Award Number: W81XWH-10-1-0773

TITLE: Mechanical Loading for Peripheral Nerve Stabilization and Regeneration

PRINCIPAL INVESTIGATOR: Sameer B. Shah

CONTRACTING ORGANIZATION: University of California, San Diego , CA 92093

REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                            Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                           Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 October 2012	Annual	15-09-2011 – 14-09-2012

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Mechanical Loading for Peripheral Nerve Stabilization and Regeneration	
	5b. GRANT NUMBER W81XWH-10-1-0773
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Sameer B. Shah, Ph.D. (PI)	
Ting-Hsien Chuang, Ph.D. (Postdoc)	**5e. TASK NUMBER**
Robin Wilson (Student Research Scientist)	
	5f. WORK UNIT NUMBER
E-Mail: sbshah@ucsd.edu	

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
University of California, San Diego 9500 Gilman Drive, MC 0934 La Jolla, CA 92093-0934	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Peripheral nerve damage is one consequence of injury to the extremities of soldiers by improvised explosive devices (IEDs). The degree of functional recovery from peripheral nerve damage is often poor, particularly for severed nerves. The result can be impaired motor function, sensory loss, and chronic pain with inappropriate autonomic responses. Consequently, strategies for enhancing nervous function are of high military relevance. Towards the development of more effective nerve regeneration strategies, this proposal addresses the hypothesis that moderate tensile loading (stretch) of peripheral nerves can stabilize nerve degradation and also promote accelerated regeneration. Our project aims are to 1) To examine the impact of low levels of tensile loading on the Wallerian degeneration of proximal and distal stumps of severed peripheral nerves and 2) To examine the impact of moderate levels of tensile loading on promoting the outgrowth and functional connectivity of severed peripheral nerves. To meet these aims, in the second project period, based on results from initial in vivo implantations, we modified our two nerve lengthening devices, one a fixed length and one extensible, and are currently performing long-term implantations to test the effectiveness of these devices for nerve regeneration.

15. SUBJECT TERMS
Peripheral nerve, nerve regeneration, biomedical device, biomechanics, rat nerve injury

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	22	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION

Peripheral nerve damage is one consequence of injury to the extremities of soldiers by improvised explosive devices (IEDs) [1, 2]. The degree of functional recovery from peripheral nerve damage is often poor, particularly for severed nerves. The result can be impaired motor function, sensory loss, and chronic pain with inappropriate autonomic responses. Consequently, strategies for enhancing nervous function are of high military relevance. A variety of chemical guidance cues and synthetic scaffolds have been incorporated into strategies for neuronal regeneration and outgrowth, but have not yet yielded adequate growth rates or been limited by insufficient source tissue[3]. The mechanical environment of a neuron is a key determinant of its growth and function [4-9]. This aspect of neuronal outgrowth has been underutilized in nerve regeneration strategies.

Towards the development of more effective nerve regeneration strategies, this proposal addresses the hypothesis that moderate tensile loading of peripheral nerves can stabilize nerve degradation and also promote accelerated regeneration. This hypothesis is motivated by observations that nerves are under tension physiologically, indicated by elastic recoil following transection, and also by observations that moderate levels of tensile loading can accelerate neuronal growth, both in vitro and in vivo [4-9]. Our project aims are to *1) To examine the impact of low levels of tensile loading on the Wallerian degeneration of proximal and distal stumps of severed peripheral nerves and 2) To examine the impact of moderate levels of tensile loading on promoting the outgrowth and functional connectivity of severed peripheral nerves.*

In our most recent project period, we implanted two different biomedical devices, one a fixed length and one extensible into a rat sciatic nerve defect. We describe in the body of this report our initial characterization of the in vivo response.

BODY

Completed milestones and specific outcomes/comparisons for each milestone based on the original statement of work are summarized below. We have also included the remaining milestones as originally listed, some commentary on unexpected pitfalls, and based on these unexpected hurdles, recommended changes in the experimental plan to be requested.

Detailed results from our design, fabrication and implantation of our devices are below the completed milestones, and are also summarized in the appended draft of a manuscript in its second revision, following promising reviews, for submission to Tissue Engineering C.

Completed milestones per SOW revised following transfer from Maryland to UCSD (same as original, with modifications for UCSD IACUC approval and ACURO animal protocol approval)

Milestones and deliverables for Animal protocols:

    0.1. Approval of animal protocol by UCSD IACUC (APPROVED – UCSD IACUC PROTOCOL S11274).

    0.2. Approval of animal protocol by ACURO (APPROVED)

Milestones and deliverables for Aim 1 (fixed nerve length):
  1.1. Successful fabrication and in situ testing of nerve stabilizing device.
     a. Fabrication of collagen matrix guidance channel
     b. Fabrication of PMMA fixator
     c. Assembly of device within nerve gap created in rat cadaver, including coupling of guidance channel to nerve stumps with spiral cuff
  1.2. Successful in vivo implantation of nerve stabilizing device for short durations (48 hours).
     a. Implantation of device into anesthetized animal
     b. Removal of device and tissue harvest at 48 hour time point
     c. Freezing and sectioning of tissue for analysis in Milestone 1.4
  1.3. Successful in vivo implantation of nerve stabilizing device for longer durations (1-6 weeks).
     a. Implantation of device into anesthetized animal
     b. Removal of device and tissue harvest at 1, 3, and 6 week time points
     c. Freezing and sectioning of tissue for analysis in Milestone 1.4

Milestones 1.2 and 1.3 are partially complete – we have demonstrated successful surgical implantation of the device into animals for up to 6 weeks, and begun processing tissue for histological analysis in pilot studies. We continue to increase sample sizes per the original experimental plan; however, as detailed in unanticipated pitfalls and troubleshooting, for analysis and statistical comparison of various parameters. Consequently, only 1.2.c and 1.3.c will be included in the work plan.

Milestones and deliverables for Aim 2:

2.1. Successful fabrication and in situ testing of nerve lengthening devices with both actuation mechanisms.
   a. Fabrication of collagen matrix guidance channel (same as for Aim 1)
   b. Fabrication of PMMA fixators, including actuation assemblies
   c. Assembly of device within nerve gap created in rat cadaver
   d. Successful application of nerve stretch with at least one of the actuation devices in a rat cadaver

Remaining milestones per original SOW.

Remaining milestones and deliverables for Aim 1 (fixed nerve length):

1.2. Successful in vivo implantation of nerve stabilizing device for short durations (48 hours).
   c. Freezing and sectioning of tissue for analysis in Milestone 1.4
1.3. Successful in vivo implantation of nerve stabilizing device for longer durations (1-6 weeks).
   c. Freezing and sectioning of tissue for analysis in Milestone 1.4
1.4. Comparison of nerve outgrowth, cytoskeletal stability, organelle accumulation, and myelination between 10 mm (no device), 10 mm (device), and 15 mm (device) groups at all four time points. The following measurements will be compared using a 2-way ANOVA, considering gap length vs. time.
   a. Comparison of nerve outgrowth across groups: distance of farthest neuron terminal beyond original stump ending or, if applicable, distance over which degeneration has occurred.
   b. Comparison of cytoskeletal stability: proportion of axonal length containing properly aligned neurofilament, tubulin, or actin staining. Alternately, the density of cytoskeleton-containing spheroids (indicating instability) along the nerve.
   c. Comparison of organelle accumulation: density of mitochondrial or CGRP staining in stump ending.
   d. Comparison of myelination: nerve fiber diameter and myelin diameter. Position along nerve length may be a covariate (ANCOVA).

Remaining milestones and deliverables for Aim 2 (actuation devices):

2.2. Successful in vivo implantation of nerve lengthening device for longer durations (1-6 weeks)
   a. Implantation of devices into anesthetized animals
   b. Removal of device and tissue harvest at 1, 3, and 6 week time points
   c. Freezing and sectioning of tissue for analysis in Milestone 2.4

2.3. Statistical comparison of nerve outgrowth, cytoskeletal stability, organelle accumulation, myelination, and toe spreading (function) between 10 mm (no device), 10 mm (device), and 15 mm (device) groups for both actuation mechanisms at all three time points. The following measurements will be compared using a 2-way ANOVA, considering gap length vs. time, for each actuation mechanism.
   a. Comparison of nerve outgrowth across groups: distance of farthest neuron terminal beyond original stump ending or, if applicable, distance over which degeneration has occurred.
   b. Comparison of cytoskeletal stability: proportion of axonal length containing properly aligned neurofilament, tubulin, or actin staining. Alternately, the density of cytoskeleton-containing spheroids.
   c. Comparison of organelle accumulation: density of mitochondrial/CGRP staining in stump ending
   d. Comparison of myelination: nerve fiber diameter and myelin diameter. Position along nerve length may be a covariate (ANCOVA)
   e. Comparison of functional recovery: measured gap between 1st and 5th toes during walking track analysis.

Pitfalls and deviations from original strategy
As indicated in project period 1:
We have made two minor deviations in the design of the fixator device, both regarding material choice. Instead of PMMA, the backbone and telescoping mechanisms of the devices have been fabricated out of stainless steel, which is also FDA approved for implantation. The advantages of this material are the ready availability of prefabricated stainless steel rods and tubes of narrow diameter. In addition, due to the unpredictability of collagen expansion following hydration, we have fabricated tissue engineered guidance channels from PLGA, which is also an FDA-approved biocompatible scaffold used previously in a nerve regeneration model [10, 11].

Project period 2:
The PI has relocated his laboratory to UCSD, within the department of Orthopaedic Surgery. Collaborations with relevant

investigators at the University of Maryland and at the FDA remain intact, and the key postdoc working on the project, Dr. Ting-Hsien Chuang, also moved to UCSD. As a result of this change of institution, animal studies were placed on hold until the approval of the animal protocol at UCSD (and subsequent approval by ACURO). A no-cost extension was requested and granted through April 2013.

Observations from the large number in vivo implantations since the initial project period were quite informative. Feasibility was demonstrated for long-term implantations for both static and dynamics devices. Not surprisingly for a novel technology, though, several minor design changes were required to improve the performance of the device.

First, devices translated more than expected, despite the tight constraints of the nerve bed. This resulted in an inability to ascertain the exact distribution of strain in the proximal and distal stump. Consequently, simple anchors (1 mm in length) into the underlying muscle were fabricated out of stainless steel. These successfully tethered the device to the muscle bed, prevented translation, and confirmed the accurate application of desired stain onto the nerve stumps.

Second, a simple non-ratchet lengthening device was tested, and a ratchet-based lengthening device was designed. After in situ testing, it was apparent that we could achieve the benefits of both devices by simply extending the guidewire housing slightly extracorporeally, to provide a fixed frame of reference against which the guidewire may be actuated. The simpler device is adequate to test our hypotheses, and a request will be made to modify the SOW accordingly .

Modified devices are being used across all implantation groups, and do not appear to require an SOW modification, given the current language of the SOW.

Third, 15mm gaps have been determined to be unfeasible for device implantation within the rat. This length was chosen based on literature reports of passive nerve scaffolds being implanted in such gaps, but in the typical large adult rat, including those >400g, this length requires either the severing of individual distal branches of the sciatic nerve or more invasive dissection further proximally. In either case, sufficient nerve does not exist for cuff deployment and proper device orientation. A request will be made to replace it in the static group, with the new proposed 1.4.

> New Proposed 1.4: Comparison of nerve outgrowth, cytoskeletal stability, organelle accumulation, and myelination between 10 mm (no device), 10 mm (device, 10% strain; physiological), and 10 mm (device, 20% strain; superphysiological) groups at all four time points. The following measurements will be compared using a 2-way ANOVA, considering intervention vs. time.

In the dynamic group, the group will be eliminated outright.

> New proposed 2.3: Statistical comparison of nerve outgrowth, cytoskeletal stability, organelle accumulation, myelination, and toe spreading (function) between 10 mm (no device), 10 mm (device) at all time points. The following measurements will be compared using a 2-way ANOVA, considering intervention and time.

Fourth, the biggest unanticipated hurdle was the surprisingly high rate of autotagy (self-mutilation) among injured rats at time points >2 weeks. We did not observe this problem at earlier time points, for unknown reasons. However, up to 40% of animals demonstrated some form of toe or hindlimb chewing, rendering these animals un-analyzable. While this outcome is not unheard of for nerve (or spinal cord) injury, it was nonetheless unanticipated given the large number of short-time point surgeries completed. Consultation with the veterinary staff and a deeper examination of autotagy literature led to our recent modification of pre-op and post-op dosages for buprenorphine and metacam, extending the latter to the experimental endpoint rather than cutting it off at 1.5 weeks, as originally recommended by the veterinary staff. This phenomenon is often equated to a phantom limb phenomenon in humans; therefore, it may not be a response to pain, in animals. Therefore, in addition, bitter apple delivered within a New Skin liquid bandage will also be applied topically, as a deterrent. This constituted a minor amendment within our IACUC, and a similar request will be made to ACURO. The downside of the observed autotagy was the inability to use many of these animals for analysis; these experiments are being repeated under new dosing guidelines.

These changes do not affect the general testing of our proposed hypotheses; a separate study, outside of the scope of this proposal, will examine the implementation of our device in longer deficits, within a more amenable rabbit sciatic nerve model. Practically speaking, the elimination of one actuation mechanism in vivo and the elimination of the 15mm actuation group allows us to stay on budget and time-frame, in light of the unanticipated device redesign and probems with autotagy.

Detailed results.

Device fabrication

The device is composed of three major components: self-sizing silicone spiral nerve cuffs, poly(lactic co-glycolic) acid (PLGA) NGCs, and a mechanical backbone to which the cuffs and nerve guidance channels are attached (Fig. 1).
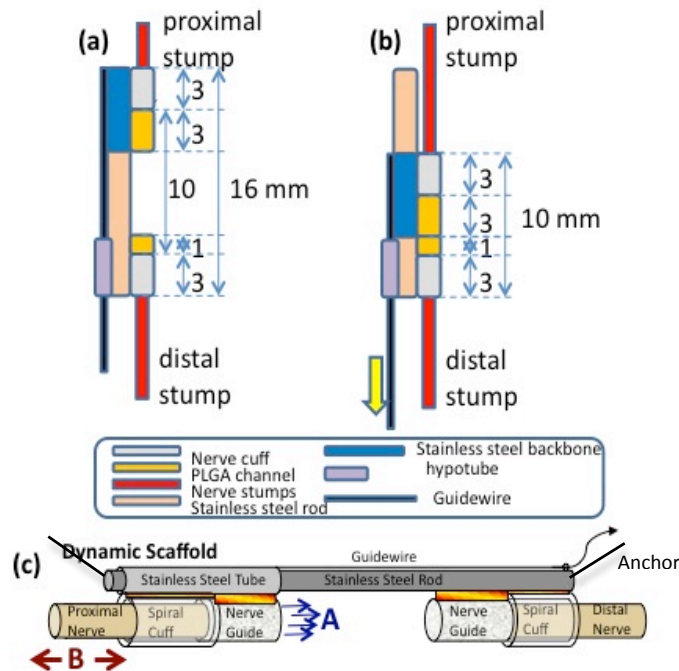

Fig. 1 Device design The nerve stretching device is composed of spiral nerve cuffs, PLGA nerve guidance channel and stainless steel backbone **(a)** before stretching; **(b)** being stretched; **(c)** Two regions of regeneration, A: Enhanced axonal outgrowth into a tissue engineered nerve guide/scaffold; B: Lengthening of intact regions of nerve stumps – cf. limb lengthening

Spiral nerve cuffs

The fabrication of spiral nerve cuffs is shown in Fig 2a; two silicone sheets, one stretched to a specific strain, were glued together and clamped between two metal slabs. After curing, the compound sheet curled into spiral shape (Fig. 2b). In addition, the microgroove pattern was successfully transferred to the compound silicone sheet (Fig. 2c). The curled sheet was cut into small segments of 3 mm to serve as nerve cuffs. Measurements of the inner diameter of the spiral nerve cuffs showed a decreasing trend of inner diameter with increasing percent strain in the silicone sheet. In the test of efficacy of nerve gripping, cuffs of 70% and 100% pre-loaded strain (average inner diameter of 1.35mm and 0.85mm, respectively) successfully held the nerve without slippage, though self-sizing was more reliable in the 100% strain cuff. The inner surface of spiral nerve cuff was in close contact with the nerve stump but caused minimal nerve compression, based on observation at high magnification through a dissecting microscope. The 100% strain cuff was used in the following studies based on its reliable self-sizing to the nerve.
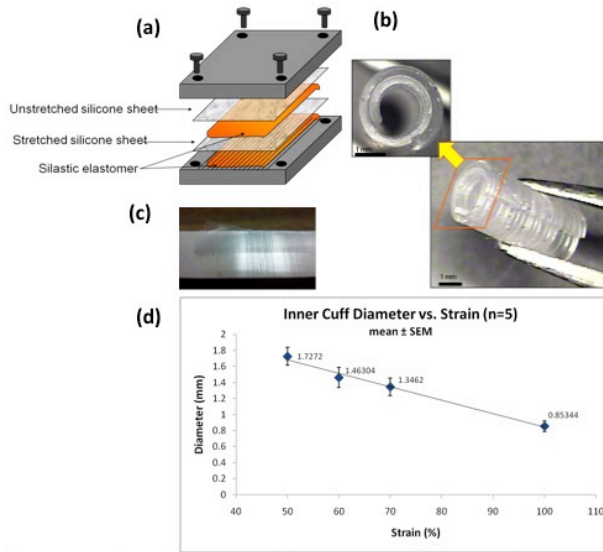
Fig. 2 Fabrication of spiral nerve cuff (a) two layers of silicone sheets (with one being stretched) are glued together; **(c)**the microgroove pattern on the slab is transferred to the cuff; **(b)**the resultant curled nerve cuff after curing; **(d)** Inner diameter decreases with increasing % strain of pre-stretched silicone sheet

PLGA nerve guidance channel

Due to phase separation, white solid state PLGA precipitated gradually onto the water-eluding alginate hydrogel. After retrieval of the alginate rod, a hollow PLGA tube was produced (Fig. 3a). The PLGA tube was then cut into small segments to serve as NGCs (Fig. 3b). As expected based on previous studies, cross-sectional SEM images showed that the PLGA tubes had a porous structure (Fig. 3c). (42)
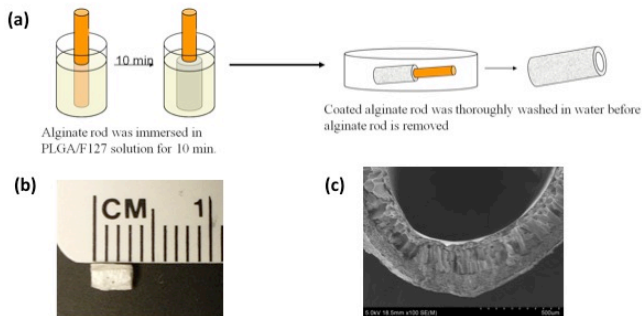

Fig. 3 Fabrication of PLGA nerve guidance channel (a) Tubular PLGA layer is formed due to phase separation when water diffuses out the alginate rod **(b)** PLGA tube is cut into short segments to serve as nerve guidance channel **(c)** Cross section SEM picture of PLGA tube

PLGA cytotoxicity

For the test on extracts cytotoxicity method, SH-SY5Y cell fed with medium pre-incubated with PLGA showed similar morphology (Fig. 4a) to that of control (Fig. 4b). Live/Dead® cell viability assay also showed that only very few dead cells were present (Fig. 4c; Fig. 4d as control). From both results, no signs of cytotoxicity were observed. For cell proliferation on PLGA, the experimental group displayed no significant difference compared to the control group (Fig. 4e). For direct observation of morphology of cell grown on PLGA, wheat germ agglutinin (WGA) staining was performed. From the fluorescent microscopy images, we observed SH-SY5Y cells spread and extended outward (Fig 5a). In light of neuronal preferences for a protein-coated substrate, we also seeded cells on PLGA coated with laminin. Neurites appeared more robust and cells appeared more dense on laminin-coated PLGA (Fig. 5b). Thus, overall, PLGA induced no significant effects on viability or morphology.
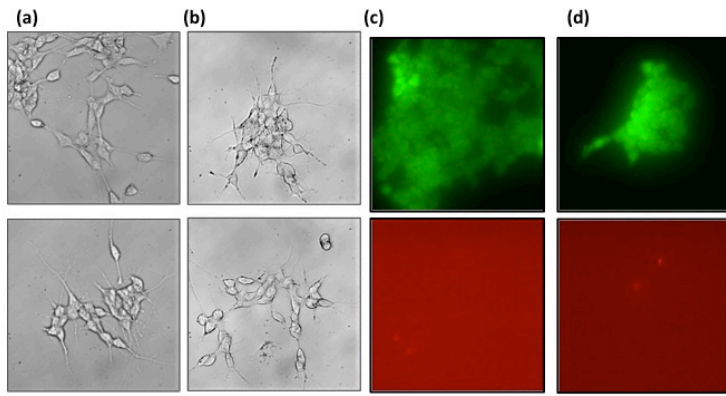
Fig. 4 PLGA cytotoxicity test, indirect contact method (a), (b) culture medium was pre-incubated with PLGA at 37˚C for 3 days to test if any leachable substance from PLGA would have cytotoxic effects on the cells. No signs of PLGA cytotoxicity was observed in terms of cell morphology (b). Live/Dead® cell viability assay showedvVery few dead cells were found in either (c)control or (d)PLGA group (cells fed with PLGA-incubated culture media)
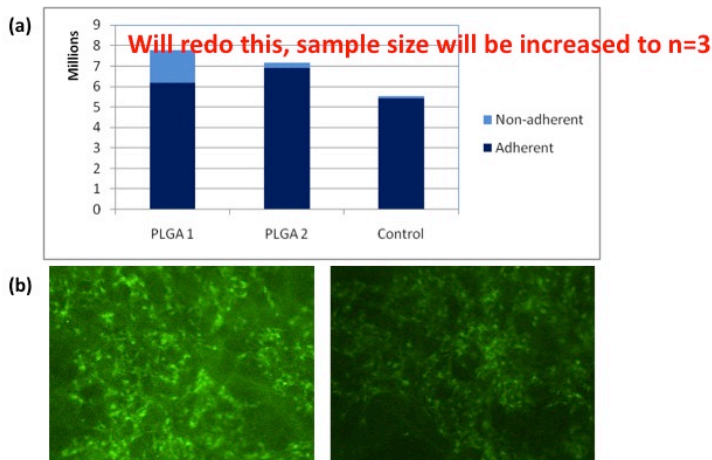
.

Fig. 5 PLGA cytotoxicity test, direct contact method (a)1x10^6 SY5Y cells were seeded in each PLGA coated or control petri dishes and culture for 5 days. PLGA group was no different form control in terms of cell proliferation. **(b)** visualization of cell morphology by wheat germ agglutinin fluorescent staining

In situ implantation

The assembled device is shown *ex vivo* in Fig. 6a (before stretching nerve) and Fig. 6b (after stretching nerve). Deployment of the device was first performed in a rat cadaver. The device was implanted appositional to the transected nerve with the nerve stumps wrapped into the spiral cuffs (Fig. 6c). By pulling the guidewire attached to the hypotube sliding over the rod, the nerve stump of proximal end was successfully stretched and placed under tension (Fig. 6d). Following positioning, to prevent translation, the device was fixed to the underlying muscle with medical grade super glue or stainless steel anchors.
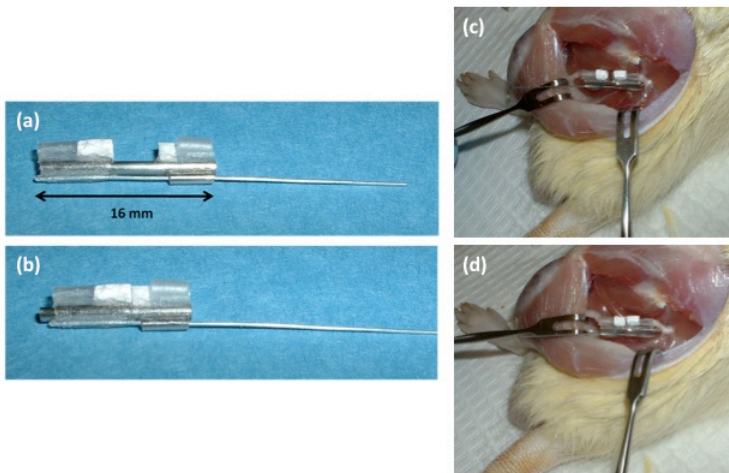
Fig. 6 Outlook of the device and implantation in rat cadaver. (a), (c) fully extended device **(b), (d)** device after stretching Note that implanted device can stretch the nerve stumps without slippage

In vivo implantation

We then tested whether the device enabled nerve regeneration. The device was successfully deployed across a sciatic nerve defect in anesthetized rat (n=5), demonstrating feasibility and repeatability of device implantation *in vivo*. In all animals, the animal tolerated the device for up to two weeks without obvious signs of infection. Slight fibrosis was observed (Fig 7b), but the device backbone was excised cleanly (Fig 7c), and both nerve stumps remained within the confinement of the cuffs without slippage. The proximal stump extended 6 mm beyond the cuff (into the scaffold; Fig 7e). These results contrasted sharply with the response in non-device controls (n=3), which revealed a variable regenerative response, including misalignment, a bulging proximal stump, and considerable fatty infiltration and fibrosis distally (two examples in Fig 7e-f). Immunohistological assessment confirmed successful outgrowth of neurons into the guidance channel as well as neuronal maturity, based on positive staining of phosphorylated neurofilaments (SMI31) and aligned Schwann cells (S100 antibody) (Fig 8a-b). Such alignment and staining was similar to that in contralateral controls (Fig 8c-d). Based on these promising results, studies are currently underway to use this device to test hypotheses regarding the role of mechanical loading in nerve regeneration.
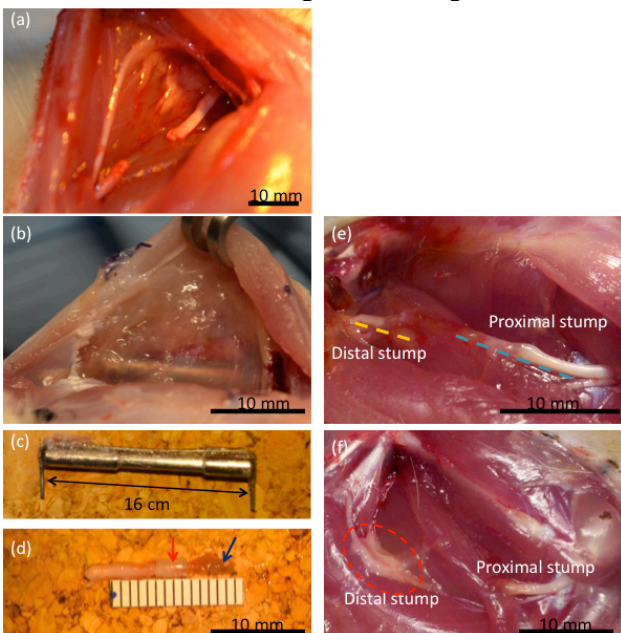

Figure 7. (a) sciatic nerve defect; (b) fibrotic encapsulation of the device; (c) cleanly excised device; (d) successfully regenerating proximal nerve stump; (e) poorly aligned regeneration in absence of device.
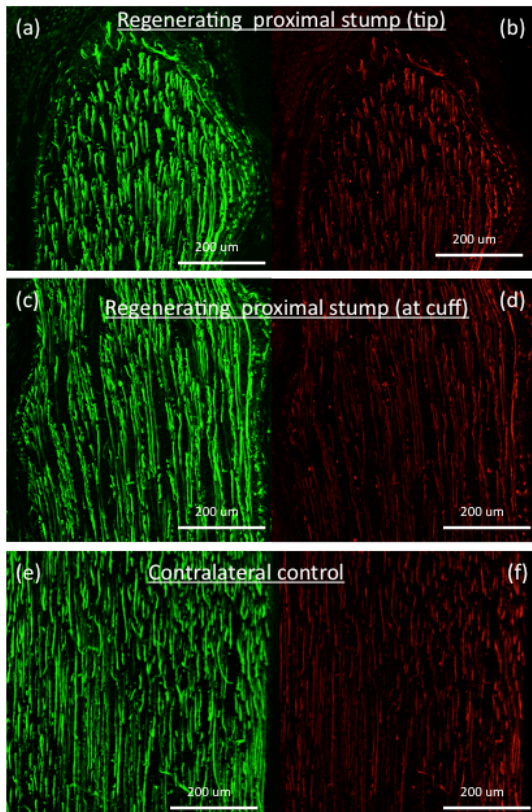
Figure 8: (a)-(d) successfully regenerating proximal nerve and (e)-(f) contralateral control reveal well-aligned phosphorylated neurofilaments (a,c,e) and Schwann cells (b,d,f).

KEY RESEARCH ACCOMPLISHMENTS
- Design and fabrication of two novel biomedical devices for lengthening peripheral nerves in vivo, as a strategy for accelerating nerve regeneration.
- Successful implantation of devices in a rat sciatic nerve injury model for up to six weeks.
- Successful observation of neural regeneration at as early as 2 weeks.

REPORTABLE OUTCOMES
- *Conference abstract:* Wilson, R., Fisher, J.P., Shah, S.B. (2010) "Peripheral Nerve Regeneration Using a Tension-inducing Scaffold." BMES 2010 Annual Meeting, Austin, Texas.
- *Conference abstract:* Chuang, T.-H., Wilson, R., Fisher, J.P., Shah, S.B. (2011) "A Novel Internal Fixator Device for Peripheral Nerve Regeneration." BMES 2011 Annual Meeting, Hartford, Connecticut.
- *Manuscript in revision:* Chuang, T.-H., Wilson, R., Fisher, J.P., Shah, S.B. (2011) "A novel biomedical device for tensile loading of peripheral nerves to promote regeneration." *(Manuscript in revision, submitted to Tissue Engineering Part C (Methods).)*
- *Invention disclosure in preparation:* Chuang, T.-H., Wilson, R., Fisher, J.P., Shah, S.B. (2011) "A biomedical device for securing and stretching peripheral nerves." *(will be submitted to University of Maryland, College Park OTC and UCSD Tech Transfer office concurrent with manuscript acceptance.)*

CONCLUSION

Most strategies for transected peripheral nerve repair focus on bridging the gap in a tension-free setting. The role of a biomechanical stimulus has not been broadly explored. We present a novel biomedical device that is able to impose tensile loading to severed nerve stumps towards the acceleration of peripheral nerve regeneration. The primary innovation of our multi-disciplinary proposal lies in the integration of tensile loading (stretch) and tissue engineering strategies for peripheral nerve regeneration. By imposing tensile loads in parallel with existing tissue engineering strategies for nerve repair (e.g., a nerve guidance channel), we anticipate significantly accelerated nerve regeneration. Progress to date has demonstrated the feasibility of such a strategy and early promising regenerative outcomes, providing us confidence for executing and interpreting in vivo studies in the next project period.

REFERENCES

1. Covey, D.C. (2006). Combat orthopaedics: a view from the trenches. J Am Acad Orthop Surg *14*, S10-17.
2. Hofmeister, E.P., Mazurek, M., and Ingari, J. (2007). Injuries sustained to the upper extremity due to modern warfare and the evolution of care. J Hand Surg Am *32*, 1141-1147.
3. Schmidt, C.E., and Leach, J.B. (2003). Neural tissue engineering: strategies for repair and regeneration. Annu Rev Biomed Eng *5*, 293-347.
4. Abe, I., Ochiai, N., Ichimura, H., Tsujino, A., Sun, J., and Hara, Y. (2004). Internodes can nearly double in length with gradual elongation of the adult rat sciatic nerve. J Orthop Res *22*, 571-577.
5. Bray, D. (1984). Axonal growth in response to experimentally applied mechanical tension. Dev Biol *102*, 379-389.
6. Bueno, F.R., and Shah, S.B. (2008). Implications of tensile loading for the tissue engineering of nerves. Tissue Eng Part B Rev *14*, 219-233.
7. Pfister, B.J., Iwata, A., Meaney, D.F., and Smith, D.H. (2004). Extreme stretch growth of integrated axons. J Neurosci *24*, 7978-7983.
8. Topp, K.S., and Boyd, B.S. (2006). Structure and biomechanics of peripheral nerves: nerve responses to physical stresses and implications for physical therapist practice. Phys Ther *86*, 92-109.
9. Zheng, J., Lamoureux, P., Santiago, V., Dennerll, T., Buxbaum, R.E., and Heidemann, S.R. (1991). Tensile regulation of axonal elongation and initiation. J Neurosci *11*, 1117-1125.
10. Oh, S.H., Kim, J.H., Song, K.S., Jeon, B.H., Yoon, J.H., Seo, T.B., Namgung, U., Lee, I.W., and Lee, J.H. (2008). Peripheral nerve regeneration within an asymmetrically porous PLGA/Pluronic F127 nerve guide conduit. Biomaterials *29*, 1601-1609.
11. Oh, S.H., and Lee, J.H. (2007). Fabrication and characterization of hydrophilized porous PLGA nerve guide conduits by a modified immersion precipitation method. J Biomed Mater Res A *80*, 530-538.

APPENDICES

Appended is a draft of the manuscript to be submitted. It provides additional details on methodology and key results in support of milestones achieved. Figures are numbered identical to those provided in the text above, and therefore are not included again.

A novel internal fixator device for peripheral nerve regeneration

Ting-Hsien Chuang[1,2‡], Robin E. Wilson[3‡], John P. Fisher[1], Sameer B. Shah[1,2*]

[1] Fischell Dept. of Bioengineering, University of Maryland, College Park, MD 20742

[2] Dept. of Orthopaedic Surgery, University of California, San Diego, La Jolla, CA 92093

[3] Dept. of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106

* Author to whom correspondence should be addressed

‡ Contributed equally to this work

9500 Gilman Drive
Mail Code 0863
La Jolla, CA 92093
Phone: (858) 822-0720
Fax: (858) 822-3807
Email: sbshah@ucsd.edu

Abstract

Recovery from peripheral nerve damage, especially for a transected nerve, is rarely complete, resulting in impaired motor function, sensory loss, and chronic pain with inappropriate autonomic responses. In consequence, strategies for enhancing peripheral nerve repair are of high clinical importance. Tension is a key determinant of neuronal growth and function. In vitro and in vivo experiments have shown that moderate levels of imposed tension (strain) encourage axonal outgrowth; however, few strategies of peripheral nerve repair emphasize the mechanical environment of the injured nerve.

Towards the development of more effective nerve regeneration strategies, we hypothesize that moderate tensile loading of peripheral nerves may stabilize nerve degeneration and promote regeneration. Here we present a novel design of a tension-inducing device with the aim of achieving more effective peripheral nerve repair. By imposing mechanical loads in parallel with existing tissue engineering strategies for nerve repair, we anticipate significantly accelerated nerve regeneration in two regimes of nerve extension – traditional extension through axonal outgrowth into a scaffold as well as novel extension in intact regions of the proximal nerve.

Spiral silicone nerve cuffs were fabricated to grip nerve stumps without slippage and tension was successfully induced during the actuation of device. In addition, tubular poly(lactic co-glycolic) acid (PLGA) constructs were placed appositionally to guide nerve extension.

PLGA nerve guidance channel cytotoxicity was tested on neuron-like SH-SY5Y cells using direct contact and extract methods and no signs of cytotoxic effects were seen in terms of cell morphology and proliferation. We confirmed the feasibility of implanting our device across a sciatic nerve gap and lengthening the proximal nerve stump in a rat cadaver. Nerve outgrowth into the guidance scaffold was observed following device extraction at a two-week time point, and regeneration of mature neurons was indicated by positive staining for phosphorylated neurofilaments and myelin. The successful fabrication and implementation of our device provides a novel method for examining mechanical influences on nerve regeneration.

Introduction
Peripheral nerve injury may result from trauma, cancer, or congenital defects (1-3), with the severity of injury categorized by the degree and reversibility of structural changes to the nerve (4, 5). Recovery from peripheral nerve damage, especially for a transected nerve, is rarely complete, resulting in impaired motor function, sensory loss, and chronic pain with inappropriate autonomic responses that may seriously impair the quality of life. It is estimated that more than 50,000 peripheral nerve repair procedures are performed annually in the United States alone, imposing a financial burden of 7 billion dollars per year (2). In consequence, strategies for enhancing peripheral nerve repair are of high clinical importance.

Short gaps (<10mm) may be readily bridged by surgical reconnection of stumps(6) or through a variety of grafts or nerve guidance channels(NGCs)(3, 7-10). Such nerve grafts can be autologous (11, 12) or non-autologous (13, 14). The use of non-nerve biological tissues such as skeletal muscle (15, 16), veins (17-19) and tendon (20) has also been reported. NGCs of natural or synthetic biomaterials have been designed with increasing complexity, including several that incorporate chemical and biological cues to encourage nerve regeneration. For instance, Schwann or stem cells have been incorporated into grafts along with neurotrophic factors that promote neurite extension into the artificial conduit (21-24). Improved fabrication technology has also enabled the construction of nerve guidance channels with a variety of geometries, porosities, and material properties (3, 25). Despite such developments, engineered NGCs do not perform as well as autologous nerve grafts, which are currently the gold standard of nerve graft transplantation. However, the availability of autologus grafts is often limited; moreover, donor site morbidity, which develops with the tissue harvest and size mismatch between defect nerve and graft dimension, may pose additional problems. In addition, even autologous grafts are not effective for bridging gaps longer than 10mm, because degeneration outpaces the maximal neuronal extension rate(~1mm/day) (26). Therefore, there is a need to develop a strategy for repairing large gaps (>10mm).

Tension is an influence on nerve growth and function that has been underexamined in the context of nerve regeneration. Nerves bear tension under several physiological scenarios. Peripheral nerves exist under tension, releasing strains of up to 11% following transection (27-29), and may deform additionally, in some cases upwards of 20%, during joint movement (30-32). During growth, axonal tension, induced either by growth cone attachment to the substrate or to other neurites, serves as a survival and stabilization signal for axons. Conversely, neurites not under tension retract (33). It has also been shown that moderate tensile loads can accelerate neuronal growth, both in vitro and in vivo (34-39).

Given this background, towards the development of more effective nerve regeneration strategies, we hypothesize that moderate tensile loading of peripheral nerves may stabilize nerve degeneration and promote regeneration. By imposing mechanical loads in parallel with existing tissue engineering strategies for nerve repair, we anticipate significantly accelerated nerve regeneration. In particular, we expect two regimes of nerve extension – traditional extension through axonal outgrowth into a scaffold as well as novel extension in intact regions of the proximal nerve (Fig 1). Based on this rationale, with the aim of achieving more effective peripheral nerve repair, we present the novel design, fabrication, and preliminary implementation of a modular tension-inducing internal fixator device that integrates engineered guidance scaffolds.

Materials and Methods
Device fabrication
The device is composed of three major components: self-sizing silicone spiral nerve cuffs, poly(lactic co-glycolic) acid (PLGA) NGCs, and a mechanical backbone to which the cuffs and nerve guidance channels are attached (Fig. 1).

Spiral nerve cuffs:
In order to impose tensile loading on the transected nerve, self-sizing spiral nerve cuffs were created to firmly grip the proximal and distal nerve stumps, without exerting excessive compression. We modified a protocol for the fabrication of spiral cuff electrodes (40); however, no electrode was embedded in our cuffs. In brief, two Silastic® silicone sheets (Dow Corning) of 0.005 inches in thickness, with the first silicone sheet being stretched to a specified strain while the second sheet remained unstretched, were bound together using a silicone adhesive (biomedical grade Silastic® elastomer MDX4-4210, Dow Corning). The compound silicone sheet was clamped between two stainless steel slabs and cured at 60°C for 2 hours. In order to increase friction between

the cuff and nerve stump, a layer of silicone adhesive was applied onto a grooved slab before clamping the two silicone sheets in between. After curing, the microgroove pattern was thus transferred to the compound silicone sheet, thereby creating a rough interior surface. The resultant spiral sheet was cut to a length of 0.5 cm and a width corresponding to approximately one and a quarter spirals around a typical rat sciatic nerve, to serve as nerve cuffs.

Nerve Guidance channel:
Preparation of the nerve guides involved a modified phase inversion technique (41). PLGA (lactic to glycolic acid mol ratio of 75:25, M_w =66,000-107,000, Sigma) was dissolved in tetraglycol (Sigma) at 60°C (10 wt%) and then Pluronic® F-127 was added (3 wt%) to increase the hydrophilicity. Alginate hydrogel rods were formed by first dissolving alginate (Sigma) in water (4 wt%) and injecting the solution into 2% $CaCl_2$ with a syringe (needle gauge 14). After saturation, the alginate hydrogel was immersed in the PLGA / Pluronic® F-127 solution. Due to phase separation between polymer (PLGA/F127) and non-solvent (water in alginate hydrogel), PLGA precipitated onto the alginate rod as water diffused out of the hydrogel. The construct was washed thoroughly in water for 24 hours to remove excess tetraglycol. After retrieval of the alginate rod; the resultant PLGA hollow tubes were dried at room temperature and cut into the desired length to serve as nerve guidance channels.

Mechanical backbone:
A stainless steel rod of 1.57 mm in diameter (Component Supply Co.) was used as the backbone of the device. This material was selected due to its strength and previous applications in implanted devices. The end of the device to be affixed to the proximal stump consisted of one spiral nerve cuff and a PLGA nerve guidance channel fixed onto a 14 gauge thin wall stainless steel hypotube (Component Supply Co.), designed to slide along the stainless steel rod. The hypotube had a guidewire attached, enabling us to stretch the proximal nerve stump. A second spiral nerve cuff and PLGA nerve guidance channel were fixed to the distal terminal of the inner stainless steel rod. Medical grade n-butyl cyanoacrylate adhesive was used to glue the cuffs, guidance channel and guidewire to the mechanical backbone of the device.

Characterization of spiral nerve cuffs
To find the correlation between pre-loaded strain in the silicone sheet and degree of spiral curl, the inner diameters of nerve cuffs of 50%, 60%, 70% and 100% strains were measured and compared. Inner diameter was defined as the distance from the inner most tip of the curvature to the point which was 180° of turn from the starting point. Evaluation of no-slip gripping of nerve was performed in situ.

PLGA cytotoxicity
Both direct contact and test on extract methods of in vitro cytotoxicity test were performed on SH-SY5Y cells (ATCC # CRL-2266) derived from human neuroblastoma. The test on extracts method was performed as suggested in the ISO 10993-5 standard, to test the impact of leachable factors from PLGA on cell viability. Cell culture medium (90% MEM/F12, 10% fetal bovine serum) was incubated in PLGA-coated petri dishes at 37°C for 24 hours and then was used to feed SH-SY5Y cells. Cellular proliferation was compared by cell counting followed by t-test. Cell viability was tested using Live/Dead© fluorescence assay. Morphology of cells fed with PLGA-incubated medium was observed under an inverted light microscope. For the direct contact study, visualization of morphology of cells grown on PLGA using traditional transmitted light microscopy was difficult due to the opaque nature of the thick PLGA layer. Therefore, SH-SY5Y cells were first seeded onto discs coated with PLGA or PLGA followed by laminin in a petri dish filled with culture medium. Discs were then inverted onto coverslips for imaging. For identifying the contours of the neuronal cells, they were stained with Alexa Fluor® 488 conjugated wheat germ agglutinin, which binds to sialic acid and N-acetylglucosaminyl sugar residues which reside on the cell membrane. Subsequent imaging was performed on an inverted widefield fluorescence microscope (Nikon TE-2000U) using filters appropriate for FITC visualization. To examine PLGA cytotoxicity using the extract method, SH-SY5Y cell number was quantified by first trypsinizing adherent cells and then counting the proportion that excluded trypan blue.

Device implantation in situ:
Animal use protocols were approved by the University of Maryland, College Park Institutional Animal Care & Use Committee. For *in situ* studies, adult Sprague Dawley rats weighing about 0.5kg were sacrificed by CO_2 euthanization. The sciatic nerve was exposed by separating branches of the hamstring muscles. A small segment of sciatic nerve of about 10mm was removed, and then the device was implanted appositional to the nerve

stumps, and oriented along the original axis of the nerve within the nerve bed. Proximal and distal nerve stumps were wrapped with the spiral nerve cuffs. The guidewire was pulled to stretch the proximal stump of the sciatic nerve proximal stump to the desired length. Once the device was positioned appropriately, it was prevented from translating by fixing it to the underlying muscle bed with < 1µl medical adhesive or steel anchors.

Device implantation in vivo and immunohistochemistry:

In vivo studies were approved by UMCP and UCSD IACUC. Adult Sprague Dawley rats weighing between 425-500g were anesthetized using 5% isoflurane, and then injected with analgesic (0.05 mg/kg buprenorphine) and antibiotics (5 mg/kg Baytril®). Anesthesia was maintained by 2% isoflurane throughout the surgery. The surgical site was shaved and sterilized, and the sciatic nerve was exposed and severed as above. The device was implanted, the incision to the muscle was closed by 4-0 Vicryl® suture and the incision to the skin with 3-0 Prolene® monofilament suture. The rat was kept for two weeks with full access to food and water, and then was sacrificed to examine response to device implantation. For comparison, we also examined the response to a 10 mm sciatic nerve gap without device implantation . The length of the regenerating proximal nerve stump beyond the cuff (i.e., into the channel) was measured. The regenerating nerves were then rapidly frozen in liquid nitrogen-chilled isopentane for histology preparation. Because the PLGA scaffold adhered poorly to the glass slide during transfer, frequently lifting tissue off of the slide as well, it was removed prior to freezing. However, the position of the scaffold along the nerve was marked, and used as a frame of reference to assess regeneration. The regenerating nerve was embedded in Optimal Cutting Temperature (Sakura Fintek USA Inc.; Torrance, CA) and cut into 10um sections for immunostaining. Primary antibodies used were mouse anti-rat SMI-31 (axon: phosphorylated neurofilament) monoclonal antibody (SMI-31, Covance; Princeton, NJ) diluted at 1:200 and rabbit anti-rat S-100 (myelin) polyclonal antibody (Sigma-Aldrich; St. Louis, MO) diluted at 1:200. Secondary antibodies used were Alexa-Flour 488 conjugated goat anti-mouse and Alexa-Flour 594 conjugated goat anti-rabbit diluted at 1:200 (Invitrogen; Carlsbad, CA). The immunostained tissue slices were preserved with a drop of VectaShield mounting media (Vector Laboratories; Burlingame, CA) and then coverslipped.

Imaging

Immunofluorescence was imaged by confocal microscopy using an inverted microscope scanning system (Leica TCS SP5 LSM) with a 10X NA 0.4 objective. Excitation wavelengths were 488 nm and 594nm; the corresponding emission wavelengths were 500-580 nm and 605-700 nm, respectively.

Results

Spiral nerve cuffs

The fabrication of spiral nerve cuffs is shown in Fig 2a; two silicone sheets, one stretched to a specific strain, were glued together and clamped between two metal slabs. After curing, the compound sheet curled into spiral shape (Fig. 2b). In addition, the microgroove pattern was successfully transferred to the compound silicone sheet (Fig. 2c). The curled sheet was cut into small segments of 3 mm to serve as nerve cuffs. Measurements of the inner diameter of the spiral nerve cuffs showed a decreasing trend of inner diameter with increasing percent strain in the silicone sheet. In the test of efficacy of nerve gripping, cuffs of 70% and 100% pre-loaded strain (average inner diameter of 1.35mm and 0.85mm, respectively) successfully held the nerve without slippage, though self-sizing was more reliable in the 100% strain cuff. The inner surface of spiral nerve cuff was in close contact with the nerve stump but caused minimal nerve compression, based on observation at high magnification through a dissecting microscope. The 100% strain cuff was used in the following studies based on its reliable self-sizing to the nerve.

PLGA nerve guidance channel

Due to phase separation, white solid state PLGA precipitated gradually onto the water-eluding alginate hydrogel. After retrieval of the alginate rod, a hollow PLGA tube was produced (Fig. 3a). The PLGA tube was then cut into small segments to serve as NGCs (Fig. 3b). As expected based on previous studies, cross-sectional SEM images showed that the PLGA tubes had a porous structure (Fig. 3c). (42)

PLGA cytotoxicity

For the test on extracts cytotoxicity method, SH-SY5Y cell fed with medium pre-incubated with PLGA showed similar morphology (Fig. 4a) to that of control (Fig. 4b). Live/Dead® cell viability assay also showed that only

very few dead cells were present (Fig. 4c; Fig. 4d as control). From both results, no signs of cytotoxicity were observed. For cell proliferation on PLGA, the experimental group displayed no significant difference compared to the control group (Fig. 4e). For direct observation of morphology of cell grown on PLGA, wheat germ agglutinin (WGA) staining was performed. From the fluorescent microscopy images, we observed SH-SY5Y cells spread and extended outward (Fig 5a). In light of neuronal preferences for a protein-coated substrate, we also seeded cells on PLGA coated with laminin. Neurites appeared more robust and cells appeared more dense on laminin-coated PLGA (Fig. 5b). Thus, overall, PLGA induced no significant effects on viability or morphology.
.

In situ implantation

The assembled device is shown *ex vivo* in Fig. 6a (before stretching nerve) and Fig. 6b (after stretching nerve). Deployment of the device was first performed in a rat cadaver. The device was implanted appositional to the transected nerve with the nerve stumps wrapped into the spiral cuffs (Fig. 6c). By pulling the guidewire attached to the hypotube sliding over the rod, the nerve stump of proximal end was successfully stretched and placed under tension (Fig. 6d). Following positioning, to prevent translation, the device was fixed to the underlying muscle with medical grade super glue or stainless steel anchors.

In vivo implantation

We then tested whether the device enabled nerve regeneration. The device was successfully deployed across a sciatic nerve defect in anesthetized rat (n=5), demonstrating feasibility and repeatability of device implantation *in vivo*. In all animals, the animal tolerated the device for up to two weeks without obvious signs of infection. Slight fibrosis was observed (Fig 7b), but the device backbone was excised cleanly (Fig 7c), and both nerve stumps remained within the confinement of the cuffs without slippage. The proximal stump extended 6 mm beyond the cuff (into the scaffold; Fig 7e). These results contrasted sharply with the response in non-device controls (n=3), which revealed a variable regenerative response, including misalignment, a bulging proximal stump, and considerable fatty infiltration and fibrosis distally (two examples in Fig 7e-f). Immunohistological assessment confirmed successful outgrowth of neurons into the guidance channel as well as neuronal maturity, based on positive staining of phosphorylated neurofilaments (SMI31) and aligned Schwann cells (S100 antibody) (Fig 8a-b). Such alignment and staining was similar to that in contralateral controls (Fig 8c-d). Based on these promising results, studies are currently underway to use this device to test hypotheses regarding the role of mechanical loading in nerve regeneration.

Discussion

Towards accelerated peripheral nerve regeneration, we have successfully designed, fabricated, and implanted across a rat sciatic nerve gap a modular device that enables simultaneous lengthening of the proximal nerve stump and axonal outgrowth into an engineered scaffold. The novelty of our approach lies in the application of tensile loading (stretch) as a strategy to accelerate peripheral nerve regeneration, and the seamless integration of this strategy with existing tissue engineering strategies for nerve repair.

A role of tension in nerve repair

Although several recent studies implicate tension as a key regulator of neuronal survival, the role of tension in peripheral nerve repair is debatable. In clinical practice, tension-free repair remains the gold standard (43, 44). The rationale behinds this is that tension has been attributed to hampering nerve regeneration, possibly by promoting scar tissue formation and adhesion (45, 46) or by impairing blood supply (47, 48); studies showed blood flow reduced approximately 50% when nerves are stretched to 10% (49, 50). However, Sunderland et al. showed that modest levels of tension were well tolerated in rat sciatic nerve regeneration model(51), and robust nerve regeneration were observed at 4 weeks in all except the 9mm-repair group. Moreover, Smith and colleagues demonstrated the capacity of integrated axons to undergo substantial growth via continuous mechanical tension; indeed a stretch-induced axonal growth of 1 cm in length by 10 days of stretch was achieved (52). The most compelling evidence for a role of tension in nerve regeneration may be found in animal and human models of limb lengthening, where nerves tolerate substantial deformations during the lengthening process (34, 53-62).

Therefore, the more appropriate debate should be on defining an appropriate threshold beyond which tension is detrimental. We hypothesize that by maintaining physiological strain during the lengthening process, nerve regeneration will be accelerated.

Device features and characterization

Our device, which includes silicone spiral nerve cuffs and PLGA nerve guidance channels attached to a stainless steel backbone, is capable of stretching nerve without slippage, thus inducing moderate strain to the nerve stumps. This is made possible by the design of spiral cuffs patterned with microgrooves on the inner surface, which successfully held the nerve during deformation. Upon self-sizing, the cuffs had an average inner diameter similar to that of rat sciatic nerve, with minimal apparent nerve compression. This is consistent with the original implementation of similar cuffs used as implantable electrodes (40).

Though any scaffold may be integrated into our device, we initially selected tubular PLGA/Pluronic F127 conduits as nerve guidance channels. This material was selected based on its hydrophilicity, our ability to carefully control its geometry, even in hydrous conditions, as well as its promise as a guidance channel (41). We confirmed that the resultant PLGA nerve guidance had a porous structure (Figure 3), which is likely to be permeable to nutrients. We extended the previous fabrication and characterization of PLGA nerve guidance channels by performing direct and indirect contact cytotoxicity tests. Though PLGA is a well-established biomaterial, our use of neuronal cells in toxicity testing extends previous studies on fibroblasts, and is directly relevant to our intended application for neuronal regeneration. For both direct contact and test on extracts methods, no signs of cytotoxicity effects were found in terms of cell proliferation and cell viability. With respect to morphology, SH-SY5Y cells on PLGA exhibited adherent cell bodies with projections extending outward, similar to neuronal cells cultured in tissue culture dishes. A substantial improvement in neuronal adherence and neurite morphology was observed when neurons were plated on PLGA coated with laminin. Consequently, this minor, but important change in surface coating should be incorporated into implanted PLGA nerve guidance channels.

Device implantation and surgical implementation

We confirmed the feasibility of implanting our device across a sciatic nerve gap and lengthening the proximal nerve stump in a rat cadaver. We demonstrated that the spiral nerve cuffs were able to hold severed nerve stumps without slippage, and that the proximal end of the sciatic nerve stump could be placed under tension by maneuvering the guidewire. Meanwhile, the tips of the nerve stumps were apposed to the tubular PLGA conduit, to guide axonal outgrowth.

Preliminary results in vivo were promising; they revealed that rats did not show signs of infection, and survived the two weeks before sacrifice. Moreover, the proximal nerve stump extended about 6 mm beyond the cuff within two weeks. This result contrasted with misalignment and shorter stumps observed in the absence of device implantation. We further identified mature axons and myelin within the elongating tissue, based on the positive staining of phosphorylated neurofilaments and S100. Thus, while it is premature to speculate on the relative utility of this device compared to other regenerative strategies, our in vivo results demonstrate the feasibility of device implantation and its usage to test nerve regeneration.

A comment is necessary regarding the implementation of our device in such studies. In practice, because the proximal and distal guidance channels will collide following sufficient lengthening, an ideal scenario will be to have the channels meet shortly before axons exit the proximal channel. Based on literature values, dimensions of the prototype device assume conservative lengthening rates of 1mm/day and axonal outgrowth rates of 0.3mm/day. However, these rates will eventually be optimized based on empirically determined thresholds for lengthening and axonal outgrowth rates into the scaffold.

Finally, we would be remiss if we did not point out some potential pitfalls of implanting our device in vivo. Most anticipated issues are likely to arise due to inflammatory and fibrotic processes. These include jamming of the telescoping mechanism of the hypotube and the inner rod owing to fibrous tissue infiltration, cell infiltration into the nerve gap, and scar formation. We speculate that due to frequent actuation, the jamming of the telescoping mechanism is unlikely and fibrosis will be no more of an issue with our device than any other implanted scaffolds. However, we will test the utility of medical grade expanded polytetrafluoroethylene (ePTFE) sleeves as a protective sheath surrounding the device. Another potential drawback is possible infection at the site where guidewire extends from the animal's body. Such a scenario would be treated with antibiotics; however, it should be noted that many peripheral nerve devices, including spiral cuff electrodes (40), have been successfully implanted with leads exiting the body. Therefore, we speculate that this risk is minimal. Finally, in

order to successfully stretch the proximal nerve stump with the device, the mechanical backbone must be secured to a reference position. In our initial studies, we have secured the device to the underlying muscle using a small volume of tissue adhesive or thin stainless steel anchors. Such anchorage may result in a focal loss of muscle function. This does not appear to be the case based on preliminary short-term survival surgeries; however, should complications arise, anchorage to the femur provides another more invasive, but reasonable, option.

Comparison to other strategies for nerve regeneration

Our lengthening device is intended to be used in parallel with other guidance strategies, including autologous grafts, synthetic grafts, acellular guidance matrices, and seeded matrices. Consequently, a direct comparison between a passive scaffold and the same scaffold incorporated within our device would be most appropriate. For the device introduced in this study, the appropriate comparisons would be an uncoated passive PLGA scaffold [33] and a passive PLGA scaffold coated with laminin. Differential responses between our device and these scaffolds would reflect effects of mechanical extension of intact regions of the proximal nerve superposed on outgrowth into the scaffold (Fig 1).

To our knowledge, only one other group has directly evaluated tensile loading as a regenerative strategy. The Ochiai group proposed an intriguing series of papers (63-65) that implemented a direct lengthening device to lengthen proximal and distal nerve stumps. Regeneration rates were equal or greater than those observed with an autologous graft, lending strong support to the hypothesis that mechanical loading can be beneficial to outgrowth. However, three key issues detract from the translational viability of this technology. First, based on their proposed design, nerves are readily lengthened, but the device configuration is not amenable to reattachment (i.e., there is nowhere for outgrowing axons to go). Second, because this design requires a complex external fixator mounted within the adjacent femur, more invasive surgery is required. Finally, nerve ends are secured with suture for tensioning; such attachment is likely to impose compression or uneven strain distributions owing to the discrete sites of attachment, again diminishing the likelihood of successful axonal outgrowth. Through the use of an internal fixator and self-sizing cuffs, we believe that our design has accounted for the most pressing of these issues.

Though at an early stage of development, the successful fabrication and implementation of our device provides considerable enthusiasm for this device and the broader strategy of mechanical influences on nerve regeneration.

Acknowledgements

References

1. Burnett, M.G., and Zager, E.L. Pathophysiology of peripheral nerve injury: a brief review. Neurosurg Focus 16, E1, 2004.
2. Evans, G.R. Peripheral nerve injury: a review and approach to tissue engineered constructs. Anat Rec 263, 396, 2001.
3. Schmidt, C.E., and Leach, J.B. Neural tissue engineering: strategies for repair and regeneration. Annu Rev Biomed Eng 5, 293, 2003.
4. Seddon, H.J. Three types of nerve injury. Brain 66, 247, 1943.
5. Sunderland, S. A classification of peripheral nerve injury produced by loss of function. Brain 74, 491, 1951.
6. Scherman, P., Kanje, M., and Dahlin, L.B. Bridging short nerve defects by direct repair under tension, nerve grafts or longitudinal sutures. Restor Neurol Neurosci 22, 65, 2004.
7. Dahlin, L., Johansson, F., Lindwall, C., and Kanje, M. Chapter 28: Future perspective in peripheral nerve reconstruction. Int Rev Neurobiol 87, 507, 2009.
8. Terenghi, G., Wiberg, M., and Kingham, P.J. Chapter 21: Use of stem cells for improving nerve regeneration. Int Rev Neurobiol 87, 393, 2009.
9. Zacchigna, S., and Giacca, M. Chapter 20: Gene therapy perspectives for nerve repair. Int Rev Neurobiol 87, 381, 2009.
10. Donat, J.R., and Wisniewski, H.M. The spatio-temporal pattern of Wallerian degeneration in mammalian peripheral nerves. Brain Res 53, 41, 1973.
11. Atchabahian, A., Genden, E.M., MacKinnon, S.E., Doolabh, V.B., and Hunter, D.A. Regeneration through long nerve grafts in the swine model. Microsurgery 18, 379, 1998.
12. Millesi, H., Meissl, G., and Berger, A. The interfascicular nerve-grafting of the median and ulnar nerves. J Bone Joint Surg Am 54, 727, 1972.

13.   Strasberg, S.R., Mackinnon, S.E., Genden, E.M., Bain, J.R., Purcell, C.M., Hunter, D.A., and Hay, J.B. Long-segment nerve allograft regeneration in the sheep model: experimental study and review of the literature. J Reconstr Microsurg 12, 529, 1996.

14.   Bora, F.W., Jr., Bednar, J.M., Osterman, A.L., Brown, M.J., and Sumner, A.J. Prosthetic nerve grafts: a resorbable tube as an alternative to autogenous nerve grafting. J Hand Surg Am 12, 685, 1987.

15.   Fawcett, J.W., and Keynes, R.J. Muscle basal lamina: a new graft material for peripheral nerve repair. J Neurosurg 65, 354, 1986.

16.   Gattuso, J.M., Glasby, M.A., Gschmeissner, S.E., and Norris, R.W. A comparison of immediate and delayed repair of peripheral nerves using freeze-thawed autologous skeletal muscle grafts--in the rat. Br J Plast Surg 42, 306, 1989.

17.   Chiu, D.T., Janecka, I., Krizek, T.J., Wolff, M., and Lovelace, R.E. Autogenous vein graft as a conduit for nerve regeneration. Surgery 91, 226, 1982.

18.   Suematsu, N., Atsuta, Y., and Hirayama, T. Vein graft for repair of peripheral nerve gap. J Reconstr Microsurg 4, 313, 1988.

19.   Strauch, B., Ferder, M., Lovelle-Allen, S., Moore, K., Kim, D.J., and Llena, J. Determining the maximal length of a vein conduit used as an interposition graft for nerve regeneration. J Reconstr Microsurg 12, 521, 1996.

20.   Brandt, J., Dahlin, L.B., and Lundborg, G. Autologous tendons used as grafts for bridging peripheral nerve defects. J Hand Surg Br 24, 284, 1999.

21.   Ao, Q., Fung, C.K., Tsui, A.Y., Cai, S., Zuo, H.C., Chan, Y.S., and Shum, D.K. The regeneration of transected sciatic nerves of adult rats using chitosan nerve conduits seeded with bone marrow stromal cell-derived Schwann cells. Biomaterials 32, 787, 2011.

22.   Harvey, A.R., Chen, M., Plant, G.W., and Dyson, S.E. Regrowth of axons within Schwann cell-filled polycarbonate tubes implanted into the damaged optic tract and cerebral cortex of rats. Restor Neurol Neurosci 6, 221, 1994.

23.   Hsu, S.H., Su, C.H., and Chiu, I.M. A novel approach to align adult neural stem cells on micropatterned conduits for peripheral nerve regeneration: a feasibility study. Artif Organs 33, 26, 2009.

24.   Marchesi, C., Pluderi, M., Colleoni, F., Belicchi, M., Meregalli, M., Farini, A., Parolini, D., Draghi, L., Fruguglietti, M.E., Gavina, M., Porretti, L., Cattaneo, A., Battistelli, M., Prelle, A., Moggio, M., Borsa, S., Bello, L., Spagnoli, D., Gaini, S.M., Tanzi, M.C., Bresolin, N., Grimoldi, N., and Torrente, Y. Skin-derived stem cells transplanted into resorbable guides provide functional nerve regeneration after sciatic nerve resection. Glia 55, 425, 2007.

25.   Bell, J., and Haycock, J.W. Next Generation Nerve Guides - Materials, Fabrication, Growth Factors and Cell Delivery. Tissue Eng Part B Rev.

26.   Lundborg, G., Dahlin, L.B., Danielsen, N., Gelberman, R.H., Longo, F.M., Powell, H.C., and Varon, S. Nerve regeneration in silicone chambers: influence of gap length and of distal stump components. Exp Neurol 76, 361, 1982.

27.   Brown, R., Pedowitz, R., Rydevik, B., Woo, S., Hargens, A., Massie, J., Kwan, M., and Garfin, S.R. Effects of acute graded strain on efferent conduction properties in the rabbit tibial nerve. Clin Orthop Relat Res, 288, 1993.

28.   Kwan, M.K., Wall, E.J., Massie, J., and Garfin, S.R. Strain, stress and stretch of peripheral nerve. Rabbit experiments in vitro and in vivo. Acta Orthop Scand 63, 267, 1992.

29.   Rydevik, B.L., Kwan, M.K., Myers, R.R., Brown, R.A., Triggs, K.J., Woo, S.L., and Garfin, S.R. An in vitro mechanical and histological study of acute stretching on rabbit tibial nerve. J Orthop Res 8, 694, 1990.

30.   Phillips, J.B., Smit, X., De Zoysa, N., Afoke, A., and Brown, R.A. Peripheral nerves in the rat exhibit localized heterogeneity of tensile properties during limb movement. J Physiol 557, 879, 2004.

31.   Wright, T.W., Glowczewskie, F., Wheeler, D., Miller, G., and Cowin, D. Excursion and strain of the median nerve. J Bone Joint Surg Am 78, 1897, 1996.

32.   Wright, T.W., Glowczewskie, F., Jr., Cowin, D., and Wheeler, D.L. Ulnar nerve excursion and strain at the elbow and wrist associated with upper extremity motion. J Hand Surg Am 26, 655, 2001.

33.   Anava, S., Greenbaum, A., Ben Jacob, E., Hanein, Y., and Ayali, A. The regulative role of neurite mechanical tension in network development. Biophys J 96, 1661, 2009.

34.   Abe, I., Ochiai, N., Ichimura, H., Tsujino, A., Sun, J., and Hara, Y. Internodes can nearly double in length with gradual elongation of the adult rat sciatic nerve. J Orthop Res 22, 571, 2004.

35.   Bray, D. Axonal growth in response to experimentally applied mechanical tension. Dev Biol 102, 379, 1984.

36.   Bueno, F.R., and Shah, S.B. Implications of tensile loading for the tissue engineering of nerves. Tissue Eng Part B Rev 14, 219, 2008.

37.   Pfister, B.J., Iwata, A., Meaney, D.F., and Smith, D.H. Extreme stretch growth of integrated axons. J Neurosci 24, 7978, 2004.

38.   Topp, K.S., and Boyd, B.S. Structure and biomechanics of peripheral nerves: nerve responses to physical stresses and implications for physical therapist practice. Phys Ther 86, 92, 2006.

39.   Zheng, J., Lamoureux, P., Santiago, V., Dennerll, T., Buxbaum, R.E., and Heidemann, S.R. Tensile regulation of axonal elongation and initiation. J Neurosci 11, 1117, 1991.

40.   Naples, G.G., Mortimer, J.T., Scheiner, A., and Sweeney, J.D. A spiral nerve cuff electrode for peripheral nerve stimulation. IEEE Trans Biomed Eng 35, 905, 1988.

41.   Oh, S.H., Kim, J.H., Song, K.S., Jeon, B.H., Yoon, J.H., Seo, T.B., Namgung, U., Lee, I.W., and Lee, J.H. Peripheral nerve regeneration within an asymmetrically porous PLGA/Pluronic F127 nerve guide conduit. Biomaterials 29, 1601, 2008.

42.   Oh, S.H., and Lee, J.H. Fabrication and characterization of hydrophilized porous PLGA nerve guide conduits by a modified immersion precipitation method. J Biomed Mater Res A 80, 530, 2007.

43.   McDonald, D.S., and Bell, M.S. Peripheral nerve gap repair facilitated by a dynamic tension device. Can J Plast Surg 18, e17, 2010.

44. Rivlin, M., Sheikh, E., Isaac, R., and Beredjiklian, P.K. The role of nerve allografts and conduits for nerve injuries. Hand Clin 26, 435, 2010.
45. Millesi, H. Reappraisal of nerve repair. Surg Clin North Am 61, 321, 1981.
46. Millesi, H. The nerve gap. Theory and clinical practice. Hand Clin 2, 651, 1986.
47. Flores, A.J., Lavernia, C.J., and Owens, P.W. Anatomy and physiology of peripheral nerve injury and repair. Am J Orthop (Belle Mead NJ) 29, 167, 2000.
48. Lundborg, G. Intraneural microcirculation. Orthop Clin North Am 19, 1, 1988.
49. Clark, W.L., Trumble, T.E., Swiontkowski, M.F., and Tencer, A.F. Nerve tension and blood flow in a rat model of immediate and delayed repairs. J Hand Surg Am 17, 677, 1992.
50. Driscoll, P.J., Glasby, M.A., and Lawson, G.M. An in vivo study of peripheral nerves in continuity: biomechanical and physiological responses to elongation. J Orthop Res 20, 370, 2002.
51. Sunderland, I.R., Brenner, M.J., Singham, J., Rickman, S.R., Hunter, D.A., and Mackinnon, S.E. Effect of tension on nerve regeneration in rat sciatic nerve transection model. Ann Plast Surg 53, 382, 2004.
52. Smith, D.H., Wolf, J.A., and Meaney, D.F. A new strategy to produce sustained growth of central nervous system axons: continuous mechanical tension. Tissue Eng 7, 131, 2001.
53. Abe, I., Tsujino, A., Hara, Y., Ichimura, H., and Ochiai, N. Paranodal demyelination by gradual nerve stretch can be repaired by elongation of internodes. Acta Neuropathol (Berl) 104, 505, 2002.
54. Abe, I., Tsujino, A., Hara, Y., and Ochiai, N. Effect of the rate of prestretching a peripheral nerve on regeneration potential after transection and repair. J Orthop Sci 8, 693, 2003.
55. Bora, F.W., Jr., Richardson, S., and Black, J. The biomechanical responses to tension in a peripheral nerve. J Hand Surg [Am] 5, 21, 1980.
56. Ichimura, H., Shiga, T., Abe, I., Hara, Y., Terui, N., Tsujino, A., and Ochiai, N. Distribution of sodium channels during nerve elongation in rat peripheral nerve. J Orthop Sci 10, 214, 2005.
57. Ikeda, K., Tomita, K., and Tanaka, S. Experimental study of peripheral nerve injury during gradual limb elongation. Hand Surg 5, 41, 2000.
58. Yokota, A., Doi, M., Ohtsuka, H., and Abe, M. Nerve conduction and microanatomy in the rabbit sciatic nerve after gradual limb lengthening-distraction neurogenesis. J Orthop Res 21, 36, 2003.
59. Li, J., and Shi, R. A device for the electrophysiological recording of peripheral nerves in response to stretch. J Neurosci Methods 154, 102, 2006.
60. Jou, I.M., Lai, K.A., Shen, C.L., and Yamano, Y. Changes in conduction, blood flow, histology, and neurological status following acute nerve-stretch injury induced by femoral lengthening. J Orthop Res 18, 149, 2000.
61. Lee, C., Ma, J., Deal, D.N., Smith, B.P., Koman, L.A., Smith, T.L., and Shilt, J.S. Neuromuscular recovery after distraction osteogenesis at different frequencies in a rabbit model. J Pediatr Orthop 26, 628, 2006.
62. Spiegel, D.A., Seaber, A.V., Chen, L.E., and Urbaniak, J.R. Recovery following stretch injury to the sciatic nerve of the rat: an in vivo study. J Reconstr Microsurg 9, 69, 1993.
63. Yamada, Y., Nishiura, Y., Saijilafu, Hara, Y., Ichimura, H., Yoshii, Y., and Ochiai, N. Repair of peripheral nerve defect by direct gradual lengthening of the distal nerve stump in rats: cellular reaction. Scand J Plast Reconstr Surg Hand Surg 43, 297, 2009.
64. Sharula, Hara, Y., Nishiura, Y., Saijilafu, Kubota, S., and Ochiai, N. Repair of the sciatic nerve defect with a direct gradual lengthening of proximal and distal nerve stumps in rabbits. Plast Reconstr Surg 125, 846, 2010.
65. Nishiura, Y., Hara, Y., Yoshii, Y., and Ochiai, N. Simultaneous gradual lengthening of both proximal and distal nerve stumps for repair of peripheral nerve defect in rats. Muscle Nerve 38, 1474, 2008.