Quantitative analysis of human mesenchymal stem cell alignment by electrospun polymer nanofibrous scaffolds

Nicole Green[1], Joel Wise[2], Dr. Michael Cho[2], Dr. Constantine Megaridis[3]
[1]Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL
[2]Department of Bioengineering, University of Illinois at Chicago, Chicago, IL
[3]Department of Mechanical and Industrial Engineering, University of Illinois at Chicago, Chicago, IL

1.1. Project Overview

One growing research area as of late has been the synthesis of biocompatible tissues for use in vivo. It is specifically of interest to utilize oriented nanofibrous biocompatible scaffolds to engineer functional tissue constructs that require cell and ECM alignment.

All scaffolds will be made by electrospinning, a technique that creates a non-woven fiber mat made of polymer fibers of nanoscale dimensions. Polymer solutions or melts, liquid crystals, suspensions of solid particles or emulsions, can be electrospun by applying a voltage of about 1 kV/cm to a pendant droplet (see Figure 1). In the case of a polymer solution, the electric force results in an electrically charged jet of the solution flowing out from the droplet. After the jet flows away from the droplet in a nearly straight line, it bends into complex path while other changes in shape occur, during which electrical forces stretch and thin it by very large ratios. After the solvent evaporates, birefringement nanofibers are left (Reneker et al. 2000). Depending on the method of collection of the jet, specific fiber orientations (random or aligned) can be achieved.

Past research has shown that aligned biocompatible scaffolds have the potential to align blood vessels and neurons. The combination of hMSCs and scaffolds for tissue engineering is also of much interest. It is known that differentiation of hMSCs into tissue-specific cell types (chondrogenic, adipogenic, osteogenic, etc.) can be induced by biological or physical signals. The advantages of using hMSCs in lieu of primary autologous cells are that they have higher capability of regeneration, will lead to greater integrity and functionality of the engineered tissue, have the potential for multifunctional tissue constructs, and reduce the risk of rejection and failure.

1.2. Summer Focus

During my time at the University of Illinois at Chicago, I was responsible for quantifying the orientation of hMSCs cultured on different PCL nanofibrous scaffolds and testing cell viability during a long-term culture. Undifferentiated stem cells were seeded onto nanofiber scaffolds, and images were taken every 3-4 days up to 18 days. Using MetaMorph® software, the images were analyzed, focusing on the orientation of the cells with respect to the fibers.
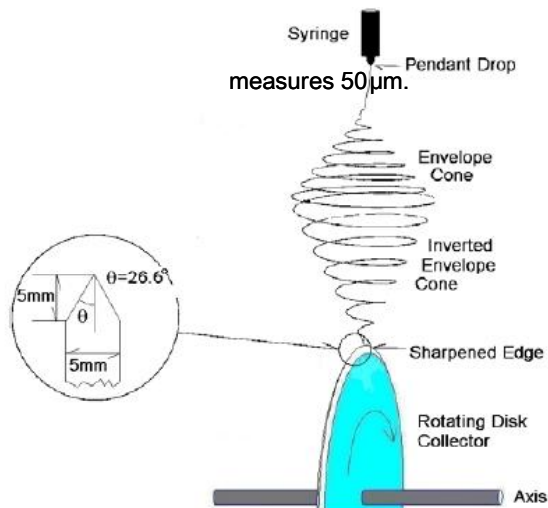
Figure 1: Electrospinning Setup. Drawing of the electrospinning process shows the double-cone envelope of the jet. The collector disk is equipped with a table that assists to collects the nanofibers.
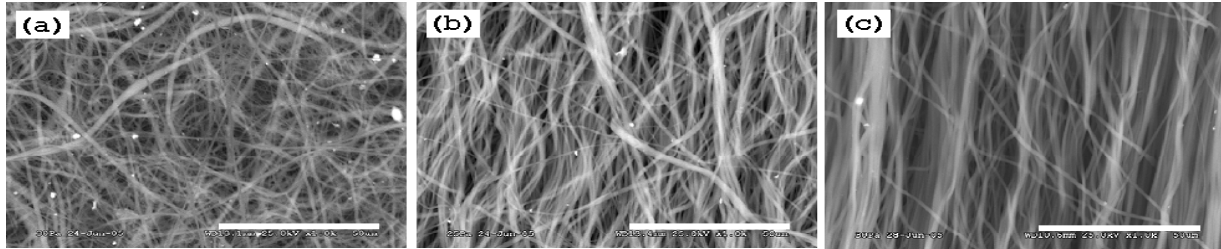
Figure 2: SEM images of PCL Nanofiber Scaffolds. Images of random (a), ribbon (b) and rope(c) scaffolds at 1000x magnification. Both ribbon and rope scaffolds show general orientation of fibers in the vertical direction. Scale bar measures 50 μm.

2. Materials and Methods

2.1. PCL scaffolds

Electrospun PCL nanofiber scaffolds used in the research thus far were obtained from the laboratory of Drs. Yarin and Zussman (Technicon, Israel). The diameter of the nanofibers is on the order of several hundred nanometers. Samples from three different types of fiber collection methods were used: random, ribbon, and rope. The random mat was spun onto a flat aluminum substrate. The aligned ribbon scaffold was collected on a yarn using the rotating disk collector similar to that in Figure 1. The aligned rope was colleted on the edge of the rotating disk. Scanning electron microscopy (SEM) was used to obtain images of each type of scaffold (see Figure 2).

2.2. Human mesenchymal stem cell culture

The hMSCs were obtained from the NIH-funded Adult Mesenchymal Stem Cell Resource location at Tulane University (copies of Agreement of Transfer of Materials available). Cells were cultured in complete media consisting of Dulbecco's Modified Eagle's Medium supplemented with 15% fetal bovine serum (FBS), 2mM L-glutamine, 1% antibiotics, antimyotics (final concentration: penicillin 100 units/ml, streptomycin 100 micrograms/ml and amphotericin B 0.25 micrograms/ml). Cells were cultured in a 5% CO_2 incubator at 37°C. The hMSCs (passage 6) were seeded onto pieces of the PCL nanofiber scaffolds at a density of 7.5×10^4 cells/cm^2.

2.3. Preparation of PCL nanofiber scaffolds for cell seeding

From the bulk material of electrospun nanofiber mats, small discs with areas of approximately 0.3 cm^2 were cut out for cell-seeding experiments. All scaffolds were soaked in 70% ethanol for 1 hour, and then dried and sterilized under UV light for 6 hours on each side. All scaffolds were pre-wetted by soaking in complete cell culture media with serum for 48 hours to result in a more hydrophilic surface for optimal cell adhesion. Cell solution was pipetted directly onto the scaffolds and incubated for 1 hour. After cells were seeded onto the scaffolds, media was added to the samples and changed every 2-3 days for long term culturing.

2.4. Live/Dead cell viability

Samples were imaged on Day 1 (24 hours after initial stem cell seeding onto scaffolds), Day 4, Day 8, Day 12, Day 15, and Day 18. On each day, one set of samples was stained with a live/dead cell viability kit (Molecular Probes, L-3224). Briefly, calcein AM enters live cells and reacts with intracellular esterase to produce a bright green fluorescence, while Ethidium homodimer-1 enters only dead cells with damaged membranes and produces a bright red fluorescence upon binding to nucleic acids. For three different images taken from each sample, the percentage of live and dead cells and the total number of cells were measured using MetaMorph® software to ensure that stem cells were healthy and proliferating throughout the duration of the experiment. The percentage of live cells in each type of scaffold on Days 4 and 18 was calculated.

2.5. CellTracker^{TM} for cell orientation analysis

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On each day, another set of samples were stained with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes, C-7025) and then fixed in 3.7% formaldehyde. The CellTracker^{TM} reagent diffuses through the cell membranes and is transformed into cell-impermeant reaction products. CMFDA is colorless and non-fluorescent until the acetate groups are cleaved by the intracellular esterases, which allows a very bright green fluorescence (500-530 emission) to be produced when imaged with a blue laser (488 nm excitation). The concentration used in our samples was 15 μM. The bright green fluorescent cells imaged on each sample allowed for reliable image analysis of cell orientation using MetaMorph® software.

2.6. Laser scanning confocal microscopy

A BioRad Radiance 2000 Multiphoton/Laser Scanning Confocal Microscope was used to produce images for analysis. Laser scanning confocal microscopy is advantageous because of its unique optical sectioning capabilities. When the scanning laser is focused within a 3-D sample, the system produces an image of exclusively the focal plane. This is possible because of a pinhole aperture in the detection system, which rejects signals from objects outside the focal plane. This technique can be used to examine both fluorescent specimens and reflective materials simultaneously using two different detectors. CMFDA-stained cells were imaged as bright green with fluorescence mode using a blue laser. In reflection mode, white PCL nanofibers were imaged using the same blue laser. Therefore, we were able to image the cells and fibers from the same exact location on the sample. Images were taken from at least five different locations on each sample, and the data from analysis was averaged. MetaMorph® software was used quantify the angle of the cells and fibers with respect to the horizontal axis. Average standard deviations of cell angles for each type of scaffold at each point in time were calculated.

3. Results

The standard deviation of the cell angles was calculated to prove that the random scaffold was not capable of aligning cells as well as the aligned ribbon and rope scaffolds (see Figure 3). Cell orientation does not show any time-dependence. Cells seeded on the random scaffold have an average standard deviation of 42.6°, while cells on the aligned ribbon and rope scaffolds have average standard deviations of 27.4° and 23.7°, respectively. Unpaired, two-tailed t-test statistical analysis returned a p-value of .00449 when comparing the random and ribbon standard deviations, and a p-value of .000997 when comparing the random and rope standard deviations. Both p-values are less than .05, which means that for over 95% of the time, the same results can be expected. So, cells on the ribbon and rope scaffolds consistently have lower standard deviations than the cells on the random scaffold.

Live and dead cells were counted, and the percentage of live cells was calculated for Day 4 and Day 18 (Table 1). The stem cells used were undifferentiated and proliferated quickly, causing the samples to quickly become overcrowded. The quick confluency may help to explain the lower than expected percentage of live cells. Over time the total number of cells viewed increased and the percentages stayed relatively consistent. A PCL scaffold of any orientation should be a reasonable choice for short or long-term cell growth. PCL is biocompatible and known to be good for cell culture, so any cell viability problems are not attributed to the scaffold material.

4. Conclusion

Overall, the organization of nanofibers does have an effect on cell orientation, and aligned scaffolds orient cells better than random scaffolds.
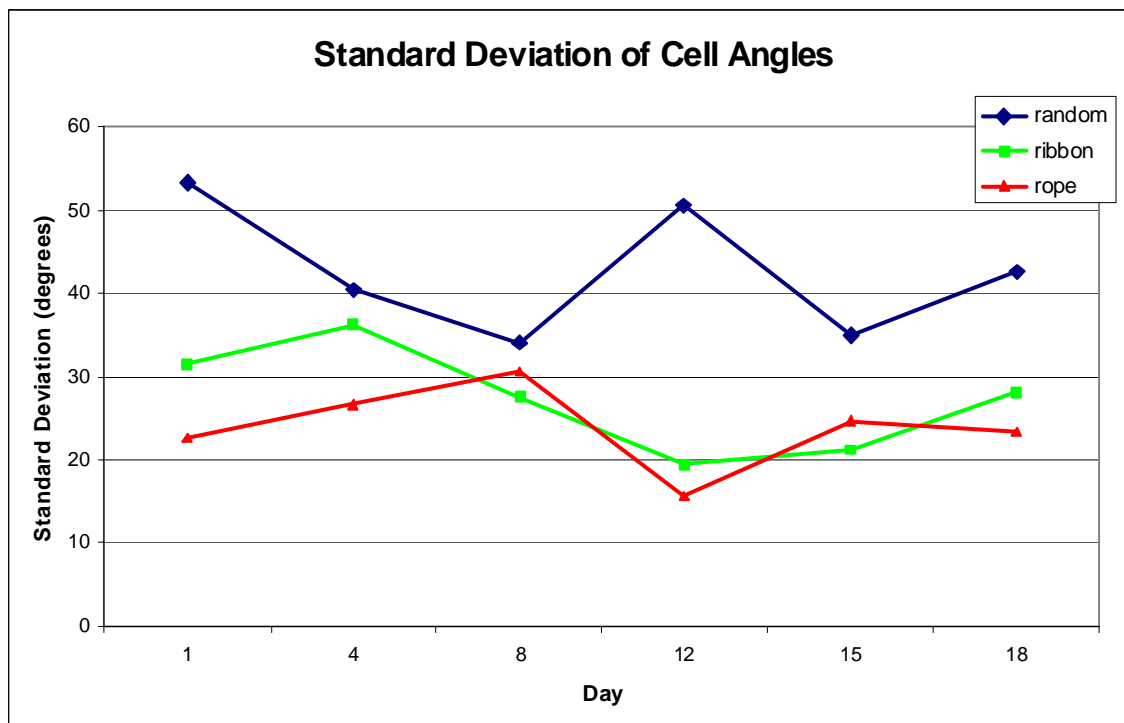
Figure 3: Standard Deviation of Cell Angles. Cells on ribbon scaffold (blue) have consistently larger standard deviation than cells on ribbon (green) or rope (red) scaffolds.

Day	Sample	% Live	Total # Cells Viewed
4	random	76%	318
	oriented	80%	525
18	random	73%	1487
	oriented	76%	913

Table 1. hMSC viability represented by the percentage of live cells and the total number of live/dead cells viewed.

References

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