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14. ABSTRACT

Each year, more than 34 million musculoskeletal injuries or organ repair or replacement surgeries, and over 300,000 cases of peripheral nerve injuries resulting in upper extremity paralytic syndrome, are reported in the U.S. alone. Active duty military personnel are more prone to orthopedic injuries involving large extremity nerve injuries than civilians. The objective of the present proposal is to develop a novel structured nanofibrous biodegradable nerve graft system that present ECM protein, neurotrophic factor, and pre-seeded with bone marrow stromal cells in rotating bioreactors for enhancing peripheral nerve regeneration to the level comparable to autograft. In the second year of this project, we assessed the release kinetics of nerve growth factor and determined that PCL-PEG-BSA-NGF provided a favorable environment as observed by PC-12 neurite extension. As compared to static culture conditions, the dynamic culture in rotating bioreactors stimulated the proliferation and differentiation of BMSCs seeded on nerve grafts. Based on our current progress with the *in vivo* animal studies, we have observed that the autograft group yielded better results as compared to the nerve graft (without any cells, growth factors, and proteins). While our *in vitro* data showed the efficacy of the nerve graft could be improved with various additives, we have not yet included these groups in the *in vivo* studies. As such, we expect the performance of the nerve graft to improve in the continuation of the following *in vivo* experiments for incorporating additives into the nerve grafts.

15. SUBJECT TERMS

Tissue engineering, Nerve regeneration, Extracellular matrix, Growth factors, Bone marrow stromal cells, Bioreactor, Nanofibers

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Table of Contents

Page

1. **INTRODUCTION:**

The peripheral nervous system is made up of the nerves and ganglia outside of the central nervous system, which is the brain and spinal cord. The function of the peripheral nervous system is to bridge the central nervous system with the rest of body. Peripheral nerve injury (PNI) affects approximately 200,000 patients in the United States, with greater numbers reported globally[1]. PNI can be severe enough to result in a loss of function to certain parts of the body especially if there is a gap in the nerve. Peripheral nerve injuries (PNI), often caused by trauma or iatrogenic injury[2], often require surgical intervention. The gold standard to repair nerve injuries is through two methods: direct coaptation of the proximal and distal stump when the nerve gap is <4mm and when the nerve gap is >4mm, application of an autograft, in which a patient's own nerve from a secondary location on the body is applied to the primary site of injury[3].

Active duty military personnel are more prone to orthopedic injuries involving large extremity nerve injuries than civilians[4]. Autografts are often associated with the limited availability and risks of immunogenicity, respectively. Due to the inherent limitations of using autografts, we proposed a tissue engineering approach to design a novel nerve guidance conduit based on the encouraging findings from our previous studies. The objective of the present proposal is to develop a novel structured nanofibrous biodegradable nerve graft system that present extracellular matrix (ECM) protein, nerve growth factor, and pre-seeded with bone marrow stromal cells (BMSCs) in rotating bioreactors for enhancing peripheral nerve regeneration to the level comparable to autograft. We hypothesize that the proposed novel structured nanofibrous biodegradable grafts will provide the micro environment, bioactivity, transport features and mechanics ideal for enhancing the rate of nerve regeneration and healing critical sized nerve defects. We further hypothesize that the grafts seeded with BMSCs will accelerate the tissue regeneration, resulting in formed tissue possessing the biochemical composition and mechanical properties of native tissue.

2. **KEYWORDS:** Nerve regeneration, nerve guidance conduit, bone marrow stromal cells, rat sciatic nerve, nerve growth factor.

3. **OVERALL PROJECT SUMMARY:**

In this annual period from September, 2014 to September 2015, we have successfully completed all the milestones in the proposed SOW for this period of time. We further investigated the incorporation of nerve growth factors into the nerve grafts and assessed its release, bioactivity and neurite stimulation effects. We seeded bone marrow stromal cells onto nerve grafts, and evaluated the influence of seeding density on cell proliferation and differentiation. We further seeded bone marrow stromal cells onto nerve grafts in bioreactors and evaluated the influence of rotating speed of bioreactors on cell proliferation and differentiation and neurite stimulation. We implanted three groups of nerve grafts into animals in a 15 mm long critical sized rat sciatic nerve injury model for 12 weeks, and characterized nerve regeneration through functional test, electrophysiological test and histological analysis. We investigated composite scaffold of polcaprolactone (PCL) nanofiber matrices with sodium alginate (Na-Alg) coating and blending approach for PCL- gelatin, and electrically conducting polymers for improving various properties for nerve conduits. Specifically, we have completed the following:

Incorporation of nerve growth factors into nerve grafts:

- Based on the successful design of nerve grafts in our previous annual report, we further optimized and characterized NGF incorporation and subsequent release from the novel structured nerve grafts. The amount of NGF to be incorporated, its stability, and bioactivity was assessed using well established laboratory protocols.
    - *Nerve growth factor delivery:* Nerve growth factor was loaded into PCL nanofibers, and the release kinetics was adjusted by adding small amount of chitosan as described in preliminary studies. The NGF loaded nerve grafts were placed in phosphate buffered saline at 37°C. Samples of buffer were taken at 2, 4, 6, 12, 18, and 24 hours, and at 2, 3, 5, 7, 10, 14, 21, and 28 days, and analyzed using ELISA for concentration to determine the nature of the release.

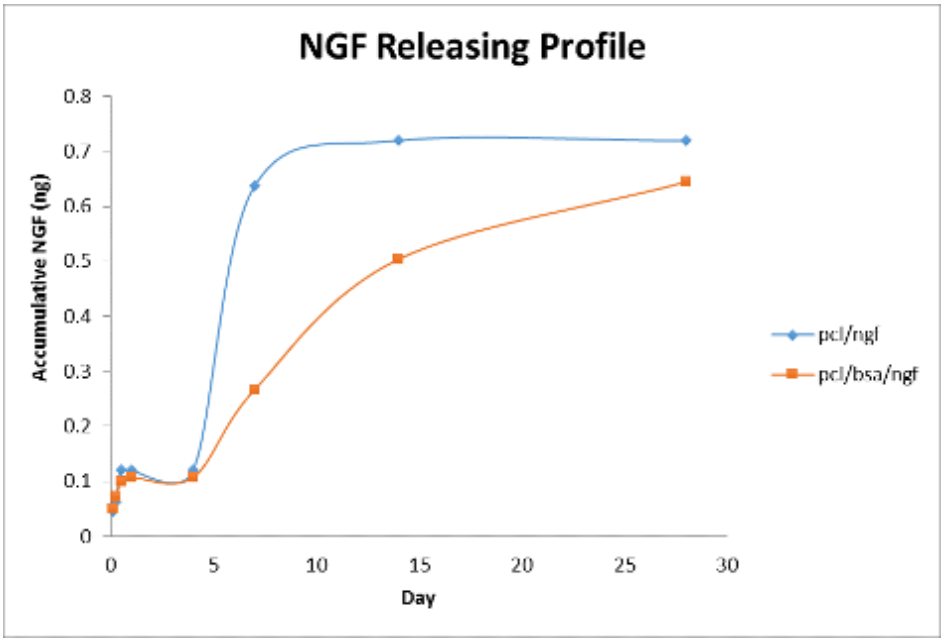


Figure 1. Comparison of NGF Release from Nanofibers coupled with and without Bovine Serum Albumin (BSA).

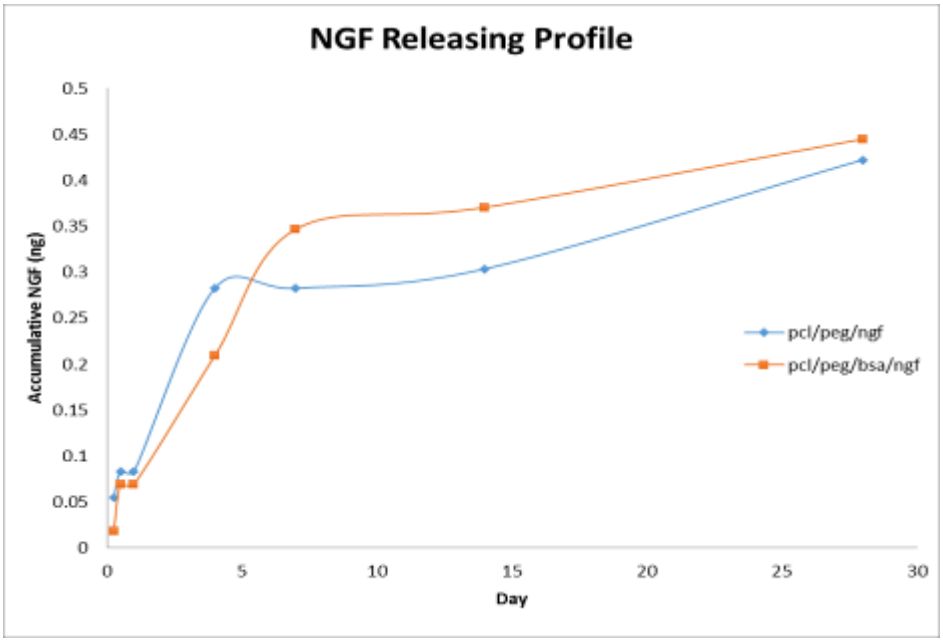


Figure 2. Comparison of NGF Release from Nanofibers cross-linked with PEG and coupled with and without Bovine Serum Albumin (BSA).

As shown in Figure 1 and 2, the release trend changes when PEG is incorporated with the nanofibers. The PEG clearly suppressed the burst release of the NGF as compared the PCL-NGF group, which depletes its NGF supply within 14 days. The BSA aids in reducing burst release of the NGF.

To further assess the techniques we incorporated NGF into PCL we seeded neuron like cell (neuronal cell line derived from a pheochromocytoma of the rat adrenal medulla – PC-12 cells) on the nerve scaffolds and observed variation in neurite extension over a 28 day period.

- *Bioassay for the released nerve growth factor:* To determine the bioactivity of the NGF released from nerve grafts, we used a PC-12 cell based bioassay. The adding of NGF was necessary for neurite outgrowth from PC-12 cells. We assessed if the released NGF had bioactivity by observing neurite outgrowth from PC-12 cells. Supernatant from nerve grafts both loaded with and free of

nerve growth factor after release study at different time points as described above were added into cultured PC-12 cells, and neurite extension was examined by staining for neurafilament and observing under a microscope.

- *Neurite outgrowth stimulating activity:* PC-12 cells will be cultured on the nerve grafts containing NGF and stained for evaluating neurite outgrowth from neurons. Neurite length and percentage of PC-12 cells with neurite outgrowth will be quantitatively determined.

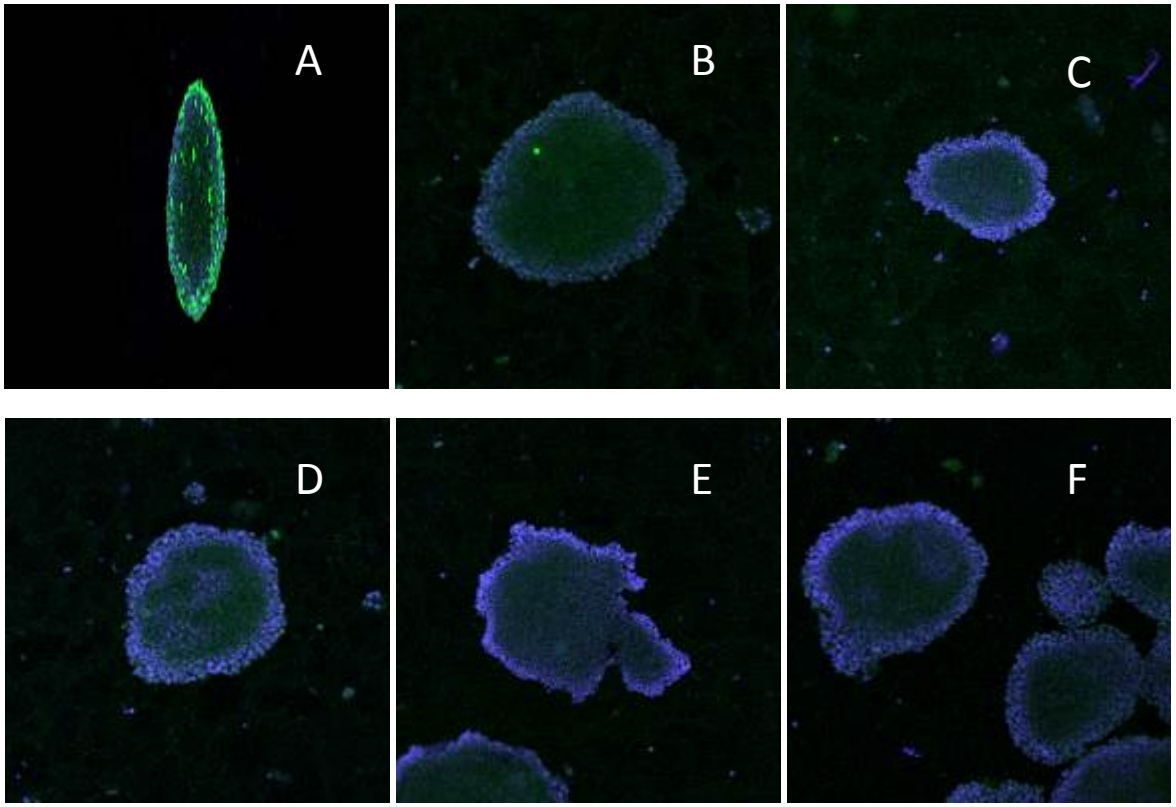
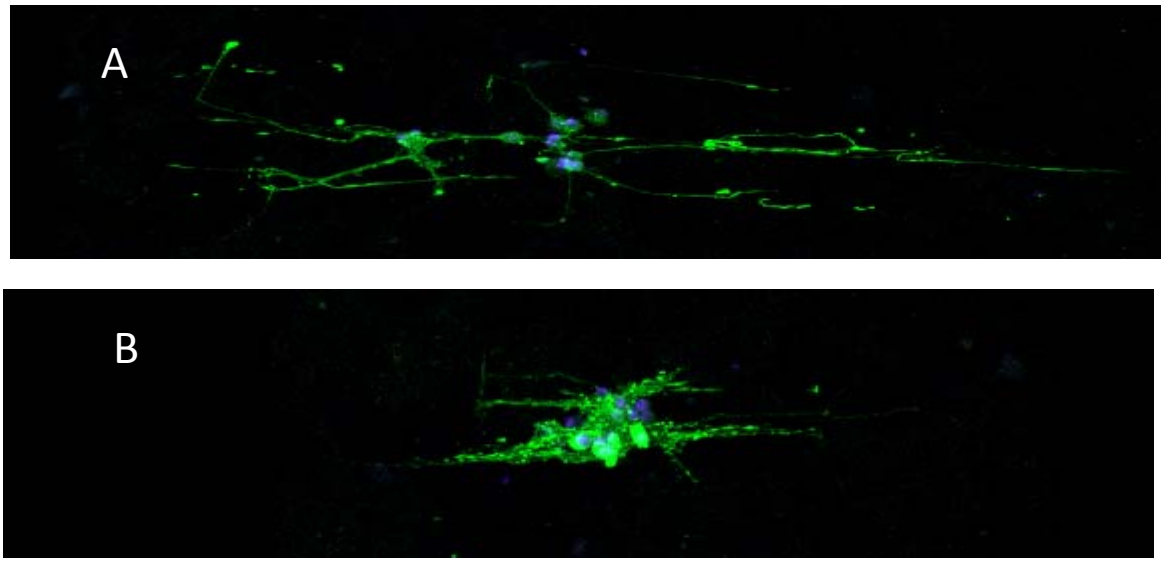


Figure 3. PC-12 Culture for a 7 day period. PCL (A), PCL with 50 ng NGF by pipetting (B), PCL/NGF through blending in the nanofibers (C), PCL/BSA/NGF (D), PCL/PEG/NGF (E), and PCL/PEG/BSA/NGF (F)



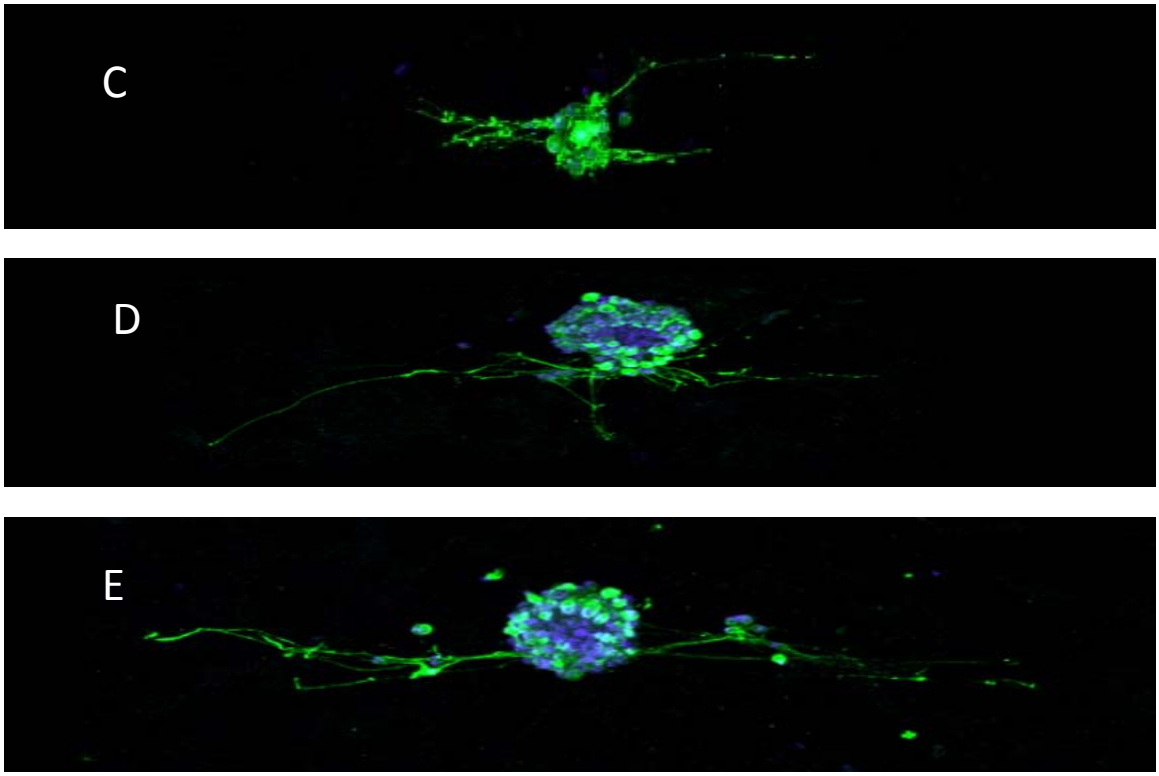
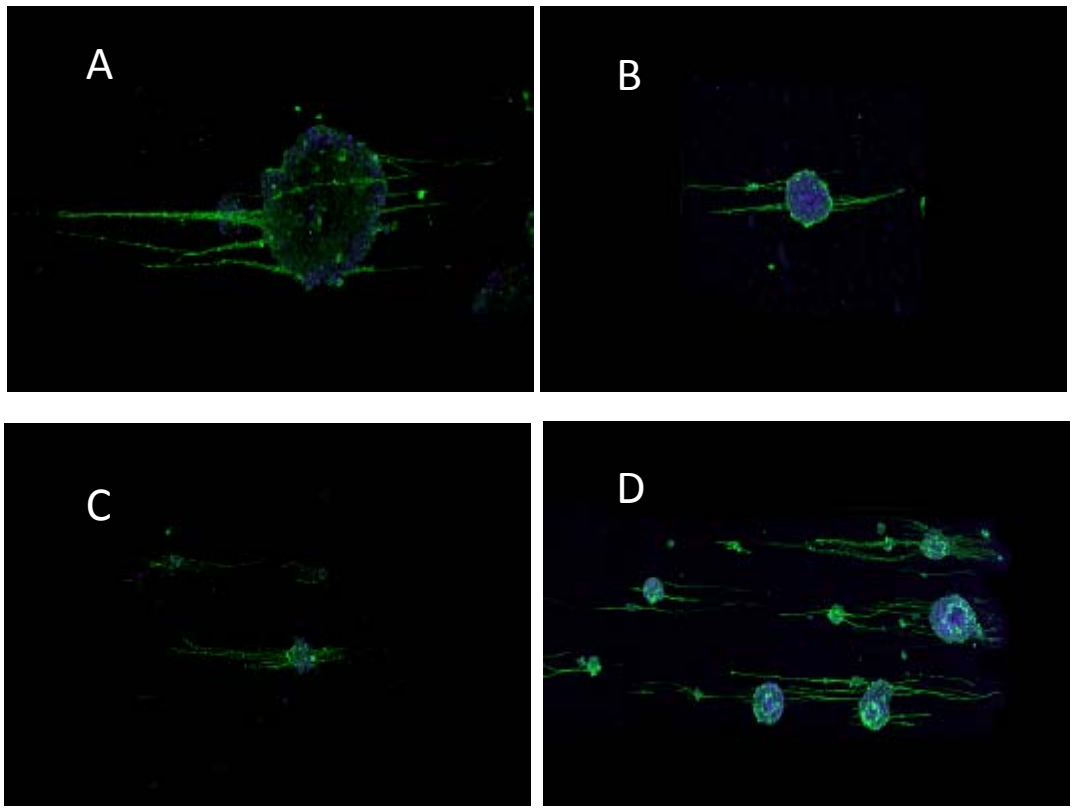


Figure 4. PC-12 Culture for a 14 day period PCL with 50 ng NGF by pipetting (A), PCL/NGF through blending in the nanofibers (B), PCL/BSA/NGF (C), PCL/PEG/NGF (D), and PCL/PEG/BSA/NGF (E)



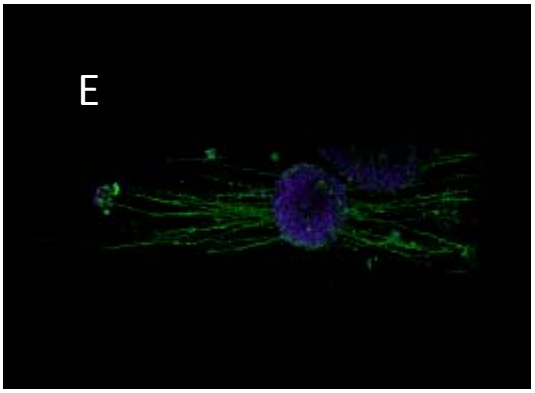


Figure 5. PC-12 Culture for a 21 day period. PCL with 50 ng NGF by pipetting (A), PCL/NGF through blending in the nanofibers (B), PCL/BSA/NGF (C), PCL/PEG/NGF (D), and PCL/PEG/BSA/NGF (E)

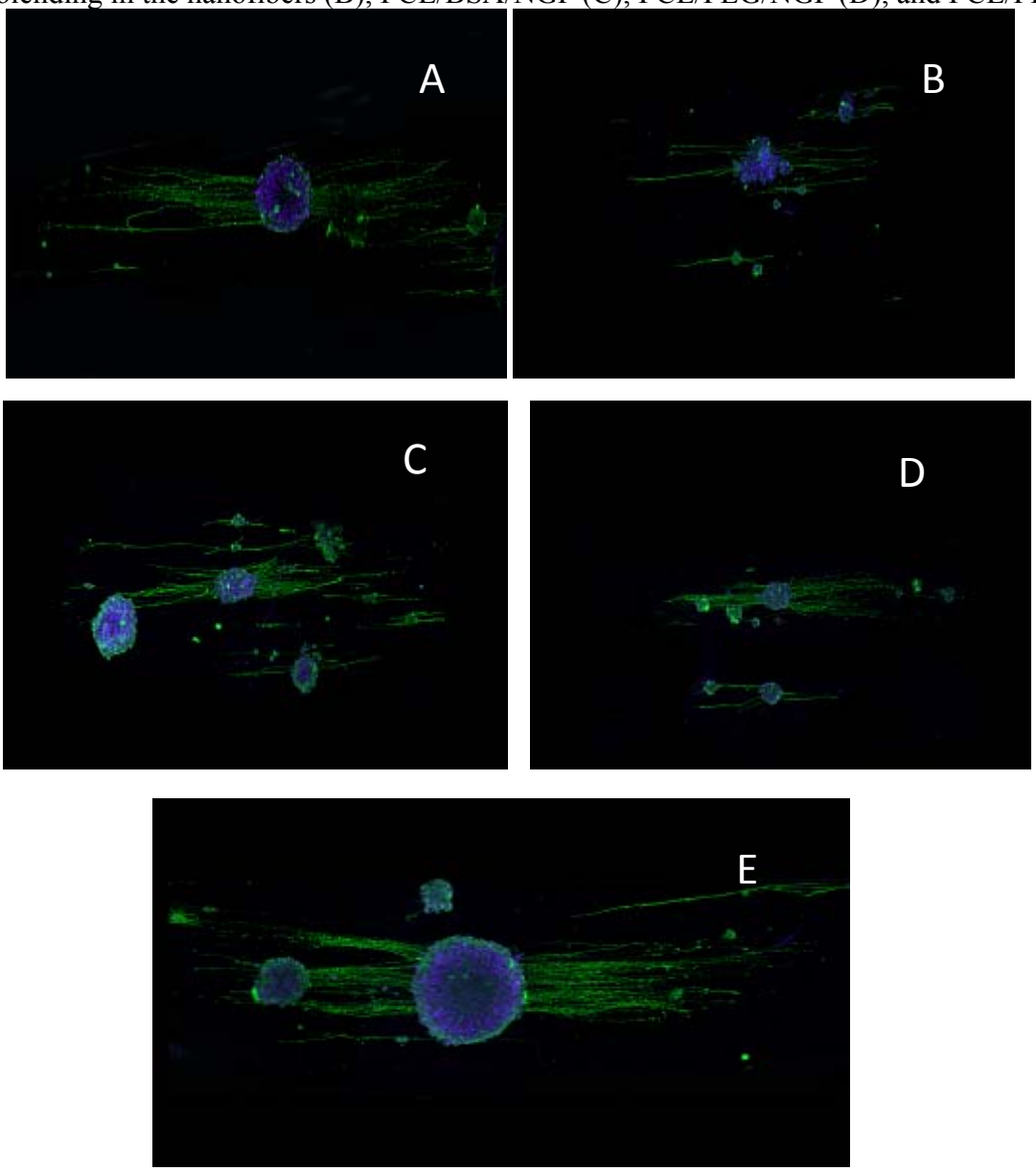


Figure 6. PC-12 Culture for a 28 day period. PCL with 50 ng NGF by pipetting (A), PCL/NGF through blending in the nanofibers (B), PCL/BSA/NGF (C), PCL/PEG/NGF (D), and PCL/PEG/BSA/NGF (E)

As shown in Figure 3, 4, 5 and 6, the PCL/PEG/BSA/NGF consistently performed better than the other groups in regard to neurite elongation. While BSA improves the NGF release and PEG aids cellular attachment, they complement each when applied together which improves neurite elongation. The addition of BSA to PCL provided a hydrophilic characteristic to an otherwise hydrophobic polymer. The hydrophilic

characteristic provided for uniform loading of the NGF and allowed the NGF to attach to the BSA. Thus the BSA reduced the burst release of the NGF and allowed a more consistent release for a longer duration.

PCL inherently does not contain any functional groups, which is why poly-ethylene glycol (PEG) is applied to crosslink PCL. A crosslinked PCL becomes more favorable for NGF release and cellular attachment. Initially we had only proposed to do PCL-BSA-NGF technique, while here we have explored the application of PEG and PEG with BSA. This coupling as shown above provides a more sustained release of NGF, is better for cellular attachment, and yields longer neurite elongation.

Animal protocol approval:

- A specific task was for obtaining the institutional animal care and use committee (IACUC) approval, and subsequent submission, review, and approval of all IACUC documents to the US Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office (ACURO).

We have prepared the animal protocol (2013-001) and received approval from our IACUC on 08-02-2013. We also submitted it to USAMRMC ACURO on 11-13-2013, and received the approval from ACURO for this protocol at December 30, 2013.

We made an amendment for the protocol (2013-001 (A01)) and get approval by Stevens IACUC on October 24, 2014. We submitted the protocol amendment for ACURO approval on November 19, 2014, and received received approval from ACURO for the protocol amendment by December 30, 2014.

Seeding bone marrow stromal cells onto nerve grafts:

- *BMSC isolation and culture:* BMSCs were isolated from the rats and cultured. The bone marrow stromal cells (BMSCs) were isolated from rat's tibia and femur. The adult male Sprague-Dawley rats (250-300 grams) were used.

Bone marrow stromal cell isolation and culture: For rat BMSCs isolation and culture, the tibia and femur of young male rats were used to isolate marrow. The animals were euthanized by administering an overdose of CO_2 asphyxiation. Bone marrow cavity contents were aseptically removed and harvested in Dulbecco's Modified Eagle's Medium (DMEM) and 50 g/mL gentamicin. Marrow were passed through 16 and 20 gauge needles and re-suspended in two medium conditions. Cells were placed in DMEM supplemented with 10% fetal bovine serum, 2.5 mM L-glutamine, 50 g/mL gentamicin. Cells were plated in flasks in 20 ml of media and cultured in a humidified 37°C/5% carbon dioxide (CO_2) incubator. BMSCs were selected based on their ability to adhere to the flask; non-adherent hematopoietic cells were removed upon refeeding after 3 days. The culture medium was replaced three times a week. Cell passaging was carried out by incubating for 5-10 minutes in calcium and magnesium-free Tyrode's solution containing 0.25% trypsin and replanted the cells in fresh medium at one-third their confluent densities. To increase the number of cells, the cells were lifted with trypsin, re-plated, and maintained in culture until they formed confluent monolayers in 75 cm^2 flasks. After the second passage, confluent monolayers of fat mesenchymal cells (total culture duration 5-7 days) were cryopreserved in liquid nitrogen until they were used. Passages 2-4 were used in the experiments.

We isolated and cultured primary bone marrow stromal cells from 4 male Sprague Dawley rats. As shown in Figure 7, we cultured them to the point of confluence upon which we passaged as well as froze the cells for safe keeping.

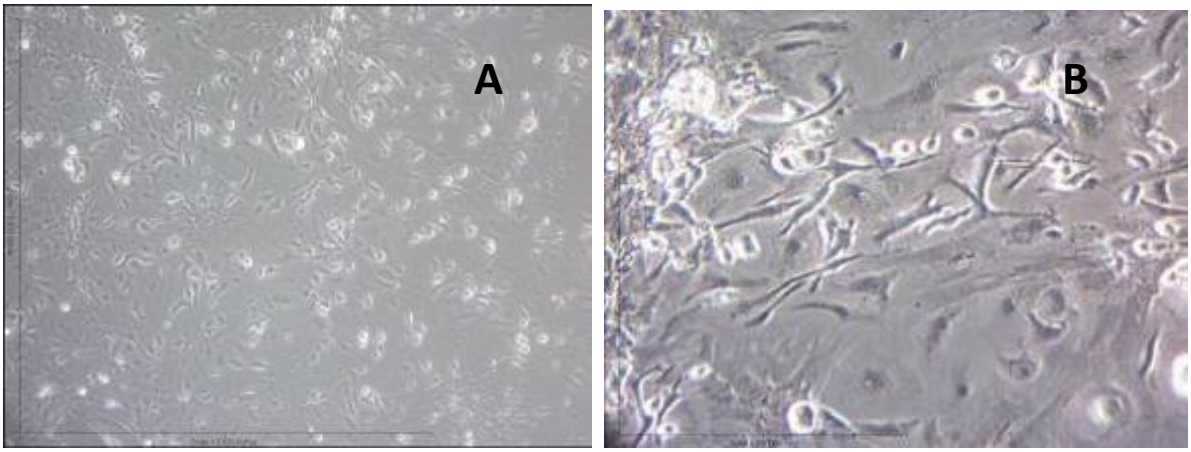


Figure 7. Bone Marrow Stromal Cell (BMSC) Culture at 10x (A) and 25x (B).

- *Influence of seeding density for BMSC on nerve grafts:* BMSCs were seeded onto the nerve grafts for promoting neurite outgrowth. Cell density is an important culture variable for phenotypic expression. We quantified and characterized the BMSCs after seeding onto scaffolds at three different cell seeding densities (low: $0.5x10^4$, medium: $1x10^4$ cells/cm^2, high: $1x10^5$ cells/cm^2) corresponding to 0.5X, 1X and 10X confluency. Cell attachment and morphology were observed using confocal microscopy. Cell proliferation was studied at different time points (1, 7, 14 and 21 days) by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTS) assay. Cell differentiation was studied at different time point by immunostaining for the protein S-100. Relative expression of genes for Schwann cell phenotypic expression in nerve grafts was also measured using Quantitative Real-time Polymer chain reaction (PCR).

    - *Cell proliferation:* We fabricated 72 nerve grafts and divided them into three groups of 24. For the first group we seeded $1X10^4$ cells/scaffold, the second group we seeded $5X10^4$ cells/scaffold, and for the third group we seeded $1X10^5$ cells/scaffold. The time points for this task are 1, 7, 14, and 21 days.

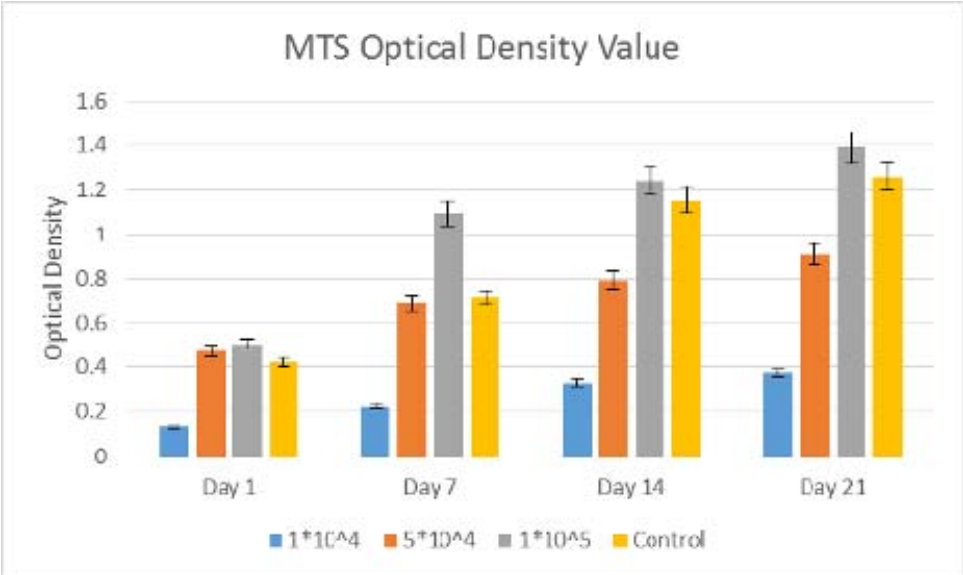


Figure 8. The MTS results for BMSCs seeded on nerve grafts in tissue culture plates.

For the nerve grafts in the tissue culture plate three different cell densities were studied: 1*10^4, 5*10^4, and 1*10^5. The nerve grafts were of the same type for each cell density. Once the BMSCs were seeded onto the nerve grafts, they were treated with differentiation medium allowing them to

mature into Schwann Cells. The differentiation medium was added on Day 1. As shown in Figure 8, cell proliferation increases with respect to time.

- *Confocal imaging:*

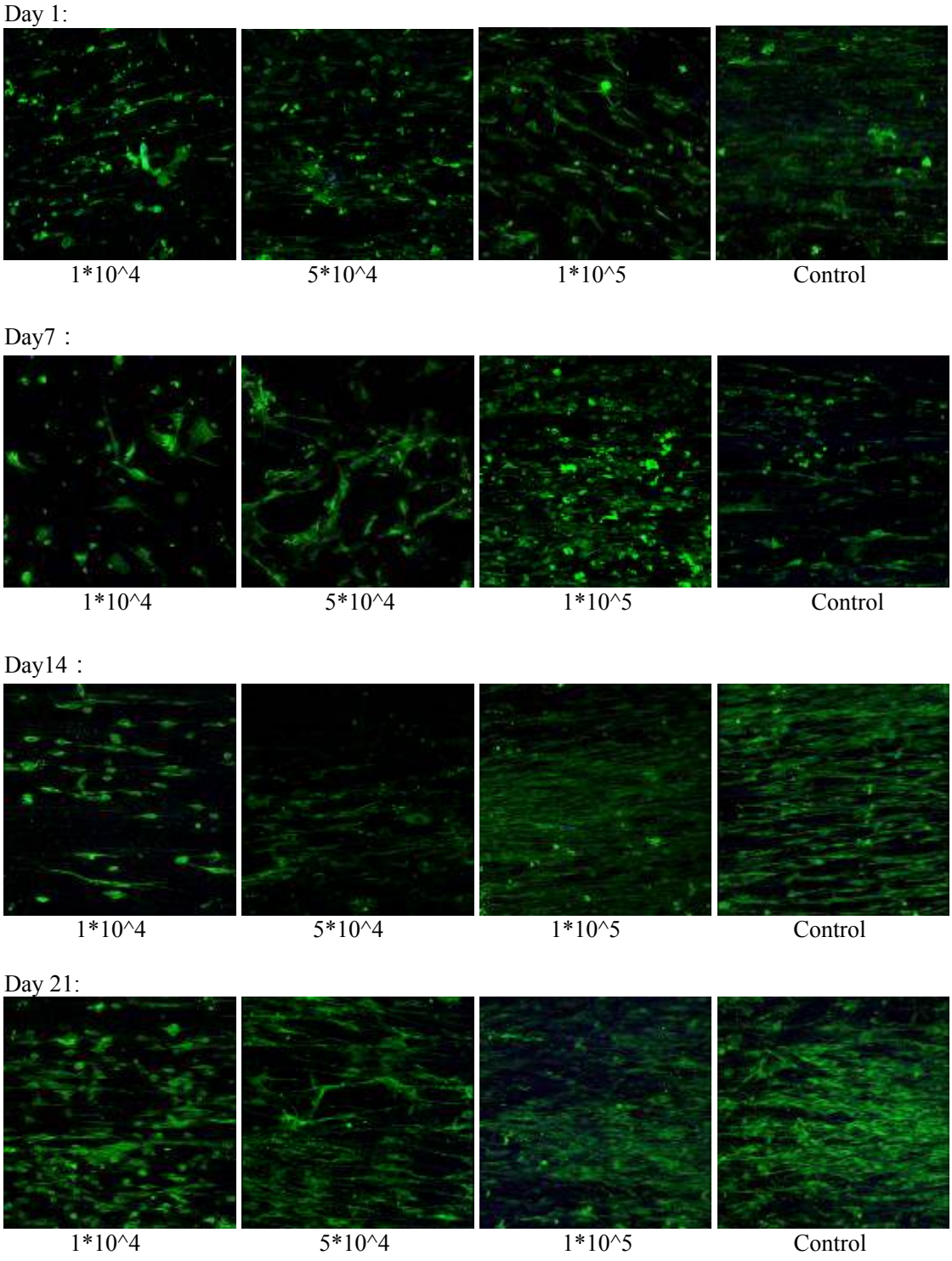
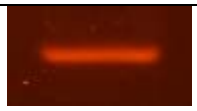
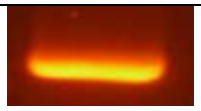
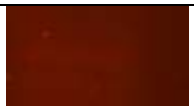
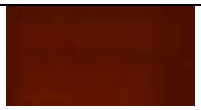
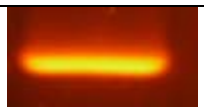


Figure 9. Confocal imaging of BMSCs on nerve grafts with different seeding densities.

As shown in Figure 9, BMSC cell differentiation was indicated by positive staining of S-100. We further focused on the PCR analysis by identifying more sensitive primers for characterizing the differentiation of BMSC on nerve grafts.

- *RT-PCR for Schwann cells and BMSCs:* Shown below are the RT-PCR results of Schwann cells and BMSCs cultured in a flask. The RT-PCR is an assay to determine cell type based on which biomarkers are expressed at the time points of interest. It is well known that BMSCs differentiate into Schwann cells with sufficient time, however it was critical to determine which biomarkers are expressed to distinguish whether the cell was a Schwann cell or BMSC. Once BMSCs differentiate, they express the same biomarkers as Schwann Cells.

Table 1. The RT-PCR results of Schwann cells and BMSCs.

	Schwann cells	BMSCs
GAPDH		
MBP		
NGF		
NGL-1		
NGL-2		
MPZ		

Based on the RT-PCR results shown in Table 1, it was concluded that the NGL-1, NGL-2, and MPZ biomarkers were applicable for distinguishing whether a cell was a Schwann cell or a BMSC. These are the only biomarkers that were not expressed by BMSCs in RT-PCR, as such it provides a clear distinction between both cell types.

- *Q-PCR:* Based on our current assessment, we moved forward and performed Quantitative-Polymerase Chain Reaction (Q-PCR). Our current study allowed us to better understand which biomarkers to focus on and further grasp the distinction between Schwann cells and BMSCs. We are now better prepared to perform future tasks. We designed new primers for GFAP, MPZ, P75, and Sox 10. The designed sequences for biomarkers are noted below in Table 2.

  We used quantitative PCR (Q-PCR) to identify the expression level of each sample, and applied the standard curve for comparison of samples to a control group. We developed the standard curve by diluting a 50ng/µl RNA of Control group to 5ng/µl, 0.5ng/µl, 0.05ng/µl, 0.005ng/µl, 0.0005ng/µl and 0.00005ng/µl. We then used these RNA dilutions to build the threshold cycle (Ct) standard curve.

Table 2. New Primer Design

Primers	Orientation	Sequences
GFAP	Forward	agaaaaccgcatcaccattc
	Reverse	gcacacctcacatcacatcc
MPZ	Forward	CTGGTCCAGTGAATGGGTCT
	Reverse	GTTGACCCTTGGCATAGTGG
P75 (nerve growth factor receptor)	Forward	AGCAGACCCATACGCAGACT
	Reverse	TATCCCCGTTGAGCAGTTTC
Sox 10	Forward	TAGCCTTGAAGGGCAGAAAA
	Reverse	GGAGGGGAGTGGGTACTCTT

We used the melting curve, which is provided by the software (DNA Engine Opticon 2 System for Real Time PCR Detection) to determine if the expression curve included byproduct. Since there was only one peak in the melting curve figure, this confirms our expression curve is derived from only one product and does not have any contamination or byproduct.

We observed an expression difference of Neuregulin-1(Nrg-1). Neuregulin-1 (Nrg1) controls Schwann Cell proliferation and differentiation depending on the cellular environment and the particular stage of Schwann Cell maturation. The Nrg-1 was used in Q-PCR because it is a biomarker for Schwann cells and can be used to assess differentiation of BMSCs to BMSC derived Schwann Cell-like cells.

We performed Q-PCR on the bioreactor groups, tissue culture plates, and the control on day 14 and day 21. We selected day 14 and day 21 for Q-PCR based on our quick study with the bioreactor since cell differentiation could not be assessed at day 1 and day 7.

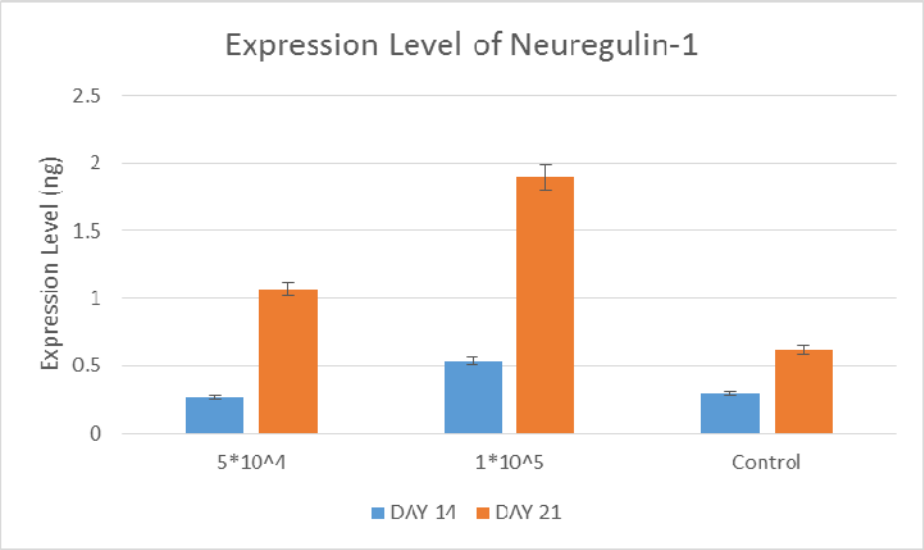


Figure 10. Expression Level of Neuregulin-1 for Task #2b.

While the tissue culture plate groups were cultured with differentiation medium, the control group was cultured with complete medium alone. By retaining the control group BMSCs in an undifferentiated state, it allowed us to compare the gene expression level for the BMSC derived Schwann Cell-like cells to that of the BMSCs for the Q-PCR.

The Expression Level of Neuregulin-1 bar graph above illustrates the quantified day 14 and day 21 expression curves for the listed groups (Figure 10). The control group had the lowest Nrg-1 expression level due to the fact that no differentiation medium was applied for the control group. Expression increased for all groups. The data for the TCP groups yielded a higher expression level for 1X10^5 at day 14 and day 21 compared to the control. This observation is consistent throughout all of the assays that were performed.

Seeding BMSCs onto nerve grafts and cultured in rotating bioreactors:

- BMSCs were seeded onto nerve grafts and cultured in rotating bioreactors. The cell proliferation and differentiation was characterized as described below. The nerve grafts were placed in bioreactors for further improving their performance. The nerve grafts were seeded with primary rat BMSCs and examined for cellular adhesion, proliferation, phenotypic expression, and neurite outgrowth stimulating activity in bioreactors. Specifically, we evaluated the bioreactor rotating speed and culturing durations on cellular responses. Cell proliferation and differentiation was evaluated for 1, 7, 14 and 21 days. The rotating speed of bioreactors is an important factor to determine the transport of nutrients and cellular response in bioreactors. In addition, the shear stress was generated in the rotating bioreactors and affects the behavior of bone marrow stromal cells. The shear stress in the bioreactors increases with the increase of rotating speed. Cells in the bioreactors may be damaged by very high shear stress. To study the influence of rotating speed of bioreactors, we evaluated three different rotating speeds (low: 16 rpm, medium: 24 rpm, high: 32 rpm) on cell proliferation and differentiation.

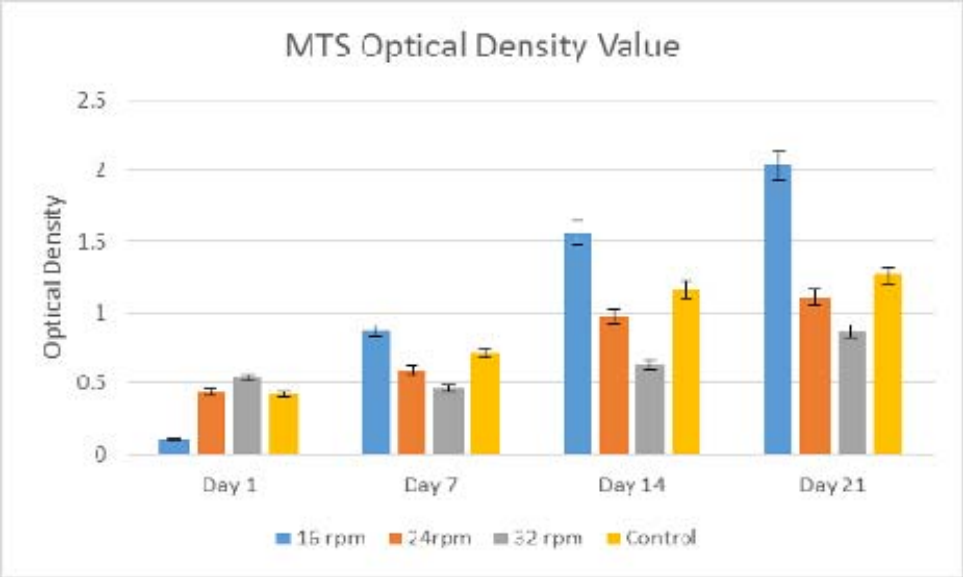


Figure 11. The MTS results for BMSCs seeded on a bioreactor.

- *Cell proliferation:* For each of the nerve grafts seeded in a bioreactor 5*10^4 BMSCs were seeded and the bioreactor was rotated at 16 rpm, 24rpm, and 32 rpm. The nerve grafts were of the same type for each group. Once the BMSCs were seeded onto the nerve grafts, they were treated with differentiation medium allowing them to mature into Schwann Cells. The differentiation medium was added on Day 1.

  As shown in Figure 11, cell proliferation increases with respect to time. The nerve graft seeded with 5*10^4 BMSCs in a bioreactor at 16 rpm showed the highest cell proliferation rate, according to weekly confocal imaging and MTS data throughout the 21 day period. The confocal images also supported use of bioreactors for greater cell infiltration.

- *Quantitative PCR:* While the bioreactor and tissue culture plate groups were cultured with differentiation medium, the control group was cultured with complete medium alone. By retaining the control group BMSCs in an undifferentiated state, we were able to compare the gene expression level for the BMSC derived Schwann Cell-like cells to that of the BMSCs for the Q-PCR.

  The Expression Level of Neuregulin-1 bar graph above illustrates the quantified day 14 and day 21 expression curves for the listed groups (Figure 12). The control group had the lowest Nrg-1 expression level due to the fact that no differentiation medium was applied for the control group. Expression increased for all groups. The data for the TCP groups yielded a higher expression level for 1X10^5 at day 14 and day 21, however the bioreactor group 16 rpm performed better than the TCP and the control.

This observation is consistent throughout all of the assays that were performed. The critical difference between the bioreactor and TCP is that the bioreactor allows for cell attachment and growth for a greater surface area on the scaffold due to the reduced impact of gravity. This allows a more favorable environment for cells to attach, grow, proliferate, and infiltrate in comparison to the opportunities available in the TCP.

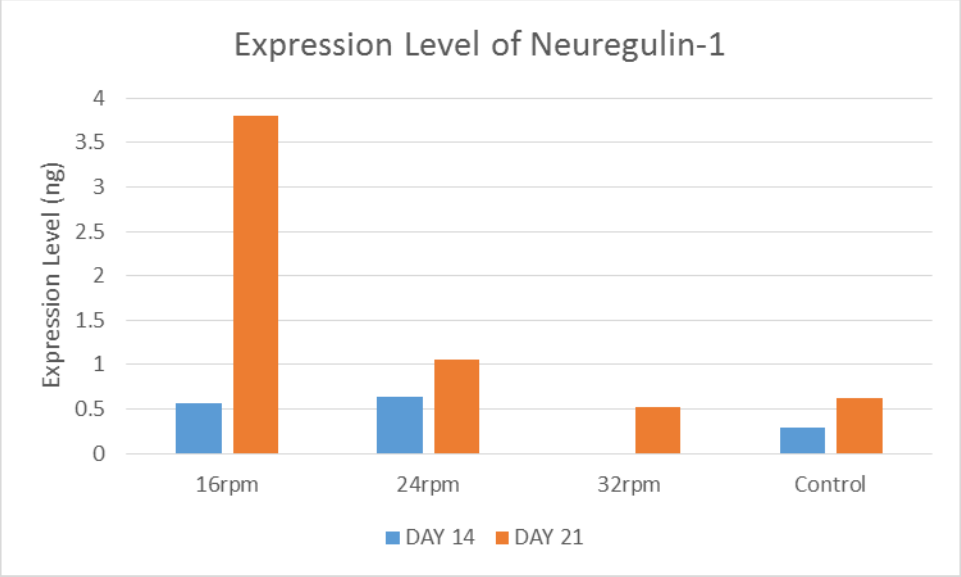


Figure 12. Expression Level (ng) of Neuregulin-1 assessed using Q-PCR.

Based on the results above, we concluded that a bioreactor provides a more favorable environment for cell culture than a tissue culture plate. From the MTS analysis and confocal images at day 21 it was evident that the data for 5*10^4 and 1*10^5 groups were statistically insignificant. While the proliferation was higher up until day 21 for the 1*10^5 group compared to the 5*10^4 group, the day 21 showed with sufficient time the 5*10^4 seeding density achieved comparable proliferation as that of the 1*10^5 seeding density.

In the bioreactor study a 16rpm was determined to provide a more suitable condition for cell culture than 24rpm and 32rpm. This is based on the MTS data and confocal images. While the proliferation for 16rpm up until day 21 is lower and/or similar to 24rpm and 32rpm the day 21 data show higher proliferation for 16rpm.

The bioreactor provided a more favorable environment for cell culture compared to the tissue culture plate. Based on the MTS data and cell proliferation in the confocal images the bioreactor at 16 rpm provided a more favorable environment than any other group we studied, especially at the day 21 time point.

- *Neurite stimulation:* BMSCs seeded on nerve grafts were assessed for promoting neurite extension from PC-12 cells as described below.
  - Neurite outgrowth stimulating activity: The nerve grafts optimized in the above adhesion/proliferation and differentiation studies were used to evaluate neurite outgrowth stimulating effects from PC-12 cells cultured on the nerve grafts by staining for neurofilament. Specifically, PC 12 cells were cultured on the nerve graft with optimal cell proliferation and differentiation as determined in static and dynamic bioreactor cultures, and stained for neurofilament for evaluating neurite outgrowth. Neurite length and percentage of PC 12 cells with neurite outgrowth will be quantitatively determined by analyzing the images from confocal laser scanning microscopy.

We seeded BMSCs on nerve grafts. The following groups were studied: a control which was treated only with complete medium and nerve grafts in a bioreactor (at 16rpm; 3D group) and nerve grafts in a tissue culture plate (both groups treated with differentiation medium; TCP group). We seeded 5*10^4 BMSCs on nerve grafts for 7 days and 21 days. At the 7 day and 21 day time point, we seeded PC-12 cells on the set of nerve grafts for which BMSCs were cultured for 7 days and the set that had been cultured for 21 days. The BMSCs and PC-12 cells were co-cultured. In one set of groups the medium contained NGF and in another set the medium did not contain NGF, as such this resulted in a total of six groups. The justification for using these groups stemmed from the results of the BMSC proliferation. Since it was determined that the 16rpm group was best from the BMSC study above, we used this speed for the bioreactor for the PC-12 study. This allowed us to assess the efficacy of the nerve grafts in aiding neurite extension of the PC-12 cells. The groups were assessed at day 11 following the PC-12 seeding. The neurite extension was assessed images using a confocal microscope.

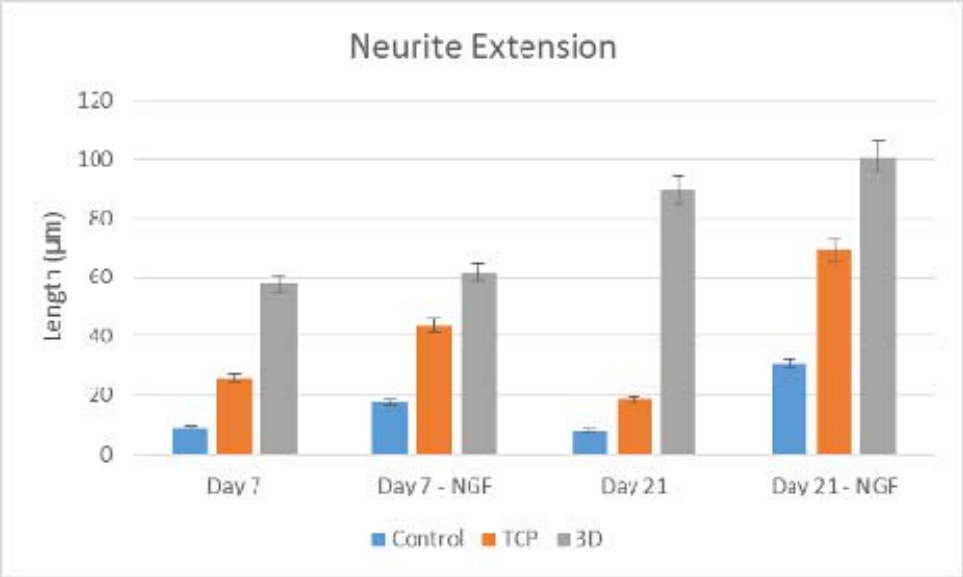


Figure 13. Neurite Extension of PC-12 cells on BMSCs and Nerve Grafts cultured in different environments.

Based on the confocal images, the neurite extension was assessed to be best for the bioreactor group for which the medium contained NGF (Figure 13). For the day 7 results the bioreactor groups (with NGF and without NGF) were statistically insignificant and the control group with NGF and the TCP group without NGF were statistically insignificant. This was observed again for the results at the day 21 time point. From these results we assume that after differentiation the BMSCs secrete sufficient NGF for PC-12 neurite outgrowth. In conclusion, a bioreactor provides a more favorable environment for differentiated BMSCs to secrete NGF.

***In vivo* animal experiments:**

- *Preparation of nerve grafts:* Fabrication of optimized novel structured scaffolds with and without biological factors based on the results obtained from in vitro studies as described below.

  The following types of nerve grafts have been prepared so far: 1) Nerve graft alone (n=9); 2) Nerve graft loaded with nerve growth factors (n=9).

We fabricated nerve grafts for implantation (Figure 14). The following nerve grafts were fabricated: 0.1mm spiral nerve graft with inner aligned nanofibers (16 min) wrapped with an outer tube (50µm thickness) (SAT) and 0.1mm spiral nerve graft with inner aligned nanofibers (16 min – loaded with NGF) all wrapped with an outer tube (50µm thickness) (SAT+NGF). This task is currently ongoing. Below is an illustration of the wall thickness and cross section, respectively, of the fabricated nerve grafts for implantation.

Figures 14. Illustration of the thickness of the 0.1mm wall (left) and gap width (right).

- *Surgery and characterization:* The animal study protocol was approved by the Animal Care and Use Review Office at the Stevens Institute of Technology and at the Department of Defense and the Institutional Animal Care and Use Committee. Male Sprague Dawley rats weighing between 251-275 grams were used for this animal study. The animals were anaesthetized using a combination of oxygen and isoflourane. After incision of the skin, the right sciatic nerve was exposed and then severed for the removal of 15mm near the obturator tendon in the mid-thigh for the implanation of the aforementioned nerve grafts. The proximal and distal nerve stumps were secured into the conduit using 10-0 nylon microsutures. The muscle and skin were then closed using 4-0 resorbable sutures.

    - Sciatic functional index: We performed a functional assay, the walking track analysis, to measure the sciatic functional index (SFI). This assay is widely used by investigators in the field of peripheral nerve injury to assess nerve regeneration. The paw prints from the experimental and control hind limbs were compared. As the SFI value approaches zero, the corresponding functional recovery is better. Preoperatively, the right hind paws of the rats were painted with trypan blue dye, and the right hind paw prints were recorded by training the animal to walk in a box. Every two weeks post-surgery the same procedure was repeated and the right hind paw prints were again recorded. Three parameters were derived from the paw prints: print length (PL), toe spread (TS; distance from toe 1 to toe 5), and intermediate toe spread (IT; distance from toe 2 to toe 4). The parameters from paw prints taken before and after surgery were considered to be normal and experimental respectively. The sciatic functional index was calculated by using the following formula[3]:

SFI = -38.3 [(EPL – NPL)/NPL] + 109.5 [(ETS – NTS)/NTS] + 13.3 [(EIT – NIT)/NIT] – 8.8.

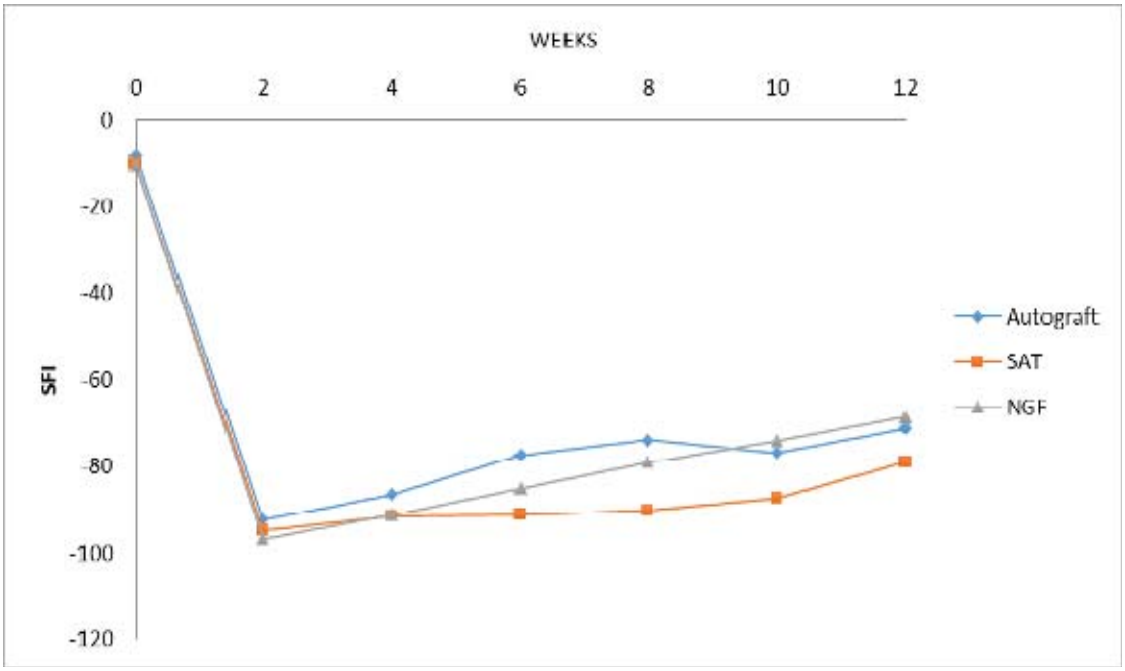


Figure 15. The SFI recovery over 12 Weeks

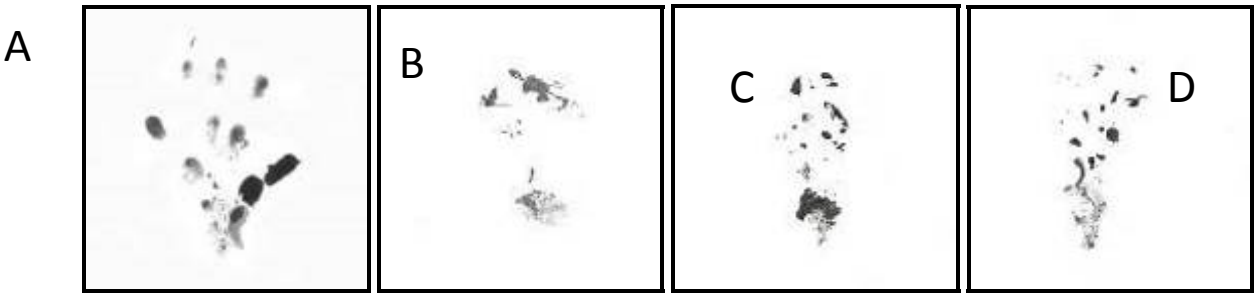


Figure 16. The footprints from the groups studied for SFI: normal footprint pre-surgery (A), autograft, (B), SAT (C), and SAT+NGF (D) .

The normal SFI was recored for the animals prior to the surgery and every 2 weeks post-surgery the SFI was calculated. As can be seen from Figure 15 and 16, the autograft had better recovery than the 0.1mm spiral nerve graft with inner aligned nanofibers (16 min) and wrapped with an outer tube (50µm thickness). The autograft may have had better recovery due to the fact that it provides the ideal cross sectional surface area, and contains the growth factors, proteins, and cells ideal for recovery, which is why it is the gold standard in the field. The favorable environment provided by the autograft is what we are trying to emulate in our project.

o Electro physiologic test: Electro physiologic studies were performed to assess the restoration of functionality of the regenerated nerve through the implants at the 12 week time point after implantation. Each rat was placed under anesthesia again during the course of the data collection. The sciatic nerve was exposed at the surgical site. A recording needle electrode was placed in the gastrocnemius muscle and stimulation electrodes were placed directly posterior to the tibia; the sciatic nerve was stimulated with two stainless wire electrodes connected to an electrical stimulator. A ground electrode was placed in the surrounding muscle tissues to remove conduction of stimulation through muscle tissues. The amplitude and nerve conduction velocity (NCV) of the evoked compound muscle action potentials (CMAPs) were recorded. As shown in Figure 17, the electrophysiology test results from the autograft and the 0.1mm spiral nerve graft with inner aligned nanofibers (16 min) wrapped with an outer tube (50µm thickness) were completed. The signal is stronger for the autograft group thus indicating a greater presence of myelinated axons, and potentially axons with a greater diameter and a thicker myelin sheath. This is evident based on the amplitude of the signal and the latency values[5-7].

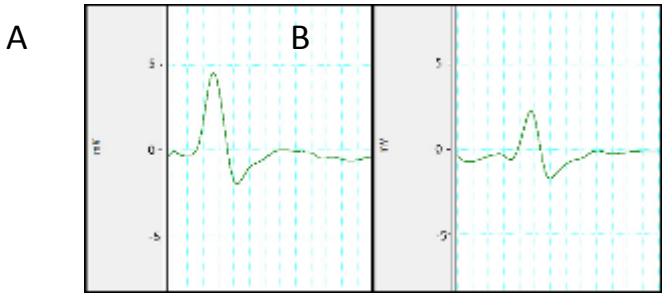


Figure 17. The results of the electrophysiology test. Autograft (A) and and 0.1mm spiral nerve graft with inner aligned nanofibers (16 min) wrapped with an outer tube (50µm thickness) (B).

Table 3. The results of the Pinch Test

Pinch Test Results		
Autograft	9/9	100%
SAT	4/9	44.4%
SAT+NGF	7/9	77.8%

o Pinch test: The pinch test was performed on the animals following the electrosphyiological assessment. The nerve trunk distal to the conduit was pinched with a pair of forceps. Contraction of the muscle on the back or movement of the leg indicated the presence of a regenerated nerve inside the conduit. The pain reflex was observed. This assessment was critical to evaluate the presence of myelinated axons in the regenerated nerve and as well as if they are functional[8]. As shown in table 3, the autograft group has the highest number os animals pass the pinch test (100%), follow by the nerve graft with NGF group (77.8%), and nerve graft alone group (44.4%).

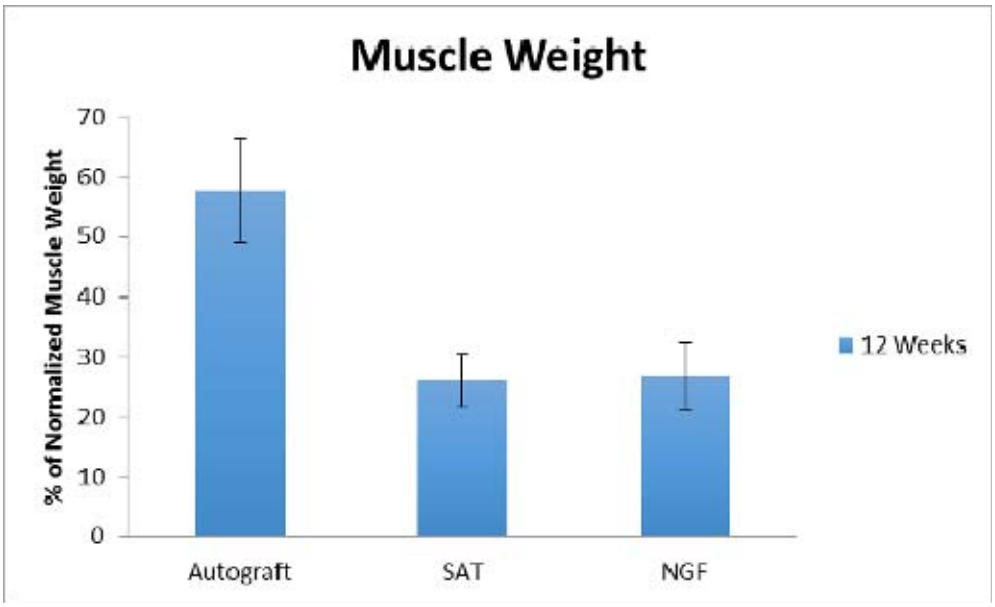
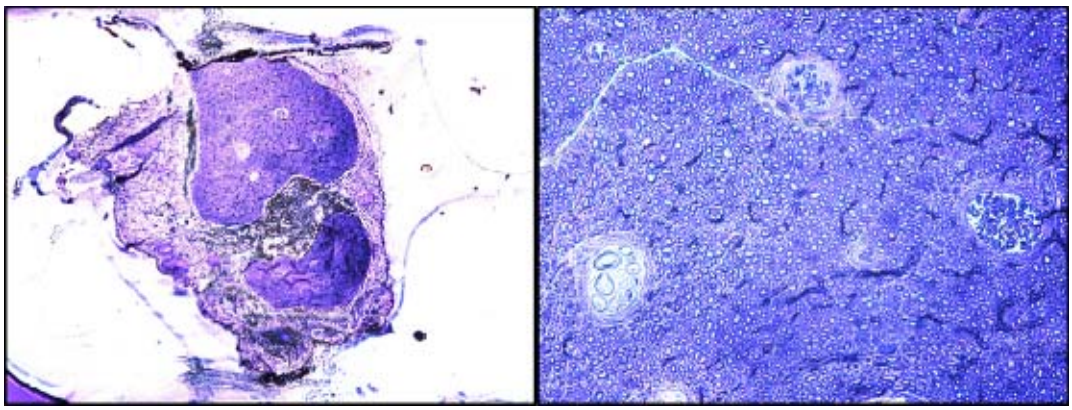


Figure 18. The Relative Gastrocnemius Muscle Weight over 12 Weeks

o Gastrocnemius muscle mass measurement: It has been noted that gastrocnemius muscle mass is proportional to the degree of sciatic nerve innervation and is an indicator of the functional activity of sciatic nerves. Therefore gastrocnemius muscle mass provides indirect evidence for evaluating functional sciatic nerve regeneration since it undergoes atrophy after sciatic nerve injury; its mass is proportional to extent of sciatic nerve innervation. The relative gastrocnemius muscle weight (RGMW) is defined as the ratio of the gastrocnemius muscle weight from the experimental (right) side to that of the normal (left) side. RGMW is used as a parameter to represent the "functional" consequences of sciatic nerve regeneration[9-12]. The results indicated that the autograft group has signigicantly higher recovery of gastrocnemius muscle mass as compared to the nerve graft with NGF group and nerve graft alone group (Figure 18).

A



B

Figure 19. The histology image for the autograft at 12 weeks. Here we see the overall cross section (A) at 4x and a 20x image (B) focused on a specific region of the cross section.

o Histology: At the specified time point of 12-weeks, the rats were euthanized via carbon dioxide asphyxiation. The surgical site was reopened to explant the NGCs for a histological analysis. The

NGCs, which housed the regenerated nerve, were placed in 4% paraformaldehyde. Specimens (2 mm) at the midpoint of the NGCs were collected and embedded in epoxy. Sections (900 nm) were cut with a glass knife on an ultra-microtome and then stained with 1% toluidine blue solution[13-15]. Currently, the histological analysis is still ongoing (Figure 19).

Based on the assessments above, it can be concluded that for a critical gap length of 15mm in a rat sciatic nerve the autograft still provides a more favorable environment than a nerve guidance conduit synthesized in a laboratory. It has been noted an autograft contains appropriate structures, growth factors, cells, and proteins all of which are crucial for successful for nerve regeneration. Based on these features, the autograft served as the positive control. As we continue our *in vivo* studies we will incorporate more additives using different techniques to achieve results comparable to the autograft and in turn provide a more favorable environment for nerve regeneration.

Moreover, composite scaffold PCL nanofiber matrices with Na-Alg coating and blending approach for PCL- gelatin, and electrically conducting polymers were investigated for improving various properties for nerve conduits.

- *Incorporate ECM protein laminin onto nerve grafts:* ECM protein laminin was incorporated onto the nerve grafts using blending technique. In addition to the laminin, cells were treated using NGF (17.5 μg/ml from stock of 1 mg/ml was added in 50 ml cell culture medium) in cell culture medium. Nerve grafts loaded with laminin were incubated with hBMSCs and samples and treated with NGF cell culture media. Samples were collected at various time points for the microscopy. The nature and action of nerve growth factor in in vitro environment on nerve grafts was studied using confocal microscopy.

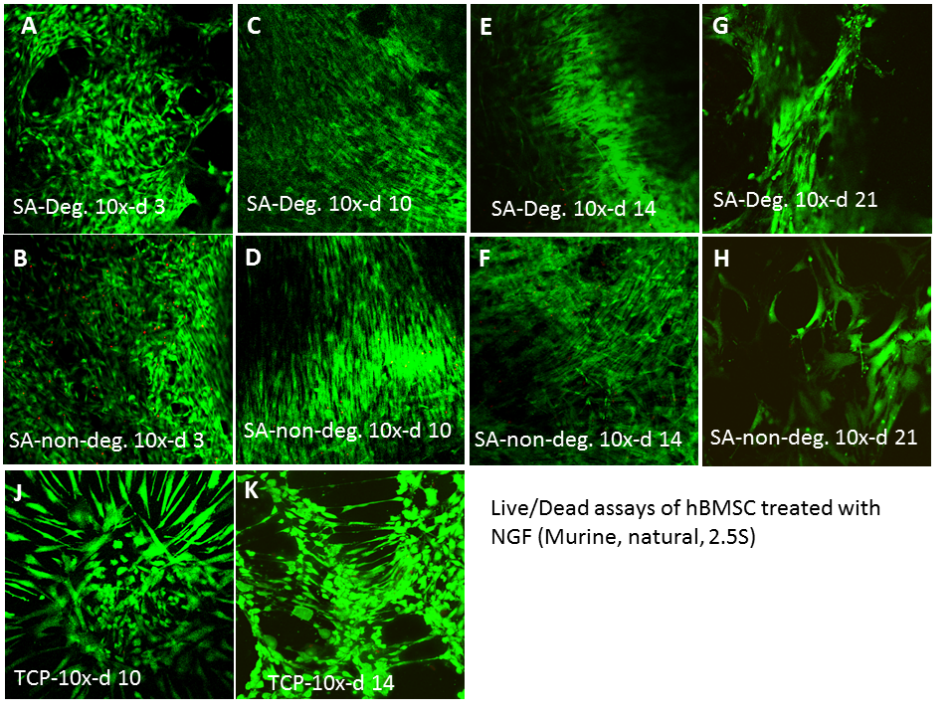


Figure 20: PCL-sodium alginate aligned nerve grafts loaded with laminin seeded with hBMSC and treated with NGF (Murine, natural, 2.5S) and Live/Dead assays were performed at each time point. Study indicated cell proliferation with respect to time also the neurite extension was minimal on day 3 as compared to day 14 and day 21.

Due to the non-availability of any active groups in PCL we adopted a blending technique for the loading of laminin to the PCL-sodium alginate nerve grafts. To create nanofibers, PCL solution was electrospun at 20 kV, 1 ml/hr and a distance of 20 cm using custom made aluminium mold to obtain aligned nanofibers. For laminin loading, to the sodium alginate solutions 40 ug/ml laminin

was dissolved and the solution was coated on PCL nanofibers. Coated fibers were dried at room temperature and stored at 4°C until further use.

Nerve growth factor treatment: Nerve growth factor was added to the cell culture medium and samples were treated.

Neurite outgrowth stimulating activity: To determine the neurite outgrowth in presence of NGF, images were obtained for Live/Dead assay using confocal microscopy.

Study indicated cell proliferation with respect to time also the neurite extension was minimal on day 3 as compared to day 14 and day 21 (Figure 20).

- *Cell proliferation studies:* Cell proliferation studies indicated that, sodium alginate coated PCL nanofibers had enhanced cell proliferations as compared to non-coated PCL nanofibers. When compared between degraded sodium alginate and non-degraded sodium alginate coated PCL nanofibers for cell proliferations, the PCL nanofibers coated with degraded sodium alginate had enhanced cell proliferations over PCL nanofibers coated with non-degraded sodium alginate. Comparative results are as shown in Figure 21.

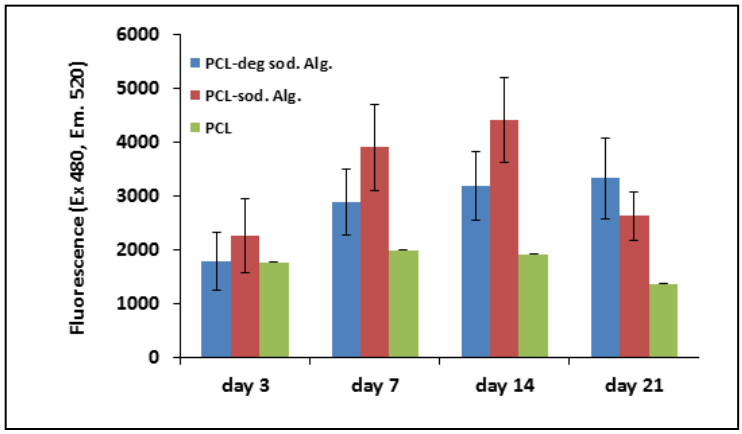


Figure 21: DNA assay indicating the cell proliferation over a period of 21 days on neat and sodium alginate coated PCL matrices. The progressive bone marrow stromal cell growth with respect to time ensured the matrix compatibility with the cells.

- *Ionically conductive polymers for nerve regeneration:* Ionically conductive polymers: PCL nanofibers were coated with ionically modified hydrogels and conductivities were tested. Studies indicated that PCL nanofibers coated with ionically modified hydrogels had significantly less resistance as compared to unmodified hydrogel coated PCL nanofibers as well as only PCL nanofibers. These studies indicate that ionically modified hydrogel could be useful for the differentiation of BMSCs to the neural cells under electrical stimulations. Results of ionic conductivity measurements are as shown in figure 22.

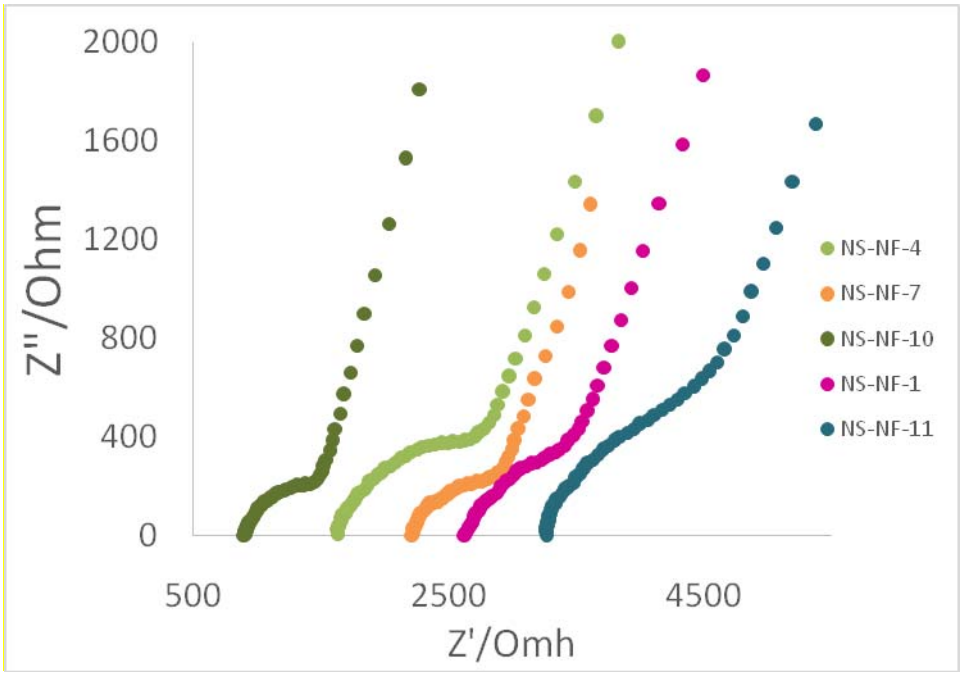


Figure 22: Electric conductivity measurements of PCL nanofibers coated with unmodified hydrogel (NS-NF-1), with sulfonate modified hydrogel (NS-NF-4), with carboxylate modified hydrogel (NS-NF-7), with phosphate-modified hydrogel (NS-NF-10), and without coating (control).

- *Cell proliferation studies:* Cell proliferation studies indicated that, ionically modified hydrogel coated PCL nanofibers had excellent cell proliferations. Results are as shown in Figure 23B. Also upon electrical stimulations, neurite extensions were noticed as shown in figure 23E.

- *Neurite outgrowth stimulating activity:* Electrical stimulations were performed by applying direct current (DC) of 400 mV for 10 minutes every 24 hrs. Studies were continued for 5 days and then were analyzed by immunostainings for neuronal protein expressions.

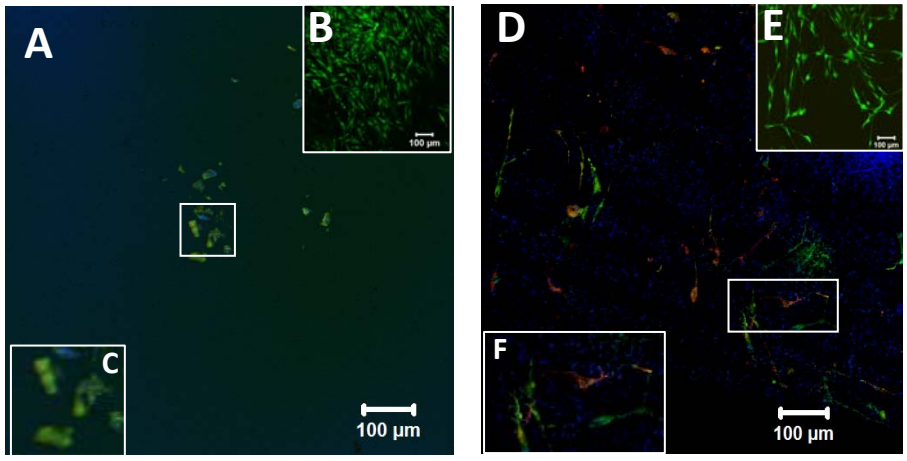


Figure 23. Cells stained for tubulin (green) and MAP2 (red) (A-C) control without stimulations, (D-F) upon electrical stimulation at 400 mV DC-day5. Image (C,F) in inset indicates stained images, and (B,E) in inset indicates Live/Dead assays of cells before and after electrical stimulations.

- *Immunostainings:* To determine the neurite outgrowth in presence of electrical stimulations, samples were stained for nerve specific proteins such as tubulin and MAP 2. Images are as indicated in figure 23D-F.

Electrical stimulation studies indicted expressions of tubulin and MAP 2 upon electrical stimulations for 5 days. Whereas there were no tubulin and MAP 2 expressions noticed in control samples.

Cell viability studies indicated excellent cell attachment and proliferations on ionic chitosan. Also neurite extensions and expression of neuronal markers were prevalent upon electrical stimulations. These observations conclude that ionic chitosan's are an excellent material to the use for nerve regeneration applications.

4. KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

- We quantitatively assessed the release kinetics of the nerve growth factor by adding chitosan and observed the neurite extension of PC12 cells. We compared the following groups: PCL, PCL-NGF, PCL-BSA-NGF, PCL-PEG-NGF, and PCL-PEG-BSA-NGF. PCL-NGF resulted in poor PCL-12 extension, PCL-BSA-NGF showed a better NGF release based on PC-12 neurite extension, and PCL-PEG-NGF improved cell attachment. Based on these characteristics the PCL-PEG-BSA-NGF group yielded the best results.

- We seeded BMSCs onto nerve grafts at different seeding densities of $1X10^4/ cm^2$, $5X10^4/ cm^2$, and $1X10^5/ cm^2$, and observed that the seeding density of $1X10^5/ cm^2$ resulted in highest proliferation and differentiation of BMSCs.

- The bone marrow stromal cells (BMSCs), which had been isolated and cultured from rats were seeded on nerve grafts in tissue culture plates and bioreactors. We seeded different densities of BMSCs on nerve grafts to assess cell adhesion, proliferation, differentiation, gene expression, and cell morphology. The results indicated that the bioreactor culture condition in 3D significantly stimulated BMSC proliferation and differentiation.

- The rotating speed at 16 rpm in bioreactors stimulated better proliferation and differentiation of BMSCs in 3D, and stimulated better neurite extension from PC 12 cells.

- We cultured BMSCs on nerve grafts in bioreactors for days, and we then seeded PC-12 cells and co-cultured the BMSCs and PC-12 cells on the nerve grafts for another 11 days. We assessed neurite extension of the PC-12 cells on the nerve grafts using confocal microscopy. The results indicated that BMSCs cultured in bioreactors stimulated neurite extension from PC 12 cells.

- We have prepared the animal protocol and received approval from our IACUC on 08-02-2013. We also have put the approved animal protocol in the forms requested by the USAMRMC ACURO, and submitted it to USAMRMC ACURO on 11-13-2013, and received the approval from ACURO for this protocol at December 30, 2013. We made an amendment for the protocol and get approval by Stevens IACUC on October 24, 2014. We submitted the protocol amendment for ACURO approval on November 19, 2014, and received the approval from ACURO for this protocol amendment at December 30, 2014.

- We implanted three different nerve grafts: Autograft, 0.1mm spiral nerve graft with inner aligned nanofibers (16 min) wrapped with an outer tube (50µm thickness), and 0.1mm spiral nerve graft with inner aligned nanofibers (16 min – loaded with nerve growth factor) and wrapped with an outer tube (50µm thickness). We then assessed nerve regeneration using functional tests and histology analysis, the preliminary observation indicated that the autograft had better recovery among the three groups.

- Protocols and methodology was developed to coat PCL nanofiber matrices with a natural polymer. In the composite approach aligned and randomly and aligned nano fiber matrices were coated with sodium alginate (Na-Alg) of varying molecular weight in an effort to improve mechanical properties and cell attachment and phenotype development. Na-Alg coating was used to encapsulate bioactive factors specially laminin and NGF and their release to promote neuronal development. Based on the mechanical properties sodium alginate degraded for 1 hr was chosen for further experiments such as BMSCs seeding, attachment, microscopy and proliferation. Results for cell attachments and proliferations are published in *Polymers for Advanced Technology 2015* (DOI: 10.1002/pat.3594)**.**

- Ionically conductive hydrogels are developed and ionic conductivities of these hydrogels coated on PCL nanofibers are tested**.**

- Live/Dead assays were performed on ionically conductive hydrogels using BMSCs.

- Cell differentiations to the neuronal phenotypes were studied by applying electrical stimulations (400 mV for 10 minutes every 24 hrs). Samples without electrical stimulations served as a control.

5. CONCLUSION:

Since initiating this project we have observed that in fabricating our spiral scaffold that a 0.1 mm wall thickness is more desirable than a 0.2 mm wall thickness as it can allow more axon infiltration and yield more layers for the spiral structured scaffold thus allowing a greater surface area for nerve regeneration. We assessed the release kinetics of nerve growth factor and determined that PCL-PEG-BSA-NGF provided a favorable environment as observed by PC-12 neurite extension. The BMSCs, when seeded on nerve grafts placed in a bioreactor, cultured much better than when the nerve grafts were placed in a TCP. The completion of the *in vitro* studies allowed us to progress to the *in vivo* studies, which are currently on going. Based on our current progress with the *in vivo* studies we have observed that relative to the nerve graft (without any cells, growth factors, and proteins) the autograft yielded better results. While our *in vitro* data showed the efficacy of the nerve graft could be improved with various additives we have not yet included these groups in the *in vivo* studies. As such we expect the performance of the nerve graft to improve in our *in vivo* experiments as continue to incorporate additives. Cell viability studies indicated excellent cell attachment and proliferations on ionic chitosan. Also neurite extensions and expression of neuronal markers were prevalent upon electrical stimulations. These observations conclude that ionic chitosan's are an excellent material to their use for nerve regeneration applications.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press:

(2) Peer-Reviewed Scientific Journals:

(3) Invited Articles:

(4) Abstracts:

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Peer reviewed journal articles

1. Shelke N.B., Lee P., Anderson M., Mistry M., Nagarale R.K., Ma X.M., Yu X., Kumbar S.G., Neural Tissue Engineering: Nanofiber-Hydrogel Based Composite Scaffolds (*Polym. Adv. Technol.*, DOI: 10.1002/pat.3594). 2015.
2. Evaluating nerve guidance conduits for peripheral nerve injuries: a novel normalization method. Munish Shah, Wei Chang, and Xiaojun Yu. *Neural Regeneration Research. 9(22): 1959-1960,* 2014.
3. Anderson M., Shelke N.B., Manoukian O.S., Yu X., McCullough L.D, Kumbar S.G.*, Peripheral Nerve Regeneration Strategies: Electrically Stimulating Polymer Based Nerve Growth Conduits (Accepted: Critical Reviews™ in Biomedical Engineering Journal, 2015)
4. Bioactive Polymeric Nanofiber Dressings for Wound Healing. Guadalupe E., Ramos D.M., Shelke N.B., James R., Gibney C., and Kumbar, S.G. *J. Appl. Polym. Sci.* 2015, 132, 41879.
5. Gelatin Nanofiber Matrices Derived from Schiff Base Derivative for Tissue Engineering Applications. Jaiswal D., James, R., Shelke N.B., Harmon M.D., Brown J. L., Hussain F., and Kumbar, S.G.* *J. Biomed. Nanotechnol.,* 11,1-14, 2015, doi:10.1166/jbn.2015.2100
6. Innovative Regenerative Engineering Technologies for Soft Tissue Regeneration. James, R., Harmon M.D., Kumbar, S.G. Laurencin, C.T. *Technology and Innovation-J. Nat. Aca. Inventors* 16 (3-4), 195-214,2014
7. Synthesis and Characterization of Electrically Conducting Polymers for Regenerative Engineering Applications: Sulfonated Ionic Membranes. James, R., Nagarale, R.K., Sachan, V., Badalucco, C., Bhattacharya P., and Kumbar, S.G. *Polym. Adv. Tech.,* 25 1439–1445, 2014, DOI: 10.1002/pat.3385
8. Development of Redox-conducting Polymer Electrodes for Non-Gassing Electro-Osmotic Pumps: A Novel Approach" Bhattacharya P., Nagarale, R.K., Sachan, V., Singh A., Jahan K., and Kumbar, S.G. *J Electrochemical Soc.,* 161 (13), H3029-H3034 (2014).

Conference Proceedings/Abstracts/Presentations

1. Xiaojun Yu. "Tissue Engineering for Peripheral Nerve Regeneration" Math Bio Seminar, Department of Mathematical Sciences- New Jersey Institute of Technology, November 4, 2014.
2. Wei Chang, Paul Lee, Munish Shah, Sangamesh G Kumbar, Cato Laurencin, Xiaojun Yu. "Development of A Novel Structured Nanofibrous Nerve Guidance Conduit for Nerve Regeneration" J&J 2015 Engineering Showcase - Johnson & Johnson World Headquarter, New Jersey, March 10, 2015 (Poster presentation).
3. Manoukian OS, Marin C, Ahmad A, James R, Kumbar S.G. "Biodegradable Injectable Implants for Long-Term Delivery of Contraceptives and Other Therapeutics." Abstract. Northeast Bioengineering Conference (NEBEC) 2015 (Rensselaer Polytechnic Institute, Troy, NY).
4. Manoukian OS, Marin C, Ahmad A, James R, Kumbar S.G. "Biodegradable Injectable Implants for Long-Term Delivery of Contraceptives and Other Therapeutics." Abstract. American Society for Artificial Internal Organs (ASAIO) 2015 Conference (Chicago, IL).
5. Ahmad A, Manoukian OS, Idrees S, James R, Kumbar S.G. "Novel Soft Tissue Compliant Hydrogels Extracted from Sea Weed." Abstract. U21 Undergraduate Research Conference (URC) 2015. (University of Auckland, New Zealand).
6. Alejandra, M., Ramos, D.M., Laurencin, C.T., Kumbar, S.G, "Peptide linkage of Poly(caprolactone)-Chitosan Blend Scaffolds" BMES 2014 Annual Meeting, San Antonio, Texas, October 22-25, 2014 Poster # 3118
7. Ramos, D.M., Laurencin, C.T., Kumbar, S.G, "Tendon Differentiation Using Human Recombinant Insulin" BMES 2014 Annual Meeting, San Antonio, Texas, October 22-25, 2014 Poster # 653

8. Aravamudhan, A., Ramos, D.M., Harmon, M.D., Kumbar, S.G, "Characterization of Polysaccharide Based Micro-Nano Structured Scaffolds for Osteoinductivity" BMES 2014 Annual Meeting, San Antonio, Texas, October 22-25, 2014 Poster # 492

Book Chapter

1. Shelke N.B., Anderson M., Idrees S.M., Nip J., Donde S., Gronowicz G., Kumbar, S.G. "Polyester nano-and micro-technologies for tissue engineering" in "Handbook of Polyester Drug Delivery Systems" Edited by MNV Ravikumar 2015, Pan Stanford Publishing (Accpted 2015)
2. Manoukian O.S., Ahmad A., Marin C., James R., Mazzocca, A.D., Kumbar, S.G, "Bioactive Nanofiber Dressings for Wound Healing" in "Wound Healing Biomaterials Vol 2: Functional Biomaterials" Edited by Magnus Agren 2015, Woodhead Publishing Limited (Accepted-In Presss).
3. Ramos, D.M., Peach, M.S., Mazzocca, A.D., Kumbar, S.G, "Tendon Tissue Engineering" in "Engineering Musculoskeletal Tissues and Interfaces" Edited by Nukavarapu, Freeman and Laurencin 2014, Woodhead Publishing Limited (Accepted-In press).

7. INVENTIONS, PATENTS AND LICENSES: List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

Patent:

1. Kumbar S.G., Harmon M.D., Method for making non-shrinking porous poly(lactic-co-glycolic acid) nanofiber matrices for tissue regeneration and drug delivery applications. US Provisional Application # US 62/109,238, 2015

8. REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

- We quantitatively assessed the release kinetics of the nerve growth factor by adding chitosan and observed the neurite extension of PC12 cells. We compared the following groups: PCL, PCL-NGF, PCL-BSA-NGF, PCL-PEG-NGF, and PCL-PEG-BSA-NGF. PCL-NGF resulted in poor PCL-12 extension, PCL-BSA-NGF showed a better NGF release based on PC-12 neurite extension, and PCL-PEG-NGF improved cell attachment. Based on these characteristics the PCL-PEG-BSA-NGF group yielded the best results.

- We performed Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assay on Schwann Cells (positive control) and BMSCs (negative control). Based on the results, we have designed primers for better characterizing the differentiation of BMSCs.

- We seeded BMSCs onto nerve grafts at different seeding densities of $1X10^4/ cm^2$, $5X10^4/ cm^2$, and $1X10^5/ cm^2$, and observed that the seeding density of $1X10^5/ cm^2$ resulted in highest proliferation and differentiation of BMSCs.

- We observed there was higher cell proliferation and differentiation of BMSCs seeded on nerve grafts for the 3D culture method in the rotating bioreactors than those of BMSCs seeded on nerve grafts 2D culture method. The rotating speed at 16 rpm in bioreactors stimulated better proliferation and differentiation of BMSCs in 3D, and stimulated better neurite extension from PC 12 cells.

- We implanted three different nerve grafts: Autograft, 0.1mm spiral nerve graft with inner aligned nanofibers (16 min) wrapped with an outer tube (50μm thickness), and 0.1mm spiral nerve graft with inner aligned nanofibers (16 min – loaded with nerve growth factor) and wrapped with an outer tube (50μm thickness). We then assessed nerve regeneration using functional tests and histology analysis, the preliminary observation indicated that the autograft had better recovery among the three groups.

- Protocols and methodology was developed to coat PCL nanofiber matrices with a natural polymer. In the composite approach aligned and randomly and aligned nano fiber matrices were coated with sodium alginate (Na-Alg) of varying molecular weight in an effort to improve mechanical properties and cell attachment and phenotype development. Na-Alg coating was used to encapsulate bioactive factors specially laminin and NGF and their release to promote neuronal development. Based on the mechanical properties sodium alginate degraded for 1 hr was chosen for further experiments such as BMSCs seeding, attachment, microscopy and proliferation. Results for cell attachments and proliferations are published in *Polymers for Advanced Technology 2015* (DOI: 10.1002/pat.3594)**.**

- We have developed ionically conductive hydrogels and ionic conductivities of these hydrogels coated on PCL nanofibers have tested. These hydrogels have the potential for enhancing nerve regeneration in presence of electrical stimulations.

9. OTHER ACHIEVEMENTS: This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

Funding applied for based on work supported by this award:

1. Project title: I-Corps: A Novel Structured Nanofibrous Nerve Guidance Conduit for Nerve Regeneration (PI: Xiaojun Yu)
Supporting agency: National Science Foundation
Performance period: 07/15/2015 to 12/31/2015
Level of funding: $ 50,000
Project's goals: This project is to get training through NSF for potentially commercializing a nerve conduit for repairing peripheral nerve injuries.
Status: Funded.

10. REFERENCES: List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

[1] G. R. Evans, K. Brandt, A. D. Niederbichler, P. Chauvin, S. Hermann, M. Bogle, L. Otta, B. Wang, and C. W. Patrick, "Clinical long-term in vivo evaluation of poly (l-lactic acid) porous conduits for peripheral nerve regeneration," Journal of Biomaterials Science, Polymer Edition, vol. 11, no. 8, pp. 869–878, 2000.

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[13] Wood, Matthew D., et al. "Affinity-based release of glial-derived neurotrophic factor from fibrin matrices enhances sciatic nerve regeneration." *Acta biomaterialia* 5.4 (2009): 959-968.
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[15] Jenq, C-B., and R. E. Coggeshall. "Numbers of regenerating axons in parent and tributary peripheral nerves in the rat." *Brain research* 326.1 (1985): 27-40.

11. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

None.