, the morphology and function of the target muscle are reliable parameters for assessing nerve regeneration. Herein, the wet weight and myofiber size of gastrocnemius muscle, a main target muscle of sciatic nerve, was tested to demonstrate the therapeutic effect (Figure 7A). Following this, NF immunohistochemistry and α-BTX double-staining were carried out to visualize the re-innervated axons and neuromuscular junctions with the muscle (Figure 7C). The quantification data revealed that the wet weight (Figure 7D), area of myofiber (Figure 7E) and neuromuscular junction area (Figure 7F) all showed no statistical difference between the OSSL group and the suture group, while these two groups were significantly higher than the OSS group and the HIS group. All together, these data proved that OSSL has an excellent therapeutic effect on the nerve regeneration and then the target muscle re-innervation. Although in previous studies lithium is widely used for promoting nerve regeneration, it was taken by intraperitoneal injection or oral. Herein, Lithium was loaded into the adhesive and locally released into the injury site, potentially minimizing the side effects of lithium on other tissues.

3. Conclusion

Here, we utilized the OSS hydrogel with succinyl units to construct adhesives loading lithium. The hydrogel can be controllably dissolved, providing great convenience when removal is needed. After adding lithium, the therapeutic performance of the adhesive is greatly improved, and is nearly the same as that of the suture but much better than that of the commercialized tissue glue, with less inflammatory response, and better regeneration efficacy. Hence, our view is that the adhesive might be a proper substitution for suture in tension-free nerve injuries. Moreover, this adhesive can significantly shorten the surgery time needed to reconnect the injured nerve and, furthermore, this operation does not rely on the surgery microscope and skillful operators.
Taken together, we believe OSS adhesive loading lithium can serve as a novel prospective strategy for healing the transected peripheral nerve.

4. Experimental Section

Materials: Octa-arm poly(ethylene glycol) (octa-PEG-OH, $M_w = 10$ kDa, $M_w/M_n = 1.03$) and octa-arm PEG-amine (octa-PEG-NH$_2$, $M_w = 10$ kDa, $M_w/M_n = 1.03$) were purchased from SINOPEG, China. LiCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). HIS was purchased from B. Braun, USA. Suture was purchased from Ningbo Medical Needle Co., Ltd (Ningbo, China). All chemicals and reagents utilized in the following procedures were analytical grade and used without further purification or treatment.

Synthesis of Octa-PEG-SS: The octa-PEG-SS was prepared based on the same procedure as that in a previously published work.[18] First, the intermediate product octa-arm PEG-succinic acid (octa-PEG-COOH) was prepared by reacting octa-PEG-OH (1 g, 1 equiv.), succinic anhydride (200 mg, 20 equiv.), and DMAP (112 mg, 10 equiv.) in anhydrous CH$_2$Cl$_2$ (50 mL) for 36 h. After that, the solution was washed with brine (3 × 40 mL). The collected organic layer was dried with MgSO$_4$ and concentrated under vacuum, which was further precipitated twice with an excess amount of diethyl ether to give the octa-PEG-COOH. Then the octa-PEG-COOH (1 g, 1 equiv.), EDCI (368 mg, 20 equiv.), and NHS (220 mg, 20 equiv.) were dissolved in dry CH$_2$Cl$_2$ (200 mL). The system was stirred at 37 °C for 24 h and then directly washed with brine (3 × 100 mL) at room temperature. The organic layer was collected and dried with MgSO$_4$, after which the polymer was obtained as a white solid under vacuum. The structures of the compounds were confirmed by $^1$H NMR and $^{13}$C NMR measurements in CDCl$_3$. Yield: 65%.

Preparation of the Hydrogels: First, the LiCl solution was prepared by dissolving the LiCl (0.2 g) in water (1 mL). Then, octa-PEG-NH$_2$ was dissolved in LiCl solution with the concentration of octa-PEG-NH$_2$ to be 6, 8, 12, and 16 wt%. Octa-PEG-SS was dissolved into the distilled water with the concentration to be 6, 8, 12, and 16 wt%. The adhesive OSSL was prepared by mixing the same volume of the octa-PEG-SS and LiCl-octa-PEG-NH$_2$ at the same concentrations. Also, we prepared PEG hydrogel...
without LiCl for control by just mixing the solution of octa-PEG-SS and octa-PEG-NH₂. In the following description, the PEG hydrogel without LiCl was named as OSS, while the hydrogel with LiCl was named as OSSL.

**Gelation Time Assessment:** The gelation time was measured by the inversion method. The precursor solutions were injected into glass tubes with different concentrations, after which the tubes were inverted. The time at which the gel stopped flowing was recorded as the gelation time.

**Hydrogel Extracts Preparation:** After the gelation, the OSS or OSSL with the concentration of 12% was soaked in the conditioned medium of Schwann cells (DMEM/F12 containing 3% fetal bovine serum (FBS), 3 µM forskolin (Sigma-Aldrich, St Louis, MO, USA), 10 ng mL⁻¹ heregulin (PeproTech, Rocky Hill, NJ) and 100 mg mL⁻¹ penicillin-streptomycin (Gibco, USA)) for 24 h and then the supernatant was collected to work as hydrogel extract for the following cytotoxicity assay.

**Lithium Release Assays:** The lithium released from the OSSL was measured by photometrical spectroscopy as described in the previous literature. In vitro, 20 µL OSSL was immersed in 100 µL ultrapure water in an Eppendorf tube. The tube was kept under a shaker with rotation of 20 rpm at 37 °C for 14 days. Every 24 h, the supernatant was collected with a centrifuge and fresh water was added into the tube. Then the lithium in all collected supernatants was measured by photometrical spectroscopy. In vivo, 20 µL OSSL was buried beneath the skin of the rat for 3, 7, or 14 days later, then the OSSL was taken out and minced in 20 mL ultrapure water to dissolve the residual lithium in the OSSL. The lithium in the collected solution was also measured by photometrical spectroscopy.

**Cytotoxicity Assay with Schwann Cells:** To assess the potential cytotoxicity of the developed adhesives, Live/Dead assay was conducted with primary cultured Schwann cells which are the most important host cells in the peripheral nerves. The Schwann cells were isolated from the spinal nerves of neonatal SD rats based on previously reported protocol. In brief, the collected nerves were digested with 0.25% Trypsin-EDTA (Gibco) at 37 °C for 30 min and then suspended in DMEM/F12 (Corning, NY, USA) containing 10% FBS (Corning, NY, USA). After an overnight culture on the poly-l-lysine-coated petri dish, the cultures were treated with 10 µM cytosine arabinoside (Sigma-Aldrich, St Louis, MO, USA) for 48 h to eliminate fibroblasts. Then the cells were routinely cultured with the Schwann cells medium to expand the cells. Following this, the third passaged cells were seeded at 1 × 10⁴ per well in 96-well plates and incubated overnight to allow cell adherence. The culture media were replaced with hydrogel extracts, or 30% DMSO for 24 h and then incubated for 30 min with the staining solution which was prepared with reagents A, B, and C of the Live/Dead assay kit (BestBio, Shanghai, China). After that, the cells were observed with a fluorescence microscope (Zeiss, Jena, Germany) and five images were captured at the center and four quadrants in each culture. Based on the instructions of the assay kit, the live cells were defined as Calcein-AM positive with green fluorescence and dead cells were PI positive with red fluorescence. The live cell ratio was than calculated as the number of Calcein-AM positive cells divided by the number of total cells (Calcein-AM-positive cells plus PI-positive cells).

**Animal Surgery of the Sciatic Nerve Transection and Reconnection:** Healthy adult SD rats (weight of 220–250 g) were provided by the animal center of the Southern Medical University (Guangzhou, China). The procedures of surgery, electrophysiological assessment, behavior assay and tissue collection were carried out with the approval of the Southern Medical University Animal Care and Use Committee in accordance with the guidelines for the ethical treatment of animals. All efforts were made to
minimize the number of animals used and their suffering. After the rats were anesthetized with an intraperitoneal injection of 12 mg mL\(^{-1}\) tribromoethanol (180 mg kg\(^{-1}\) body weight), the sciatic nerve in the right leg was bluntly exposed and subjected to a transection at 0.5 cm distal to the sciatic notch. Immediately after the transection, 10 µL 12 wt% octa-PEG-SS, and 10 µL 12 wt% LiCl-octa-PEG-NH\(_2\) were simultaneously injected into the interface of the proximal stump and distal stump of the transected nerve. The OSSL was gelatinized after 2 s and the cut nerve was adhered end by end. The rats treated with the above procedures were included into the OSSL group. In addition, the rats with cut nerves were adhered by the OSS hydrogel (10 µL 8% octa-PEG-SS and 10 µL 8% octa-PEG-NH\(_2\)) or 20 µL Histoacryl (cyanoacrylates solution) were included into OSS group or HIS group. While the transected nerves in the suture control group were anastomosed three stitches with 11/0 microsuture under an operating microscope. In the sham operation group, the sciatic nerve was exposed only without transection.

**Adhesives Dissolution Assessment:** To evaluate the dissolvable property of adhesives, in vitro and in vivo dissolution experiments were performed. For in vitro dissolution test, 500 µL of 12 wt% OSS or OSSL was prepared and immersed into 5 wt% cysteamine solution. The time was recorded till the complete disappearance of the OSS. For in vivo degradation, after surgery, 20 µL of OSS or OSSL was used to adhere the transected nerve. Then, a wad of cotton soaked with 1 mL 5 wt% cysteamine solution was covered on the injury site to dissolve the hydrogel. Due to the elasticity of the sciatic nerve, when the gel was dissolved, the ends of the nerve would be separated automatically, and the time was recorded. A swab soaked with cysteamine solution was also used to incessantly wipe the OSS in the injured nerve and then recorded the time needed to remove the gel from the injury site.

**Behavioral Assessment:** The behavioral assessment was carried out using previously reported methods.\(^{41-43}\) 8 weeks after the surgery, the footprint analysis was carried out using a computerized digital footprint analysis system (DigiGait, Mouse Specifics Inc., MA, USA). Before testing, the rats were trained to become used to the machine. By using a high-speed digital video recorder, the movement of the paws of rats was captured. The behavioral study was evaluated by the sciatic function index (SFI) as follows:

\[
\text{SFI} = 109.5 \times (\text{ETS} - \text{NTS}) / \text{NTS} - 38.3 \times (\text{EPL} - \text{NPL}) / \text{NPL} + 13.3 \times (\text{EITS} - \text{NITS}) / \text{NITS} - 8.8
\]

The symbols in the formula are: 1) distance from the heel to the third toe, the print length (PL); 2) distance from the first to the fifth toe, the toe spread (TS); and 3) distance from the second to the fourth toe, the intermediate toe spread (ITS). All three measurements were taken from the experimental (E) and normal (N) sides. The SFI oscillates around 0 for normal nerve function, whereas for SFI around −100 represents total dysfunction.\(^{44,45}\)
Electrophysiological Test: The electrophysiological test was performed as per the previous studies.[41–43] Briefly, 6 h after the behavioral assessment, the animals were anesthetized with 12 mg mL\(^{-1}\) tribromoethanol (90 mg kg\(^{-1}\) body weight). Then, the involved sciatic nerve was exposed, stimulating electrodes were applied to the host nerve trunk 3 mm proximal to the injury site, and a pair of electrodes was placed in the intrinsic foot muscle to record the compound muscle action potential with a set of electrophysiological recorders (Axon Digidata 1550 Digitizer, Molecular Devices). The amplitude and latency of each animal were recorded and analyzed.

Tissue Collection: 1 week post-surgery, five rats of each group were euthanized by intraperitoneal injection of overdose pentobarbital (50 mg kg\(^{-1}\)), followed by transcardiac perfusion with 4% paraformaldehyde (PFA, in 0.1 M PBS), and then the sciatic nerves were collected for GAP43 and ED1 immunohistochemistry. 8 weeks post-surgery, immediately after the electrophysiological recording was completed, the rats (\(n = 6\) per group) were intracardially perfused. Then, the sciatic nerves were harvested for ED1 immunohistochemistry and Masson’s trichrome staining. Meanwhile, the nerve targeted gastrocnemius muscles were collected to assay the wet weight of whole muscle, the size of the myofibers, and the density of neuromuscular junctions.

Immunofluorescence Staining: The collected nerves were post fixed in 4% PFA for 24 h, cryoprotected in 30% sucrose overnight at 4 °C, and longitudinally sectioned on a cryostat (Leica, Germany) at the thickness of 10 µm. Then the sections were immunostained with the routine procedures: following permeabilization with 0.5% Triton X-100 (Sigma, USA) for 30 min, the sections were blocked with 5% bovine serum albumin (GBCBIO) in 0.01 M PBS for 1 h. Then the sections of 1 week post-surgery were incubated overnight at 4 °C with primary antibodies of GAP43 (1:400, Abcam; marker of regenerating axons) and ED1 (1:500, AbD Serotec; marker of macrophages), and the sections of 8 weeks post-surgery were incubated with ED1. Alexa 488 or 568 fluorescent conjugated secondary antibodies (1:400, Life Technologies, CA, USA) were applied for 2 h at room temperature. Digital images were captured under a fluorescence microscope (Zeiss, Jena, Germany) and processed with montagesplicing using Photoshop CS8 software. To quantify the regenerating axons in the distal trunk of the injured nerve, 3 vertical lines were placed at 2, 4, and 6 mm distal to the injury site, then the number of GAP43 positive axons crossing these lines was measured. To evaluate the inflammation around the injury site, a rectangle image with size of 1 mm × 2 mm was captured in the central of distal trunk close to the injury site, and all the ED1 positive macrophages in the image were counted.

Masson’s Trichrome Staining: To illustrate the fibrosis in the lesion area, a classic Masson’s trichrome staining was done on the sections of sciatic nerves as per a previous description.[46–48] Briefly, the sections were rinsed three times with distilled water, then successively stained with Mayer’s haematoxylin for 5 min, incubated in 0.5% hydrochloric acid in 70% alcohol for 5 s, washed in running tap water for 30 s and rinsed twice in distilled water, stained with acid ponceau for 5 min, rinsed three times in distilled water, incubated in 1% phosphomolybdic acid aqueous solution for 5 min, stained with 1% aniline blue for 5 min, and dissolved in 1% glacial acetic acid for 5 min. Finally, the sections were dehydrated with
ethanol and hyalurinized with xylene. In this staining, collagen fibers were stained blue, nerve fibers and cytoplasm were stained red, and the nuclei were stained dark purple.

**Evaluation of Gastrocnemius:** Since the level of myoatrophy and the reinnervation in the target muscle was well accepted as reliable indexes to reflex the nerve regeneration,[3,4,20] the gastrocnemius muscles harvested from the perfused rats of 8 weeks post injury were evaluated as previously reported.[3,41] Briefly, the wet weight of each gastrocnemius muscle was measured after the connective tissue was trimmed off and the trimmed wet weight of the surgery gastrocnemius was divided by the wet weight of the contralateral gastrocnemius and then multiplied 100 (%).

\[ \text{Wet weight ratio (\%)} = \frac{\text{weight of the surgery gastrocnemius}}{\text{weight of the contralateral gastrocnemius}} \times 100 \]

(2)

After weighing, two set of sections were prepared from the mid-belly of each gastrocnemius muscle. One set was longitudinally sectioned with a thickness of 80 μm for double staining with NF-200 and α-bungarotoxin (α-BTX) as described in a previous report,[3,41] to show the regenerated axons and neuromuscular junctions in the target muscles. Another set was transversely sectioned with a thickness of 10 μm for routine haematoxylin–eosin (H&E) staining to show the outline of the myofibers. As described in previous publications,[3,41] six random non-overlapping fields of five sections from each animal were captured, and the area of myofibers in the transverse section and neuromuscular junctions in the longitudinal section were counted by using Image-Pro Plus 6.0 software.

**Statistical Analysis:** All the data were used during the processing. Data were analyzed by using two tail Student t-tests with SPSS statistics 19 and Excel. \( p < 0.05 \) was defined as significant for all statistical tests. Data are presented as means ± SD.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

adhesives, poly(ethylene glycol), peripheral nerve repair, succinyl unit

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