Electrospinning collagen, gelatin, and PLLA/laminin nanofibers for the production of a bioengineered cornea

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Background

Corneal damage is a leading cause of vision loss worldwide, second only to cataracts.[1] One possible solution is tissue engineering replacement corneas. However, the cornea contains living cells that need particular conditions unmet by standard cell culture procedures in order to maintain a properly transparent phenotype. The cornea is composed of an extracellular matrix of collagen nanofibers. These collagen nanofibers are highly aligned, with uniform fiber diameter. This structure provides support as well as an input signal to cells.[2] Changes to the structure of the matrix, including fiber alignment and fiber diameter, can change how cells bind to the material and thus affect cell phenotype.[3] Nanofiber mats mimicking the structure of the native corneal extracellular matrix can be fabricated using an electrospinning process, in which a voltage differential deposits nanofibers of a polymer solution on a grounded plate. The volatile solvent evaporates, leaving mats of nanofibers. Rotation of the grounded collection plate can create samples with high fiber alignment.

Objectives

We aim to determine the impact of substrate composition on the protein expression of corneal keratocytes in vitro by varying the composition and structure of the growth substrate. Electrospinning was used as a means to produce a substrate of similar material and structure to the native cornea. The primary objectives were:

1. To optimize protocols for electrospinning mats of collagen, gelatin, PLLA, and PLLA/laminin;
2. To control for fiber diameter by manipulating the concentration of the gelatin, PLLA, and PLLA/laminin solutions to create nanofibers of a statistically similar diameter to that of the collagen nanofibers;
3. To generate aligned samples of each substrate to determine the effect of alignment on cell protein expression;
4. To successfully develop a protocol for crosslinking these mats and seeding corneal fibroblasts on them.

Materials and Methods

Four different compositions were tested: collagen, gelatin, poly(L-lactic) acid (PLLA), and blended PLLA/laminin, in which the synthetic polymer PLLA was blended with laminin to increase cell attachment and viability. Nanofiber mats were created by electrospinning solutions of polymer dissolved in acetic acid (for collagen, gelatin samples) or Hexafluoro-2-Propanol (HFP) (for PLLA, PLLA/laminin samples). Concentrations were tested until the populations matched the fiber diameter of the 7% collagen solution.

- Collagen was dissolved in a 7%w/v solution with acetic acid. The solution was covered and heated at 35°C for 10 minutes, stirred with heat for 20 minutes, then stirred overnight.
- Gelatin was dissolved in a 7, 8.5, or 10%w/v solution with acetic acid. The solution was covered and heated at 35°C while stirring for 15 minutes.
- PLLA was dissolved in a 2.5 or 4%w/v solution with HFP. The solution was stirred and heated (covered) at 35°C for 20 minutes and used as a control for PLLA/laminin. PLLA/laminin samples used a 250:1 ratio[4], at the same concentration as PLLA samples.
- Solutions were housed in a 3mL syringe and 18 gauge needle. The flow rate of the syringe was held constant at 0.1 mL/hr, and charged by a 10.5 kV power source (Figure 1). Fibers collected on grounded copper foil, and were removed for imaging and crosslinking using a razor blade and tweezers. Samples for imaging were sputter coated for 90 seconds at 30mA.
- Fiber mats were vapor and liquid crosslinked in glutaraldehyde and ethanolamine in preparation for cell culture.
- Samples were imaged on a Hitachi SU-70 scanning electron microscope at 10k magnification. Three images each were taken at two separate areas on the sample, and fiber diameter was measured at 10 random points per image using Matlab. These values were averaged and each sample treated as a distinct data point.
- Sample sets were found to lack normal distribution, so groups were compared using a Mann-Whitney U test, $\alpha=.05$.

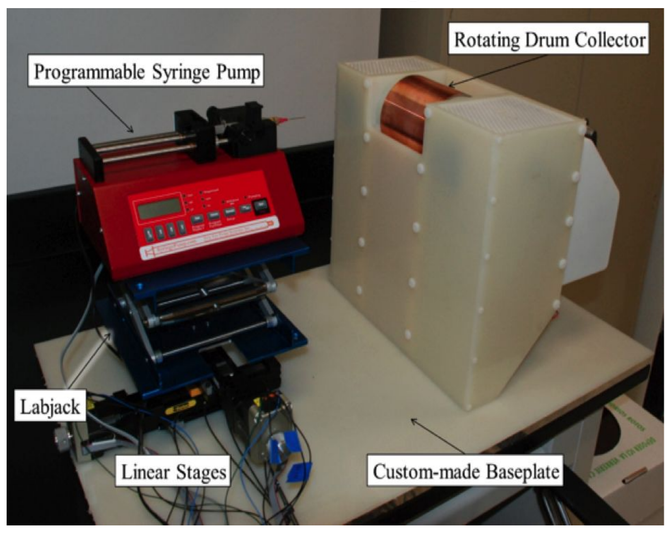

Figure 1. Electrospinning apparatus setup.

Results

As seen in Table 1, A 7% w/v solution of collagen was spun to create reference mats, which were determined to have an average fiber diameter of 171nm. To maintain constant fiber diameter between polymers, multiple concentrations of gelatin and PLLA were tested until the average fiber diameter of the groups were found to be statistically similar (Figure 3). A gelatin solution of 8.5%, and PLLA or PLLA/laminin solutions of 4% were found to be statistically similar (*p=.25, .44, .89, respectively*). Untreated electrospun fiber mats had an opaque white appearance and were thick and stable enough to be easily removed from the copper collection surface. PLLA samples were opaque when submersed in milliQ water or PBS, suggesting that they would not form a suitable substrate upon which to grow a transparent cornea. Scanning electron microscope images of electrospun samples taken before and after crosslinking are seen in Figure 2. Collagen and gelatin crosslinked samples exhibited the characteristic structure of crosslinked fiber mats and did not dissolve in water. However, PLLA containing samples did not appear to have noticeable difference in fiber structure.

Table 1: Fiber Diameter and Sample Size

Composition (%w/v)	***Average Diameter (nm)***	***St. Dev. (nm)***	***Sample Size***
Collagen 7%	171	50	14
Gelatin 7%	114	31	5
Gelatin 10%	254	46	8
Gelatin 8.5%	183	31	7
PLLA 2.5%	107	0.14	2
PLLA 4%	146	33	6
Laminin/ PLLA 4%	163	15	4

Results

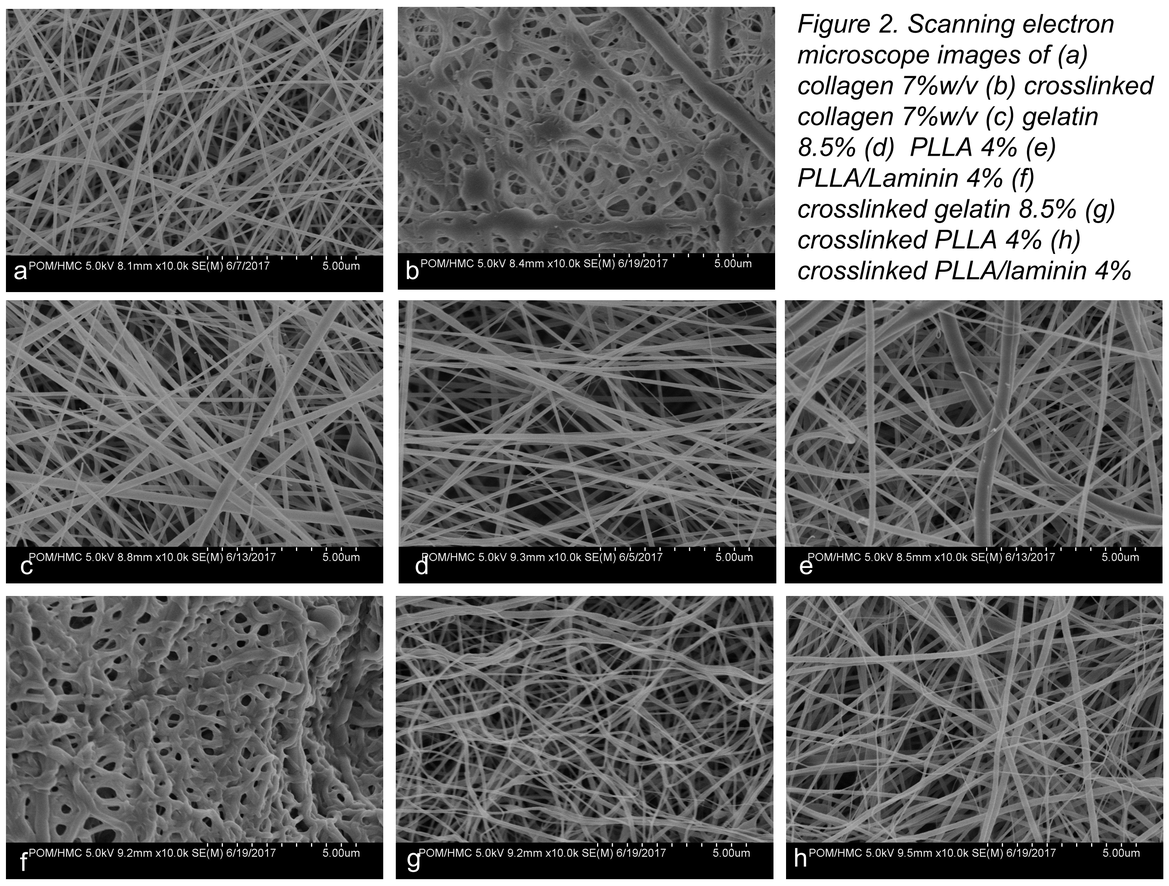

Figure 2. Scanning electron microscope images of (a) collagen 7%w/v (b) crosslinked collagen 7%w/v (c) gelatin 8.5% (d) PLLA 4% (e) PLLA/Laminin 4% (f) crosslinked gelatin 8.5% (g) crosslinked PLLA 4% (h) crosslinked PLLA/laminin 4%

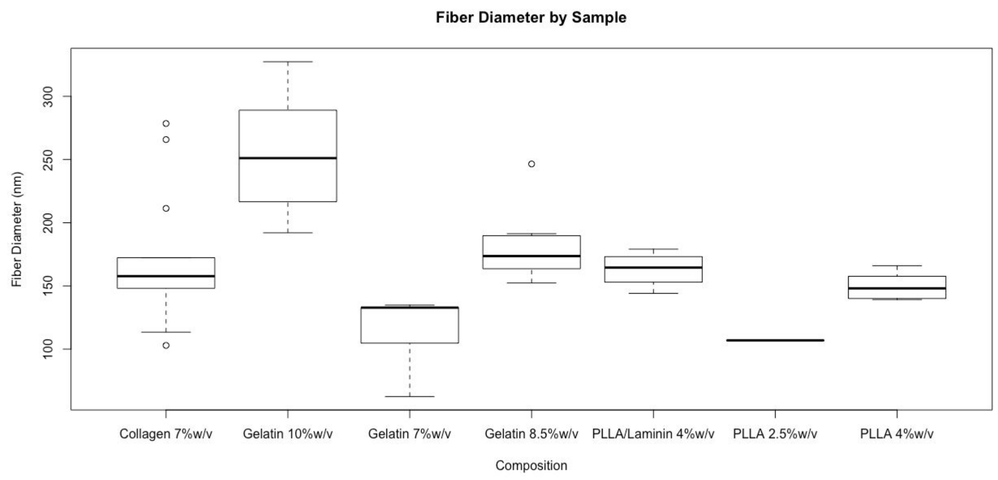

Figure 3: Fiber Diameter of samples by composition. Collagen 7%w/v samples were found to be statistically similar to Gelatin 8.5%w/v, PLLA 4%w/v, and PLLA/Laminin 4%w/v.

Discussion

Fiber mats of statistically similar fiber diameter were successfully electrospun from collagen, gelatin, PLLA, and PLLA/laminin. These studies will eliminate fiber diameter as a possible input signal affecting cell response. Cross-linking successfully prevented the mats from dissolving, and modified the structure of the collagen and gelatin mats. However, PLLA and PLLA/laminin nanofibers did not appear to be successfully crosslinked. In addition, PLLA and PLLA/laminin mats were opaque throughout crosslinking. Moving forward, we will seed cells on these mats and analyze cellular response in order to determine which polymer is optimal for the eventual synthesis of a bioengineered cornea. This study will also answer fundamental questions of cellular response to nanoscale extracellular matrix features, which will inform tissue engineering studies more broadly.

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