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Promotion of human Schwann cell proliferation using heparin/ collagen coated nerve conduits

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Abstract

Often in the aftermath of an injury or surgery, the sense of touch and muscle control is lost in the affected area as nerves are damaged or severed and fail to grow back completely. The regeneration of the nerve cells can be promoted by treating the nerves with nerve conduits. Nerve conduits are hollow cylinders of bio-compatible materials that can be surgically implanted to the disconnected nerve to promote and direct the growth of nerves. The objectives of this research are to investigate the ability of nerve conduits treated with layer-by-layer coatings to promote the growth of Schwann cells, to manufacture nerve conduits in the lab, and to compare the performance the home-made conduits to those available on the market. Nerve conduits were manufactured by electrospinning solutions of collagen to create a solid film of collagen fibers only several millimeters thick. The film can be shaped into a cylindrical nerve conduit. The nerve conduits are then submerged in alternating solutions of collagen and heparin to create a thin, polyelectrolytic coating on the surface of the fibers. The viability of the nerve conduits are evaluated by growing cultures of human Schwann cells on the conduits and then measuring and comparing the proliferation of the cultures on the conduits with and without coatings. In reference to the stated objectives of the research: It was shown in each of the experiments that the conduits with coatings performed better than those without coatings, sometimes by as much as double the number of cells grown. Nerve conduits were consistently and successfully manufactured in the lab via electrospinning, but upon treatment with the coatings they lost much of their physical integrity. It follows that the nerve conduits manufactured in the lab consistently performed worse than those available on the market. Nerve autografts are currently the most widely used method of nerve repair, but they have limitations including limited supply of donor nerves, mismatch between nerve and graft dimensions, and neuroma. Nerve conduits are a promising alternative to grafts, and in conclusion the results of the research indicate that the addition of heparin and collagen coatings to nerve conduits will increase their efficacy, making them a more viable option for nerve repair.

1. Introduction

Peripheral neuropathy is a health condition that results from disconnects in peripheral nerves, nerves which are outside of the central nervous system. The peripheral nervous system consists of essentially a network of cables and wires of varying sizes like the cardiovascular system's arteries, veins, and capillaries. Physical damage from injury and surgery has the potential to create disconnects in the "cables." The disconnects causes loss of signals in the nerves, signaling when there should not be any, and distortion of signals.[3] Some of the resulting symptoms of peripheral neuropathy include numbness, pain, and loss of motor control. Nerves that are injured but not cut have high chances of healing, but full recovery from a disconnected nerve is not as likely. Because this type of injury can be difficult to completely remediate it is commonly a chronic issue for the affected persons. Nerve grafts or autografts are currently the gold standard of peripheral nerve repair and can significantly increase the healing ability of nerves in disconnects. They effectively bridge the gap in the disconnected nerve, do not cause a reaction in the immune system, and can be used to treat nerve disconnects of more than several centimeters. There are few drawbacks to the autograft approach, the first being that there are limited options for the donor nerve as it must be taken from somewhere else in the body which also leads to donor site morbidity. Secondly, the donor nerve will not always match the dimensions of the damaged nerve, which can cause complications in healing as well as issues in nerve signaling. And thirdly, autografts can lead to neuroma (nerve tumors) which can cause even more discomfort and pain in the affected area.

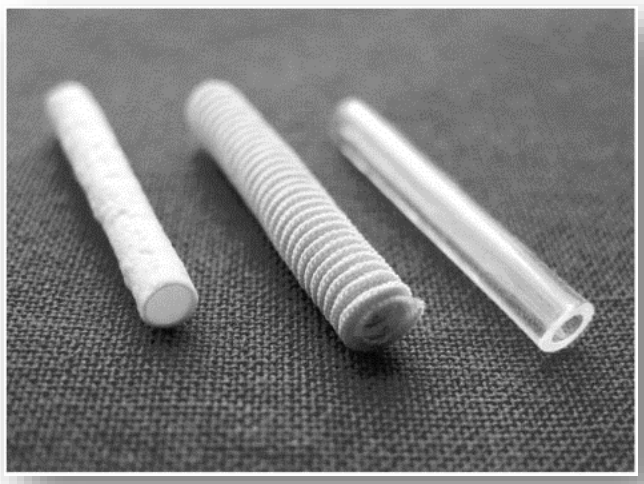

Figure 1: Nerve conduit: (left) Collagen I NeuraGen; (middle) PGA NeuroTube; (right) PLACL Neurolac
From: "Strategies for Regeneration of Components of Nervous System: Scaffolds, Cells and Biomolecules."

Nerve guide conduits (also referred to as nerve guides, nerve conduits, and NGCs) are an alternative to autografts that ideally provide the same effectiveness of autografts while eliminating the drawbacks. There are a variety of kinds of nerve guides, and they can be classified as biological or synthetic.[1] Examples of biological nerve guides are repurposed blood vessels and muscle tissues. An

example of synthetic nerve conduits are three tubes shown above. There are many different materials that could be used to make synthetic nerve conduits. “Synthetic polymers like poly (glycolic acid) (PGA), poly (L-lacticacid) (PLLA) and poly (caprolactone) (PCL) and their copolymers have been extensively used to produce nanofibers via electrospinning method for nerve tissue applications.”[5] In this research we used synthetic nerve guides that were electrospun using collagen type 1. In addition to the nerve conduits that we made we also used nerve conduits available on the market; Integra LifeSciences donated nerve conduits for us to use including their NeuraGen and NeuroFlex products, which are also made with Collagen 1. Integra LifeSciences’ nerve conduits were some of the first approved by the FDA in the early 2000’s and have further proven themselves through their extensive use since. “Pre-clinical and clinical studies have been performed on the efficiency of this device (NeuroGen), found comparable to the gold standard (autografts) in defects size up 20 mm”.[2] As stated earlier, nerve conduits would *ideally* have all the benefits and none of the drawbacks of autografts, but nerve conduits have their limitations as well. Their effectiveness is limited to applications to nerve disconnects less than 20 millimeters in length. They also have not been successful in “large diameter nerve deficits”.[6] Pushing the boundaries in either scenario reduces the effectiveness of the nerve conduit and has the potential to cause further complications for the patient.

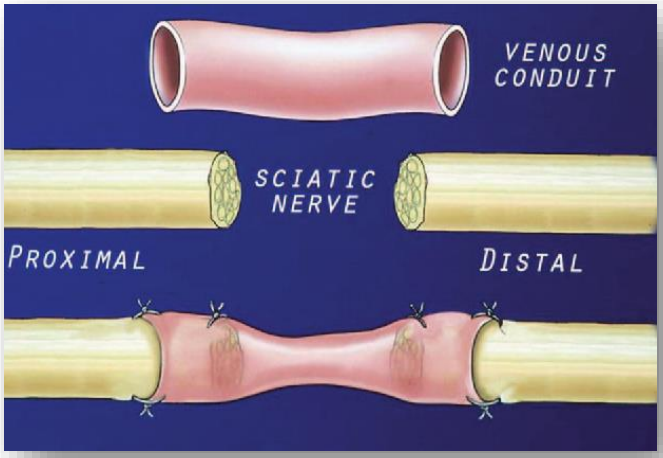

Figure 2: Illustration of a biological nerve guide being used to mend the gap in a disconnected nerve
From: Semantics Scholar

For this research, the modification to the nerve conduits that is hypothesized to increase their efficacy is the coating of the nerve conduits with thin films via the Layer-by-Layer (LBL) method. The thin films consist of alternating layers of charged molecules on top of a surface meant to be in contact with a biological system. The film of electrically charged molecules creates unique surface properties that are conducive to cell attachment and growth, and it can serve as a repository for additional chemicals like proteins or drugs.[7] The LBL method is executed in our case by submerging the nerve conduits in alternating solutions of heparin and collagen. Heparin and collagen molecules will accumulate via diffusion onto the surface of the nerve conduit to build up a thin layer polyelectrolytic material. The single collagen molecule is a triple helix of interconnected polypeptide chains called a tropocollagen. Tropocollagens arrange themselves into structures call fibrils, and many fibrils together make fibers. Collagen is found in muscles, cartilage, bone, and the extracellular matrix.[9] Collagen thus is highly biocompatible and can provide strong structural support in the LBL scaffolding. Heparin is a polysaccharide with a strong negative charge. It is commonly prescribed as a blood thinner, but heparin has also been shown to improve the growth of axons in peripheral nerve damage.[7] The combination of strong collagen layers with active heparin layers is known to increase the proliferation,

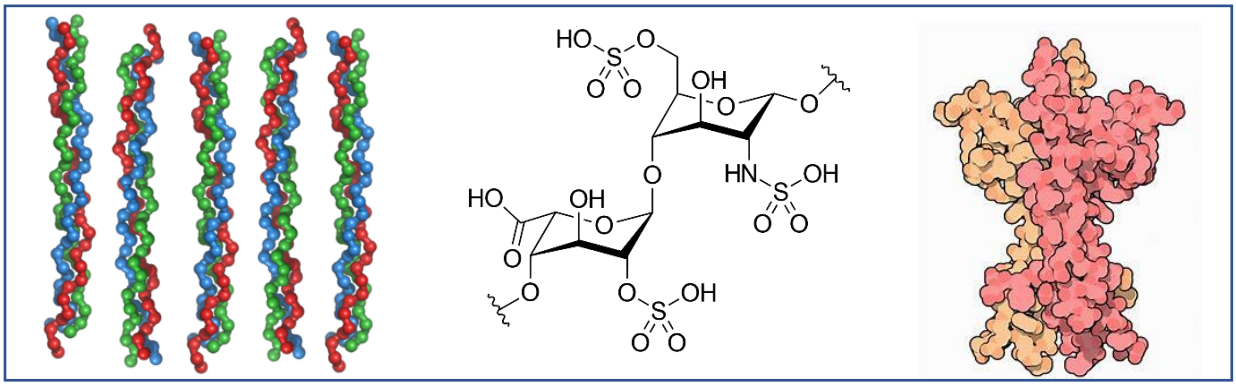

Figure 3: A single collagen protein (left), a section of a chain of heparin molecules (middle), and Nerve Growth Factor (right)
From: ResearchGate

adhesion, and viability of Schwann cells.[7] Nerve growth factor (NGF) is a protien that "is involved in the development and maintenance of the nervous system. NGF binds with high affinity to the extracellular region of the tyrosine kinase receptor TrkA."[10] It is used in this research to boost the proliferation of Schwann cells.

Schwann cells are glial (non-impulse producing) cells in the peripheral nervous system and are chiefly responsible for forming the myelin sheath around the axons of nerve cells. The nerve cell can be likened to a wire where the axon is the conductive metal, and the Schwann cells are the insulating plastic material. Myelin's physical and chemical properties allow it to reduce the mass transfer of ions in and out of the axon membrane. By decreasing the mass transfer across the surface of the axon the speed of propagation of signals through the axon is increased. In addition to increasing the effectiveness of nerve signals, "Schwann cells contribute greatly to the replacement of injured neurons and allow the extension of the axon."[7] When there is an injury to the peripheral nervous system, Schwann cells will

release chemicals and neurotrophic factors that signal for the immune response and boost nerve regeneration. Because Schwann cells play an essential role in the healing process for nerves, they were used in this research project to evaluate the effectiveness of the nerve guides.

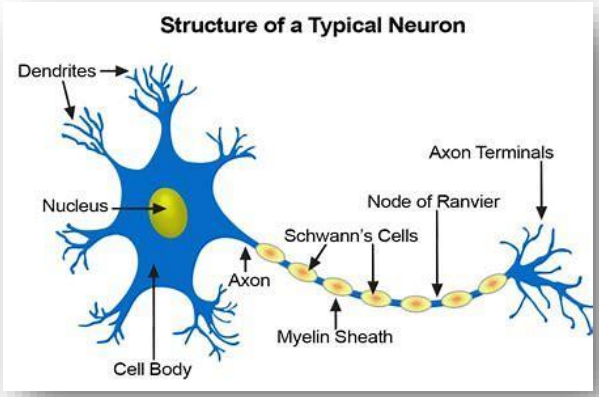

Figure 4: Structure of a typical neuron with Schwann cells colored in yellow
From: The National Center for Biotechnology Information (NCBI)

2. Problem Statement, Objectives, and Scope

Peripheral neuropathy affects more than 20 million people in the United States and medical expenses on peripheral nerve damage and nerve regeneration treatments for those individuals exceeds $150 billion annually.[3, 7] In light of the drawbacks mentioned for the current "gold standard" of nerve repair, nerve autografts, there is a significant need and market for the development of a nerve guide conduit that is as effective and less problematic than autografts. The hypothesis of this research is if Schwann cells are grown on nerve growth conduits with LBL coatings of heparin and collagen, then cell viability will be increased compared to nerve conduits without LBL coatings. It follows that the overall objective of this research is to investigate the ability of the nerve conduits treated LBL coatings to promote the growth of Schwann cells. Sub-tasks related to this objective that need to be met in order to say that the objective has been appropriately addressed are to 1) confirm the presence of LBL coatings on the nerve conduits, 2) confirm cell attachment to nerve conduits, and 3) to measure the viability of cells that have grown on the nerve conduits. Two secondary objectives of this research are to 4) manufacture nerve guide conduits in the lab and then 5) to compare the performance of the home-made ones to the nerve conduits provided by Integra which serve as a representative of the nerve conduits available on the market. The scope of this project is limited to in-vitro testing of Schwann cell growth on the nerve conduits.

3. Materials and Methods

Cell culture

Human Schwann cells from a 28-year-old male were used for the experiments. The cells used were from passages 18 through 21. The Schwann cells were given 90% Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal bovine serum (FBS) as food and were incubated in a humid incubator at 37 degrees Celsius and 5% CO_2.

Electrospinning collagen

Electrospinning is a common technique in biomaterial fabrication. The general methodology for electrospinning and crosslinking the collagen fibers that served as the material for the nerve conduits was developed by Drs. Jorge Almodovar and David Castilla in their paper "Engineering of a stable collagen nanofibrous scaffold with tunable fiber diameter, alignment, and mechanical properties." For this method, 0.6 grams of lyophilized type 1 collagen derived from cow tendon is cut into tiny pieces and mixed with 3 mL of 90% acetic acid (v/v in pure water) inside of a 20 mL vial. The vial is transferred to a hotplate where it is magnetically stirred and heated at 100 Celsius for 1.5 hours or until all the collagen has been dissolved. The final concentration of the solution comes to 20% w/v or 0.2 grams/mL. If the solution has been prepared properly it will have a slight yellow color and a noticeably high viscosity. Tilting the vial and observing a slow response (slow compared to water) of the fluid to the incline is sufficient to confirm a good viscosity. Each properly prepared vial will contain approximately 3 mL of solution, thus multiple vials must be prepared to have enough solution for making a significantly thick nerve conduit fiber. In our experiments we used 8.5 and 10.5 mL of the collagen solution. At this point a 10cc syringe is filled with the solution which can be transferred into the syringe pump of the electrospinning apparatus, and the rotating drum collector is covered with aluminum foil so that the fibers can be easily removed from the apparatus. The power supply generates an electric field with a strength of 45 kilovolts between the needle of the syringe pump and the rotating drum collector. The solution is pumped out of the syringe at a rate of 3.0 mL/hour.

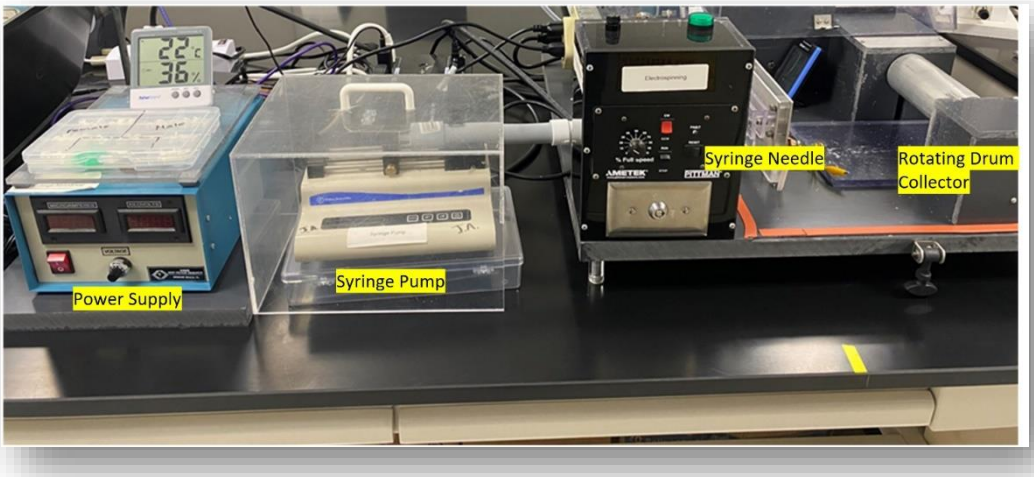

Figure 5: The electrospinning apparatus

Depending on the starting volume in the syringe, the electrospinning process can take up to several hours to complete. As the solution is pushed out of the syringe the collagen is forced onto the collector by the electric field where it forms into a film of nanofibers. After all the solution has been used the newly created fiber material can be taken out of the electrospinner and stored in a vacuum sealed container.

Glutaraldehyde crosslinking

Cross-links are bonds that connect chains of polymers. The cross-linked polymer chains cause a change in the physical properties of the polymer material such as increasing stiffness or decreasing solubility. There are many different methods and chemical agents to use in polymer cross-linking processes, but the method used for crosslinking in this research includes the reaction of 50% glutaraldehyde with the collagen in the fibers. The electrospinning apparatus used consists of a plastic box that serves as the reaction chamber, an Erlenmeyer flask to hold the supply of glutaraldehyde, and a compressor pump. The compressor pump pushes air up through the bottom of the Erlenmeyer flask to create aerated glutaraldehyde. The aerated glutaraldehyde then flows through the reaction chamber and across the fibers to form the cross-links. This is done for 15 to 20 minutes or until the material becomes insoluble in water. Crosslinking for too long will result in a very stiff material.

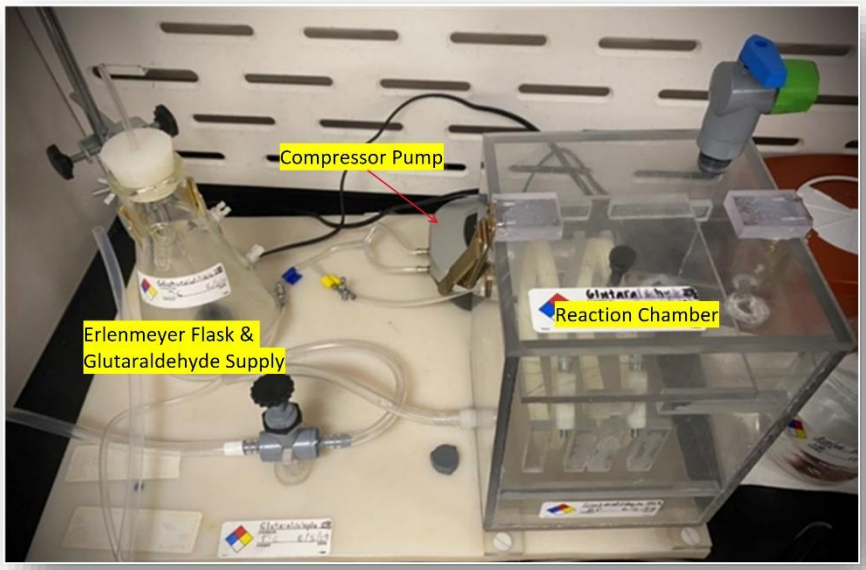

Figure 6: The cross-linking apparatus

Layer by layer coatings fabrication

The layer-by-layer process used in this research is the same used in the 2019 paper, “Real-time monitoring of human Schwann cells on heparin-collagen coatings reveals enhanced adhesion and growth factor response” by Luis Pinzon. In this process, sodium heparin, lyophilized collagen 1, and 50% polyethyleneimine (PEI) in water were prepared in a sodium acetate buffer (pH=5 for heparin and PEI and pH=4 for collagen), each to a concentration of 1mg/mL. The steps for creating the layers on the

nerve conduits via submersion are as follows: 15 minutes submerged in PEI to create a positively charged foundational layer followed by 5 minutes washing, then 5 minutes submerged in collagen followed by 3 minutes of washing, and then 5 minutes submerged in heparin followed by 3 minutes of washing. Completion of these steps will create one bilayer. These steps are repeated 6 times to complete the LBL coating. Upon completion of the procedure the samples are set out to dry.

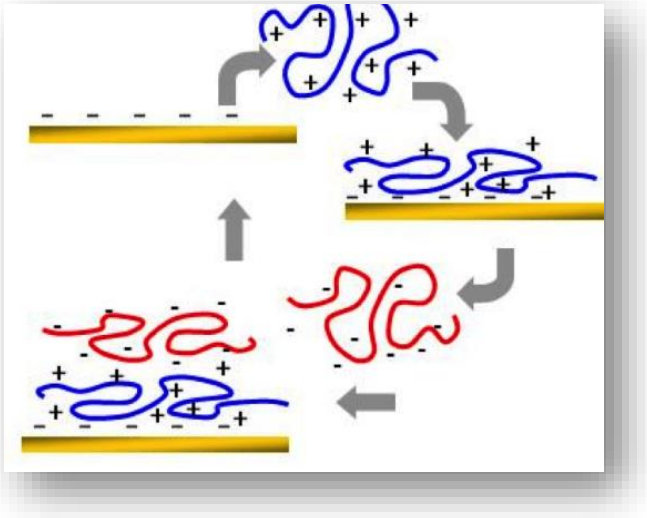

Figure 7: Visualization of the LBL process.

Qualitative confirmation of LBL coatings on nerve conduits

Scanning Electron Microscope (SEM) imaging and colorimetric tests were used to confirm the presence of the LBL coatings on the nerve conduits. Azure A's color turns from blue to purple in the presence of heparin. A drop of Azure A (80 ug/mL) was added to samples with and without LBL coatings.

Experimental Conditions

A total of four experiments were performed in this research project. The first two experiments included testing only the nerve guides from Integra, while the second two experiments tested the electrospun fibers in addition to the nerve guides. All experiments were carried out in-vitro in 96 well plates. Material from the Integra nerve conduits and electrospun fibers were cut into small circles and placed at the bottom of the wells. Before cells were seeded to the wells they were counted in a hemocytometer and diluted to a target concentration of 25000 cells/square centimeter. 20 microliters of cells suspended in cell media (DMEM + 10% FBS) was added to each well. 15 microliters of NGF at a concentration of 10 nanograms/mL was added to half of the wells (shown as a plus sign in figure 8). And finally, each well was completed with cell media to a total volume 200 microliters. After seeding the cells, the 96 well plate was covered and placed in the incubator. The test groups for the experiments are included in the bulleted list and figure below.

- TCPS (cells grown on the bottom of the well)
- TCPS + NGF
- Fibers
- Fibers + NGF
- Fibers + LBL
- Fibers + LBL + NGF
- NGC
- NGC + NGF
- NGC + LBL
- NGC + LBL + NGF

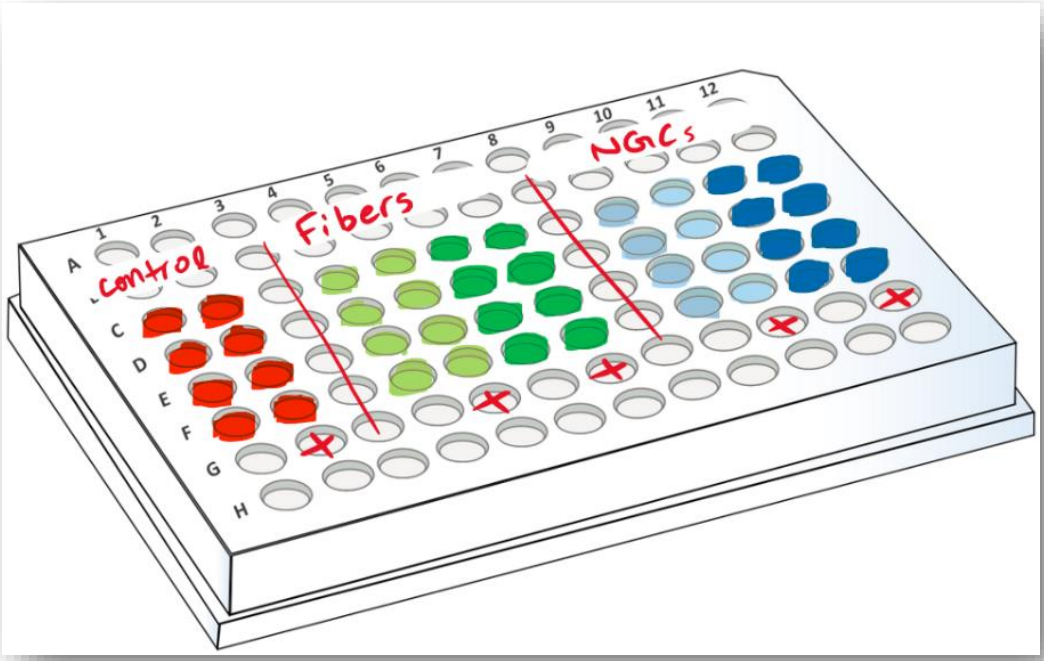

Figure 8: Experimental set-up

Figure 9: Two adjacent wells containing electrospun fiber without LBL (top) and an electrospun fiber with LBL (bottom).

Schwann cell viability measurements

Quantitative measurements for the viability of the human Schwann cells were taken using the BioTek Synergy|LX multiplate reader and PrestoBlue cell viability agent. The multiplate reader detects the intensity of fluorescent light radiating from each sample. At the end of the 4 days the cell medium mixture was removed, and 90 microliters of cell media and 10 microliters of PrestoBlue Reagent were added to each well. When PrestoBlue is in contact with the cells it becomes reduced and takes on fluorescent properties.[7] The more reduced PrestoBlue there is in a sample the higher the intensity of the fluorescence readings and thus cells there are on the sample.

Qualitative confirmation of cell attachment to nerve conduits

To see the cells attached to the nerve conduits they were stained using Hoechst 33 342 dye which attaches to the nucleic acids of the DNA. The working volume for each step in the process is 500 microliters. The staining process starts by incubating the cells in 4% formaldehyde in 1X phosphate buffered saline (PBS) for at least 15 minutes. After incubation, the formaldehyde is removed, and the cells are washed several times with PBS for 3 minutes each wash. The cells are then treated with Triton X-100 for 10 minutes which makes the cell membranes permeable to the dye.[7] After being permeabilized the cells are washed again and are ready to be stained. Once the Hoechst solution has been added it takes 10 minutes to complete the staining. The Hoechst solution will degrade when exposed to light, so this step is done with dimmed lights. From this step on, the cells must be covered with aluminum foil when not being observed. A final washing step is performed, and the cells are ready to be seen. A Leica inverted fluorescence microscope with standard DAPI filter was used to see the cells.

4. Results Discussion

Layer by layer coating on nerve conduits

The layer-by-layer method was carried out on the electrospun fibers and the nerve conduits donated to us by Integra. SEM images were taken of nerve conduit samples with and without LBL to inspect the morphology of the fibers and the extent to which the polyelectrolyte coating attached to the nerve conduit. The SEM images of the Integra nerve conduit are shown in figure 10. Although the difference between the smaples with and without LBL coatings is not profound in these images, in the images of nerve conduit with no coating the nanofibers are quite frayed, while in the conduits with coatings less of the frayed fibers are visible as layers of heparin and collagen are covering them.

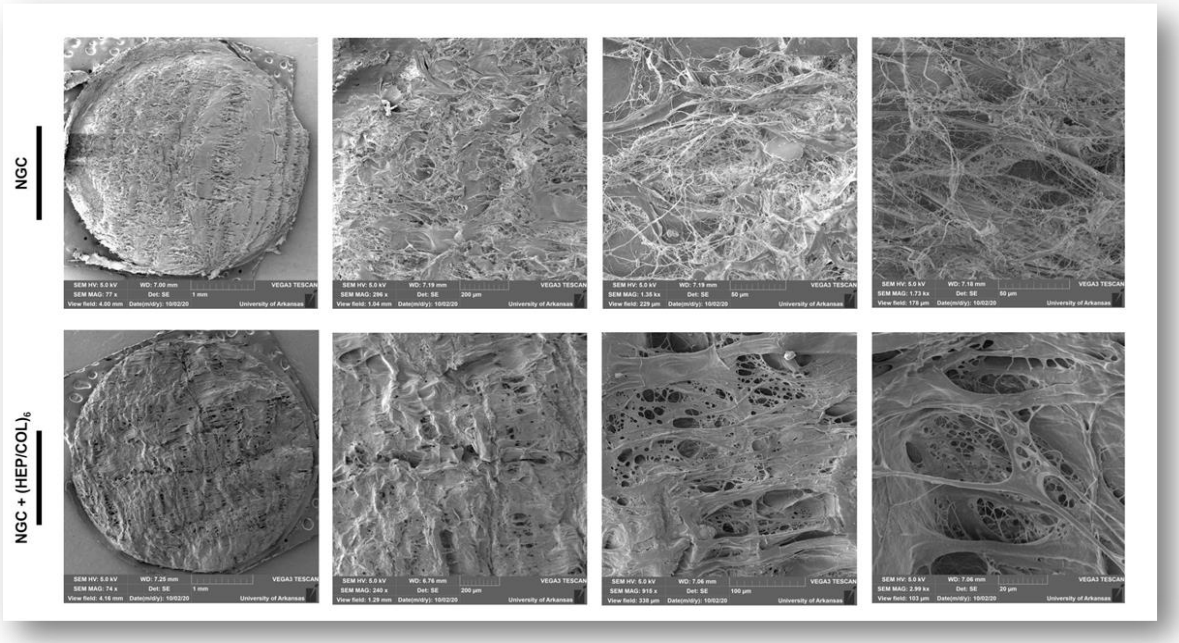

Figure 10: SEM images of the Integra nerve conduit without LBL (top) and with LBL (bottom) at varying zooms

The Integra nerve conduit's physical properties such as stiffness and thickness remained virtually unchanged after the addition of the coatings. Conversely, the electrospun fiber's poperties changed significantly upon addition of the coatings. After the electrospun fiber samples finished drying following the LBL procedure, they had turned into a translucent, plastic material that was extremely rigid. This material would snap and break under stress whereas the Integra nerve conduits and fibers without LBL would tear under stress. This happened each time an electrospun fiber was coated with LBL.

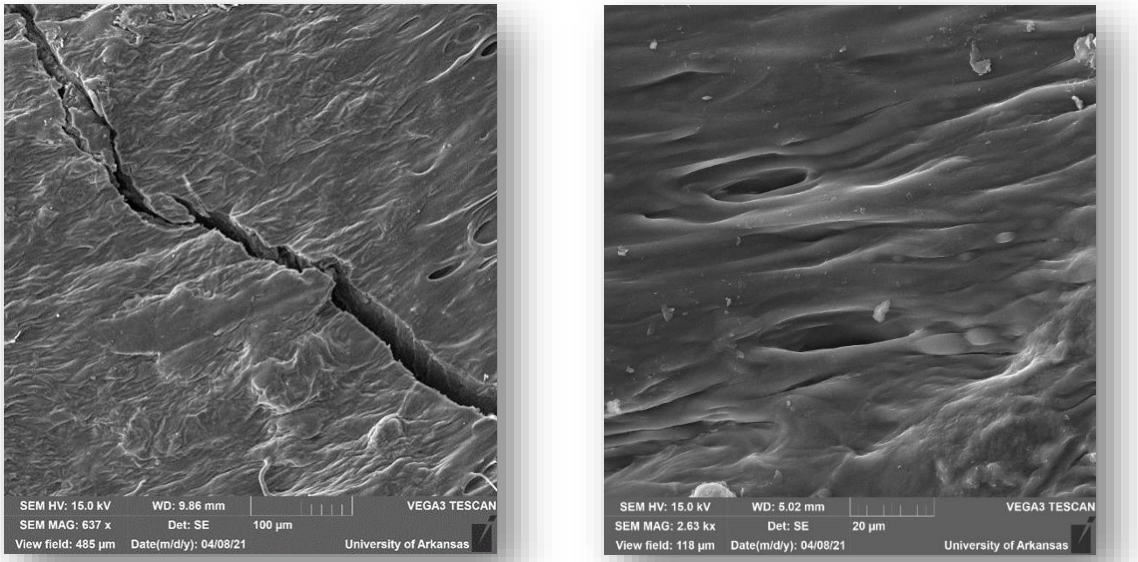

Figure 11: SEM images of the electrospun fibers with LBL at two different zoom levels.

The presence of the coatings can clearly be seen in figure 11 in that the individual fibers have been totally covered by the LBL coating. The presence of the coatings was also confirmed via colorimetry with Azure A dye. The process and results are simply illustrated in figure 12.

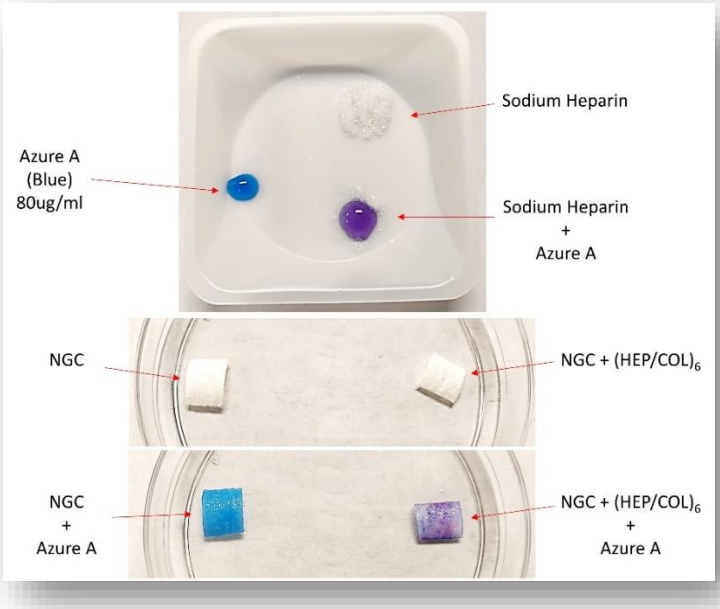

Figure 12: Results of addition of Azure A to LBL coated nerve conduits

Cell attachment results

Cell attachment was successfully confirmed using the nuclear staining method described in the methods section.

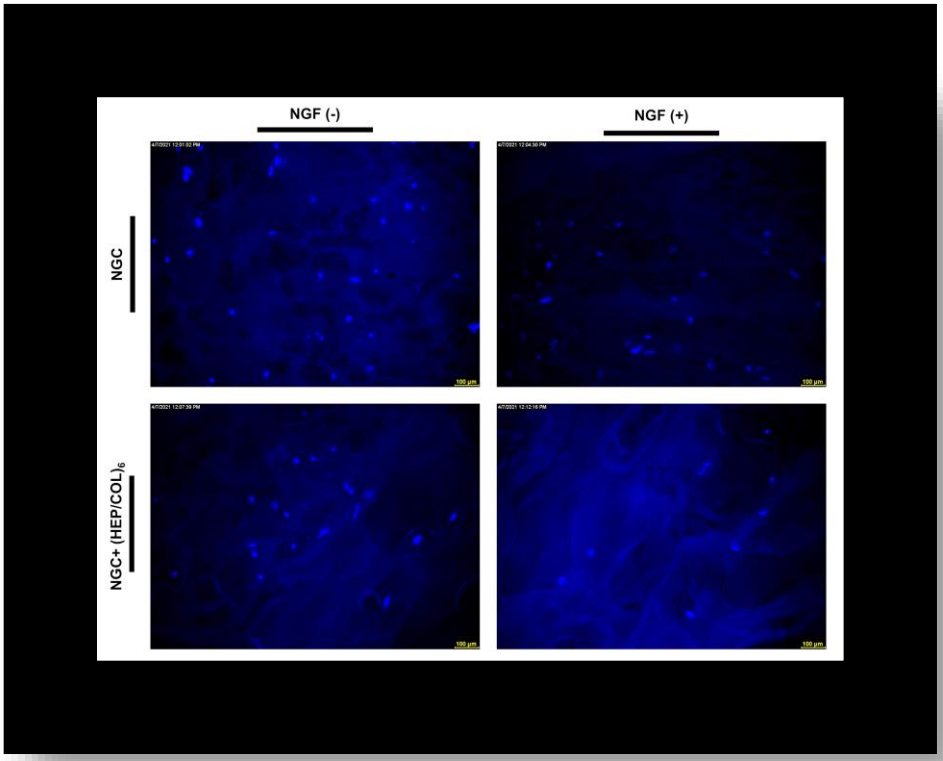

Figure 13: light blue dots are the cell nuclei and the darker blue background is the nerve conduit material

Cell viability results

Data for the flourenscene intensity reading from each well was recorded and copied into an excel spreadsheet. Calculations were performed on each test group to reveal the average intensity and standard deviation for each test group. A test group would be a single column of wells as shown in figure 8. The average performance of each test group for all four experiments is shown below.

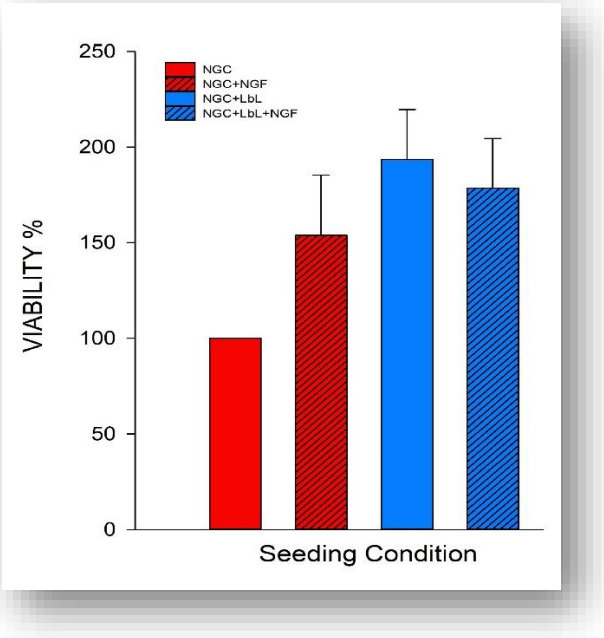

Figure 14: Average test group viability +/- the standard deviation of n=12 samples.

For every experiment the nerve conduits with LBL performed the best out of the test groups. It is curious that the last group, the nerve conduits with LBL and NGF, did not perform as well as the group without NGF. This does resemble preliminary experiments with Schwann cells and heparin/collagen LBL films in Dr. Almodovar's lab as shown in figure 15. These experiments were made to track real time monitoring of Schwann cell growth on heparin/collagen films. The data below is from those experiments, and shows that during the first day of the experiment the LBL+NGF group starts out with the highest rate of proliferation, but ends up dropping off before evening out at a lower amount of cell growth than the group without NGF. This could be because the high rate of profileration during the first day uses up more cell media so that down the road the cells do not have enough food to maintain their numbers.

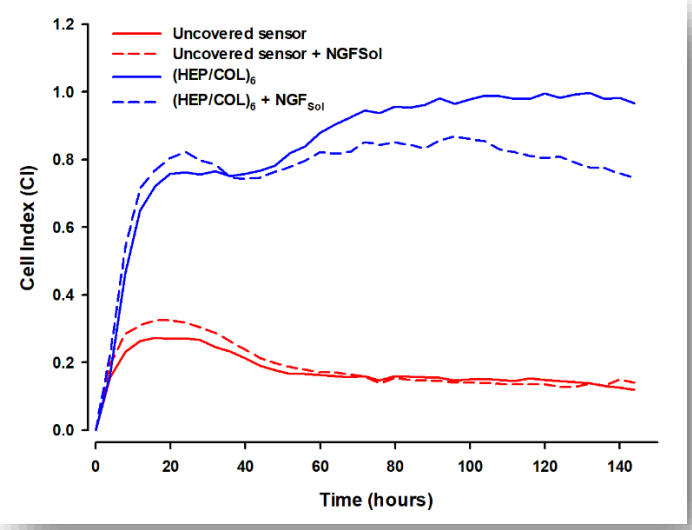

Figure 15: Data for real time monitoring of Schwann cell growth on heparin/collagen LBLs
From: Pinzon-Herrera, Luis, et al. "Real-Time Monitoring of Human Schwann Cells on Heparin-Collagen Coatings Reveals Enhanced Adhesion and Growth Factor Response."

Electrospinning results

The nerve growth conduits made using the electrospinning process as described in the materials and methods section were created using 8.5 mL and 10.5 mL of the collagen in acetic acid solution. These volumes resulted in a much higher quality for the nanofiber material as compared to previous attempts using 3 and 5.5 mL of solution. The fibers were stiff but could be deformed with minimal effort. Only a slight stiffness is desirable for the fibers as they would need to be formed into a cylindrical shape if they were to be used in an in-vivo experiment. The macroscopic visual and physical properties of the electrospun fibers were like that of the nerve conduits provided by Integra.

Figure 16: A section of the fibers made from 8.5 mL of solution

Crosslinking results

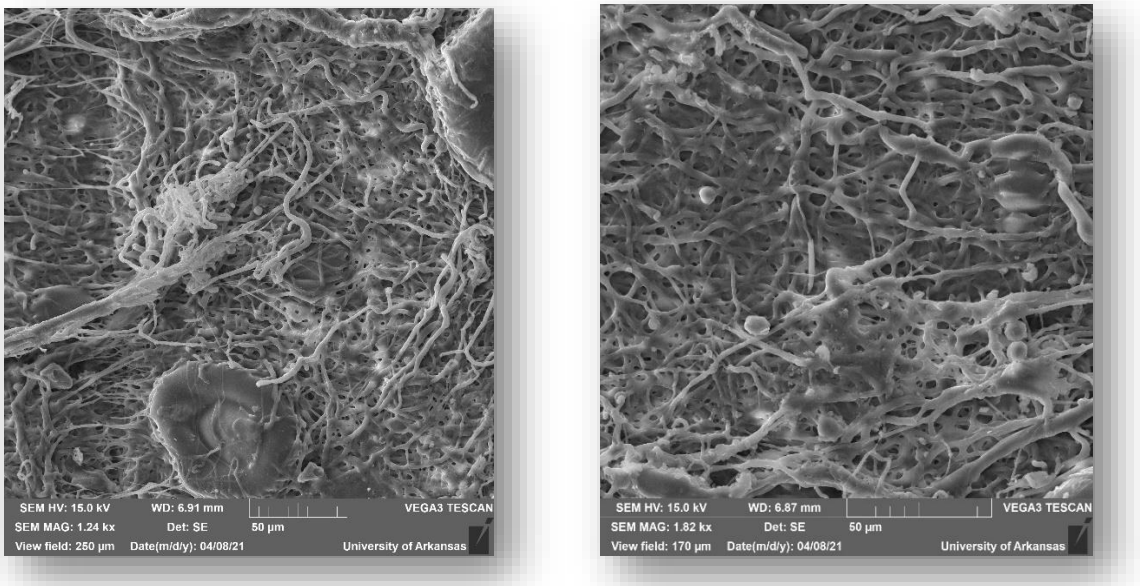

Figure 17: SEM images of the electrospun fibers.

After crosslinking, the fibers became insoluble in water at room temperature. It can be seen from the SEM images above that the electrospun fibers are tightly packed. On the left image there is shape that resembles a red blood cell. This could be a spot on the fiber where a portion of the solution from the syringe pump did not completely solidify into fibers before it reached the collector. It looks like it hit the collector as a liquid droplet and then solidified as it was flattening, like in a slow motion video of a raindrop flattening when it hits the ground, except for imagine if the raindrop froze mid-flatten.

Performance comparison between the electrospun fibers and market NGCs

Notice that in the cell viability results data from only four test groups is presented, but in the experimental conditions part of the methods section there are eight test groups listed (excluding the two control groups). This is because the electrospun fibers became a hydrogel material after the four days of cells growing on them in cell media and were not able to be used to collect flourescent data.

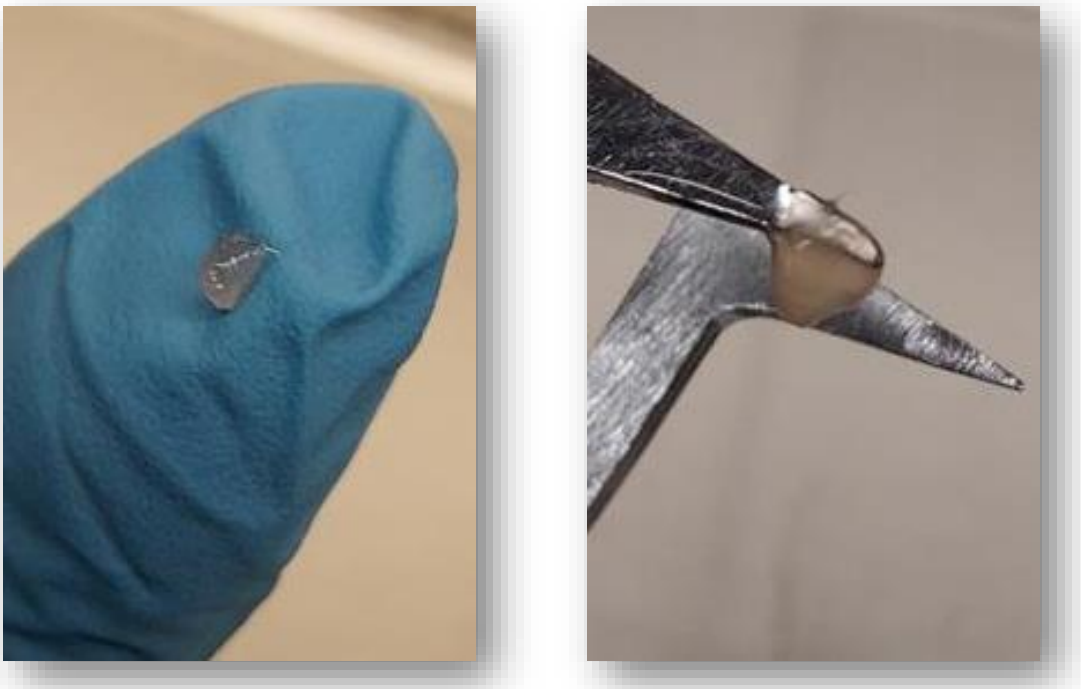

Figure 18: Electrospun fibers turned hydrogel after the experiment

5. Conclusions and Future Work Recommendations

The overarching research objective was appropriately addressed as each of the sub-tasks related to the overarching objective were completed with favorable results overall. The conclusion to the overarching objective was that the LBL coatings do indeed enhance growth of Schwann cells on the nerve conduits. The obvious next step for this research is to compare the nerve conduits with and without LBL in-vivo. A possible in-vivo experimental setup could be cutting the sciatic nerve in the legs of lab rats and then comparing the ability of nerve conduits with and without LBL to heal the disconnected nerve. The secondary objective of this research was also successfully addressed, but not with as favorable results. The electrospun fibers that served as the home-made nerve conduits became non-viable after application of the coatings as they turned into rigid plastic material, and then failed even further when they turned to hydrogel during each experiment. The first issue could be avoided by not drying the electrospun fibers after the LBL process and instead storing them in phosphate buffered saline until it is time for the experiment. This solves the turning into plastic problem, but it probably would not keep the fibers from turning into hydrogel during the experiment; it may even make it worse. A potentially better way to avoid this issue would be to change the LBL process so that instead of submerging the samples in solutions of collagen and heparin, the collagen and heparin could be electrospun onto the fibers. Electrospinning the collagen as a LBL coating has been shown to work already, but electrospinning heparin would not be as easy. It has been done, specifically in reference 11, where solutions of heparin were electrospun onto an artificial vascular graft. They used a more exotic electrospinning apparatus to spin the heparin inside of a second solution so that heparin would comprise the core of the nanofibers. This method would not be as applicable to the LBL method though.

If electrospinning heparin were to be tried, the concentration of the heparin solution used for LBL would have to be increased.

In conclusion, the heparin/collagen LBL coatings successfully enhanced the ability of the nerve conduit material to promote Schwann cell growth and suggests that nerve conduits could one day fully replace autografts.

6. Acknowledgements

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