Texas Tech University

Electrospun Nanofiber Scaffold made of PCL and Collagen for HUVEC Viability

IE 5356 Biomedical Design and Manufacturing

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1. Introduction

The goal of tissue engineering to create an engineered organ scaffold with a viable and functioning cellular matrix. Tissue engineering has a been a talk amongst the medical and biotechnology community for a long period, when tissue engineering scientists in the 1970s and 1980s were working to create organ constructs for the use of transplantation into patients [1]. From then until now, these efforts are still being made for transplantation and for creating ex-vivo scaffolds of the human anatomy that can be used for diagnosis and treatment of infectious diseases and ailments.

The building of scaffolds for the making of human parts is not a new process; one of the most groundbreaking and controversial news of the tissue engineering community came from Drs. Joseph and Charles Vacanti and the making of the "Vacanti Rat." This rat has been in news headlines for years, because of an image that circulated of the growth of an artificial human ear on the back of the rat in 1997 [2]. In an interview conducted by Newsweek with Dr. Joseph Vacanti, he states that the ear was created by creating an artificial scaffold in the shape and size of a human ear, out of man-made material that was bioabsorbable and biocompatible. Once this scaffold was made, it was seeded with cartilage cells and put into an incubator. Afterwards, this living construct was then implanted within the rat [2]. This research shows the beginning steps of creating engineered organs, but as time as progressed, the need for vascularity and cell viability in constructs is still in the works of being addressed. The basic constituents of a tissue engineered construct is a scaffold, then seeded with cells, then introduced with growth factors. Varying factors of a tissue engineered scaffold are currently being studied, like material porosity, pore size, and overall fabrication of the scaffold for the betterment of hosting cell adhesion, proliferation, and extracellular matrix development [3]. Currently, most studies being done on in-vitro cell performance are done from a two-dimensional perspective, meaning from a petri dish or from cell cultures. Researchers have proposed cells on 3D printed scaffolds, but there is a lack of success in translating the success of cells from a 2D surface to a 3D surface. Because of this, the progression of bringing tissue engineering to human application has been slow.

Many challenges have been faced in the tissue engineering community, mainly with the issue faced when creating a 3D matrix of cells. Problems arise when trying to convert a 2D system of cell growth to 3D; cells will tend to portray a different set of behaviors, that have led to a lack of viability and bioactivity of cells when trying to be transplanted in-vivo. When removing cells from their microenvironment, they tend to lose their functionality because of the lack of neighboring cells within proximity [4]. Therefore, in this study, we are attempting to create a 3D tubular construct from polycaprolactone (PCL) with a collagen extracellular matrix for hopes of cell adhesion and increased functionality ex-vivo. This study will add insightful knowledge to the tissue engineering community for benefit of the ongoing efforts at creating 3D scaffolds. Within this 3D engineered scaffold, we will be assessing cell behavior in terms of alignment on nanofibers, its 3D morphology and orientation, cell viability, and the overall survival of the construct in a simulated, 37°C cell environment.

For this study, PCL was chosen as the biomaterial for the nanofiber scaffold. Polycaprolactone is a commonly used synthetic polyester in biomedical applications. It is hydrophobic, semi crystalline, biocompatible, and has slow degradation properties [5]. It is commonly used specifically in wound dressings, cardiovascular tissue engineering, nerve regeneration, and bone engineering [5]. Because of these properties, PCL is a good candidate

for creating fibrous scaffolds via electrospinning. Using solvents like chloroform, DCM, formic acid, or DMF, PCL can be used in an electrospinning process to create fibrous nanofiber scaffolds

2. Electrospinning Process

Electrospinning creates porous nanofiber constructs from any soluble polymer; this process has many implications in various industries, like tissue engineering. These fibers result from an internal electric field that is deposited to the polymer solution. At a critical voltage, the repulsive force created secedes the surface tension of the solution within the syringe, and this results in the solution to then drip out [6]. As the solution is released from the needle tip, the solvent is evaporated; during this process, polymer chains are entangled, which is what prompts fiber formation. Once the fibers are formed, they catch onto a grounded base, which has zero potential. This process is visually represented by Figure 1.

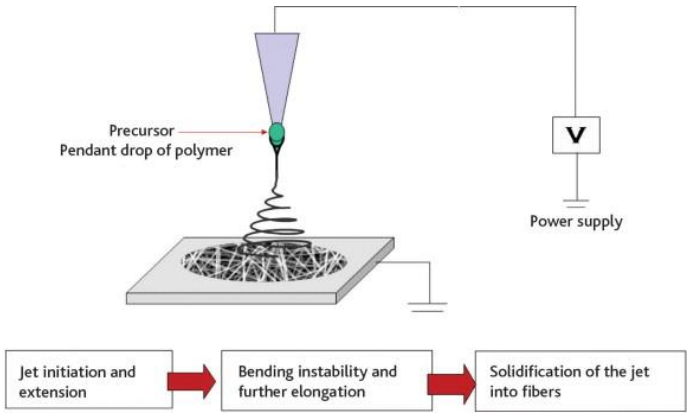

Figure 1: Electrospinning Process [6]

3. Materials

3.1 Electrospinning and Fiber Rolling

An electrospinning machine will be needed to conduct this experiment; for this study, a TL-Pro-BM Electrospinning Machine was used, that hosts a maximum voltage of 220-240V. To convert the energy, a power transformer is used. Both devices are imported from China. In this procedure, the polymer used is 15% polycaprolactone (PCL), dissolved in a 1:1 chloroform to DMF solvent. A syringe that can fit into the apparatus within the machine is used for deposition of the PCL solution; the syringe used is a 6 ml syringe. The ground is created from using positive and negative electrode ends that are clamped to create an uncharged field. The electrodes are clamped unto aluminum foil beneath the metal bar set up, which is stainless steel bars set 4 cm apart. A ruler will be needed to measure the distance from the ground to the needle tip of the syringe. A thermometer is used to assess for constant temperature within the machine. To roll the nanofiber, a metal wire with a diameter of ~0.4-0.5 μm will be used.

3.2 Cell Seeding

Human umbilical valvular endothelial cells (HUVECs) will be used in this study. A cell size of 10^5cells is to be used for maximum cell proximity. HUVEC cells should undergo 2-3 passages in their appropriate medium prior to seeding onto nanofiber construct to ensure optimum cell behavior.

3.3 Surface Coating and Modification

A method adopted from Sousa et al will be used for the surface treatment of the nanofiber construct prior to cell seeding [7]. For this surface treatment, phosphate buffered saline (PBS) with 5 mg/ml carbodiimide water-soluble (1-ethyl-3(3-dimethylaminopropyl) will be made, along with 0.5 mg/ml collagen in PBS. These solutions are to be stored in a 4°C refrigerator.

4. Methods

The 15% PCL solution is filled to the 4 mL mark in a syringe and is deposited from a 13 mm, stainless steel, blunt-tipped needle. The electrospinning device is powered by a step-up transformer and a double-powered programmable syringe pump. A 10 mL syringe filled with water is placed into one of the syringe pump chambers. The flow rate of the syringe pump can be adjusted to pump the water at a certain speed (milliliters per hour), which in turn pushes the PCL/solvent solution out at a certain speed. Whilst the electrospinning is being conducted, the internal temperature of the cabinet should be kept stable. The internal syringe is attached to the base of the syringe with the PCL solution and it is locked in place via a syringe holder. Near the needle of the bottom syringe, there is terminal present where a positive or negative electrode that provides voltage to the needle is attached. Once this is attached, the stage with the syringe set-up can be adjusted to a height of ~14-15 cm from the base, where the spun fibers will be collected. In order to ensure for fiber alignment, there is a parallel plate method that was used. This method is the arrangement of 2 stainless steel bars that are positioned 4 cm apart. The fibers, once spun, will fall and attach between the bars, and the residual fibers will collect on top of the metal bars. Once the apparatus has been set up, the electrospinning machine will be turned on to a voltage between the range of 10.4-12 kv at a pump rate of 0.4-0.5 ml/h. As the spinning is going on, these two parameters will possibly be adjusted according to the consistency of the fiber making. If the fibers are not continuously depositing from the needle tip, or there is dripping of PCL occurring, then the pump rate should be increased, or the voltage should be decreased. The sample should be run for approximately 40 mins at these parameters.

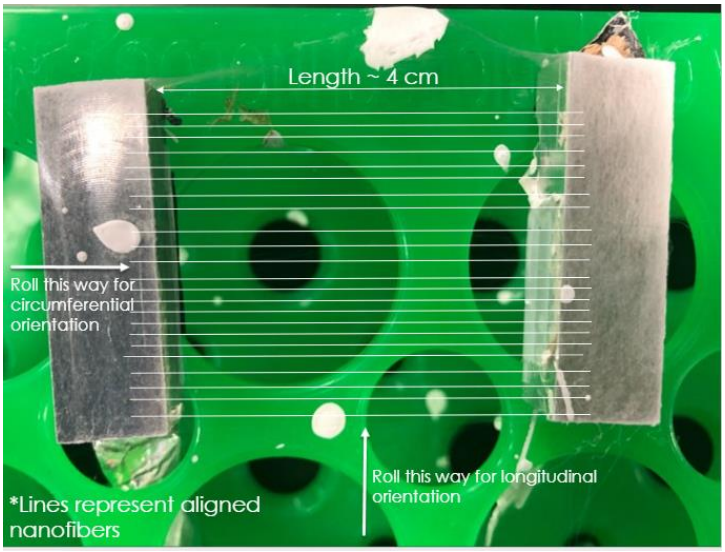

Figure 2: Demonstration of fibers on metal bar construct, with rolling mechanism

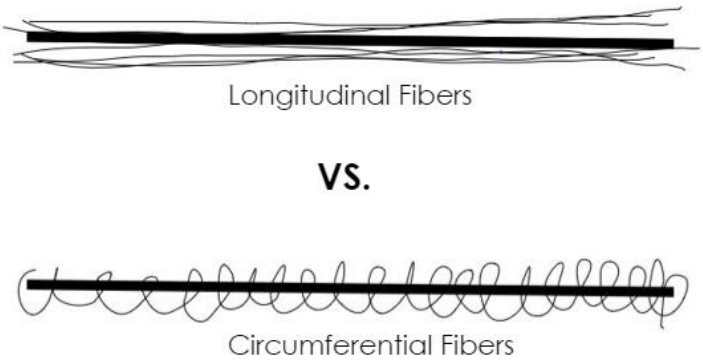

Figure 3: Difference between longitudinal and circumferentially rolled nanofibers onto wire

Once the fibers have been created, the metal wire should be wet with isopropanol or ethanol to create a wet surface for the fibers to stick to while rolling. The metal wire will be brought underneath the fibers and rolled both circumferentially (perpendicular to wire) and longitudinally (along the same axis as the wire). This method is shown in Figures 2 and 3.

In order to create a surface that will encourage cell adhesion, the surface treatment of the nanofibers with collagen will be done. This is done by using the method adopted by Sousa et al. The nanofibers on the needle will be treated with phosphate buffered saline (PBS) with 5 mg/ml carbodiimide water-soluble (1-ethyl-3(3-dimethylaminopropyl) and stored in a 4°C refrigerator for 1 hour. This is to activate the carboxyl groups of the PCL so that collagen can attach [7]. Once this has been done, then the nanofibers will be treated with 0.5 mg/ml collagen in PBS for 5 hours, stored in a 4°C refrigerator. The fibers will remain in the collagen solution until it is ready for cell seeding.

Once cells have reached an optimum confluency after a passage of 2-3x, they will be detached using trypsin, counted, and seeded with the nanofibers. The nanofibers in the cell medium will then be incubated overnight for cell attachment, at a temperature of 37°C; circumferential and longitudinally rolled nanofibers will be incubated in separate plates. The next day, the remaining cells that are not attached will be re-plated into separate wells for future use.

5. Results

After electrospinning for approximately 40 mins and rolling the nanofibers onto the wire, microscopy images were taken at magnifications ranging between 10x-40x, to assess for fiber alignment along the wire surface. Along with images taken from the rolled nanofibers, nanofibers were also gathered on top of a glass slide, in order to assess nanofiber diameters and length. These images were then analyzed using ImageJ software. Values obtained from the ImageJ analysis were then transformed to a histogram, that displays the varying lengths of the nanofibers. These images can be found in Appendix A.

The surface treatment of the nanofibers was done not only to create an adhesive surface for cell attachment, but also to promote the fibers to slide off the wire construct. Unfortunately, the wires were not able to come out, therefore leaving the inner surface of the tubular structure blocked off from cells being able to grow inside.

6. Discussion

6.1 Result of rolling of nanofibers

The goal of rolling the fibers is to maintain a tube-like structure that would mimic vessels and would provide more surface area for cells to grow onto. The images obtained from microscopy do not show any sign of consistent alignment in either parallel or perpendicular direction, relative to the wire. The fibers were also not compacted well along the wire, which can create a tube that will lose structure once it is off the metal wire. Three trials of creating nanofibers and rolling them were done, with each one improving in direction and orientation of fibers along the wire. To get the fibers off the wire, minimal manipulation of the construct was desirable to not disrupt the nanofiber structure. However, when the fibers were attempted to be taken off, there was no avail. Instead, the fibers seemed to be breaking the more we attempted to slide them off. Wetting agents like ethanol, isopropanol, and deionized water were used to soak the fibers in, with hopes of lubricating the surface, however, there was no success. This observation suggests that there needs to be another method for removing the fibers, that may have to consider the material properties of PCL. Polycaprolactone is a semi-crystalline polyester, meaning that absorption of water and hydrolysis occur at a slow rate. In order to "loosen" the hold of the fibers on the wire, there is a possibility for looking at agents that will "swell" the material and make it malleable enough to slide off the wire.

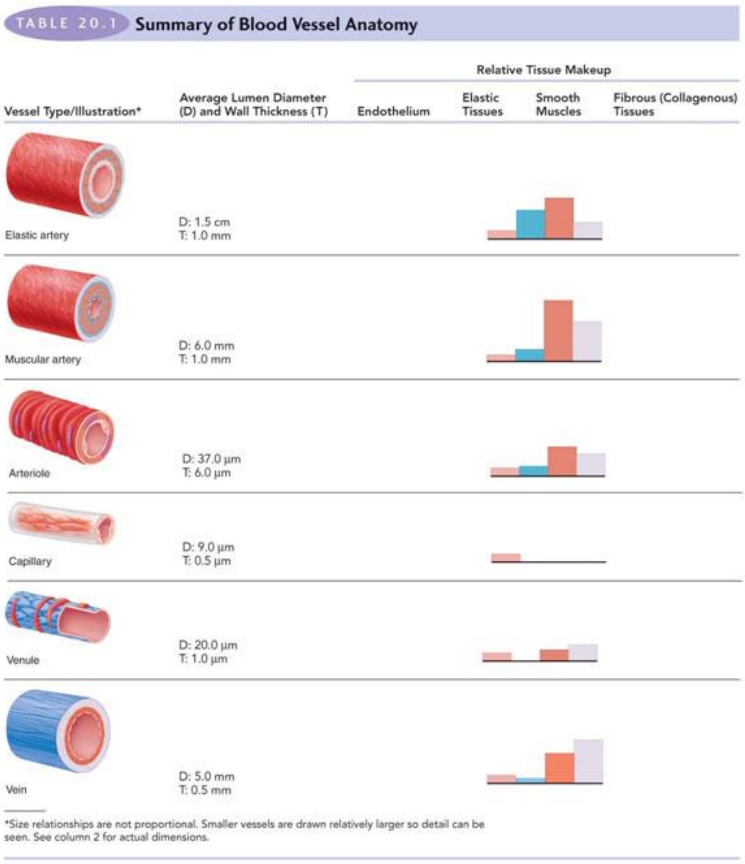

Figure 4: Blood Vessel Anatomy [8]

6.2 Choosing the wire size

Choosing the size of wire to roll the nanofibers onto determines the overall diameter of our construct. With our construct, we are attempting to create 'vessel-like' structures that mimic the size and strength characteristics of native blood vessels. The most attainable diameter for our project was capillary and arteriole diameters (Figure 4). These structures have a realistic amount of endothelium, which the PCL and collagen will mimic, and have an attainable cell layer thickness. The use of HUVECs will create an additional thickness to the diameter, depending on the orientation of the cells. If cells are growing longitudinally, they will exhibit a more elongated shape, and therefore will flatten, due to there being more surface area in that direction. On the circumferential construct, cells will probably be more bulbous and not as elongated due to

a shorter distance for them to grow. This variation in cell alignment can affect the strength of the fiber construct due to amounts of cells that are contacting the PCL/collagen surface.

6.3 Image Analysis

ImageJ was used to determine the orientation of the nanofibers collected on the glass slide. The need for orientation is necessary as orientation salience function is a kind of histogram and may be normalized accordingly. In fact, it is a weighted histogram, i.e. intensity/contrast of the structures are considered. Porosity of the structure is preferred for multiple reasons; for deposition of the nanoparticles on the fiber substrate, for controlling molecular release, and for diffusion of nutrients to the cells that will be seeded into the construct [10]. Using ImageJ, the porosity percentage, or the amount of area that is not covered by nanofibers, is calculated from the following images located in Appendix A:

10x: 100 – 61.843 = **38.157%**

20x: 100 – 65.903 = **34.097%**

40x: 100 – 64.960 = **35.04%**

Another interesting aspect of this experiment is the question of what the maximum length of PCL nanofibers is under optimum experimental conditions [11]. The question can be analyzed in some different ways whether the length of the fiber is the continuous correct orientation of the fibers or the adhesion of the fibers extended to the two parallel bars [12]. It is seen that some fibers were extended across the two bars but are seen broken due to inconsistency in the fiber deposition, which was caused by variability in the voltage and pump rate output [13]. The data suggests that the electrical properties of the jet and the electric field influence both fiber length and fiber diameter as indicated by the observed effect of applied voltage and pump rate on maximum fiber length [14]. There are forces seen which have been acting to the adhesion of the plate, the weight of the fiber, electrostatic repulsion of other factors and the collision seen from other fibers [15]. Figure 4 displays the different forces that are acting on the nanofibers during the electrospinning process.

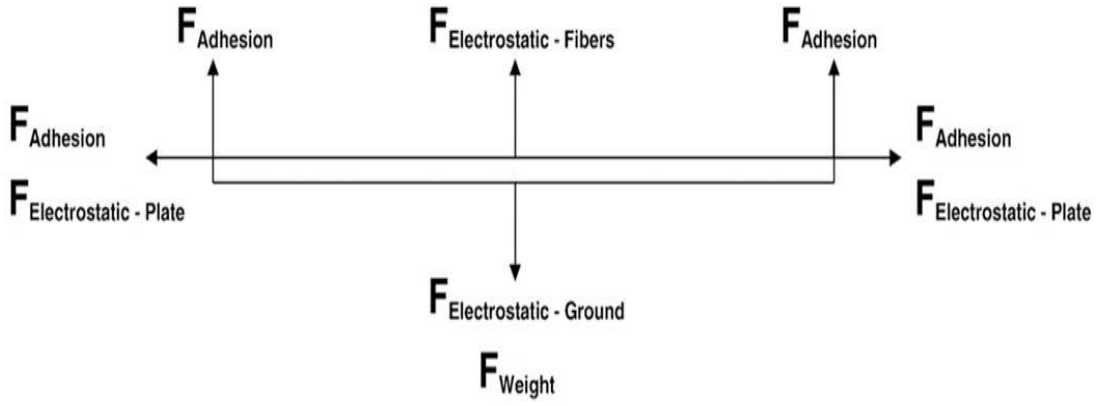

Figure 5: Forces on Nanofibers during electrospinning proces

7. Conclusion

To conclude, electrospinning of nanofiber constructs has a variety of implications in the bioengineering industry. With this study, we hope to construct tubular nanofiber constructs that come together to mimic a 3D human model of the vasculature that could then be used for diagnostic or implantation purposes. With our data, it can be concluded that continued study of nanofiber characteristics needs to be done to create a construct of high mechanical strength and promotes cell viability. In future studies, we will further test the ratio of collagen to PCL for its effect on cell viability and overall construct conductivity. Due to time constraints and the inability of HUVEC cells to reach optimum confluency, cell seeding did not occur prior to the writing of this report. Therefore, in our future studies, we will calculate our cell seed size, which will be dependent on the overall surface area of our construct and the average cell diameter size. Timing of incubation is another parameter that can be tested to assess cell growth and activity within various time frames. Another matter of question that this study brought about is the question of how to remove the fibers from the wire. The idea of the percentage of PCL swelling over time has been tested by Zargarian and Haddadi-Asl, using deionized water for the benefit of drug uptake for drug delivery scaffolds. Their results show that PCL immersed in deionized water for up to 80 hours showed an increase in swelling ratio of 50% for PCL. These methods will be adapted for future studies.

In this paper, we determined the porosity of the nanofibers, which in turn can give us a good idea on the density of the construct and how much surface area for the cells to grow. Because the porosity analysis was on unaligned, unrolled nanofibers, we predict that upon rolling, the construct may be less porosity than was what demonstrated in this study. Due to the process of rolling the fibers onto the metal wire, there is a chance of that construct we obtain will be very densely packed with fibers, in comparison to the random orientation of fibers that is showcased in figured 8, 10, and 12. Keeping this in mind, for our future studies, we will conduct another porosity analysis.

Another parameter to incorporate is the use of a scanning electron microscope for further analysis of our nanofibers. With the use of SEM, we can calculate the exact diameters of our construct, which will lead to a better diagnosis of how many cells will be able to grow per nanofiber strand. All these characteristics will then contribute to our argument of creating a stronger construct with the use of cell orientation and fiber density and diameter.

Appendix A:

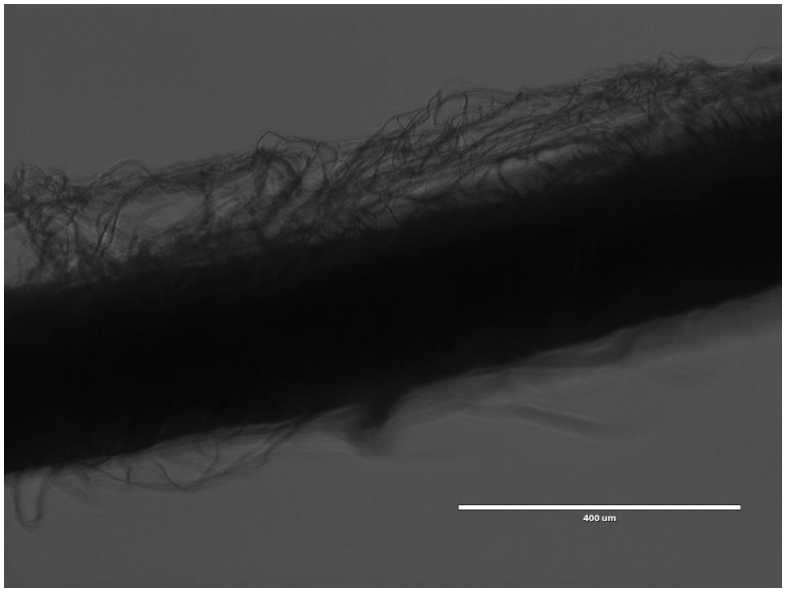

Figure 6: Nanofibers rolled onto 0.05 mm wire, circumferential roll, 10x

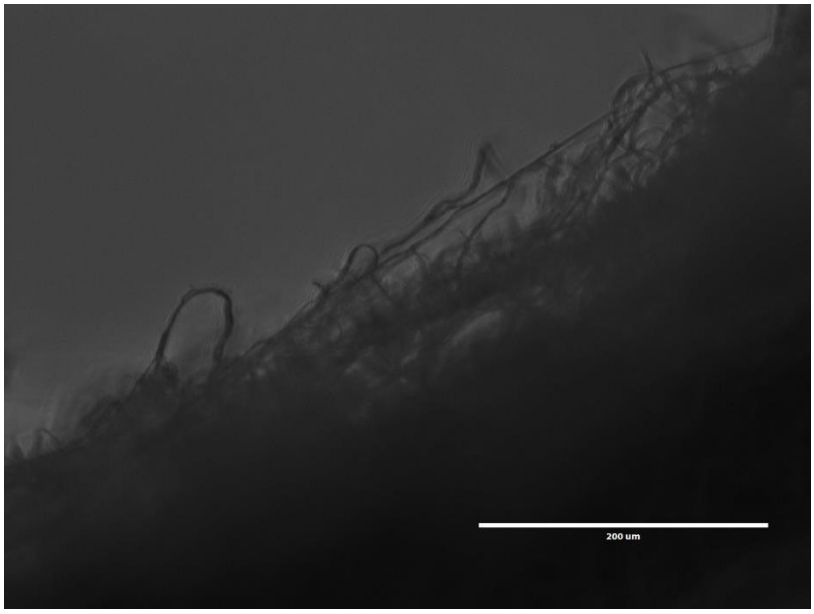

Figure 7: Nanofibers rolled onto 0.05mm wire, circumferential roll, 20x

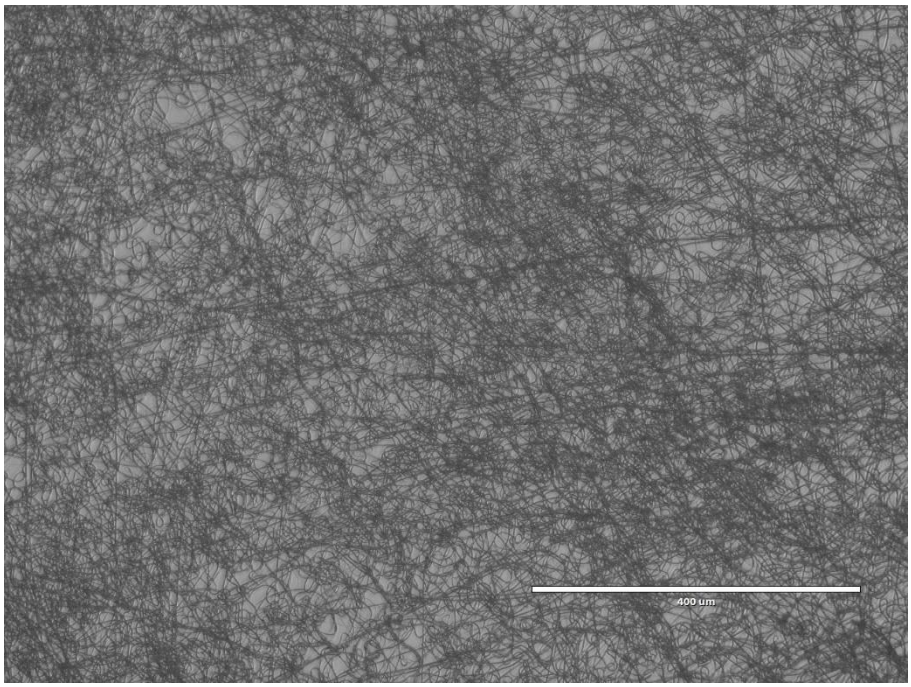

Figure 8: 10x Image of fibers on glass slide

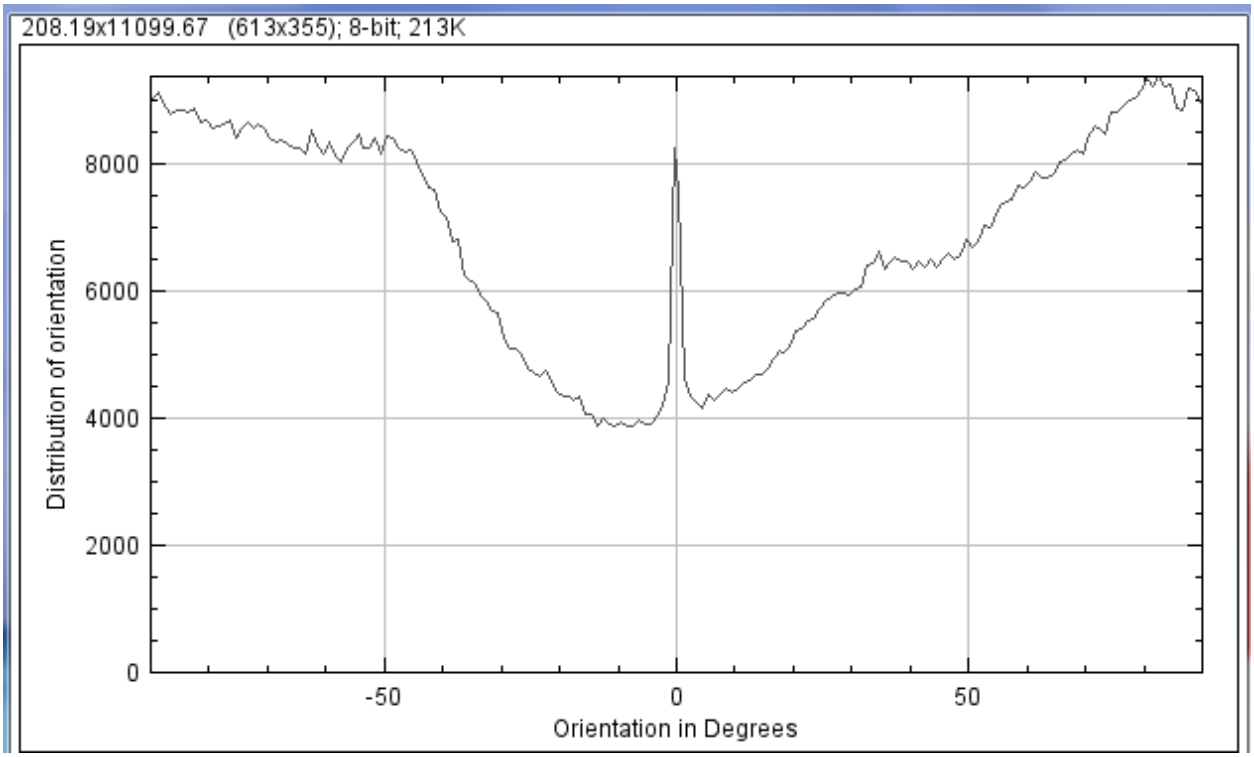

Figure 9: Orientation of 10x image, ImageJ

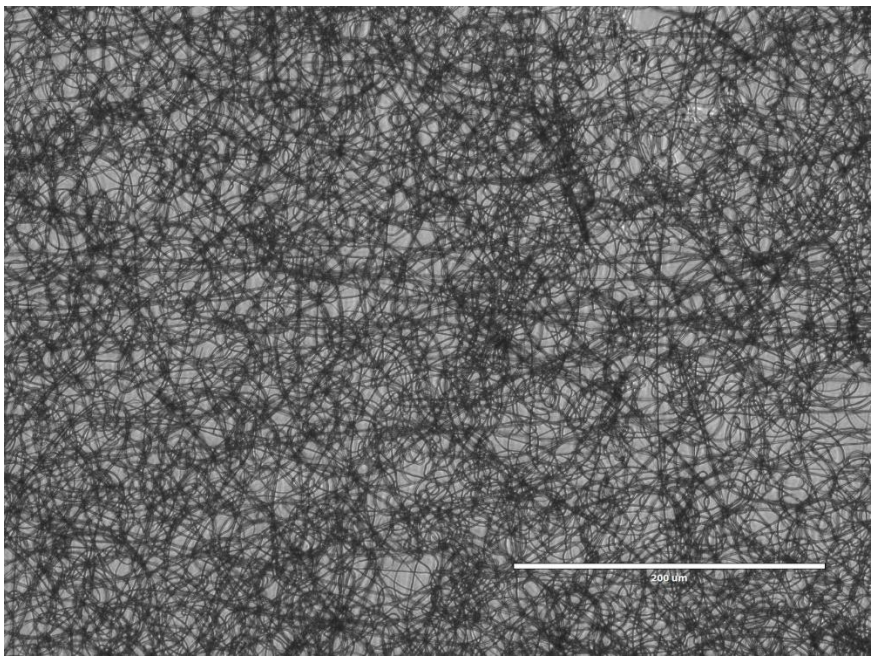

Figure 10: 20x Image of fibers on glass slide

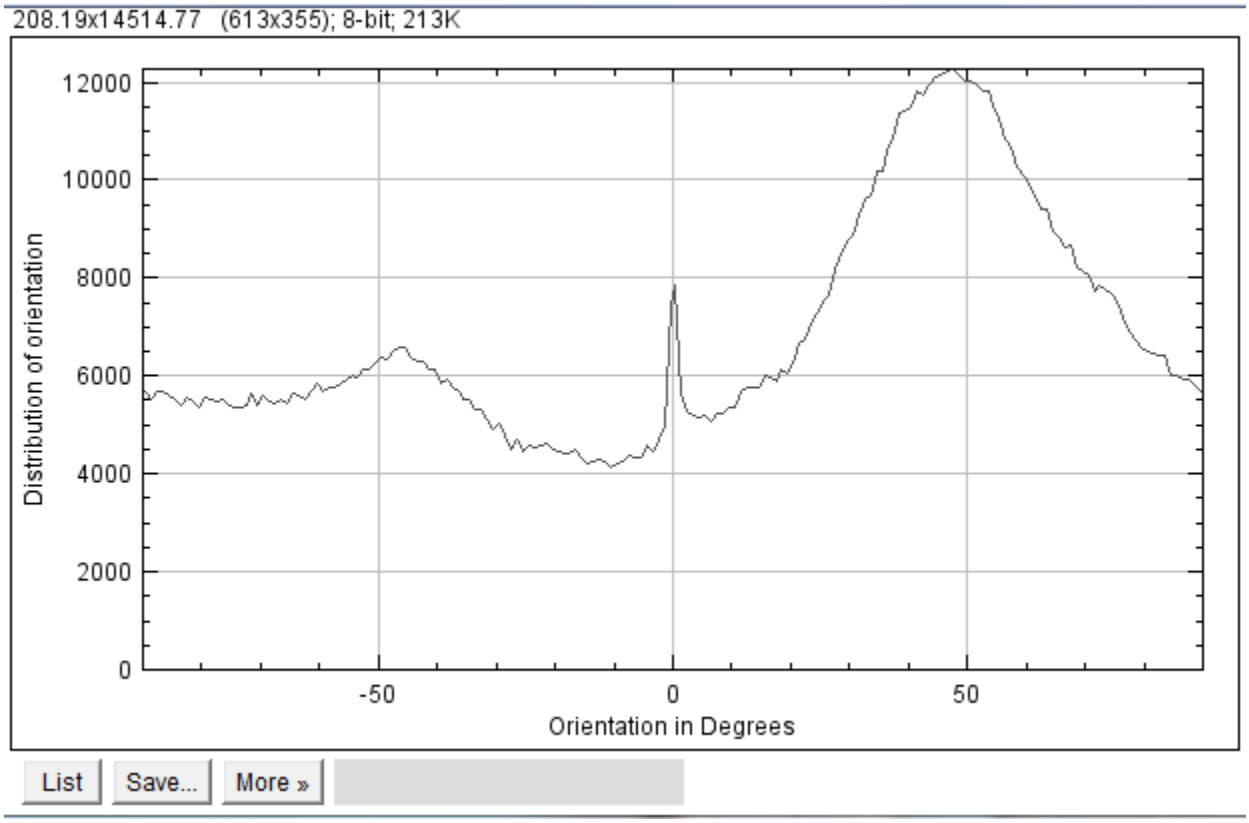

Figure 11: Orientation of 20x image, ImageJ

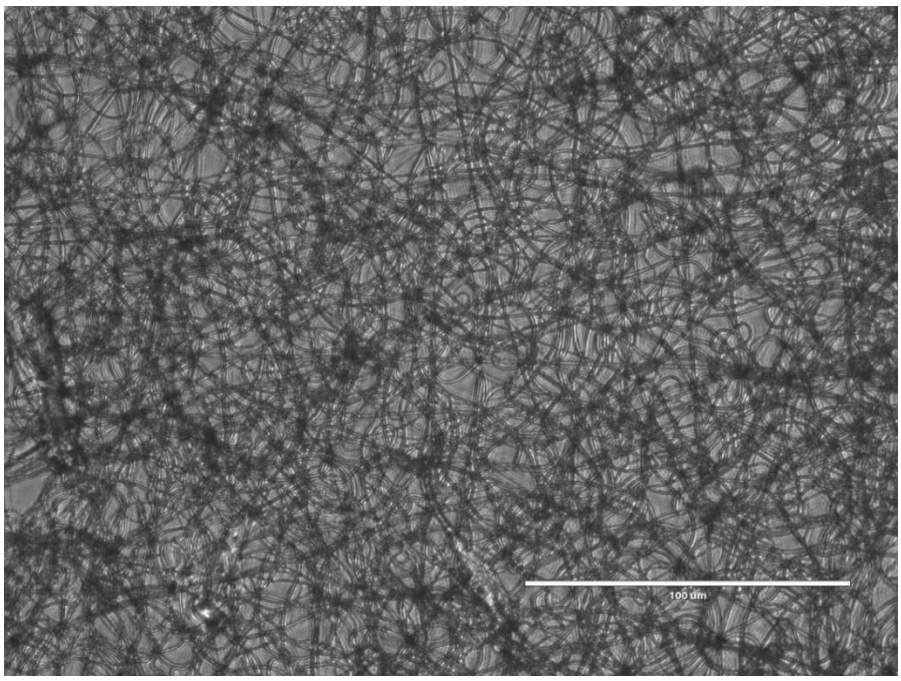

Figure 12: 40x Image of fibers on glass slide

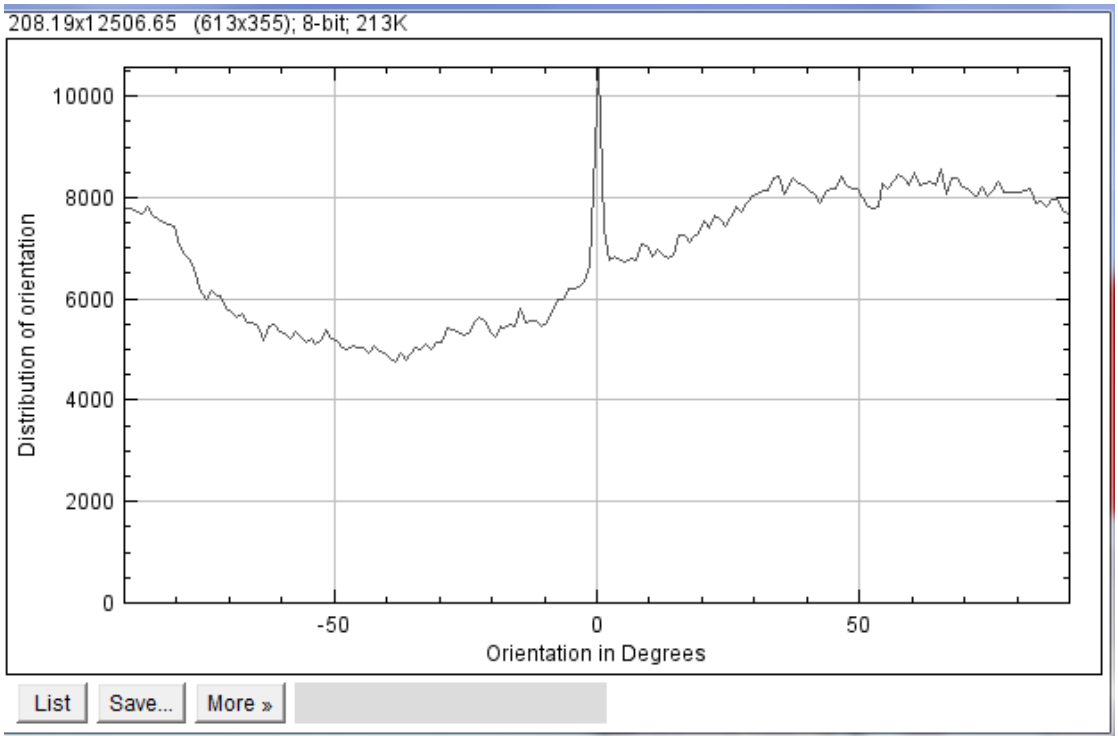

Figure 13: Orientation of 40x image, ImageJ

References

1. Trafton, A. (2012, December 14). Tissue engineering: Growing new organs, and more. Retrieved from http://news.mit.edu/2012/engineering-health-tissue-engineering-growing-organs-1214
2. Hugo, K. (2018, April 19). Remember the lab mouse with a human ear on its back? The scientist accused of "playing God" explains his work. Retrieved from https://www.newsweek.com/tissue-surgeon-ear-mouse-human-organs-transplant-cell-phones-666082
3. Carletti, E., Motta, A., & Migliaresi, C. (2010). Scaffolds for Tissue Engineering and 3D Cell Culture. Methods in Molecular Biology 3D Cell Culture, 17-39. doi:10.1007/978-1-60761-984-0_2
4. Mondal, D., Griffith, M., & Venkatraman, S. S. (2016). Polycaprolactone-based biomaterials for tissue engineering and drug delivery: Current scenario and challenges. International Journal of Polymeric Materials and Polymeric Biomaterials, 65(5), 255-265. doi:10.1080/00914037.2015.1103241
5. Katsogiannis, K. A., Vladisavljević, G. T., & Georgiadou, S. (2015). Porous electrospun polycaprolactone (PCL) fibres by phase separation. European Polymer Journal, 69, 284-295. doi:10.1016/j.eurpolymj.2015.01.028
6. Ramakrishna, S., Fujihara, K., Teo, W., Yong, T., Ma, Z., & Ramaseshan, R. (2006). Electrospun nanofibers: Solving global issues. Materials Today, 9(3), 40-50. doi:10.1016/s1369-7021(06)71389-x
7. Sousa, I., Mendes, A., & Bartolo, P. J. (2013). PCL scaffolds with collagen bioactivator for applications in Tissue Engineering. *Procedia Engineering*, 279-284.
8. Blood Vessels. (2019). Retrieved from Antranik Strength and Flexibility Expert
9. Diameter of Human Umbilical Vein Endothelial Cells (HUVEC). (n.d.). Retrieved from https://bionumbers.hms.harvard.edu/bionumber.aspx?id=108899
10. 10. Qi, Z. Highly porous fibers prepared by electrospinning a ternary system of nonsolvent/solvent/poly(Llactic acid). *Materials Letters*, 2009, 63, page 415
11. Effect of electrospinning parameters on the nanofiber diameter and length Vince Beachleya and Xuejun Wena,b,c,* aClemson–MUSC Bioengineering program, Department of Bioengineering, Clemson University, Charleston, SC, 29425, USA bDepartment of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, SC, 29425, USA cDepartment
12. Chua KN, et al. Biomaterials. 2006;27(36):6043Stitzel J, et al. Biomaterials. 2006;27(7):1088.
13. 12.Li D, Wang Yuliang, Xia Younan. Nano Lett. 2003;3(8):1167
14. Teo W, Ramakrishna S. Nanotechnology. 2005;16:1878
15. Zong K, Fang, Ran, Hsiao, Chi Polymer. 2002;43:4403.
16. Dong L, Huang, Yang, Zheng Mater. Lett. 2007;61:2556
17. Ahn P, Kim, Hwang, Lee, Shin, Lee Curr. Appl. Phys. 2006;6:1030