

ELECTROSPINNING NATURAL POLYMERS FOR TISSUE ENGINEERING APPLICATIONS

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ABSTRACT

Electrospinning is an efficient method by which to produce scaffolds composed of nanoscale to microscale fibers, which are comparable to the fiber diameters of native components of the extracellular matrix. In electrospinning, a polymer solution is pumped through a syringe that is connected to a high voltage source. As a droplet forms at the tip of the needle, electrostatic repulsions form long fibers that are collected onto a grounded metal plate in the form of a nanofibrous mat. These structures can be used in tissue engineering and drug delivery applications. Natural polymers are candidate materials to develop as electrospun scaffolds. Fibrinogen is one such protein that is present in blood plasma. Physiologically, the reaction between fibrinogen and thrombin leads to the assembly of fibrous structures that play a key role in wound healing. Collagen is another natural polymer that is an essential component of the extracellular matrix. It is largely responsible for the mechanical integrity of the extracellular matrix, making it ideal to be electrospun as a scaffold. The feasibility of electrospinning fibrinogen and collagen into viable nanofibrous scaffolds was explored in this study. Both proteins were produced as nanofibrous mats when certain electrospinning parameters were applied. While non-uniformities were observed in these structures, the presence of nanofibers throughout the mats and the versatility of the electrospinning process suggest that uniform nanofibrous scaffolds can be formed from these proteins. Finally, because fibrinogen and collagen are naturally occurring, these scaffolds have much potential to support cell adhesion and growth for use in tissue engineering applications.

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1. INTRODUCTION

Tissue engineering is a rapidly expanding field that tackles the problem of organ and tissue shortage. The traditional tissue engineering paradigm attempts to replace damaged tissue with scaffolds that provide a structure for cell attachment and growth at an injury site [1]. This scaffold typically biodegrades or integrates itself into the host tissue as new extracellular matrix proliferates throughout the site, resulting in mechanically and biologically functional tissue. It has been generally recognized that scaffold architecture profoundly influences cell behavior, and thus tissue engineering constructs composed of a large number of different materials through various fabrication methods have been studied. However, nanofibrous scaffolds have unique advantages over microscale fibrous structures that make them promising for tissue engineering applications.

2. NANOTECHNOLOGY & TISSUE ENGINEERING APPLICATIONS

Nanotechnology is an area of study that has garnered much recent attention in the scientific research community due to its broad applications and powerful potential. In 1974, the term “nanotechnology” was first defined by Tokyo Science University Professor Norio Taniguchi as follows: “Nano-technology’ mainly consists of the processing of, separation, consolidation, and deformation of materials by one atom or by one molecule” [2]. Generally, the theme of nanotechnology consists of the control of matter with atomic precision, resulting in devices or materials with enhanced properties that may serve to revolutionize many technologies.

Nanofibrous scaffolds, specifically those produced through a fabrication process called electrospinning, are structures composed of individual continuous nanofibers. These particular scaffolds have many potential advantages over scaffolds of a larger scale, especially in the context of tissue engineering. Since scaffold architecture greatly affects cell behavior, a prime objective of the tissue engineering scaffold is the close simulation of the natural extracellular matrix. For instance, my advisors have found that annulus fibrosus cells are able to sense their three-dimensional environment, and respond accordingly to their geometrical surroundings [3]. It has been found in previous studies that cells seeded electrospun nanofibrous scaffolds produce a greater quantity of extracellular matrix than those seeded on microfibrillar scaffolds [4]. This is likely due to the various properties of the nanofibrous scaffold, including high porosity, improved mechanical properties, and morphological similarities to components of the native extracellular matrix [1]. Each of these characteristics has been suggested to facilitate a greater degree of cell adhesion, cell proliferation, and mechanical integrity of the scaffold.

A wide variety of biomaterials have been used in the synthesis of nanofibrous scaffolds for tissue engineering applications, including natural, synthetic, biodegradable, and non-biodegradable polymers [1]. Biocompatibility is also a key requirement in the selection of a biomaterial. In addition, nanofibers have been manipulated into aligned and non-aligned scaffolds and seeded with cells to observe the biological, chemical, and physical

responses of the extracellular matrix. These scaffolds have generally been found to be highly promising in the broader application of tissue engineering.

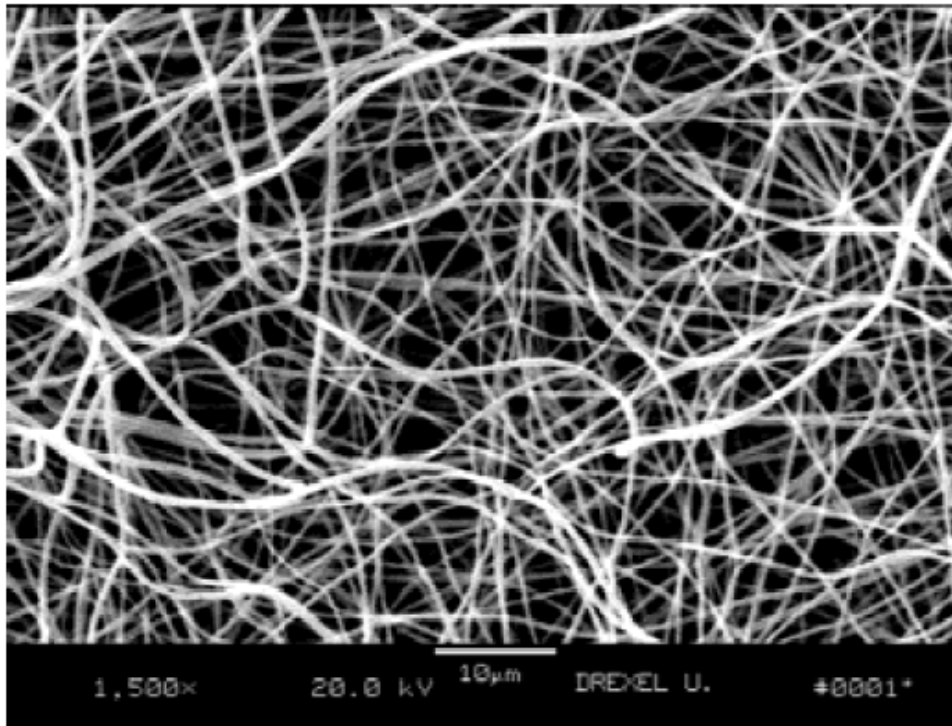


Figure 1: SEM micrograph of an electrospun PLGA nanofibrous structure composed of non-aligned fibers. Scale bar, 10 μm [5].

3. THE ELECTROSPINNING PROCESS

Electrospinning is a simple and efficient method by which to produce scaffolds composed of nanoscale to microscale fibers that are comparable to the fiber diameters of essential components of the extracellular matrix. In electrospinning, a polymer solution is pumped at a constant rate through a syringe with a small-diameter needle that is connected to a high-voltage source. This polymer solution usually consists of a natural or synthetic polymer combined with a volatile organic solvent. When this voltage source is turned on, an electric field is created between the needle and a metallic collecting plate. As the polymer solution is extruded through the syringe, a droplet forms on the tip of the needle and deforms into a conical shape as the applied voltage increases and causes electrostatic repulsion on the polymer droplet. This conical structure, called a Taylor cone, becomes increasingly unstable over time. As instability increases and the organic solvent evaporates, the Taylor cone divides geometrically into nanofiber jets, which are subsequently collected onto the grounded metal plate [1]. The final product is a mat composed of individual continuous nanofibers that can be used in many different applications, including drug delivery and tissue engineering.

The morphology of electrospun structures is greatly affected by four main adjustable parameters: a) flow rate of the polymer solution through the syringe, b) concentration of

the polymer solution, c) voltage applied to the needle, and d) the working distance between the needle and the collecting plate. Ideally, a scaffold for tissue engineering should be composed of uniform nanofibers with no beads. This uniformity is thought to facilitate an optimal environment in which cells can grow, communicate, and differentiate [1]. The four parameters for electrospinning can determine whether the final electrospun scaffold is composed of nanofibers, as opposed to beads or non-uniform polymer formations. For example, it has been found that a higher concentration polymer solution exhibiting high viscosity is more likely to form fibers than is a non-viscous solution [1].

Moreover, other factors such as the choice of solvent and solubility of the polymer can present challenges to the process, as some polymers may be difficult to dissolve. Harsh organic solvents are commonly used to solubilize such polymers. The vaporization of this organic solvent also facilitates the formation of fibers during electrospinning [1]. Together, many factors can profoundly affect the results of the electrospinning process, making its optimization a challenge to understand and execute.

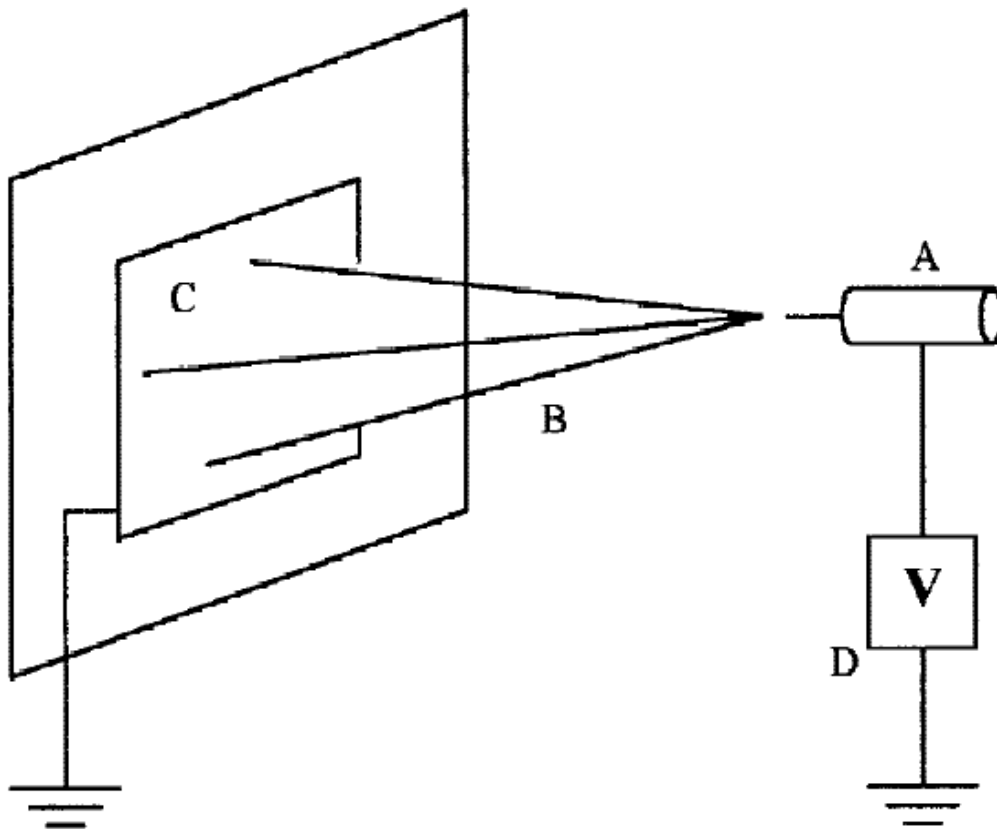


Figure 2: General schematic of an electrospinning apparatus consisting of a syringe with polymer solution (A), nanofiber jet (B), grounded collecting plate (C), and high-voltage power supply (D) [5].

4. FIBRINOGEN AND COLLAGEN: NATURAL POLYMERS

Natural polymers are candidate materials to be developed as electrospun nanofibrous scaffolds. Because these polymers are naturally occurring as opposed to synthetic, they have many potential advantages over other commonly used scaffolds. Scaffolds electrospun from natural polymers are thought to possess desirable qualities in terms of biocompatibility and biodegradability. Moreover, because they occur physiologically, it has been suggested that they mimic the native extracellular matrix to a great extent, allowing for a higher degree of tissue regeneration and functionality [6].

Fibrinogen is a natural polymer that is present in blood plasma. It is a globular protein composed of six chains – two A α , two B β , and two γ chains that are linked by disulfide bonds [7]. Physiologically, the reaction between fibrinogen and thrombin leads to the assembly of fibrous clots composed of fibrin that play a key role in wound healing [7]. Because its role in the body as a tissue regeneration mechanism is analogous to the objective of tissue engineering, fibrinogen has much potential as a novel tissue engineering scaffold that has been electrospun and studied only to a limited extent at the time of writing.

Collagen is another natural polymer that has been more commonly electrospun than fibrinogen. It is a major structural protein of the extracellular matrix, and makes up roughly one-quarter of all proteins in the human body [8]. Collagen is composed of three fibers wound into a coiled-coil triple helix. Its extremely stable structure gives it great mechanical integrity, and accordingly, collagen acts as a resilient support mechanism for cells that make up tissue [8]. As a tissue engineering scaffold, collagen can be isolated from a great number of sources, is highly conserved, and is relatively biocompatible, and thus is thought to have potential as a functional structure for tissue regeneration [9].

While other natural polymers have been electrospun and studied, this particular study explored the feasibility of electrospinning fibrinogen into viable nanofibrous scaffolds that can support cell adhesion and proliferation. The electrospinning of collagen was also investigated, as a means to understand the mechanism by which fibrinogen could potentially be electrospun into nanofibers.

5. METHOD

The electrospinning apparatus was constructed using a high-voltage power source, flow pump, and copper plate (Figure 3). Polymer solutions were loaded into plastic syringes, and subjected to the four adjustable electrospinning parameters: a) flow rate of the polymer solution through the syringe, b) concentration of the polymer solution, c) voltage applied to the needle, and d) working distance between the needle and the collecting plate. This general apparatus was used for electrospinning fibrinogen and collagen throughout the length of the study, although the four parameters were constantly varied. After electrospinning, samples were examined either through a scanning electron microscope or through a fluorescent microscope at high magnification.

The following set of parameters previously suggested by existing work to produce nanofibrous fibrinogen was first used as a control [7]:

- 22 kV voltage
- 2.0 mL/hr flow rate
- 10.0 cm working distance
- 71 mg/mL fibrinogen solution in H₂O

The first three parameters in the preceding list were easily accommodated using our electrospinning apparatus. However, because fibrinogen was in powder form, a method needed to be developed to obtain a desired solution. Distilled water was sprinkled slowly onto the fibrinogen powder and allowed to dissolve over roughly 12 hours without heat or agitation. A bicinchoninic acid (BCA) assay was then performed on the fibrinogen solution to quantify the amount of protein in the solution. In this biochemical assay, BCA is added to samples of unknown protein concentrations. The resulting color change of green to purple caused by the reaction between acid and protein is measured using colorimetric techniques, ultimately allowing the user to quantify the amount of protein present in a given sample.

Organic solvents are commonly added to polymer solutions for electrospinning. They are highly volatile (easily vaporized), and their presence in solution and subsequent vaporization during the electrospinning process is thought to facilitate the formation of nanofibers [1]. After the fibrinogen solution was prepared, previous work called for the addition of a harsh organic solvent called 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at 9 parts to 1 part fibrinogen solution [7]. Due to its corrosiveness and polarity, HFIP is commonly used as a solvent for polymer solutions, including those that are not soluble in many common organic solvents.

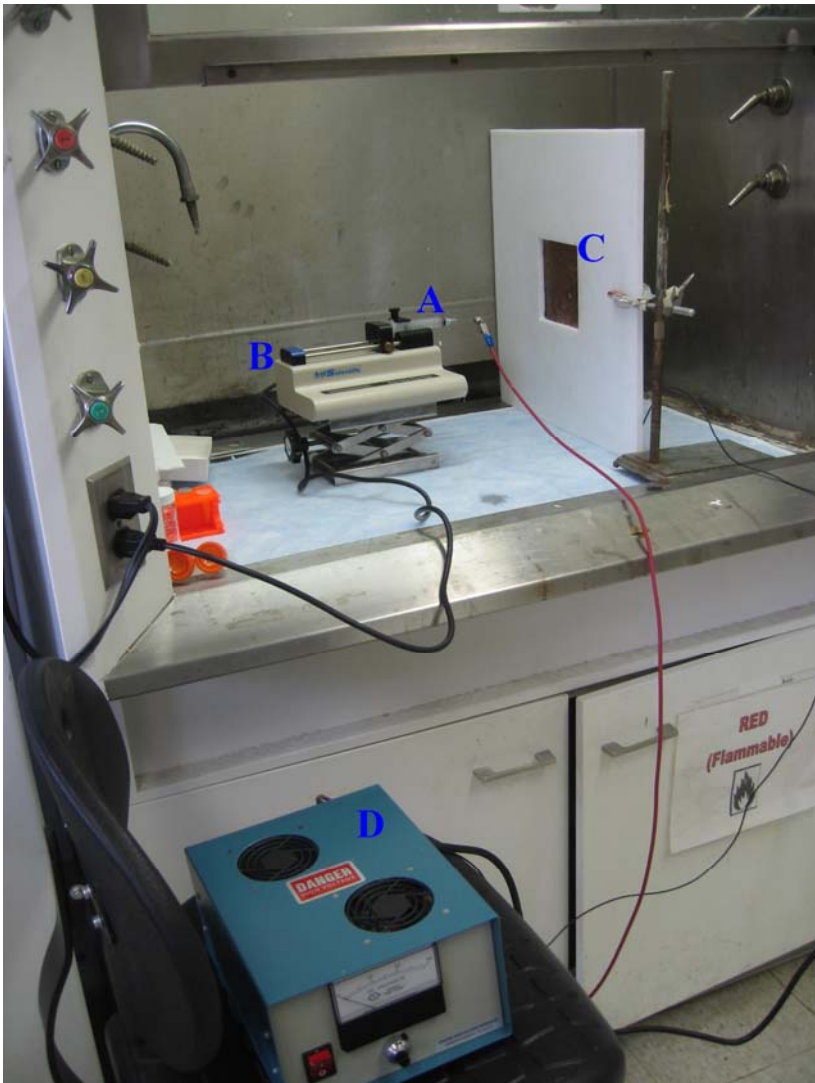


Figure 3: Photograph of the electrospinning apparatus used in this study. A polymer solution was subjected to a flow rate using a syringe pump (B). A power source (D) was used to apply a high voltage to the polymer as it was extruded through the syringe (A). The final product was collected onto a grounded copper plate (C).

5.1. Preliminary Results & Challenges

The following general morphologies were observed by applying electrospinning parameters in the ranges of the control parameters, specifically 18-26 kV voltage, 0.5-3.5 mL/hour flow rate, 2-18 cm working distance, and 71-94 mg/mL fibrinogen solution.

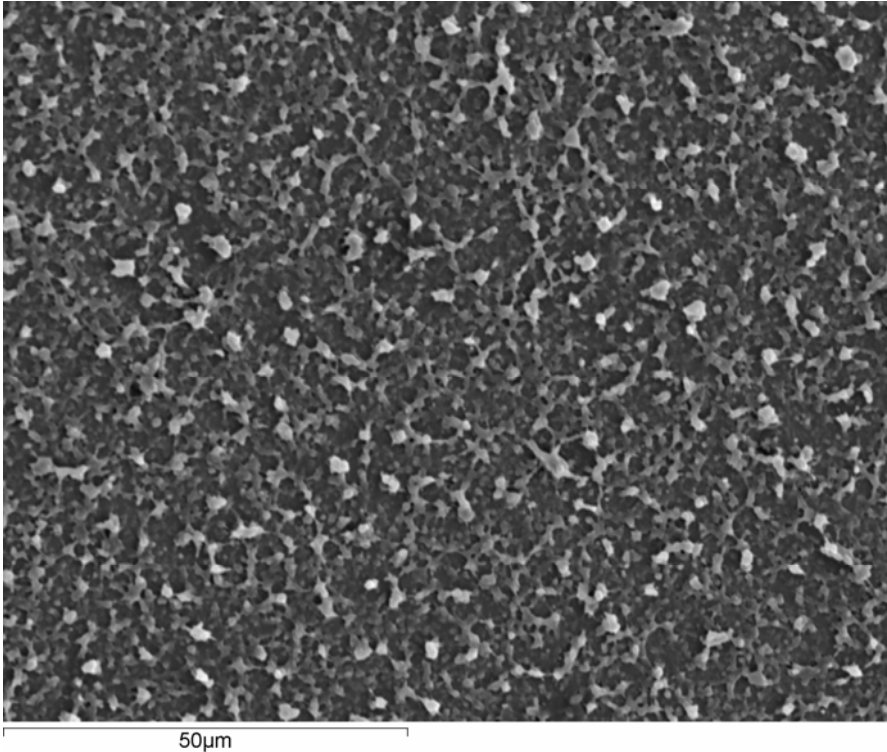


Figure 4: Scanning electron micrograph of an electrospun fibrinogen structure at control parameters. Magnification: 1000X.

When compared to nanofibers as shown in Figure 1, fibrinogen electrospun under these parameters exhibited a significant difference: no fibers were present. Instead, uniform beads of about 1 μm were present throughout the structure. Nanofibrous structures, by definition, typically exhibit fiber diameters of 1 μm and below. This suggested that the formation of fibers rather than beads of the same approximate size scale might be possible using a different combination of parameters.

While the voltage, working distance, and flow rate were identical to those in a previous study in which fibrinogen fibers were formed, the concentration of the fibrinogen solution was slightly modified. Existing work on electrospinning fibrinogen has shown that minimal essential medium (MEM) is needed for the formation of nanofibers as well as the dissolving of fibrinogen due to the presence of salts that can affect the morphology of an electrospun scaffold. However, a significant challenge in electrospinning fibrinogen was the difficulty of solubilizing the fibrinogen powder. MEM did not appear to dissolve fibrinogen, even at temperatures up to 50°C and agitation using a stir bar or rocker. In addition, MEM formed a precipitate when combined with the organic solvent HFIP before electrospinning. Moreover, HFIP alone did not appear to dissolve the powdered fibrinogen. The absence of salts in the fibrinogen/H₂O solution was a major concern, and other tests needed to be performed to determine whether the presence of salts was required to form a nanofibrous fibrinogen structure.

5.2. Further Results & Modifications to Approach

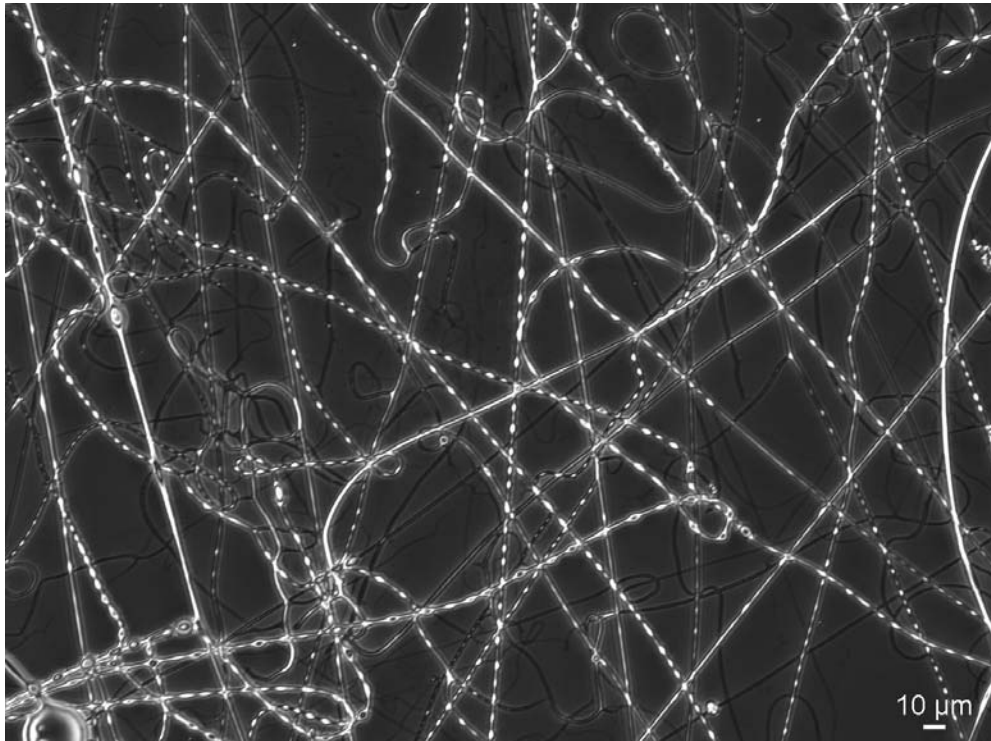
To garner a clearer understanding of the process by which natural polymers form nanofibers when subjected to electrospinning, we next sought to electrospin collagen. Because much more literature existed on nanofibrous collagen structures, specific conditions under which collagen scaffolds were electrospun into nanofibers were readily available to examine. Collagen from calf skin was obtained and subjected to the following electrospinning parameters, which were roughly suggested by existing literature:

- 15 kV voltage
- 1.0 mL/hr flow rate
- 15.0 cm working distance
- 80 mg/mL collagen solution in HFIP

Because collagen was directly dissolved in the organic solvent HFIP rather than water or MEM, the extra step of adding HFIP to the polymer solution before electrospinning was skipped. The BCA assay was used again to determine the amount of collagen present in each collagen solution that was electrospun.

At the control parameters listed above, the following structures were observed under a high-magnification fluorescent microscope.

A.



B.

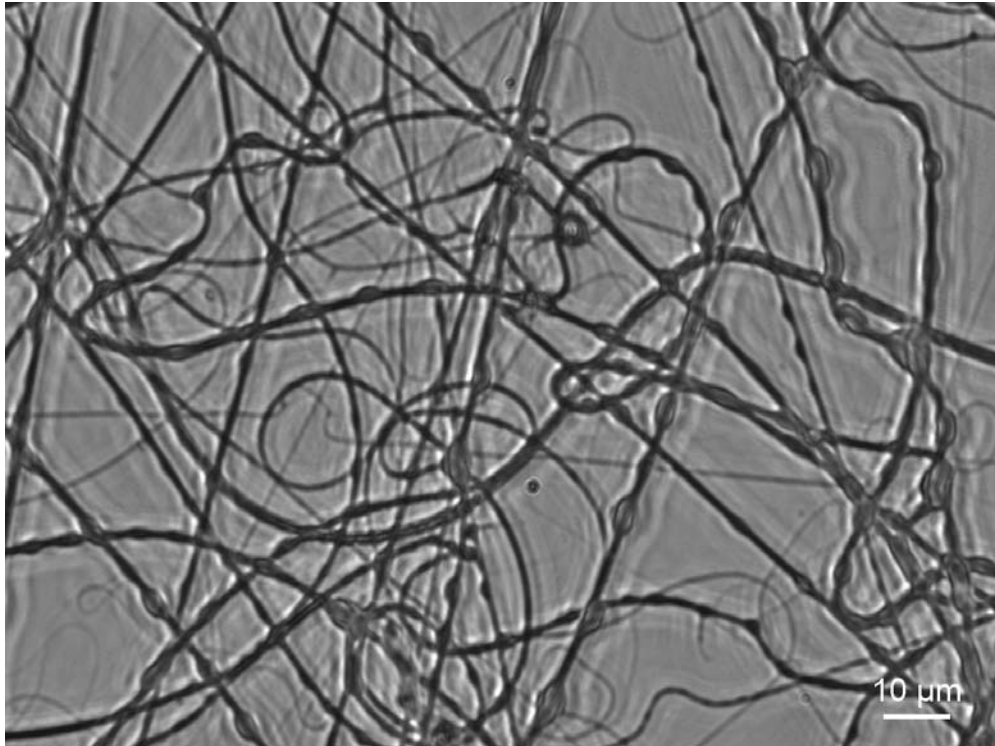


Figure 5: Micrographs of electrospun collagen structures subjected to control parameters of 15 kV, 1.0 mL/hr, 15.0 cm, and 80 mg/mL. Magnification: 20X (A) and 60X (B).

Continuous collagen nanofibers were observed in these micrographs, with an approximate fiber diameter of 1 μm . While the presence of fibers and their size scale were highly desirable, non-uniformities in the fibers were also observed in the form of beads throughout each fiber. We hypothesized this to be related to the slight difficulty of dissolving collagen in HFIP, although collagen was much more easily solubilized than fibrinogen. Collagen dissolved in HFIP over several minutes with constant agitation at room temperature. The resulting mixture was mostly homogeneous, although undissolved particles may have been present that could have caused beads within the electrospun fibers. With a slightly higher temperature and agitation over a longer period of time, these non-uniformities could potentially be eliminated.

Although non-uniformities were present in the electrospun scaffold, the formation of nanofibers was a significant accomplishment that allowed us to gain great insight on how to form fibrinogen nanofibers. Two key observations were made that allowed us to modify our fibrinogen electrospinning parameters accordingly. First, the collagen solution drawn into the syringe was much more viscous than the fibrinogen solution. This is attributed to the fact that fibrinogen was directly dissolved in HFIP, and therefore did not require the addition of HFIP to a solution of polymer and water. Moreover, the addition of HFIP to the fibrinogen/H₂O solution at a large proportion of 9 parts HFIP to 1 part fibrinogen/H₂O caused the fibrinogen solution to have an especially low viscosity. Next, it was noted that collagen did not require the presence of salts in order to form

nanofibers. This suggested that solubilizing fibrinogen in MEM was not necessary, and that dissolving fibrinogen in H₂O or organic solvent should have been adequate.

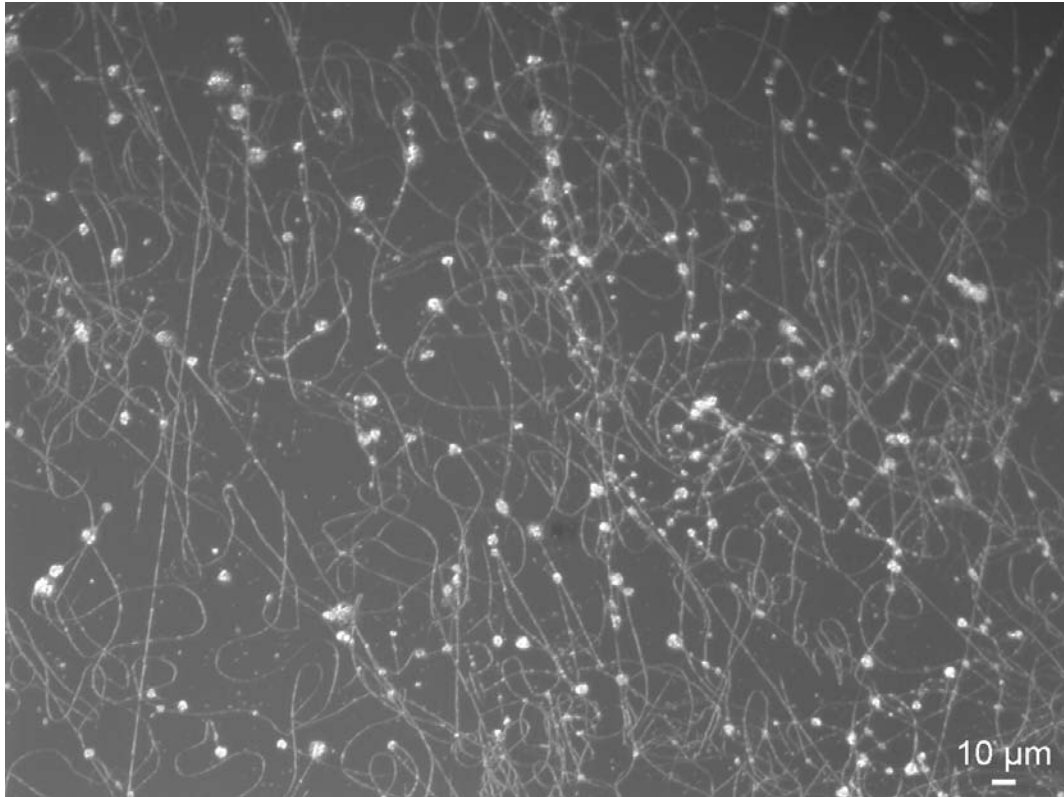
These observations led to two key modifications in our method for electrospinning fibrinogen:

1. Fibrinogen was dissolved in distilled H₂O at a higher concentration than was attempted before. Using a BCA assay, the final concentration of this solution was found to be 98 mg/mL. This solution was highly viscous.
2. Instead of adding 9 parts HFIP to 1 part fibrinogen/H₂O, the two solutions were combined in equal proportions. In this manner, the high viscosity of the 98 mg/mL fibrinogen/H₂O solution was not lowered upon the addition of HFIP as it was before.

The other control parameters (22 kV voltage, 10.0 cm working distance, and 2.0 mL/hr flow rate) remained unchanged and were applied in combination with the two changes in method. The modified fibrinogen solution was electrospun and viewed under a fluorescent microscope.

6. FINAL RESULTS & DISCUSSION

A.



B.

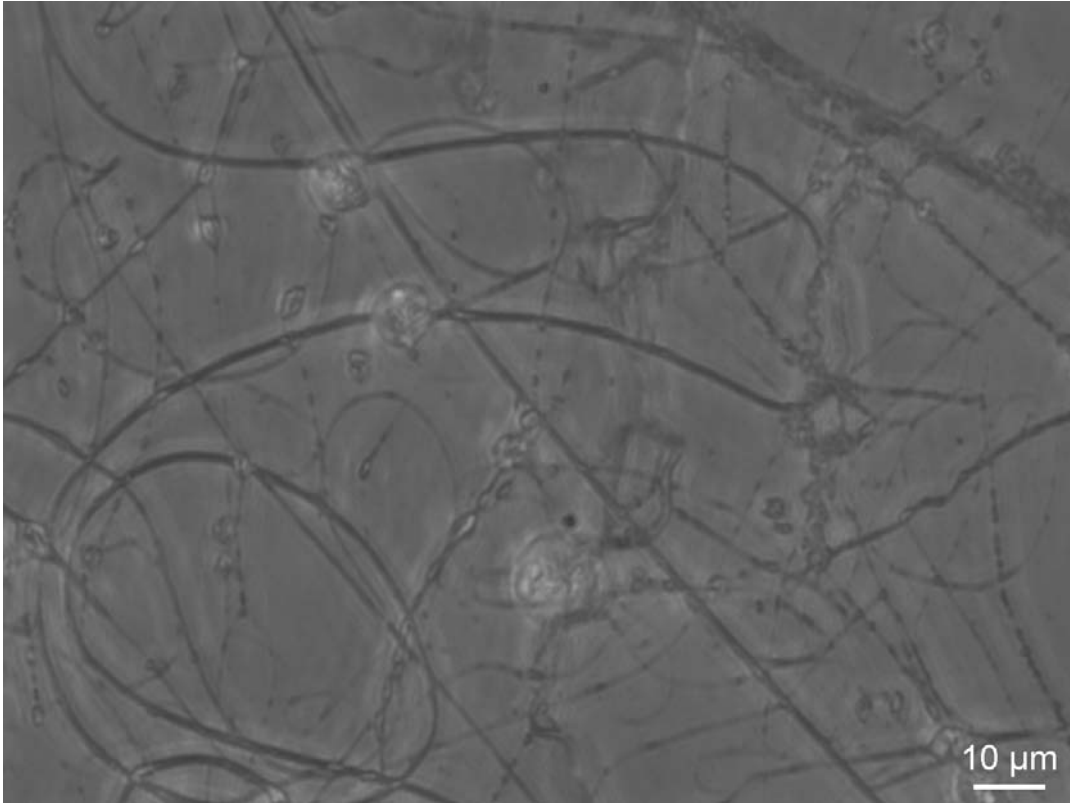


Figure 6: Micrographs of electrospun fibrinogen structures subjected to parameters of 22 kV, 2.0 mL/hr, 10.0 cm, and 98 mg/mL in distilled water with an addition of HFIP at a proportion of 1:1. Magnification: 20X (A) and 60X (B).

The modifications to the method proved to be successful, as fibers about 1 μm and smaller in diameter were formed. Non-uniformities were once again observed in the form of beads interspersed throughout the fibers. These non-uniformities differed from those observed in the electrospun collagen scaffolds in that they were larger in size (roughly 3-10 μm in the micrograph) and more randomly dispersed. Although nanofibers were formed, the presence of these beads is not desirable for any tissue engineering scaffold because they can disrupt the way cells grow, communicate, and differentiate. The beads are also inconsistent with the native extracellular matrix, which tissue engineers strive to mimic when fabricating scaffolds.

It has been suggested that a polymer solution with a higher viscosity is directly related to its ability to form nanofibers [1]. Thus, decreasing the amount of HFIP added to the fibrinogen/ H_2O solution to achieve a high viscosity fibrinogen electrospinning solution may aid in the formation of uniform nanofibers with a smaller quantity of beads present. Alternatively, the addition of more HFIP to the fibrinogen/ H_2O solution may help to solubilize any remaining particles of fibrinogen that were not initially dissolved by water. This may help to homogenize the fibrinogen electrospinning solution, facilitating the formation of uniform nanofibers without beads and other inconsistent structures. In either direction, there will be a threshold at which the formation of fibers is no longer

possible, and this information may be helpful to future work in preparing fibrinogen solutions for electrospinning.

The other three electrospinning parameters should be varied in an attempt to aid the formation of uniform nanofibers as well. First, the voltage for the structure seen in Figure 6 was set to 22 kV. Increasing this value would cause an increase in electrostatic repulsions on the polymer droplet as the solution is extruded from the syringe. These repulsions may further deform the droplet into nanofibers with smaller fiber diameters, and could therefore impede the occurrence of large beads. Next, the flow rate of the solution through the syringe directly affects the size of the polymer droplet upon which the voltage acts. A smaller droplet would experience a great amount of electrostatic repulsion on a per volume basis. Thus, decreasing the flow rate could have a similar effect as increasing the voltage: beads may be deformed into nanofibers. Finally, the length of the working distance is directly related to the amount of time that the organic solvent is able to evaporate. A greater amount of evaporation is hypothesized to lead to nanofibers with smaller fiber diameters. Therefore, increasing the working distance may allow more time for the organic solvent to evaporate, facilitating the formation of fibers and minimizing non-uniformities in the nanofiber jet as it travels to the collecting plate.

7. CONCLUSION

The formation of electrospun nanofibrous fibrinogen and collagen scaffolds was a first-time achievement in our laboratory that has much potential for future engineering endeavors, particularly in the fields of meniscus and annulus fibrosus tissue engineering. As such, many further developments await us in the process of scaffold design. Specifically, the electrospinning parameters of voltage, working distance, flow rate, and concentration can be varied in different combinations to achieve the most uniform nanofibers possible. Much was learned in the process of achieving nanofibers from these proteins. We perceived electrospinning, and the proteins themselves, to be complex and challenging to manipulate. With more work towards optimizing the electrospinning parameters of fibrinogen and collagen, the process of electrospinning natural polymers can become even more clearly understood, and this scaffold can be used in a broad range of tissue engineering studies.

8. RECOMMENDATIONS

Three main long-term goals should be subsequently achieved. First, the formation of uniform fibrinogen fibers should be achieved. As previously discussed, increasing or decreasing the amount of HFIP added, increasing the voltage, decreasing the flow rate, and increasing the working distance are all recommended. As more uniform nanofibers are achieved, scaffolds should be viewed using scanning electron microscopy to detect any smaller non-uniformities that may be present. After a uniform nanofibrous scaffold is achieved, the next goal would be to test these scaffolds mechanically. The optimized electrospinning parameters achieved in the first goal can be applied to make a great amount of randomly aligned scaffolds, as well as aligned scaffolds using a rotating mandrel in the electrospinning apparatus. Aligned scaffolds are recommended at this

point in the study, since these scaffolds exhibit greater mechanical strength and mimic the native extracellular matrix to a greater extent. After the scaffolds are confirmed to have significant mechanical integrity, the last recommended step would be to seed cells onto the scaffolds. In our laboratory, meniscus or annulus fibrosus cells would be used. After cells are allowed to grow on the scaffolds for periods of 0-56 days, the scaffolds can be tested using histological stains, biochemical assays, and mechanical tests to determine their ability to support cell adhesion and differentiation. These analyses will allow us to ascertain the potential of fibrinogen as a viable tissue engineering scaffold. These goals can also be performed upon the electrospun nanofibrous collagen scaffolds that were developed in this study.

9. ACKNOWLEDGEMENTS

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