Characterization of Chitosan Based Hybrid Nanofiber Scaffolds for Tissue Engineering

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Abstract: *Polymeric nanofibers that mimic the structure and function of the natural extracellular matrix (ECM) are of great interest in tissue engineering as scaffolding materials to restore, maintain or improve the function of human tissues. Electrospinning has been recently developed as an effective technique for nanofiber fabrication. In our study, the electropsinning method is used to create hybrid nanofibrous scaffolds made of widely accepted natural biopolymer i.e. chitosan, and polycaprolactone (PCL), a well-known synthetic biocompatible polymer. Solutions of chitosan in trifluroacetic acid and PCL in trifluroethanol were first prepared separately and mixed together to get the final electrospining solution. To achieve the fine electrospun nanofibers of chitosan/PCL composite we varied the solution concentration and adjusted electrospinning variables. The morphology of the scaffold was observed using scanning electron microscopy (SEM) and the mechanical strength and elasticity of the material measured using Microtensile test. Potential use of this nanofibrous matrix for tissue engineering was studied by examining the cellular compatibility.*

1. INTRODUCTION

There is a great interest in creating biocompatible materials for use in tissue engineering. One use for these materials is as scaffolds that closely resemble the extracellular matrix (ECM) found naturally within the body. The ECM is a structure on which cells attach and proliferate into viable tissues. For an engineered tissue to grow successfully it must have a support system similar to the ECM on which to bind. Recently, researchers have been working to develop engineered scaffolds with fibrous micro and nano-structures using biocompatible and biodegradable biopolymers [1]. Among those, chitosan extracted from crab shells is a well established natural material for use in many biomedical applications [4].

Nano-fiber scaffolds of chitosan have been created successfully using polyethylene oxide (PEO) and have been employed as scaffolds for bone tissue engineering [5]. The successful use of these biopolymers for bone tissue growth has led to research in adapting these structures for use with other tissues. This study set out to develop similar nano-fiber scaffolds with better mechanical properties that would be suitable for nerve tissue engineering which requires more flexible and elastic scaffolds.

In order for scaffolds to be useful in tissue engineering they must have a large surface area to volume ratio, be biocompatible, biodegradable, and mechanically strong, and have a controllable degradation rate in an aqueous medium. To achieve this combination of properties, a hybrid nanofibrous scaffold of the synthetic biopolymer polycrapolactone (PCL) and chitosan was prepared. PCL is a biocompatible, biodegradable polymer with good elastic and mechanical properties. The combination of these polymers yields a new polymer with improved mechanical strength compared to the individual polymers. PCL is hydrophobic and very elastic whereas chitosan is hydrophilic and too brittle.

There are several techniques for creating nanofibrous scaffolds, including drawing, self-assembly, template-directed synthesis, phase separation, and electrospinning. Due to its low cost and ease of implementation, electrospinning is often employed for creating nanofibrous scaffolds. In electrospraying, a polymer solution is contained in a syringe with a positive electrode inserted into it. The syringe is positioned some specific distance from a target connected to the negative electrode forming a ground plane. A high voltage is applied to the polymer solution, typically 5 – 30 kV, which causes the droplet at the tip of the syringe to take on a conical shape known as a Taylor Cone [6]. The Taylor Cone forms due to the competing electrostatic force and surface tension. At a high enough voltage the surface tension is overcome by the electrostatic force and at low solution viscosities droplets are collected on the target. If

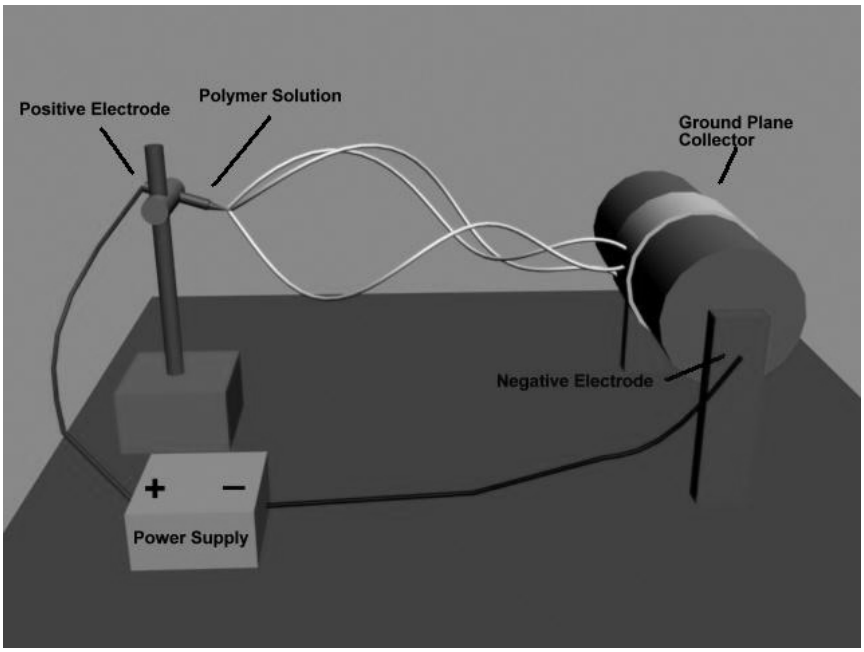

Figure 1 *Typical electrospinning setup.*

the solution viscosity is sufficiently high, fibers are collected instead of droplets and electrospinning occurs [3]. A typical electrospinning setup is shown in Figure 1.

In this study, we attempted to develop and characterize a process for creating nanofibrous scaffolds of chitosan and PCL. Solution properties, fiber morphology, material strength, and biocompatibility of the hybrid nanofibrous scaffold were characterized.

2. METHODS

2.1 Materials

Chitosan with medium molecular weight, trifluroacetic Acid (TFA), polycrapolactone (Mw= 80,000), and Trifluroethanol (TFE) were purchased from Sigma Co. Genipin and ammonium hydrohyde were obtained from Challenge Bioproducts and VWR, respectively.

2.2 Solution Preparation

5 wt% Chitosan in Trifluroacetic Acid (TFA) and 12 wt% polycrapolactone in TFE solutions were prepared separately. They were then combined in PCL/chitosan weight ratios varying from 40/60 to 80/20 2 grams of solution at a time. The resulting solutions were then vortexed for 1 min to ensure the complete mixture of polymers.

2.3 Electrospinning of Nanofibers

A DC voltage of approximately 19 kV was applied (High DC power supply, Del Electronics Corp.) between the syringe tip and a cylindrical collector. The distance between the syringe tip and collector was approximately 29 cm with the syringe angled down approximately 30 degrees below the horizontal. The positive voltage was supplied to the solution via a platinum wire, connected to the positive electrode, inserted into the solution. The cylindrical collector was electrically grounded by attachment of the negative electrode.

2.4 Viscosity Measurements

Solution viscosity was measured using a VT 550 Viscometer in a cup and rotor geometry. The solution was placed in the viscometer and two separate viscosity measurements were taken. The first test consisted of applying a constant stress of 50 MPa for 100 sec. The second test applied an initial stress of 10 MPa and slowly increased the stress to 100 MPa in steps of 0.75 MPa/sec over 100 sec.

2.5 Crosslinking of Nanofibrous Mats

The mats were first affixed to 2 cm x 2 cm cover slides and then soaked in 14 % NH_4OH for 5 min. They were then thoroughly rinsed in deionized (DI) water and soaked in 1 wt% genipin (aqueous) for 24 h. Finally, they were rinsed thoroughly in DI water for 5 min before further processing.

2.6 Characterization of Nanofibrous Structure

We used the scanning electron microscopy (SEM), specifically the JEOL 7000, with a 15 kV applied voltage to analyze the structure of the nanofibers. The fibrous mats were prepared for the SEM by sputter coating them with gold.

2.7 Tensile Strength Measurements

Tensile strength was measured for both cross-linked and non-crosslinked nanofibrous mats were conducted using a micro tensile tester as described in previously [2]. Briefly, samples were cut in a rectangular shape, measured for cross sectional area, and loaded into a load cell. The load on the sample was provided by a stepper motor controlled by a LabView program which outputted a stress-versus-strain curve from which the tensile modulus was calculated.

2.8 Cell Culture

Crosslinked nanofibrous mats were sterilized with ethanol overnight and washed with excess phosphate buffered saline (PBS). One million Schwann cells were seeded on the fiber and cultivated for 3 days before 0.5 million PC12 neuron cells were seeded on the top of Schwann cells. After 3 days of co-culture, cells were visualized with live and dead assay based on the previous method [1]. Cells were also harvested and fixed for SEM observation.

3. RESULTS AND DISCUSSION

3.1. Concentration Experiment

It was important to first establish a solution containing an optimal concentration of both PCL and chitosan. We first started with a PCL/chitosan concentration of 40/60 and observed the fiber morphology using SEM. The fibers produced by this solution had an average diameter slightly less than 0.5 μm. As we increased the concentration PCL/chitosan to 60/40 fibers with diameters of 300 – 500 nm were consistently seen under SEM (Figure 2). We continued to increase the PCL concentration to 70/30 and then to 80/20 but no improvement in fiber morphology was observed.

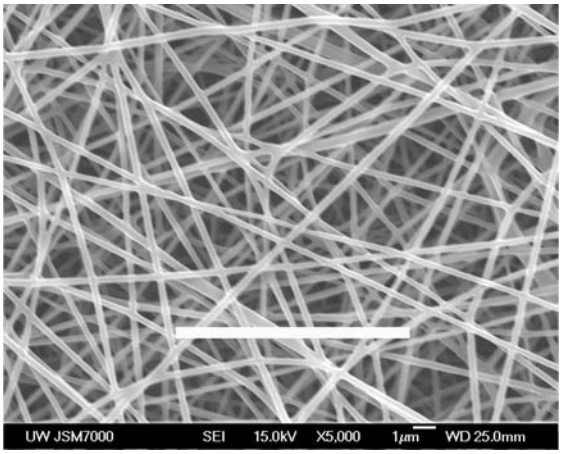

Figure 2 *Result of concentration experiment showing 60/40 PCL/chitosan nanofibers. Scale bar is 10 μm.*

3.2 Electrospinning Variables

Although the electrospinning process is well characterized there are several variables having to do with the experimental setup that need to be adjusted for a given solution to produce usable fibrous mats. The most important variable is applied voltage. Typical voltages range between 5 kV and 30 kV and are dependent on solution viscosity, the solvents being used, and the distance the syringe is from the target. If the voltage is too low there is not sufficient electrostatic force to overcome the solution's surface tension and no fibers will be collected. If the voltage is too high or the syringe too close the solvents will not have enough time to evaporate and will deposit on the collector dissolving any fibers they touch. To characterize the correct process for our solution, we first used an initial distance from target of approximately 22 cm and a constant solution viscosity and then attenuated the voltage from 17 kV to 22 kV taking samples at an increase of 1 kV. The samples were then observed under light microscopy. The best fibers were produced at a voltage of 19 kV. With the voltage now held constant we adjusted the distance between the

syringe and the collector. We found that optimal fiber deposition occurred at a distance of approximately 29 cm. With our electrospinning variables well defined we were then able to consistently create nanofibrous mats for further processing.

3.3 Viscosity Measurements

To characterize our solution we used two separate viscosity measurements taken on a large batch (60 mL) of 60/40 PCL/chitosan solution. Six samples were taken over 26 h; four at 2 h intervals, one at 14 h, and a final sample at 26 h. Each sample then had viscosity measurements taken. The test was a constant stress measurement where a constant stress of 50 Pa was applied for duration of 100 sec. These measurements were then compared for each sample showing that the solution viscosity lessens over time. The lessening viscosity means that there is a spinnable lifetime to the solution. Further exploration into this is necessary but for this study we held our spinnable lifetime to 1 h.

3.4 Crosslinking of Nanofibrous Mats

Combining the hydrophobic polymer PCL and the hydrophilic chitosan there is a chance the hydrophilic component of the hybrid fiber to be dissolved in the aqueous medium. To help overcome this, the mats were crosslinked with the fruit extract ginipin. The process was straightforward and took 24 h to complete. After completion of the crosslinking process, the mats were stabilized but at the cost of elasticity and strength.

3.5 Tensile Test

Tensile tests were performed on both cross-linked and non-crosslinked nanofibrous mats using a micro tensile test machine. The tests were run three times for each material and the results were averaged together and displayed in Figure 3. There are two items to note about this graph: first, the position where each line decreases, and second, the slope of each line. The point at which the graph turns down is the breaking point of the material. The crosslinked mat (thick) is substantially weaker than the non-crosslinked (thin). The slope represents the elasticity of the material: the steeper the slope the more brittle the material. The loss of mechanical strength is further illustrated by the calculation of the Young's Modulus. Results shows the crosslinked mats are much weaker. Both the loss of elasticity and the weakening of the crosslinked material bring up a major concern; once crosslinked, the material loses both elasticity and strength. This led us to find an alternative way to stabilize the material in an aqueous medium.

3.6 Biocompatibility Verification

Finally, we tested the biocompatibility of the material. This was accomplished by seeding a duel layer of cells on the material and allowing the cells to proliferate. The first of the two layers are Schwann cells. These were grown as an adhesion layer. The second layer consists of PC12 neuron cells. The cells were then stained with a live and dead kit for visualization; the results were shown in Figure 4A. The PC12 cells were shown grown in clusters on top of the Schwann cells adhesion layer. Figure 4B is a magnified SEM image of one of these PC12 clusters. An aggregate of PC12 cells growing on the top of a layer of smaller Schwann cells is clearly seen. Beneath both layers of cells the nanofibrous mat can be seen. The cells attached themselves on the material and were able to proliferate.

4. CONCLUSION

Nanofibrous scaffolds of hybrid chitosan/PCL were successfully created via the electrospinning process. The relevant electrospinning variables were characterized. The mechanical properties of the scaffolds were tested, specifically strength and elasticity. Biocompatibility of the material was also tested. The scaffolds were successfully crosslinked to increase aqueous stability; however, this came with a loss of both mechanical strength and elasticity. More research is being conducted on an alternative mode for achieving aqueous stability which will allow the material to maintain its mechanical properties.

	Cross-linked	Non-Cross linked
Breaking Strength (Mpa)	**0.76599**	**1.33059**
Tensile Strain	**6.23E-04**	**3.24E-03**
Young's Modulus (MPa)	**14.18**	**7.82**

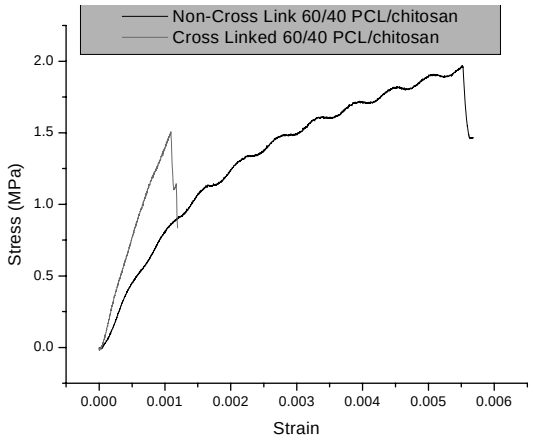

Figure 3 *Tensile test revealing breaking strength, elasticity, and Young's Modulus for both crosslinked (thin) and non-crosslinked (thick) fibrous mats.*

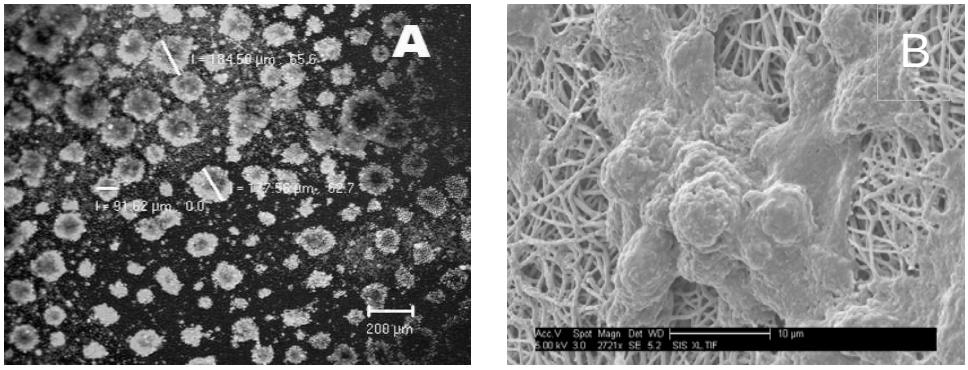

Figure 4 *Dual layered cells on 60/40 PCL/chitosan fibers. First layer consisting of an adhesion layer of Schwann cells and the second consisting of PC12 neuron cells. Figure 4A shows a live dead assay of the cells after attachment to the fibrous mat. Light contrast indicates live cells; the dark contrast indicates dead cells. Figure 4B shows a magnified SEM image of one PC12 cluster.*

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