Tissue-engineered nerve grafts using a scaffold-independent and injectable drug delivery system: a novel design with translational advantages

Yanxi Liu, Shuangjiang Yu, Xiaosong Gu, Rangjuan Cao and Shusen Cui

Objective. Currently commercially available nerve conduits have demonstrated suboptimal clinical efficacy in repairing peripheral nerve defects. Although tissue-engineered nerve grafts (TENGs) with sustained release of neurotrophic factors (NTFs) are experimentally proved to be more effective than these blank conduits, there remains a lack of clinical translation. NTFs are typically immobilized onto scaffold materials of the conduit via adsorption, specific binding or other incorporation techniques. These scaffold-based delivery strategies increase complexity and cost of conduit fabrication and lack flexibility in choosing different drugs. Therefore, to facilitate clinical translation and commercialization, we construct a TENG using a scaffold-independent drug delivery system (DDS).

Approach. This study adopted a scaffold-independent DDS based on methoxy-poly (ethylene glycol)-b-poly(γ-ethyl-L-glutamate) (mPEG-PELG) thermosensitive hydrogels that undergo sol-to-gel transition at body temperature. In addition, TENG, a chitosan scaffold filled with nerve growth factor (NGF)-loaded mPEG-PELG that gel in the lumen upon injection during surgery and function as a drug-releasing conduit-filler, was designed. Subsequently, the efficacy of DDS and therapeutic effects of TENG were assessed.

Main results. The results demonstrated that NGF-loaded mPEG-PELG controllably and sustainably released bioactive NGF for 28 d. When bridging a 10 mm rat sciatic nerve gap, the morphological, electrophysiological, and functional analyses revealed that NGF-releasing TENG (Scaffold + NGF/mPEG-PELG) achieved...
superior regenerative outcomes compared to plain scaffolds and those combined with systemic delivery of NGF (daily intramuscular injection (IM)), and its effects were relatively similar to autografts. **Significance.** This study has proposed a TENG using thermosensitive hydrogels as an injectable implant to controllably release NGF, which has promising therapeutic potential and translatability. Such TENGs obviate the need for conduit modification, complex preloading or binding mediators, therefore they allow the ease of drug switching in clinical practice and greatly simplify the manufacturing process due to the independent preparation of drug delivery system.

Keywords: peripheral nerve defect, tissue-engineered nerve graft, drug delivery system, thermosensitive hydrogel, nerve growth factor, nerve regeneration

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### 1. Introduction

Current interventions for bridging peripheral nerve defects are limited. Nerve autografting has been considered as gold standard since 1940s, but it has limitations such as donor site morbidity [1]. Alternatively, tubular nerve conduits have been developed to substitute autografts. A few of them have been approved by the Food and Drug Administration (FDA) since 1999, but they have yet to gain widespread use due to their suboptimal functional outcomes [2]. To improve the performance of these hollow conduits, researchers have explored various filler materials that provide topographical cues [3]. Recently, increasing attention has been paid to the addition of acellular and/or cellular cues via materials and tissue-engineering techniques, establishing a new research field, namely peripheral nerve tissue engineering [4].

Tissue-engineered nerve grafts (TENGs) with acellular cues, especially neurotrophic factors (NTFs), are more clinically attractive because they have a simpler path to the bedside and are more appropriate for treating acute injuries when autologous cell seeding is impractical [5, 6]. Over the past 30 years, although a number of TENGs that can controllably release NTFs have been fabricated and perform better than blank conduits, none of them extends beyond the laboratory bench [7]. This imbalance between scientific innovation and translation to patient benefit prompts us to rethink the existing design concepts of TENGs based on a translational perspective. At present, drug delivery strategy is typically applied by combining with the structure of conduits [2, 4, 8–17].

NTFs can be incorporated into scaffold materials (either wall or fillers) during conduit fabrication through blending, adsorption, affinity, covalent link, coatings, electrospinning, microparticle incorporation, etc (figure 1). However, these approaches may hinder the clinical translation and commercialization of TENGs. First, NTFs are initially dissolved to impregnate carriers or react with binding mediators, followed by drying process. Maintaining the bioactivity of these liable proteins during processing and storage may be challenging [18]. Second, a single device may not have the flexibility of delivering multiple types of drugs because different drugs require different linkers [12]. Third, the materials used for nerve scaffolds possess strict biomechanical and biocompatible requirements, thus endowing them with additional function of drug delivery can lead to a more complex and higher cost of fabrication process [19].

To overcome these limitations as well as to facilitate the clinical translation of TENGs with acellular cues, we set our sights on new biomaterial that can function as an independent drug delivery system (DDS) without the need of conduit modification and binding mediators. Specifically, injectable thermoresponsive hydrogels can serve as promising candidates. Being liquefied at room temperature, they can be easily loaded with drugs and implanted through a syringe. Upon heating to body temperature, they can gel within minutes and form a drug-releasing depot [20, 21]. Besides, the incorporation of NTFs into TENGs can bypass complex pre-loading and display longer storage stability when injected at the time of surgery.

However, no study has yet examined such applications. Therefore, the present study designed a chitosan scaffold filled with thermosensitive polypeptide-based hydrogel (mPEG-PELG) to deliver nerve growth factor (NGF), which can gel in situ in the lumen upon injection and function as a field-installed conduit-filler. In addition, the efficacy of this DDS in releasing NGF and the effect of TENG on nerve regeneration were investigated. Collectively, this preclinical study aimed to introduce the first step towards the development of a novel drug-releasing TENG that exerts both scientific and translational advantages.

### 2. Materials and methods

#### 2.1 Materials

Chitosan conduit (i.d. 2.0mm) was prepared according to our previous study [22] (for technical details, please refer to Chinese patent ZL 0110820.9). Poly (ethylene glycol) methyl ether (mPEG, $M_f = 2000$) was purchased from Sigma-Aldrich. The amino group terminated poly (ethylene glycol) methyl ether (mPEG-NH$_2$) was synthesized according to our previous study [23]. N, N-dimethylformamide (DMF) was stored over calcium hydride (CaH$_2$) and purified by vacuum
distillation. Recombinant mouse NGF and enzyme-linked immunosorbent assay (ELISA) kits were purchased from Sigma-Aldrich. PC12 cell line was obtained from Beijing Beina Biotech. Co., Ltd. Antibodies such as rabbit anti-NF200, anti-neurofilament protein NF200, anti-S100β and goat anti-rabbit IgG were purchased from BD Bioscience. All other chemicals, reagents and solvents were purchased from Sigma-Aldrich, unless stated otherwise.

2.2. Synthesis of mPEG-PELG copolymers

The synthesis of mPEG-PELG copolymers was carried out according to our previous experiment [23]. Briefly, mPEG-PELG was synthesized through ring-opening polymerization of γ-ethyl-L-glutamate N-carboxyanhydride (ELG-NCA) by utilizing amino-terminated mPEG (mPEG-NH₂) as a macro-initiator. First, mPEG-NH₂ (3.0 g, 1.5 mmol) was dissolved in toluene (300 ml), and the excess water was removed by azotropic distillation. Subsequently, anhydrous DMF (80 ml) and ELG-NCA (4.53 g, 22.5 mmol) were added into the flask. The reaction mixture was then stirred at 30 °C for 3 d under a nitrogen atmosphere. After stirring, the solution was precipitated with chilled diethyl ether. The obtained products were washed twice with diethyl ether and dried to constant weight in a vacuum at room temperature. The crude product of mPEG-PELG was dissolved in DMF and dialyzed against H₂O for 72 h at room temperature. The final mPEG-PELG copolymer was obtained through lyophilization. Then, the lyophilized powders of mPEG-PELG were dissolved in PBS (pH = 7.4) (8.0 wt %) with modest stirring, and the obtained mPEG-PELG aqueous solution was stored at 4 °C for further analysis. The synthesis route of mPEG-PELG copolymer is shown in figure S1 (stacks.iop.org/JNE/16/036030/mmedia). The structure of the copolymer was confirmed according to the 1H NMR spectra (figure S2).

2.3. In vitro release testing

To determine the optimal loading dose of NGF, 50, 100 and 150 ng of NGF were mixed with 100 µl of 8.0 wt % of

Figure 1. Current approaches to nerve conduit fabrication for the controllable release of NTFs Typically, NTFs are loaded onto the wall (left panel) or intraluminal fillers (right panel).
IRDye 800-conjugated affinity purified goat anti-mouse IgG (Aldrich). Subsequently, the membrane was incubated with 1500 µl of 1:20000 anti-ERK1/2 (Sigma-Aldrich) and anti-ERK1/2 (1: 20 000; Sigma-Aldrich) following the manufacturer’s protocol. Briefly, samples and standards were added into 96-well pre-coated with NGF antibody and incubated overnight at 4 °C. After thoroughly wash, incubate with biotinylated detection antibody for 1 h at room temperature. Repeated the wash, added HRP-Streptavidin solution and incubated for 45 min. Then, Elisa colorimetric TMB reagent was added to incubate for 30 min before adding the stop solution. Absorbance was measured at 450nm. The remaining collected medium was used for bioactivity test. For those samples with undetectable NGF, the percentage (%) of released NGF was calculated by dividing the cumulative release amount of NGF by the total NGF loaded into each device.

2.4. Bioactivity test

Prior to bioactivity test, rat PC12 cells were seeded onto 24-well culture dish at a density of 3 × 10⁴ cells/well. The cells were cultured with Nutrient Mixture F12 Ham Kaighn’s Modification (F12K) medium (Gibco) containing 15% heat-inactivated horse serum, 2.5% fetal bovine serum, 100 U ml⁻¹ of penicillin and 100 µg ml⁻¹ streptomycin, and maintained at 37 °C in a humidified 5% CO₂ atmosphere. 24 h later, the culture medium was replaced with the release medium. PC12 cells cultured in the medium collected from the hydrogel without NGF were used as controls. The percentage of differentiated cells was determined by counting 200 cells in randomly selected fields using a light microscope and computerized image analyzer (Leica). Only PC12 cells with at least one neurite longer than or equal to the cell body diameter were considered to be differentiated [14].

After 24 h’ incubation in the release medium, PC12 cells were lysed and subjected to Western blot analysis. Briefly, the proteins were separated on 10% SDS-PAGE gels, electro transferred onto PVDF membranes. After blocking, the membrane was incubated overnight at 4 °C with monoclonal antibodies, including anti-phospho ERK1/2 (1: 1500; Sigma-Aldrich) and anti-ERK1/2 (1: 20000; Sigma-Aldrich). Subsequently, the membrane was incubated with IRDye 800-conjugated affinity purified goat anti-mouse IgG (1: 5000; Odyssey) or IRDye 800-conjugated affinity purified donkey anti-rabbit IgG (1: 5000; Odyssey) secondary antibody. After incubation, the protein bands were visualized by chemiluminescence.

2.5. Surgical procedures

All animal experiments were conducted in strict accordance with the institutional guidelines of the Animal Care and Use Committee at Jilin University, China. The protocol was approved by the local Administration Committee of Experimental Animals.

Adult female SD rats, weighing 200–250g, were anesthetized by an intraperitoneal injection of 3% sodium pentobarbital solution (30 mg kg⁻¹ body weight). The sciatic nerve was exposed by making a skin incision and splitting the muscles located in the right lateral thigh. A segment of sciatic nerve was transected and removed, leaving a 10mm gap.

All rats were randomly divided into five groups: (i) Scaffold group, (ii) Scaffold + mPEG-PELG group, (iii) Scaffold + NGF/IM group, (iv) Scaffold + NGF/mPEG-PELG group, and Autograft group. In Scaffold group, the defect was bridged by a plain chitosan scaffold. In Scaffold + mPEG-PELG group, the defect was bridged using a chitosan conduit, and 100 µl of mPEG-PELG solution was added into the lumen through a 1 ml syringe to form gel in situ. In Scaffold + NGF/IM group, the defect was bridged by a chitosan scaffold, and 2 µg kg⁻¹ of NGF was administered daily via intramuscular injection (IM). In Scaffold + NGF/mPEG-PELG group, the defect was bridged using a chitosan scaffold, and 100 µl of NGF-loaded mPEG-PELG solution was added into the lumen through a 1 ml syringe to form gel in situ for 10 min. In Autograft group, the defect was bridged with the segments of transected nerves.

2.6. Immunohistochemical assessment

Twelve rats from each group were sacrificed at day 14 after surgery. The tissue admixture in the place of original nerve gap was excised, and six of them were cut into 15 µm-thick longitudinal sections on a freezing microtome and the other six were cut into 15 µm-thick transverse sections. Subsequently, the sections were incubated with rabbit anti-NF200 polyclonal antibody (1:150) at 4°C for 24h, followed by an overnight incubation at 4 °C with FITC-conjugated goat anti-rabbit IgG (1: 200). The immunohistochemically stained sections were observed under a fluorescent microscope (DMR, Leica).

Morphometric analysis was carried out to determine the length of regenerating nerve fibers and the number of regenerated nerve fibers. The length of regenerating fibers was determined by measuring the distance between the proximal end of the scaffold to the leading edge of extended axons. The number of regenerated nerve fibers was counted on the transverse section 3mm away from the proximal end of the scaffold. At 12 weeks’ post-surgery, the distal end of the nerves were excised and cut into 15 µm-thick transverse sections. Double immunohistochemical staining with anti-NF200 and...
anti-S100β (Schwann cell marker) was used to assess the distribution of regenerated nerve fibers at the distal end of the nerve graft.

2.7 Transmission electron microscopy

At 12 weeks after surgery, six rats from each group were sacrificed. The sections of regenerated nerves (located at 3 mm distal to the chitosan scaffold) were fixed in pre-chilled 2.5% glutaraldehyde for 3 h and post-fixed with 1% osmium tetroxide solution for 1 h. The fixed sections were then washed, dehydrated, embedded in Epon 812 epoxy resin, and cut into 60 nm-thick ultra-thin sections. After, staining with uranyl acetate and lead citrate, the ultra-thin sections were observed under a transmission electron microscope (JEOL Ltd, Tokyo). The number of myelin sheath layers, thickness of myelin sheath and diameter of myelinated fiber were determined from the images of each section captured in ten different random fields. In addition, the number of axons was counted at 1000× magnification. The density (myelinated axons per square millimeter of nerve bundle tissue) and percentage of myelinated nerve fibers (number of myelinated axons/total number of myelinated and unmyelinated axons) were calculated using Image-Pro Plus 6.0 software.

2.8 Electrophysiological assessment

At 12 weeks after surgery, electrophysiological tests were performed on six rats from each group. Briefly, sciatic nerve on the injured side was re-exposed under anesthesia with sodium phenobarbital. Electrical stimuli (10 mV in strength) were sequentially applied to the proximal and distal nerve stumps, and compound muscle action potentials (CMAPs) were recorded on the lateral belly of the gastrocnemius muscle. The latency of CMAPs is the conduction time from the latency of stimulus to the onset of CMAP deflection. To calculate nerve conduction velocity (NCV), the distance between the two sites was divided by the latency difference between the two sites. To calculate CMAPs generated from proximal and distal stimulations was divided by the distance between the two sites. To calculate nerve conduction velocity (NCV), the latency difference between the two sites was divided by the distance between the two sites. To calculate nerve conduction velocity (NCV), the latency difference between the two sites was divided by the distance between the two sites.

2.9 Muscle wet weight and motor endplate analysis

After completing electrophysiological tests, the rats were then used for muscle wet weight and motor endplate analysis. The gastrocnemius and anterior tibial muscles on the injured and contralateral sides were harvested and weighed immediately. The wet weight ratio was calculated as wet weight of muscle on the injured side/wet weight of muscle on the uninjured side.

For motor endplate analysis, the muscle samples were harvested from the medial belly of gastrocnemius muscles, fixed in 4% paraformaldehyde, and cut into sections. After freezing, the sections were used for measuring the neuromuscular junction connectivity and density. The frozen sections were stained with α-BTX (acetylcholine receptor labeling) and NF200 (regenerated nerve labeling). The sections were imaged using confocal microscopy, and the obtained images were merged and flattened. Multiple identified areas were randomly selected for the evaluation of the percentage of re-innervated neuromuscular junctions, by referring to a standard stereological technique [24–26].

2.10 Walking track analysis

To assess the motor functional recovery of sciatic nerve, walking track analysis was performed on six rats from each group at week 24 after surgery. The footprints of rats were recorded on a white paper, and sciatic functional index (SFI) was calculated by the following formula: SFI = −38.3 × (EPL−NPL)/NPL + 109.5 × (ETS−NTS)/NTS + 13.3 × (EIT−NIT)/NIT−8.8. PL represents the footprint length, TS indicates the total toe spread, IT is the intermediate toe spread, E refers to the injured side, and N reveals the distance on the contralateral side [27].

2.11 Statistical analysis

Statistical analyses were performed using SPSS Statistics software version 13.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) and the Student–Newman–Keuls post hoc test were used for multiple group comparison. All data were expressed as mean ± standard error (SE). P values of less than 0.05 was considered statistically significant.

3. Results

3.1 In vitro release profile of NGF

To optimize the therapeutic effects of NGF on peripheral nerve defects, the selection of an optimal dosage is of particular importance. Previous studies have reported that an optimal axonal growth response is observed at 1 to 10 ng ml$^{-1}$ of NGF doses, as a higher initial dosage can impair axonal regeneration [28–30]. In order to optimize the loading dose of NGF for its release of 1–10 ng ml$^{-1}$, an in vitro release test was conducted by loading 50, 100, and 150 ng of NGF into mPEG-PeLG aqueous solution (100 μl, 8.0 wt %). Figure 2(A) shows the daily released concentration of NGF. The release profiles of the three formulations (i.e. 0.5, 1.0 and 1.5 ng μl$^{-1}$) exhibited a similar pattern of two-stage reaction kinetics. Notably, NGF was rapidly released during the first 2 d, and its release rate slowed down and reached a standstill phase at day 7. At increased loading doses, the concentrations of NGF were found to be elevated in the release medium, and the duration of its release was prolonged. The hydrogels with 1.0 ng μl$^{-1}$ NGF released an average concentration of 13.87 ng ml$^{-1}$ at day 1 and dropped off to below 10 ng ml$^{-1}$ at day 2. During the 28 d of incubation, NGF concentration plateaued to approximately 1.5 ng ml$^{-1}$, and approximately 81.4% of NGF were released from the hydrogels. Hence, this formulation was selected for further experiments, as it could...
maintain stable concentrations within optimal therapeutic ranges during the entire course of treatment.

3.2. Bioactivities of released NGF

After verifying that NGF could be released from 1.0 ng µl⁻¹ mPEG-PELG-NGF mixture in a controlled manner at desirable concentration, the bioactivities of released NGF were evaluated with regard to the induction of neurite outgrowth from PC12 cells and the activation of downstream MAPK/ERK pathway [31]. PC12 cells were exposed to the release medium collected on day 1, 2, 4, 7, 10, 14, 21 and 28. Meanwhile, PC12 cells cultured in a medium containing 5 ng of NGF were used as a positive control. The expression levels of ERK and phosphorylated ERK (p-ERK) in PC cells treated with NGF release medium collected at day 1, 7 and 14 were detected by immunoblotting at the indicated times (figure 2(B)). Besides, PC12 cells treated with release medium from NGF-loaded hydrogel at day 1, 7 and 14 all showed differentiation phenotype (figures 2(C)–(H)). The percentages of differentiated PC12 cells treated with day 1, 7 and 14 release medium from NGF-loaded hydrogel and the control group (NGF-free hydrogel) were similarly similar, but significantly higher compared to their control (figure 2(I)). These results indicated that the released NGFs exerted their abilities to induce ERK phosphorylation and promote neurite outgrowth.

3.3. Morphological analysis of regenerated nerves

Considering the controllable release of bioactive NGF from mPEG-PELG hydrogels, TENG was constructed (figure 3) and employed as an injectable vehicle for NGF release in the subsequent in vivo experiments. In particular, Scaffold + NGF/mPEG-PELG group which used this TENG was compared with Scaffold, Scaffold + mPEG-PELG, Scaffold + NGF/IM and Autograft groups.

Immunohistochemical staining with anti-NF200 was performed on day 14 after surgery to evaluate the growth of axons. The longitudinal sections revealed the distance of axonal growth (figure 4). The conduit exhibited a green fluorescence due to the autofluorescence of chitosan, whereas the transverse sections indicated the number of regenerating axons (figure 5). Noticeably, the regenerating fibers did not reach the distal end of the 10 mm defect among the five groups. The length of regenerating fibers in Scaffold + NGF/mPEG-PELG group (0.72 ± 0.02 mm) was relatively similar to Autograft group (0.82 ± 0.03 mm). Both groups demonstrated significantly longer fibers compared to Scaffold (0.24 ± 0.02 mm), Scaffold + mPEG-PELG (0.29 ± 0.04 mm) and Scaffold + NGF/IM group (0.37 ± 0.02 mm). In addition, the inter-group differences in the number of regenerating fibers were similar to those in the length of regenerating fibers.
At week 12, the regenerated axons that extended 3 mm beyond the distal end were immunochemically stained with both anti-NF200 and anti-S100\(\beta\). The number of regenerating fibers and S100\(\beta\)-positive fibers were both significantly greater in Scaffold + NGF/mPEG-PELG and Autograft groups compared to Scaffold, Scaffold + mPEG-PELG and Scaffold + NGF/IM groups (figure 6). As S100\(\beta\) is preferentially distributed in myelin-forming Schwann cells [32], the results indicate that Scaffold + NGF/mPEG-PELG and Autograft groups may contain more myelinated nerve fibers.

### 3.4. Electron microscopy and morphometric analysis of remyelination

At 12 weeks after surgery, in order to provide a detailed examination of re-myelinated axons, the regenerated nerve fibers in the distal portion of injured nerves were observed under an electron microscope (figure 7(A)). The number of myelin sheath layers, thickness of myelin sheath, diameter of myelinated fiber, density and percentage of myelinated fibers were quantified (figures 7(B)–(F)). Morphometric analysis revealed that the five parameters were significantly greater in Scaffold + NGF/mPEG-PELG and Autograft groups compared to Scaffold, Scaffold + mPEG-PELG and Scaffold + NGF/IM groups. However, there were no significant differences between Scaffold + NGF/mPEG-PELG group and Autograft group with respect to all the five parameters.

### 3.5. Electrophysiological recovery

To assess electrophysiological recovery, both compound muscle action potential (CMAP, the estimated number of functioning axons) and nerve conduction velocity (NCV, the speed of the fastest conducting motor axons in the nerve) of regenerated sciatic nerves were measurement on week 12 after surgery.
grafting (figure 8). Notably, the CMAP amplitude and motor NCV in Scaffold + NGF/mPEG-PELG group were relatively similar to those of Autograft group (p > 0.05). Compared to these two groups, both CMAP and NCV of regenerated nerves were significantly reduced in Scaffold, Scaffold + mPEG-PELG and Scaffold + NGF/IM groups.

3.6. Motor endplate analysis and muscle wet weight

Neuromuscular junctions are the anatomical basis for the recovery of locomotor function. After surgery, the targeted muscles were changed from denervation to reinnervation. At 12 weeks, the degrees of connectivity between acetylcholine receptors and nerve fibers were further evaluated. The data were expressed as the percentage of acetylcholine receptors connected to nerve fibers (figures 9(A) and (B)). Both Autograft (35.84% ± 9.41%) and Scaffold + NGF/mPEG-PELG (31.67% ± 10.28%) groups exhibited increased percentages of reinnervated neuromuscular junction compared to Scaffold (11.84% ± 4.13%), Scaffold + mPEG-PELG (12.67% ± 4.64%) and Scaffold + NGF/IM groups (16.67% ± 5.25%). On the other hand, denervated skeletal muscles were further evaluated.

Figure 4. Immunohistochemical staining of the longitudinal sections of regenerative nerve fibers with NF200 (green) in Scaffold (A), Scaffold + mPEG-PELG (B), Scaffold + NGF/IM (C), Scaffold + NGF/mPEG-PELG (D) and Autograft (E) groups at day 14 after nerve grafting. The boxed area displays the regenerative nerve fibers at the leading edge. Scale bar, 500 μm. A higher magnification of the boxed area is shown in the right panel. (F) A histogram comparing the length of regenerative nerve fibers at day 14 after nerve grafting. Data are expressed as mean ± SE. **p < 0.01 versus Scaffold, Scaffold + mPEG-PELG, and Scaffold + NGF/IM groups.
muscles can undergo atrophy and weight loss after sciatic nerve injury. Hence, the atrophy of sciatic-innervated muscles (gastrocnemius and anterior tibial muscles) was examined and calculated as the relative wet weight ratios (the weight of the operated side divided by the weight of the contralateral side). At 12 weeks after surgery, the muscle wet weight ratios were significantly higher in both Scaffold + NGF/mPEG-PELG and Autograft groups compared to Scaffold, Scaffold + mPEG-PELG and Scaffold + NGF/IM groups. However, no significant difference was observed in the muscle wet weight ratio between Scaffold + NGF/mPEG-PELG group and Autograft group (figures 9(C) and (D)).

3.7 Functional recovery

The mean values of SFI derived from walking track analysis were used to assess the recovery of locomotor function. As shown in figure 10, SFI values were ranged from 0 (normal nerve function) to −100 (complete dysfunction). At week 24, the increased SFI values did not return to normal.
range among all the five groups. Despite that, the values of SFI in Scaffold + NGF/mPEG-PELG (−32.67 ± 3.58) and Autograft (−28.84 ± 4.16) groups were significantly higher compared to Scaffold (−64.84 ± 6.16), Scaffold + mPEG-PELG (−62.07 ± 5.58) and Scaffold + NGF/IM (−51.67 ± 4.20) groups. However, the difference between Scaffold + NGF/mPEG-PELG and Autograft groups was not statistically significant. These results indicate that the rats in both Autograft and Scaffold + NGF/mPEG-PELG groups demonstrate improved metatarsophalangeal joint dorsiflexion and toe spreading compared to the remaining groups.

4. Discussion

The in vitro findings demonstrated that mPEG-PELG hydrogels could sustainably and controllably release bioactive NGF for 28 d. In addition, the effects of Scaffold + NGF/mPEG-PELG on nerve regeneration with respect to axon outgrowth, remyelination, motor endplate reinnervation, electrophysiological and functional recovery were assessed in vivo. Notably, mPEG-PELG alone did not hamper nerve regeneration, as evidenced by the similar results obtained from Scaffold and Scaffold + mPEG-PELG groups. To our surprise, TENG (Scaffold + NGF/mPEG-PELG group) achieved outstanding performances relatively close to autografts.

The comparison between Scaffold + NGF/mPEG-PELG and Scaffold + NGF/IM revealed that the hydrogel-based delivery system was superior to IM, the most common route of NGF administration in clinical practice. However, it is not clear whether the two groups (local versus systematic administration) received equivalent doses. Since the effect of NGF is dose-dependent, it is most likely that the Scaffold + NGF/IM group achieves the same or even better results compared to the Scaffold + NGF/mPEG-PELG group, when IM dose reaches a specific point. This may be possible in the laboratory setting, but it may not have practical use in the real world due to the systemic toxicity of high-dose NGF and the inconvenience and
cost-ineffectiveness of daily IM administration. As a result, researchers have become increasingly interested in locally administrating NGF within the nerve graft, which holds great promise as a more effective and safer administration route. This study did not include an experimental group with direct local administration, which is rarely applied in clinical settings. Direct introduction of NGF solutions into the conduit only allows for a single-dose treatment at the time of surgery. Thus, it will likely be a waste if the concentration is low and the drug degrades within hours, or a threat if the concentration is high and counteractively hamper nerve regeneration.

4.1. Rationales for selecting chitosan conduit, NGF and mPEG-PELG hydrogels

The decision regarding the selection of biomaterials in TENG was made through a multidisciplinary discussion among materials scientists, engineers and clinicians. In this study, we aimed towards ‘translational innovations’ instead of ‘materials innovations’. Therefore, we constrained our selection to the existing FDA-approved materials or compounds classified as safe. The chitosan conduit used in this study, which is developed by one of our collaborators (Gu and his team), was chosen owing to its approval from the Chinese State FDA in...
Meanwhile, among all the NTFs that have been proved beneficial for nerve regeneration, NGF was chosen because it is the only approved drug for human use in China [34].

mPEG-PELG, a specific type of thermosensitive hydrogel, was selected based on the following reasons. First, it is a synthetic polymer-based hydrogel. Compared to natural hydrogels, the synthetic one lacks immunogenicity, displays higher degree of batch-to-batch consistency and offers lower production costs [20, 21]. Additionally, synthetic polymer-based hydrogels exhibit tunable properties (e.g. degradation rates, mechanical strengths and microstructures) through facile control of molecular parameters, and therefore they can be further adjusted to improve performance [21]. Moreover, unlike the traditional polyester-based hydrogels, mPEG-PELG will not trigger acidic environment due to its degradation products of neutral amino acids [35]. These may confer better protection on the stability of chitosan conduits, as chitosan is relatively soluble in weak acid [36]. Third, in previous studies, mPEG-PELG hydrogels have exhibited sustained anti-tumor drug release properties, desirable gelation behavior, favorable biodegradability and biocompatibility, as well as in vivo safety [23].

4.2. Mechanisms of drug release in thermosensitive hydrogels

Thermosensitive hydrogels are hydrophilic polymer networks that can retain a large amount of water and rapidly transform from solution to viscoelastic gel at body temperature. The ease of incorporating drug molecules into hydrogels is facilitated by the high volume of water that enhances the uptake of soluble molecules. The release of drug molecules is occurred through the diffusion of molecules as well as the swelling and erosion of hydrogels [37].

Prolonged delivery of NTFs can be achieved by embedding the drug substances into gel matrix, such as collagen, before filling them into the lumen [38–40]. This approach is considered a scaffold-independent delivery strategy. However, the previously reported matrices do not possess the injectable feature and rapid sol-to-gel transformation in comparison with thermosensitive hydrogels. For instance, after mixing...
with drugs and filling into the lumen, collagen solutions need to be incubated at 37 °C for 2 h in order to form gels [40]. Thermosensitive hydrogel solutions, on the other hand, can gel at body temperature within approximately 10 min, which offer more advantages for clinical usage. A previous study has reported that collagen solutions can be directly applied to deliver NGF without incubation [38]. Although they are injectable into the conduit, collagen solutions could not achieve desirable release profiles, as NGF is diffused and degraded more rapidly in the absence of gelling [19, 41].

4.3. Translational advantages and future prospects of thermosensitive hydrogel-based TENGs

Thermosensitive hydrogel-based TENGs possess several unique translational advantages. It obviates the need for modifying the wall materials or fabricating intraluminal structures in order to load the drug. Moreover, the drug can be delivered at the time of surgery, without being dissolved and react with its carrier during conduit fabrication. Therefore, such TENGs may require simpler manufacturing process due to the independent preparation of DDS. Furthermore, they can be used to
deliver different kinds of drugs, or even a combination of two drugs [23], without the need of specific linkers.

Injectable hydrogels have been increasingly applied in tissue engineering, such as the repair of central nervous system (spinal cord) injury [42–47]. The present study suggests that they hold tremendous potential as an effective DDS in peripheral nerve tissue engineering. Although fruitful findings have been gained in some preclinical studies, the optimization of drug loading capacity and drug release kinetics is warranted before its clinical implementation [20]. The degradation rates should be further tuned to fit the release rates of encapsulated drugs [20]. A more detailed assessment of in vivo toxicity is required to verify the safety of hydrogel polymers.

4.4. Limitations

The present research has reached its aims, but there were some unavoidable limitations. First, only a 10 mm gap was tested in rodent animal models. The results in repairing longer gap lengths might not be the same. Therefore, the efficacy of TENG should be further investigated in larger animals and with longer nerve gap (>30 mm). Second, this study lacked safety assessment. Given that PEG is FDA-approved and its degradation products are amino acids, it is unlikely to cause overt toxicity. Noticeably, mPEG-PELG hydrogels did not exert local and systemic toxicity during the delivery of chemotherapeutic drugs to melanoma and liver cancer tissues [23, 48]. However, their neurotoxicity profiles when incorporated into TENGs need further confirmation. Third, the degradation of hydrogels has yet to be tested in vivo. Previous studies have revealed that the in situ formation of mPEG-PELG gels could last for 21 d in the subcutaneous tissues [23, 48]. However, the degradation rate of mPEG-PELG is more difficult to assess in the nerve conduit, as it is not apparently seen compared to subcutaneous layer. Although the actual degradation process may be time and effort consuming to investigate, it needs to be addressed in future work. Forth, mPEG-PELG hydrogels demonstrated an initial burst release of NGF. The structure of hydrogels can be further refined to reduce the initial burst. Fifth, the data from in vitro releasing test might not accurately indicate the in vivo release kinetics. Therefore, NGF concentration-time profile in the nerve graft should be further investigated in order to completely understand the spatial-temporal drug release pattern from thermosensitive hydrogels.

5. Conclusion

In conclusion, a TENG was constructed by using thermosensitive hydrogels as an injectable implant to controllably release NGF and improve peripheral nerve regeneration. Although this method requires further refinement, such design concept is expected to accelerate the clinical translation and commercialization of TENGs compared to scaffold-based delivery strategies. TENGs with a scaffold-independent and injectable DDS can significantly simplify the manufacturing process and provide ease and versatility in clinical use.
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Conflict of interest

The authors declare no conflict of interest.

ORCID iDs

Shusen Cui https://orcid.org/0000-0003-2840-8166

References

[1] Rinker B and Vyas K S 2014 Clinical applications of autografts, conduits, and allografts in repair of nerve defects in the hand: current guidelines Clin. Plast. Surg. 41 533–50
[2] Gu X, Ding F and Williams D F 2014 Neural tissue engineering options for peripheral nerve regeneration Biomaterials 35 6143–56
[3] Sarker M D, Naghieh S, McNees A D, Schreyer D J and Chen X 2018 Regeneration of peripheral nerves by nerve guidance conduits: influence of design, biopolymers, cells, growth factors, and physical stimuli Prog. Neurobiol. 171 125–50
[4] Gu X, Ding F, Yang Y and Liu J 2011 Construction of tissue engineered nerve grafts and their application in peripheral nerve regeneration Prog. Neurobiol. 93 204–30
[5] Bell J H and Haycock J W 2012 Next generation nerve guides: materials, fabrication, growth factors, and cell delivery Tissue Eng B 18 116–28
[6] Pashuck E T and Stevens M M 2012 Designing regenerative biomaterial therapies for the clinic Sci. Transl. Med. 4 160sr4
[7] Gu X 2015 Progress and perspectives of neural tissue engineering Frontiers Med. 9 401–11
[8] Oh S H et al 2018 Enhanced peripheral nerve regeneration through asymmetically porous nerve guide conduit with nerve growth factor gradient J. Biomed. Mater. Res. A 106 52–64
[9] Yao Y et al 2017 Effect of longitudinally oriented collagen conduit combined with nerve growth factor on nerve regeneration after dog sciatic nerve injury J. Biomed. Mater. Res. B 106 1–9
[10] Chang Y C et al 2017 Multichanneled nerve guidance conduit with spatial gradients of neurotrophic factors and oriented nanotopography for repairing the peripheral nervous system ACS Appl. Mater. Interfaces 9 37623–36
[11] Wu H, Liu J, Fang Q, Xiao B and Wan Y 2017 Establishment of nerve growth factor gradients on aligned chitosan-polylactide/alginate fibers for neural tissue engineering applications Colloids Surf. B 160 598–609
[12] Madduri S and Gander B 2012 Growth factor delivery systems and repair strategies for damaged peripheral nerves J. Control. Release 161 274–82
[13] Wu H et al 2017 Multi-tube conduit-filler constructs loaded with gradient-distributed growth factors for neural tissue engineering applications J. Mech. Behav. Biomed. Mater. 77 671–82
[14] Yang Y, Zhao W, He J, Zhao Y, Ding F and Gu X 2011 Nerve conduits based on immobilization of nerve growth factor onto modified chitosan by using genipin as a crosslinking agent Eur. J. Pharm. Biopharm. 79 519–25
[15] Xia B and Lv Y 2018 Dual-delivery of VEGF and NGF by emulsion electrosprun nanofibrous scaffold for peripheral nerve regeneration Mater. Sci. Eng. C 82 253–64
[16] Zhang W et al 2017 A sequential delivery system employing the synergism of EPO and NGF promotes sciatic nerve repair Colloids Surf. B 129 327–36
[17] Whitehead T J and Avila C O C 2018 Sundararaghavan HG. Combining growth factor releasing microspheres within aligned nanofibers enhances neurite outgrowth J. Biomed. Mater. Res. A 106 17–25
[18] Ramburrun P, Kumar P, Choonara Y E, Bijukumar D, du Toit L C and Pitlay V 2014 A review of bioactive release from nerve conduits as a neurotherapeutic strategy for neuronal growth in peripheral nerve injury Biomed. Res. Int. 2014 13250
[19] Uebersax L, Merkle H P and Meinel L 2009 Biopolymer-based growth factor delivery for tissue repair: from natural concepts to engineered systems Tissue Eng. B 15 263–89
[20] Thambi T, Li Y and Lee D S 2017 Injectable hydrogels for sustained release of therapeutic agents J. Control. Release 267 57–66
[21] Li Y, Rodrigues J and Tomas H 2012 Injectable and biodegradable hydrogels: gelation, biodegradation and biomedical applications Chem. Soc. Rev. 41 2193–221
[22] Wang X, Hu W, Cao Y, Yao J, Wu J and Gu X 2005 Dog sciatic nerve regeneration across a 30-mm defect bridged by a chitosan/PGA artificial nerve graft Brain 128 1897–910
[23] Wu X, Wu Y, Ye H, Yu S, He C and Chen X 2017 Interleukin-15 and cisplatin co-encapsulated thermosensitive polypeptide hydrogels for combined immuno-chemotherapy J. Control. Release 255 81–93
[24] Hayashi A et al 2008 Axotomy or compression is required for axonal sprouting following end-to-side neurorraphy Exp. Neurol. 211 539–50
[25] Unal B et al 2005 Morphological alterations produced by zinc deficiency in rat sciatic nerve: a histological, electron microscopic, and stereological study Brain Res. 1048 228–34
[26] Guena S, Tos P, Guglielmone R, Battiston B and Giacobini-Robecchi M G 2001 Methodological issues in size estimation of myelinated nerve fibers in peripheral nerves Anat. Embryol. 204 1–10
[27] Bain J R, Mackinnon S E and Hunter D A 1989 Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat Plast. Reconstr. Surg. 83 129–38
[28] Madduri S, Feldman K, Tervoort P, Papaloizos M and Gander B 2010 Collagen nerve conduits releasing the neurotrophic factors GDNF and NGF J. Control. Release 143 168–74
[29] Madduri S, Papaloizos M and Gander B 2009 Synergistic effect of GDNF and NGF on axonal branching and elongation in vitro Neurosci. Res. 65 88–97
[30] Labroo P et al 2017 Novel drug delivering conduit for peripheral nerve regeneration J. Neural Eng. 14 066011
[31] Scuteri A et al 2010 NGF protects dorsal root ganglion neurons from oxaliplatin by modulating JNK/Sapk and ERK1/2 Neurosci. Lett. 486 141–5
[32] Mata M, Alessi D and Fink D J 1990 S100 is preferentially distributed in myelin-forming Schwann cells J. Neurocytol. 19 432–42
[33] Tang X, Qin H, Gu X and Fu X 2017 China’s landscape in regenerative medicine Biomaterials 124 78–94
[34] Zhao M, Li X Y, Xu C Y and Zou L P 2015 Efficacy and safety of nerve growth factor for the treatment of neurological diseases: a meta-analysis of 64 randomized controlled trials involving 6297 patients Neural Regen. Res. 10 819–28

https://orcid.org/0000-0003-2840-8166
[35] Park M K, Joo M H, Choi B G and Jeong B 2012 Biodegradable thermogels Acc. Chem. Res. 45 424–33
[36] Sun K and Li Z H 2011 Preparations, properties and applications of chitosan based nanofibers fabricated by electrosprining Express Polym. Lett. 5 342–61
[37] Carballo-Molina O A and Velasco I 2015 Hydrogels as scaffolds and delivery systems to enhance axonal regeneration after injuries Frontiers Cell Neurosci. 9 1–12
[38] Wells M R et al 1997 Gel matrix vehicles for growth factor application in nerve gap injuries repaired with tubes: a comparison of biomatrix, collagen, and methylcellulose Exp. Neurol. 146 395–402
[39] Jin J et al 2013 Peripheral nerve repair in rats using composite hydrogel-filled aligned nanofiber conduits with incorporated nerve growth factor Tissue Eng. A 19 2138–46
[40] Midha R, Munro C A, Dalton P D, Tator C H and Shoichet M S 2003 Growth factor enhancement of peripheral nerve regeneration through a novel synthetic hydrogel tube J. Neurosurg. Spine 99 555–65
[41] Radu F A, Bause M, Knabner P, Lee G W and Friess W C 2002 Modeling of drug release from collagen matrices J. Pharm. Sci. 91 964–72
[42] Wang W et al 2018 An injectable conductive hydrogel encapsulating plasmid DNA-eNOS and ADSCs for treating myocardial infarction Biomaterials 160 69–81
[43] Wang J, Zhang F, Tsung W P, Wan C and Wu C 2017 Fabrication of injectable high strength hydrogel based on 4-arm star PEG for cartilage tissue engineering Biomaterials 120 11–21
[44] Thompson R E et al 2018 Effect of hyaluronic acid hydrogels containing astrocyte-derived extracellular matrix and/or V2a interneurons on histologic outcomes following spinal cord injury Biomaterials 162 208–23
[45] Le L V et al 2018 Injectable hyaluronic acid based microrods provide local micromechanical and biochemical cues to attenuate cardiac fibrosis after myocardial infarction Biomaterials 169 11–21
[46] Gomes E D et al 2016 Combination of a peptide-modified gellan gum hydrogel with cell therapy in a lumbar spinal cord injury animal model Biomaterials 105 38–51
[47] Fuhrmann T et al 2016 Injectable hydrogel promotes early survival of induced pluripotent stem cell-derived oligodendrocytes and attenuates longterm teratoma formation in a spinal cord injury model Biomaterials 83 23–36
[48] Cheng Y, He C, Ding J, Xiao C, Zhuang X and Chen X 2013 Thermosensitive hydrogels based on polypeptides for localized and sustained delivery of anticancer drugs Biomaterials 34 10338–47