Nanofiber scaffolds facilitate functional regeneration of peripheral nerve injury

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Received 25 October 2011; accepted 8 August 2012

Abstract

Peripheral nerve injury still remains a refractory challenge for both clinical and basic researchers. A novel nanofiber conduit made of blood vessel and filled with amphiphilic hydrogel of self-assembling nanofiber scaffold (SAPNS) was implanted to repair a 10 mm nerve gap after sciatic nerve transection. Empty blood vessel conduit was implanted serving as control. Results showed that this novel nanofiber conduit enabled the peripheral axons to regenerate across and beyond the 10 mm gap. Motoneuron protection, axonal regeneration and remyelination were significantly enhanced with SAPNS scaffold treatments. The target reinnervation and functional recovery induced by the regenerative nerve conduit suggest that SAPNS-based conduit is highly promising application in the treatment of peripheral nerve defect.

From the Clinical Editor: In this paper by Zhan et al, a novel self-assembling nanofiber scaffold is reported to promote regeneration of peripheral nerves in a sciatic nerve injury model. The promising results and the obvious medical need raises hope for a clinical translation of this approach hopefully in the near future.

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Key words: Self-assembling peptide; Nanofiber scaffold; Peripheral nerve injury; Axonal regeneration; Remyelination

Despite rapid advances in the microsurgical techniques for the neural repair clinically, peripheral nerve injuries (PNI) still remain the common and frequently disabling cause,1 especially in the case of long distance defect in which nerve ends need to be bridged with a nerve graft. In most clinical cases nerve autografting serve as the gold standard for the peripheral nerve repair. Inevitably, autografting has been limited to multiple critical demerits including limited availability of donor graft and mismatch in size in clinical practice, additional surgery trauma at donor sites and associated functional loss of donor nerve.2 These critical issues are persisting to challenge both basic and clinical researchers. In view of this, a vast majority of PNI studies have been focused on the development of novel biomaterial-based artificial nerve constructs in the treatment of PNI. Obviously, novel biomaterials play an important role in the development of artificial peripheral nerve grafts.

Self-assembly peptide nanofiber scaffold (SAPNS) represents a promising biomaterial in the field of neural bioengineering with attractive properties like excellent interfacial compatibility as
well as bio-adhesive properties. Addition of physiological fluids to self-assembly peptide creates a three-dimensional scaffold hydrogel which is superior to the other as follows: 1) Excellent biocompatibility and biodegradability due to its naturally constituent amino acids; 2) Non-cytotoxic and immunological alert after implantation; 3) Similar to the naïve extracellular matrix (ECM). Nanofiber hydrogel scaffold is capable of supporting cell adherence, migration and proliferation. Blood vessel remains an obvious candidate of conduit material in PNI repair due to its autogenic availability and ease of harvest. Compared with the traditional vein conduits studied in the digital nerve repair clinically, the artery conduit employed in this study is capable of being more resistant to the probability of kinking or collapse, especially in the treatment of PNI with long gap defects.

Our previous reports modeling brain and spinal cord injury demonstrated that SAPNS effectively facilitated the central nervous system (CNS) repair, suppression of inflammation and anti-gliosis post-injury, and especially improving angiogenesis and axonal regeneration. Based on these previous encouraging outcomes in the treatment of CNS injury, a novel SAPNS-enhanced nanofiber artificial nerve with artery segment serving as conduit sheath was employed in the present study for the repair of PNI. Positive effects would greatly propel the development of nanofiber applications in the field of PNI repair.

Materials and methods

Animal subjects and experimental groups

Adult Sprague–Dawley(SD) female rats (67 in total) weighing 220 to 300 g (From the Experimental Animal Center of Southern Medical University) were used, of which 31 rats were assigned to provide abdominal aortas for preparations of nerve conduits (NC). The remaining 36 rats were randomly divided into three groups: (1) Artificial Nanofiber Nerve Conduit (NNC, n=18); (2) Empty Nerve Conduit (ENC, n=13); (3) Defect without treatment (n=5). As designed, 3 rats in each group of NNC and ENC were sacrificed at 2 weeks post surgery for comparisons of the early regeneration after treatment. The rest of animal subjects were allowed to survive for 16 weeks to study the long-term therapeutic efficiency.

Preparation of artificial nerve implants

The abdominal aortas were harvested after animal donors were euthanatized with overdose anesthesia (1% pentobarbital sodium with 5 mL/kg body weight, Guangzhou Chemical Reagent Plant). The descending aorta segments with dimension from 1.5 mm to 2.2 mm were placed into PBS at 4°C not in excess of 2h for use. For NNC, 1% SAPNS solution made of RADA16-I peptide (BD Biosciences, Cambridge, Massachusetts) was carefully filled into a 12 mm-long aorta conduit by a Hamilton microsyringe. Then, the aorta was immersed into DMEM culture medium (Invitrogen) for 30 min to trigger self-assembly of SAPNS for gelling, and was ready for the following transplantation. For ENC, the aorta remained un-treated before transplantation.

Nerve injury model and animal treatments

All animal subjects were administered 1% pentobarbital sodium intraperitoneally for anesthesia (3.5 mL/kg body weight). With an operating microscope the right sciatic nerve was exposed and a 10 mm gap was made at middle part of the nerve trunk. Both proximal and distal nerve stumps were sutured into the ends of the NC as far as 1 mm and the connections were sutured with 11-0 suture (Ethicon). For the negative control the injury was left alone without any treatment. Antibiotics and analgesic reagents were routinely used for post-operative health care. Guidelines for animal care and use of Southern Medical University were strictly followed. The animals were allowed standard access to food and water ad lib throughout the study.

Histomorphometry of axonal regeneration

Animal subjects were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 2 weeks or 16 weeks post-surgery. Involved sciatic nerve segments containing NC implants were harvested and followed by post-fixation, then immersed in 30% sucrose (w/v) in 0.1 M phosphate buffer at 4°C for cryosectioning.

Fifteen μm sections of sciatic nerve samples were cut and immunohistochemistry staining was processed. Briefly, every 2nd section was assigned to perform Neurofilament (NF)-200 labeling. After nonspecific antigen binding was blocked with 10% normal goat serum plus 0.25% Triton X-100 (PBS-T) for 1 h, sections were subsequently incubated with polyclonal rabbit anti-NF-200 primary antibody (1:500; Chemicon, Temecula, California) overnight at 4°C. After washing with PBS, sections were incubated with Alexa 568-conjugated goat anti-rabbit IgG (1:500; Invitrogen). Finally, Sections were coverslipped using Fluoromount G (Southern Biotechnology, Birmingham, Alabama). PBS was used to replace the primary antibody serving as negative control. Digital images were captured with fluorescence microscope (Nikon, Eclipse 80i) and processed with montage splicing using Photoshop CS5 software. Along the proximal–distal axis three vertical lines were placed within the implant at 1 mm to proximal interface, midpoint and 1 mm to the distal junction site. Numbers of NF200-labeling axons crossing these vertical lines were quantified respectively. Total axon number/animal was determined by multiplying the counted number by 2. Observers were blinded to group identity.

Detection of remyelination of the regenerated axons

Every sixth section of sciatic nerve samples was immunostained with 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) and Myelin Basic Protein (MBP) for Schwann cells and matured myelin separately. Briefly, selected sections were pooled to perform double-immunostaining with antibodies of polyclonal rabbit anti-NF-200 (1:500; Chemicon, Temecula, CA) and monoclonal mouse anti-MBP (1:500, Santa Cruz, California). Another corresponding set of sections was incubated with rabbit anti-NF-200 and monoclonal mouse anti-CNPase (1:200, Bioworld, Minnesota). After incubation with the primary antibodies overnight at 4°C, the sections were stained with the secondary antibodies of Alexa 568-conjugated goat anti-rabbit...
IgG and Alexa 488-conjugated goat anti-mouse IgG (1:500; Invitrogen). Final slides were coapposed with Fluoromount G.

Transmission electron microscopy (TEM) was conducted to confirm axonal remyelination within the nerve implants. Briefly, selective sample blocks with 1×1×1 mm³ in size at the middle part of the implant were fixed with 2.5% glutaraldehyde plus 2% paraformaldehyde for 12 h. After being post-fixed in 1% OsO₄ for 2 h at 4 °C, samples were dehydrated in graded ethanol series and embedded with Epon 812 resin (Electron Microscopy Sciences, Fort Washington, Pennsylvania) for ultrathin transverse sectioning. These cross-sections were further counterstained with 2% uranyl acetate and lead citrate as described previously. Non-overlapping images of cross-sections were evaluated. Fiber diameter and G-ratio in at least 6 random fields of view were quantified using Image J software (1.43, NIH). The G-ratio was determined by dividing axon diameter by total fiber diameter (at least 200 fibers per animal).

**Neuroinflammation revealed with immunohistochemistry**

Every fifth section of sciatic nerve samples was immunostained with ED1-Abs for macrophages and CD3-Abs for lymphocytes. The primary antibody pair of monoclonal mouse anti-ED1 (1:1000, Serotec, Raleigh, North Carolina) and polyclonal rabbit anti CD3 (1:50, Santa Cruz, California) was used to conduct double immunostaining with aforementioned procedures.

**Retrograde tracing and quantification of spinal motoneurons after treatment**

After completion of Electrophysiology measurement at 15 weeks, 2 μL of 1% FluoroGold (FG, Sigma) was injected 5 mm distal to the host-implant junction in the nerve trunk using a Hamilton syringe with 30-gauge needle. The same procedures were performed on the contralateral side with four animals to obtain reference values. Animals were allowed to survive for an additional 7 days for tracer transportation. The L3–S2 cord segments were harvested and processed with sagittal sectioning (15 μm). Polyclonal goat anti ChAT (1:500, Millipore) was used to label the motoneurons and FG-traced neurons, indicative of regenerated motoneurons. Enumeration in every other section was conducted in a blinded manner and the total number of regenerated motoneurons per animal was calculated by multiplying the counted number with 2. According to the method of Abercrombie, repeated counting of split neurons was corrected.

**Gastrocnemius muscle wet weight and muscular morphology**

Sixteen weeks after implantation, animals were euthanized with overdose of anesthesia. Bilateral gastrocnemius muscles were explanted immediately and wet muscle weight was recorded on a laboratory scale. The weight ratio of injury side/intact side was calculated as the recovery index of gastrocnemius muscle. For light microscopy, samples taken from the mid-belly of muscles were fixed with 4% PFA, dehydrated, and processed with routine paraffin embedding followed by serial longitudinal sectioning (5 μm) and Hematoxylin and Eosin (HE) staining. For TEM, samples of 1×1×1 mm³ in size were harvested from mid-belly of gastrocnemius muscle and processed as aforementioned TEM methods.

**Electrophysiology assessment**

Fifteen weeks post-treatment, animal subjects were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). The involved sciatic nerve was exposed and the peak amplitude of Nerve Compound Action Potentials (NCAP) was measured. Briefly, the stimulating electrode was placed on the proximal nerve trunk 5 mm to host–conduit junction site with recording electrode placed on the distal nerve trunk 5 mm to the conduit–host junction site. Then, a constant current of 0.4 to 0.5 mA for 0.1 ms was used and the stimulus was increased gradually till the supramaximal response was produced. NCAPs were then recorded with BL-420E Data Acquisition Analysis System for Life Science (Taimeng, China).

**Behavioral test of motor function**

The motor functional recovery of injured hindlimb was assessed with a modified SFI (Sciatic Function Index) approach at 6, 9, 15 weeks respectively post-treatment. Animal subjects were trained to walk on a paper-covered narrow runway (1 m long, 7 cm wide and 8 cm high) every other day for a period of 2 weeks before assessment. For the analyses, every animal was tested three times with 1 h interval and six steps per test were selected to measure two parameters: (1) Rotation Angle of Foot injured (RAFi), which is the angle made by a stride line and the connect line of third toe to the center of the paw pad. (2) Injured Hindlimb Synergia (IHS), which is the distance between the center pads of the injured hindlimb and ipsilateral forelimb. Since both degrees of RAFi and IHS are increased after injury, either value of RAFi and IHS would revert towards the normal value given locomotor function improved after treatments.

**Statistics analysis**

All values are presented as mean±standard deviation (SD) and SPSS 13.0 software for Windows (Chicago, IL) was used for statistical analysis. Student’s t-test was applied for 2-group comparisons and One-way ANOVA test (Bonferroni post hoc comparison) was used to analyze differences among multiple groups. P < 0.05 was considered as statistically significant difference.

**Results**

**Integrated artificial nanofiber conduit facilitated axonal regeneration and remyelination**

Nanofiber conduit connected the nerve gap with excellent peripheral nerve-like appearance without any atrophy (Figure 1, A, B). The artificial nerve implant integrated with host nerve, which was evidenced by the smooth transition of both connection zones without any significant scarring occurrence. Robust axonal regeneration was demonstrated in the NNC implant by NF-200 immunostaining at 16 weeks. The dense NF-200 labeling axons not only penetrated into nanofiber conduit across the entire length but also regrew beyond the distal interface into host nerve (Figure 1, C). In contrast, modest axonal regrowth appeared in ENC implant (Figure 1, D). Quantitative analysis demonstrated that NNC implantation
significantly improved the axon regeneration compared with the ENC one (Figure 1, E).

For comparison of the axonal regeneration at early stage after different treatments, both NC samples were checked at 2 weeks post-transplantation. Lots of NF-200 positive axons regrew into the proximal part of the nerve-conduit in NNC group accompanied with migrated Schwann cells (SCs) (Figure 2, A, C), whereas, only few of axons and associated SCs were detected in ENC group (Figure 2, B). Notably, marker of mature myelin, MBP, was not detected with the regenerated axons at 2 weeks post-injury within the conduit in either NNC groups (Figure 2, D) or in ENC group (data not shown). Interestingly, the survived axons proximal to the implants in NNC group remained myelination as shown by MBP staining (Figure 2, E), whereas significant demyelination occurred at ENC counterpart as shown by remarkable reduction in MBP signals (Figure 2, F). In the distal part of implants, no regenerated axon was detected in both NC groups at 2 weeks (data not shown). Meanwhile, implanted

Figure 1. Nanofiber nerve conduit bridged the nerve defect and greatly improved the neural regeneration. Ten mm nerve gap was bridged well with implants of NCs. (A) A sample of aorta-sheathed NC bridged the defected sciatic nerve immediate after surgery. The nerve ends (arrows) were inserted into the NC as far as 1 mm and the connections were sutured with 11-0 suture (arrowheads). (B) A sample of achieved NNC bridging host nerve stumps at 16 weeks post-implantation. Large quantities of NF200-labeling axons regenerated into the nanofiber conduit (C, 1–5), which are much higher than that of empty conduit group (D, 1′–5′). Inter-group comparisons at proximal, middle and distal part of NCs were shown in (E) using student t-test (*P < 0.05). Notably, these axons not only grew across the entire conduit but also egressed out of the distal connection sites into the distal host nerve in both experimental groups. (C 1–5) and (D 1′–5′) are higher power magnifications of corresponding boxed areas in (C) and (D) respectively. The minimal calibration of the presented ruler is 1 mm in both (A) and (B). Proximal is to the left.

SAPNS of NNC group remained partially in the conduit with rare ED1 positive macrophages or CD3 positive lymphocytes infiltrated in the scaffold (Figure 2, G), whereas the corresponding space of NC in ENC group was full of invasive lymphocyte and macrophages (Figure 2, H). The degeneration and demyelination in the counterparts of ENC group were developed remarkably as shown by much weaker NF and MBP staining (F). Moreover, with the partial SAPNS (asterisks) remaining in the conduit, much less infiltration of ED1 positive macrophages (arrows) and CD3 positive lymphocytes (arrowheads) occurred in NNC group (G) whereas the cavity of conduit in ENC group was fully occupied with invaded lymphocyte (arrowheads in H) and macrophages (arrows in H).

Retrograde labeling of spinal motoneurons

The histomorphology assessment of FG-labeling for regenerated spinal motoneurons at injury side confirmed motor axonal regeneration in both NC groups. The FG retrograde-labeling demonstrated $470.2 \pm 73.2$ and $1041.5 \pm 186.1$ motoneurons have regenerated their axons in the right ventral horn per animal in ENC and NNC groups respectively (Figure 4, A–I). The total numbers of FG-labeled motoneurons were significantly higher in the NNC treatment group compared to that of the ENC control ($P < 0.05$, Figure 4, J).

Gastrocnemius histomorphology and wet muscle weight

Sixteen weeks after implantation, there were significant steatosis occurred within the multiple interstitium between residual atrophic muscle fibers within the ENC control group (Figure 5, A, B), which represented a typical profile of atrophic muscular tissue suffered from chronic denervation. In contrast, steatosis and myoatrophy were considerably ameliorated with NNC treatment (Figure 5, C, D). More regular dense arrangements of myofibers were demonstrated in the NNC group, which appeared much closer to that of normal control (Figure 5, E, F).

Correspondingly, involved gastrocnemius muscle of injured side lost weight considerably in all injured subjects at 16 weeks. However, the weight ratio of injured/intact side in the NC groups was significantly higher than negative control group ($P < 0.01$), whereas NNC group was further higher than ENC group ($P < 0.05$) (Figure 5, G).

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Figure 2. Axonal regrowth, Schwann cell immigration and Neuroinflammation at 2 weeks after NC implantation. At 2 weeks after NC implantation, bunches of NF-200 positive axons (red) regrew into the proximal part of conduit in NNC group, which accompanied with CNPase positive (green) immigrated SCs (A). In contrast, only few axons and SCs appeared in ENC group (B). In NNC group, regenerating axons were co-localized with immigrated SCs (C) but no MBP positive signal was detected at 2 weeks (D). There were mild degeneration and demyelination that occurred in the nerve trunk proximal to the lesion sites in NNC group (E). The degeneration and demyelination in the counterparts of ENC group were developed remarkably as shown by much weaker NF and MBP staining (F). Moreover, with the partial SAPNS (asterisks) remaining in the conduit, much less infiltration of ED1 positive macrophages (arrows) and CD3 positive lymphocytes (arrowheads) occurred in NNC group (G) whereas the cavity of conduit in ENC group was fully occupied with invaded lymphocyte (arrowheads in H) and macrophages (arrows in H).
Functional recovery improved with NNC treatment

The NCAP obtained by electrophysiology proved that the nerve conduction got through the artificial nerve implant. The mean amplitude of NCAP is significantly greater in NNC group than ENC control (P < 0.01) (Figure 6, A–D). Correspondingly, the behavioral test demonstrated that both RAFi and IHS were improved by the nanofiber scaffold. Statistical analysis revealed injured hindlimb locomotor performance was significantly improved in NNC group than ENC one at different time points respectively (P < 0.05, Figure 6, E, F).

Discussion

NNC serves as an excellent implant for PNI repair

Due to inevitable limitations of autograft or allograft presented in PNI repair, a host of engineered nerve constructs has been studied attempting to replace the natural nerve grafts and achieve even better functional recovery than limited autografting. Currently utilized biomaterials could be sorted in terms of material derivation, biodegradability and fabrication properties. Ideal biomaterials for neural tissue engineering should meet the
critical requirements including excellent biocompatibility, biodegradability and neural integrity. Reported biomaterials in this scope currently involved: 1) Natural polymers, such as chitosan and collagen; 2) Synthetic polymers like polyvinyl alcohol hydrogel, polytetrafluoroethylene, polyglycolic acid polymer, poly (l-lactide-co-glycolide); polyhydroxybutyrate, and poly(lactic–caprolactone polymer. Although these biomaterials exerted certain capability of promoting axonal regeneration, multiple intrinsic deficits were explored like cytotoxic degraded products, unmatched elastic modules for nerve tissue, and inferior capabilities of modulating ECM environment and regulation of cellular interaction.

SAPNS in the present study, a self-assembling peptide named RADA16-I, is designed as alternating positive and negative L-amino acids arrangements that form an amphiphilic molecule and spontaneously self-assembled as a single nanofiber. The SAPNS further generate hydrogel scaffold with greater than 99% water content in the presence of physiological solutions or body fluids. Series studies have revealed that SAPNS featured ~10 nm in fiber diameter with pores size between 5 and 200 nm, presents a true 3-D environment similar to natural ECM architecture; therefore, it is able to support the adhesion, proliferation and differentiation of various neural cells. In line with our previous study that demonstrated the well integration of SCs and SAPNS, the present SAPNS scaffold in peripheral bridge greatly improved the immigration of host SCs and resultant remyelination. Additionally, our previous data confirmed that SAPNS can form a neural permissive 3-D

![Figure 4. FG-retrograde labeling of spinal motoneurons. At 16 weeks, FG retrograde labeling revealed that the regenerated motoneurons were greatly improved in NNC group compared to those in ENC group. (A, D, G) are FG-labeling counterparts to (B, E, H) correspondingly from group ENC, NNC and normal control respectively. (B, E, H) are representative images with ChAT immunostaining for identifying motoneurons. (C, F, I) are merged images of double labeling. Statistical analysis showed the number of regenerated motoneurons in NNC group is significantly greater than that in ENC group (One way ANOVA, *P < 0.05; **P < 0.01).](image-url)
structure, which not only significantly facilitated the adhesion, proliferation and differentiation of neural stem cell, but also robust axonal regeneration. Of note, as explored in series of related studies, the unique nanofiber-based structure provides abundant adhesive surface for neurite extension, and serves as contact guidance for the neurite outgrowth featured with multiple fiber orientation in nanoscale. Owing to such fiber-orientation guidance residing in SAPNS scaffold, the regenerative neurites get extension along linear fiber structures that significantly improve the regeneration of axons. Moreover, SAPNS can be degraded into natural L-amino acids eventually in vivo, which enable it to be immunological inert and free of chemical and biological contaminants compared with other synthesized counterparts. These endowed properties render the SAPNS superior to the other biomaterials, especially suitable for neuroregeneration applications.

Traditionally, autogenous conduits like veins are preferential to be considered as nerve guidance channel in the clinical settings since clinical application of vein graft for peripheral nerve reconstruction could be traced back to 1919. However, the inherent drawbacks of vein graft collapse plus valve-related obstruction which could compromise the vein tube with flattening and blocking nerve regeneration have not been addressed clinically and significantly weaken their clinical potential.

Figure 5. Histomorphology and quantification of gastrocnemius muscle recovery. At 16 weeks, significant steatosis (asterisks) appeared across the interstitium spaces between residual atrophic myofibers (arrows) in the ENC group (A) with according diminished cross area of myofibers in transverse section indicated by asterisk in (B). In a sharp contrast, steatosis and myoatrophy were significantly attenuated in NNC group (C and D). The myofiber profile in NNC group appeared much closer to that of normal control (E and F). Consequently, the wet weight of injured gastrocnemius muscle was improved significantly in NNC compared to ENC and negative control groups (G, ANOVA analysis, *P < 0.05, **P < 0.01). (A, C, E) are longitudinal sections with hematoxylin–eosin staining. (B, D, F) are ultrathin transverse sections for TEM.
Alternatively, artery conduits with stronger wall are capable of acting as a reliable nerve sheath to protect the regeneration pathway experimentally. Presently, segment of abdominal aorta served as nerve sheath to maintain nanofiber hydrogel, which displayed suitable biomechanical characteristics acting as nerve conduit. Our data presently demonstrated that this novel nerve conduit effectively provided a stable chamber for peripheral axon regrowth, prevented the neuroinflammation and well integrated with host tissue. Nevertheless, veins especially large vein like the great saphenous vein is still a good candidate for the conduit to be used in patients. In addition to human tissue, artificial biodegradable conduits made of collagen, PLGA or caprolactone have been approved by the US Food and Drug Administration (FDA) for the clinical application currently. Therefore, potential combinations of such artificial nerve conduit with SAPNS would be quite attractive in the further exploration with PNI models.

Collectively, the whole SAPNS nerve implant was constructed with purely natural biological materials and developed as a 3-D guidance structure of neural regeneration. In clinical practice, tension-free repair of nerve lacerations or neurotmesis that presents the most serious nerve injury featured complete severance of the peripheral nerve trunk, remains the optimal surgical treatment given that the end to end neuroanastomosis is available. When direct repair cannot be achieved, interposed nerve bridge with multiple artificial nerve guides would be an alternative to the autogenous nerve grafting. The present outcomes confirmed that this novel artificial nerve graft was capable of protecting nanofiber hydrogel content through 16 weeks after transplantation as well as excellent functional integrity with host nerve tissue, which recapitulated the critical requirements for the design of up to date bioengineered artificial peripheral nerve.

NNC promotes peripheral nerve regeneration and target reinnervation

Previous studies demonstrated SAPNS could not only repair injured optical nerve and restore visual function with brain repair, but also promote the central axonal regeneration with an acute SCI model. The present study further revealed that the SAPNS hydrogel also effectively promoted peripheral axonal regeneration over a 10 mm gap. Considerably remyelinated axons regenerated into scaffold and extended beyond the implant reentering host nerve tissue, which presents high potential of such nanofiber materials applied in the PNI repair. Owing to those nanofiber-based benefits discussed above, the intraluminal nanofiber structure exerts excellent physical guidance for the neurites extension as shown in Figure 1. Remyelinated axons displayed well-ordered arrangements with NF/MBP double...
labeling. Furthermore, spinal motor axonal regeneration was confirmed by the retrograde FG-labeling. Regenerated motoneuron pool was significantly improved with NNC compared with ENC. Interestingly, besides the self-assembly nanofibers, electrospinning is another general technique to fabricate nanosize synthetic polymeric. Recently, nanofibers made with electrospinning have been studied with PNI models.22 Comparable study using electrospin PLGA nanofiber conduit showed that the NF68-labeling axons distributed throughout the nerve conduit of 10 mm in 5 out of 11 implants at 4 weeks after implantation albeit lack of functional outcomes.23 During further study a 10 mm sciatic nerve gap was connected with PLGA/poly(-caprolactone) (PCL) nanofibers.24 At 4 months after repair myelinated axons and basement membrane component Collagen IV were identified throughout the regenerated tissue inside the conduits with 70.6% of the treated rats showing an initial reinnervation in plantar muscles by the presence of the compound muscle action potential. However, due to their intrinsic drawbacks including relatively hydrophobicity, lack of biological recognition sites, limited elastic module and acidic degraded by-products, related studies attempted to modify the material surface with natural active motif to enhance cell–material interactions.25 One of the most promising benefits of natural peptide nanofiber lies in that various novel functional motifs could be directly incorporated into SAPNS backbone to create self-assembled nanofiber scaffolds with functionalized peptides for seeding cells of interest.17,26

Functional return of axonal conduction depends on axon caliber, myelin thickness and axonal maturation since regeneration.27,28 Remyelination of axons is essential for the functional re-establishment after PNI. It is well-accepted that the G-ratio is a highly reliable value for evaluating axonal myelination.28 Assessed with TEM and quantitative analysis of myelinated fiber diameter and G-ratio after different NCs treatment, the nanofiber scaffold exhibited significant regeneration-promoting effects on both axonal regrowth and remyelination. On the other hand, FG-retrograde tracing confirmed that the spinal motoneurons regenerated axons to their peripheral targets, but implant lack of SAPNS substrates poorly supported axonal regrowth, remyelination and peripheral retrograde labeling.

Consequently, further muscle histomorphology assessment demonstrated that the targeted gastrocnemius muscles of the animals treated with NNC bridging recovered significantly better than the ENC and the wet weights reached 50%–60% of normal level. In contrast, denervated muscles lost their weight dramatically during the chronic stage. The therapeutic effects of SAPNS on muscle mass recovery partially reflected the reinnervation of injured skeletal muscles.

**NNC improves functional recovery**

Although spontaneous regeneration of peripheral nerve occurred, it could only bridge limited distance less than 10 mm in general rodent models but with poor functional recovery as shown in the control. Presently, SAPNS scaffold significantly improved the axonal regrowth in term of nerve conduction measurement since the mean amplitude of NCAP was regained considerably in NNC group compared with ENC control. This indicates that the number of regenerated axons in nanofiber conduit is much greater than that built in ENC at 16 weeks after injury. The nerve conduction outcomes were highly parallel to the axonal qualification in immunostaining evaluation, which further confirmed the encouraging effects of NNC on the peripheral axon regeneration. Of note, during our hindlimb locomotor function assessment, even as earlier as 6 weeks after treatment, significant behavioral improvements were initiated in the animals treated with nanofiber scaffolds and continued to ameliorate till experimental endpoint. Considering the 10 mm lesion gap and outcome of ENC group, longer distance lesion model should be warranted in the future study to further explore the efficacy of nanofiber scaffold on the repair of peripheral nerve injury.

In conclusion, current design of this novel NNC enables the peripheral axons to regenerate across and beyond a 10 mm gap. Motoneuron protection, axonal regeneration and remyelination are significantly enhanced with SAPNS scaffold treatments. The target reinnervation and functional recovery induced by the regenerative nerve conduit, although incomplete, suggest that SAPNS-based NC can not only improve peripheral axonal regrowth over long-distance gap in vivo, but also effectively facilitate the reinnervation of distal targets by regenerating axons. Although molecular mechanisms of nanofiber structure on the neurite outgrowth, orientation and nerve fiber maturation are warranted in the future study, the current positive outcomes enable the application of this novel SAPNS-based bioengineering construct to be a highly potential strategy for the treatment of peripheral nerve injuries.

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