
Scaffold permeability as a means to determine fiber diameter and pore size of electrospun fibrinogen

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Abstract: The purpose of this study was to construct a flowmeter that could accurately measure the hydraulic permeability of electrospun fibrinogen scaffolds, providing insight into the transport properties of electrospun scaffolds while making the measurement of their topographical features (fiber diameter and pore size) more accurate. Three different concentrations of fibrinogen were used (100, 120, and 150 mg/mL) to create scaffolds with three different fiber diameters and pore sizes. The fiber diameters and pore sizes of the electrospun scaffolds were first analyzed with scanning electron microscopy and image analysis software. The permeability of each scaffold was

measured with the flowmeter and used to calculate permeability-based fiber diameters and pore sizes, which were compared to values obtained through image analysis. Permeability measurement revealed scaffold permeability to increase with fibrinogen concentration, much like average fiber diameter and pore size. Comparison between the two measurement methods demonstrated the efficacy of the flowmeter as a way to measure scaffold features. © 2007 Wiley Periodicals, Inc. *J Biomed Mater Res* 85A: 115–126, 2008

Key words: electrospinning; fibrinogen; scaffold; permeability

INTRODUCTION

The interdisciplinary field of tissue engineering has blossomed in the brief two decades since the term was coined.^{1,2} While extensive research has been done in many areas that would fall under the umbrella name of tissue engineering, of particular interest to this study is the research done on the creation of scaffolds that mimic the native extracellular matrix (ECM). A countless number of materials, both natural and synthetic in origin, and processing techniques have been used to engineer these extracellular matrix analogues, with varying degrees of success.^{3–7} Although scaffolds may be designed and created to fit a specific application, ultimately all scaffolds regardless of application must meet a number of basic criteria to be successful. At its most fundamental, the scaffold should not elicit an immune response while remaining a viable framework for cellular infiltration/proliferation, ultimately mimicking both the fibrillar form and the complex function of the native ECM.^{3,8–12} This requires the scaffold to have an idealized combination of mechanical proper-

ties, geometry, and surface chemistry.^{9,13,14} Altered scaffold permeability and nutrient/waste diffusion, important in their own right for cell and nutrient/waste movement throughout a scaffold, can be a byproduct of tailoring the aforementioned scaffold characteristics.^{15,16}

Electrospinning is a simple process requiring little specialized equipment in order to create submicron scale, fibrous ECM analogues from a wide array of polymers. Our laboratory has used this process to create scaffolds from a number of different synthetic polymers, as well as the natural biopolymers collagen,⁴ elastin,⁶ and fibrinogen.^{7,17,18} In brief, a polymer is placed in solution and drawn into a syringe fitted with a blunt tip needle. The surface tension of the polymer solution at the tip of the needle is overcome through the application of a large electric potential. A grounded target is placed a set distance from the charged polymer solution to create a static electric field. When the electric potential reaches a critical level, the electrostatic forces overcome the surface tension at the needle tip, and a fine jet of entangled polymer chains are drawn out. This jet whips through the air toward the grounded target, creating a dry fiber through the evaporation of the polymer's solvent. This produces fibers that range from 50 nm to 10 µm in diameter, which are collected on the grounded target.^{8,13,19–21}

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As simple as electrospinning is, it offers the tissue engineer the ability to tailor several different aspects of the ECM analogue scaffolds. Fiber diameters can be controlled by altering the concentration of the polymer solution; solutions made of higher concentrations produce larger diameter fibers. Rotating the grounded target can control the alignment of the collected fibers. A high rate of rotation will produce fibers that align parallel to the direction of rotation, while a low rotational speed will create a fibrous scaffold of randomly aligned fibers. Scaffold thickness is strictly dependent on the volume of solution to be electrospun, while scaffolds of any geometry can be created by machining the desired shape into a grounded target.

The only aspect of electrospinning that is not easy to directly control is the size of the scaffold's pores. This can be controlled indirectly by creating smaller diameter fibers, as smaller fibers result in smaller, more tightly packed pores. However, it is not possible to alter pore size without changing any of the other electrospinning parameters. This is due to the fact that electrospun scaffolds do not contain true, cylindrical pores like other processing techniques. While electrospun scaffolds have porosities that are on par with scaffolds produced through other techniques, they are merely a group of nonwoven fibers lying loosely upon one another. This creates a unique situation that allows for cells to create a pore diameter of their choosing as they migrate throughout the scaffold. This dynamic architecture renders the reporting of electrospun scaffold pore diameters unnecessary, as the cells will create a pore as large or small as they please.¹² However, this makes the reporting of scaffold porosity and permeability even more important in determining the characteristics of successful electrospun ECM analogues.

The purpose of this study was to construct a flow-meter that could accurately measure the hydraulic permeability of electrospun fibrinogen scaffolds of varying fiber diameter and alignment. The quantification of electrospun fibrinogen scaffold permeability would provide further insight into the transport properties, geometry, and behavior of generic electrospun scaffolds. This permeability would then be used to calculate average fiber diameters and pore sizes for comparison with values determined by image analysis software. It was hypothesized that scaffold permeability would increase with increasing polymer concentration, as it has been previously shown that both fiber and pore size increase with concentration. In addition, scaffolds with a high degree of alignment would exhibit higher permeabilities than randomly oriented scaffolds, as alignment would reduce the tortuous nature of the electrospun scaffolds. Dry scaffolds should be more permeable than hydrated scaffolds upon initial testing, with the

dry scaffolds exhibiting a decrease in permeability as they become hydrated with increasing time, while the hydrated scaffolds would maintain a nearly constant level of permeability.

MATERIALS AND METHODS

Electrospinning

Solutions of three different concentrations of fibrinogen (100, 120, and 150 mg/mL) were made with a 90% by volume solution of 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and 10% by volume 10x MEM in a glass scintillation vial. All reagents were purchased from Sigma Aldrich. Solutions were left overnight on a shaker plate to ensure that all fibrinogen had dissolved and formed a homogenous solution. These solutions were then loaded into a 5-mL Beckton Dickinson syringe and placed in a KD Scientific syringe pump (Model 100) to be dispensed at a rate of 4 mL/h. A high voltage power supply (Spellman CZE1000R; Spellman High Voltage Electronics Corporation) was used to apply a voltage of 25 kV to a blunt tip 18 gauge needle fixed to the solution containing syringe. Solutions were electrospun onto a flat, stainless steel, grounded mandrel (2.5 cm wide $\times$ 10.2 cm long $\times$ 0.3 cm thick) placed 12 cm from the needle tip and rotating at a rate of $\sim$ 500 rpm to produce a scaffold of randomly oriented fibers. The same procedure was used in an attempt to produce aligned fiber scaffolds; however the mandrel rotational speed was increased to 3000–4000 rpm. While results would indicate that truly aligned fibers were not created, scaffolds created with a high mandrel rotational speed were referred to as aligned scaffolds for the sake of simplicity in reporting results. Immediately after electrospinning, scaffolds were cut from the mandrel and placed in a fume hood for 30 min for degassing and removal of remaining HFP.

SEM-based fiber diameter and pore size

Prior to characterization, the dry scaffolds were placed in a desiccation chamber overnight to ensure they were completely free of moisture. These samples were then gold sputter-coated (Model 550; Electron Microscope Sciences) in preparation for scanning electron microscopy (JSM-820 JE Electron Microscope; JEOL). A scanning electron micrograph (SEM) of each fibrinogen concentration, both aligned and randomly oriented, was taken at 1500 $\times$ magnification. Samples of each dry electrospun scaffold were cut from the large mats and placed in phosphate buffered saline for 24 h. SEMs of these hydrated samples were then taken on an environmental scanning electron microscope (Hitachi S-3400N) in their fully hydrated state at magnifications between 1000 $\times$ and 1500 $\times$. All micrographs were then digitized using a flatbed scanner (Hewlett-Packard Scanjet 5550c). To determine fiber and pore size, the ImageTool 3.0 image analysis software package was used (Shareware provided by University of Texas Health Science Center at San Antonio). The software was calibrated using the

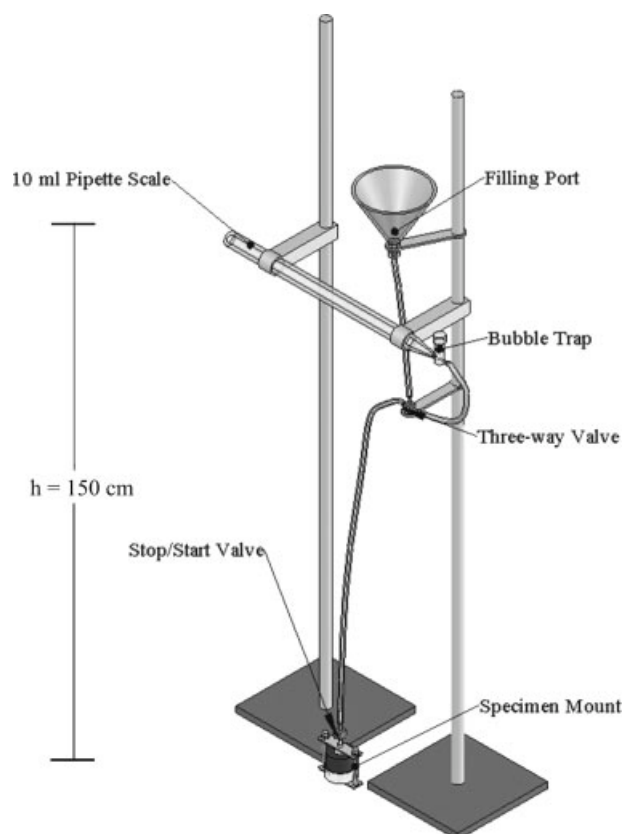


Figure 1. Diagram of the simple flowmeter used in this study. Modified from the flowmeter of Dr. Carr, designed for testing fibrin gel permeability.²³

micron scale bar of each picture. An average fiber diameter was determined by measuring the diameter of 60 different fibers, while an average pore size was determined by measuring the diameter of 60 different pores. Pores were identified as areas of void space bounded by fibers on all sides at or near the same depth of field, while their long and short diagonal axes were measured and averaged together to serve as their diameter. The same procedure and software were used for SEMs of both the dry and hydrated samples.

Permeability flowmeter design

Scaffold permeability was measured using a device and method that were modified from the work of Carr et al.^{22,23} and Wang et al.²⁴ This required the design and construction of a flowmeter and specimen mount to measure the amount of fluid passed through a fixed electrospun scaffold area over time (Figs. 1 and 2). As electrospun scaffolds have high porosities, allowing water to migrate outside of the intended area of fluid flow and into surrounding areas of the scaffold, it was necessary to create a bound area of fluid flow. The bulk of the flowmeter came directly from Dr. Carr's simplistic design, with a graduated 10-mL pipette (Fisher Scientific) placed horizontally as the measurement scale. To create a larger pressure head and push fluid through the tightly packed electro-

spun scaffolds faster, the height h (distance between horizontal pipette and scaffold) was increased from 20 to 150 cm.

The specimen-mounting portion of the flowmeter in Dr. Carr's original apparatus (glass tubing, silk screen) was designed to hold a fibrin gel, and would not support an electrospun scaffold. For this reason a novel specimen mounting apparatus had to be fabricated. To ensure that all fluid traveled through a set area of the electrospun scaffold and produced accurate permeability values, the mounting apparatus was designed to work by compressing the outer edge of the scaffold between two silicone gaskets. The compression was intended to form a tight seal and force all fluid flow through the open center of the specimen mounting apparatus. A large pore metal screen was placed on the underside of the scaffold to prevent

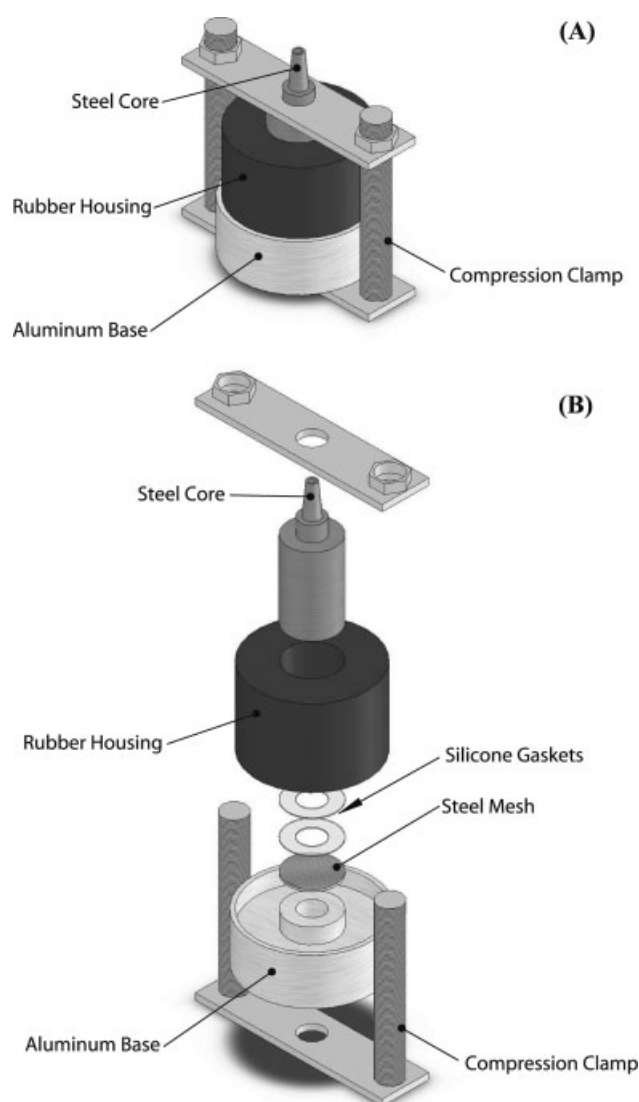


Figure 2. Flowmeter specimen mount in collapsed (A) and exploded views (B). Electrospun scaffolds are placed between the two silicone gaskets for testing in order to create a tight seal around the desired area of fluid flow. Steel mesh is placed beneath the scaffold and gaskets to reduce scaffold distension.

excessive distension of the test scaffold, which would again alter the cross-sectional area of fluid flow and effect permeability measurement.

Test of permeability flowmeter efficacy

In order to test the efficacy of the flowmeter design, in particular the design of the compression-based specimen mount, three different fibrinogen scaffolds were electrospun. A randomly oriented scaffold of each concentration whose permeability was to be tested (100, 120, and 150 mg/mL) was created and placed in the specimen mount. The flowmeter and 10-mL pipette were filled with PBS that had been dyed dark red with food coloring in order to stain the areas where fluid passed through the scaffold, the idea being that if the specimen mount functioned correctly, the only fluid flow would take place in the center of the scaffold, dying that region red. The outer edge of the scaffold, compressed between the two silicone gaskets, should not allow fluid flow and therefore remain mostly dry and undyed. Should the specimen mount not create a tight compression seal around the outside of the scaffold, dyed fluid would leak out of the area designated for fluid flow and stain the entirety of the scaffold.

Scaffold volume fraction measurement

Disks 21 mm in diameter were punched from each of the large, dry electrospun mats for permeability testing. They were individually massed, and thickness measurements were taken by placing the sample in a micrometer (Mitutoyo) between two glass cover slips. Prior to permeability testing, the volume fraction (ϕ) of each disk was determined for both dry and hydrated samples using the following equation:

$$\text{Volume Fraction} = \frac{\text{Calculated Scaffold Density}}{\text{Known Material Density}} \times 100 \quad (1)$$

The calculated scaffold density was determined by dividing the mass of the scaffold by the total volume of the scaffold, while the dry density of fibrinogen was known to be 1.38 g/cm³.¹⁸

Hydrated samples were soaked for 24 h in PBS. It has been shown previously in our laboratory that electrospun fibrinogen scaffolds experience a reduction in both diameter and thickness when placed in aqueous solutions. After 24 h in PBS, the shrunken diameter and thickness of the samples were measured to determine their wet total volume. Hydrated volume fraction was calculated using Eq. (1) in the same way as the dry volume fraction. The hydrated calculated scaffold density was determined by dividing the previously measured dry mass of the scaffolds by the altered total volume of the hydrated scaffolds, which was then divided by the dry density of fibrinogen. The dry mass and density were used because it was assumed at this point that the fibrinogen fibers were not hygroscopic and would not absorb PBS, therefore the only

difference in mass would be due to the presence of water molecules in void spaces and not a change in the actual scaffold. This assumption was later confirmed through SEM analysis.

Permeability measurement

Scaffolds both dry and hydrated were individually placed in the flowmeter's specimen mount. The flowmeter tubing and the 10-mL pipette were filled with PBS and permeability testing was begun at room temperature (~25°C). Fluid flow was measured using the incremental marks on the 10 ± 0.05 mL pipette and was recorded every minute for the first 10 min of testing. After the initial 10 min, recordings were made at 15 min and 20 min, in addition the total time for 13 mL (maximum volume of pipette) to pass through the scaffold was recorded. These time values were then used in the following Darcy equation to determine scaffold permeability²²:

$$\tau = \frac{Q\eta h_s}{Ftp} \quad (2)$$

where τ is the scaffold's permeability in darcy units (D), Q is the fluid volume passed through the scaffold in time t , η is the viscosity of the fluid (0.89 cp for water at 25°C), h_s is the scaffold thickness, F is the cross sectional area of the scaffold perpendicular to fluid flow, and p is the applied pressure head.^{22,23} The applied pressure head p was determined by using the following equation:

$$p = \rho gh \quad (3)$$

where ρ is the density of water (1000 kg/m³ at 25°C), g is the gravitational force (9.8 m/s²), and h is the total height of the system in meters (1.5 m). This pressure must then be converted from pascals (Pa) to atmospheres (atm) for use in the Darcy equation.

Permeability-based fiber diameter and pore size

The permeability values calculated previously were then used to determine the average fiber diameters and pore sizes of the electrospun scaffolds for comparison to those values measured with the ImageTool image analysis program. The scaffold's average fiber diameter can be predicted from the scaffold's permeability using the following equation²²:

$$d^2 = k\tau\phi \quad (4)$$

where k is a constant whose value is known to be 10, determined from measurements of permeation through irregularly packed metal rods of a known diameter, which has proven appropriate for use with studies of wool, cotton, cellulose fibers, and fibrin gels. For the purpose of this study it has been assumed that the differences in fiber arrangements between electrospun fibrinogen and fibrin gels are negligible, allowing for the use of this constant. The volume fraction of the scaffold is given by ϕ , which was determined prior to permeability testing, and τ is the scaffold's permeability in darcy units (D). The average

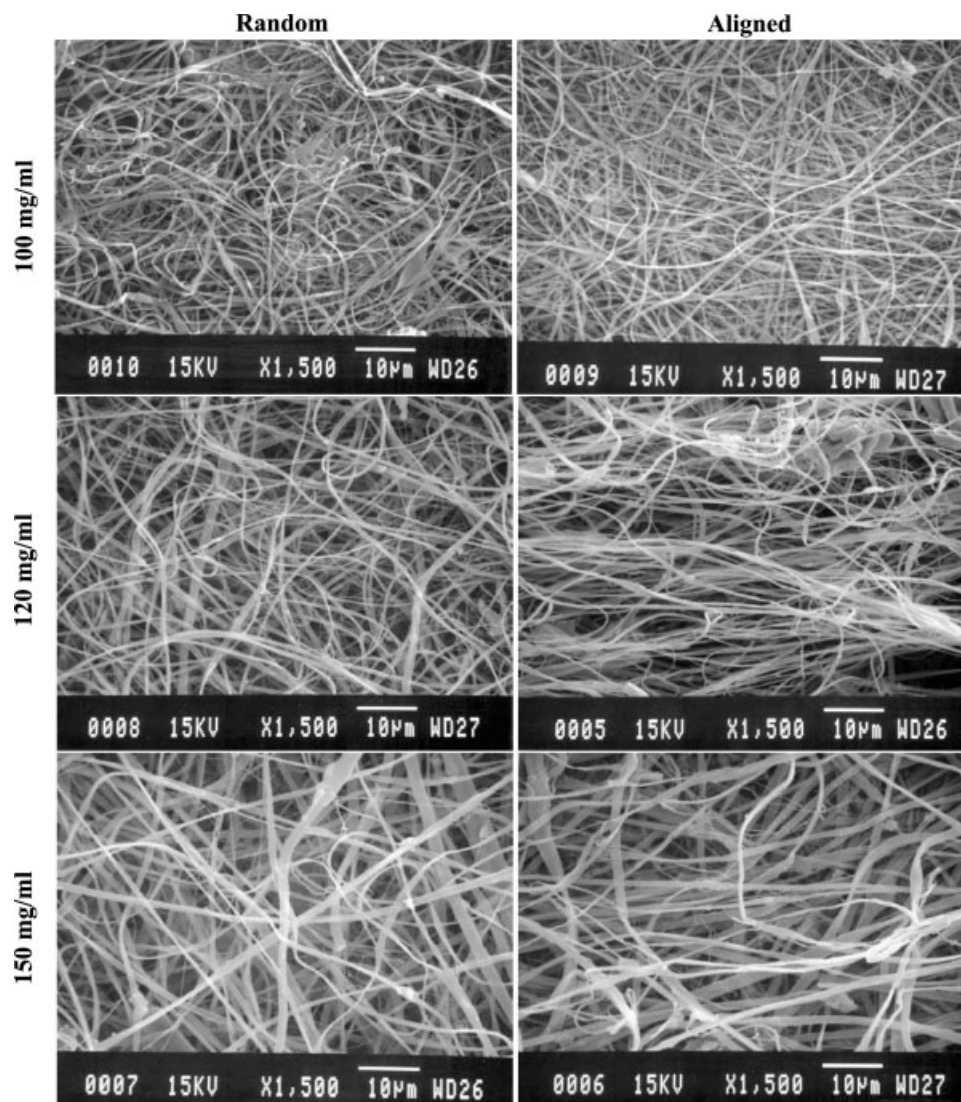


Figure 3. SEMs of dry electrospun fibrinogen scaffolds. The SEMs show scaffolds composed of both randomly oriented and aligned fibers of three different concentrations of fibrinogen: 100 mg/mL, 120 mg/mL, and 150 mg/mL. All micrographs were taken at $\times 1500$.

pore radius can be predicted directly from permeability data as follows²²:

$$r = \frac{0.5093}{\tau^{-1/2}} \quad (5)$$

From this, the average pore size can be easily calculated.

Statistical analysis

Statistical analysis was performed on mean fiber diameters and pore sizes of both dry and hydrated scaffolds of differing concentrations and fiber alignments determined through image analysis and found directly by permeability-measurement-based equations. Permeability values were compared among fibrinogen concentrations, fiber alignments, and states of hydration ($n = 3$). All statistical analyses were based on a Kruskal–Wallis one-way analysis of variance on ranks and a Tukey–Kramer pairwise multi-

ple comparison procedure ($\alpha = 0.05$, $p < 0.0001$) performed with the JMP[®]IN 4.0.3 statistical software package (SAS Institute). Graphical depictions were constructed with Microsoft Excel 2000, with error bars representing standard deviations.

RESULTS

SEM-based fiber diameter and pore size

As previously stated, the two sets of micrographs were taken with two different scanning electron microscopes, resulting in their difference in appearance (dry scaffolds in Fig. 3 and hydrated scaffolds in Fig. 4). All of the dry scaffolds were taken at $1500\times$, while the hydrated scaffolds were taken between $1000\times$ and $1500\times$.

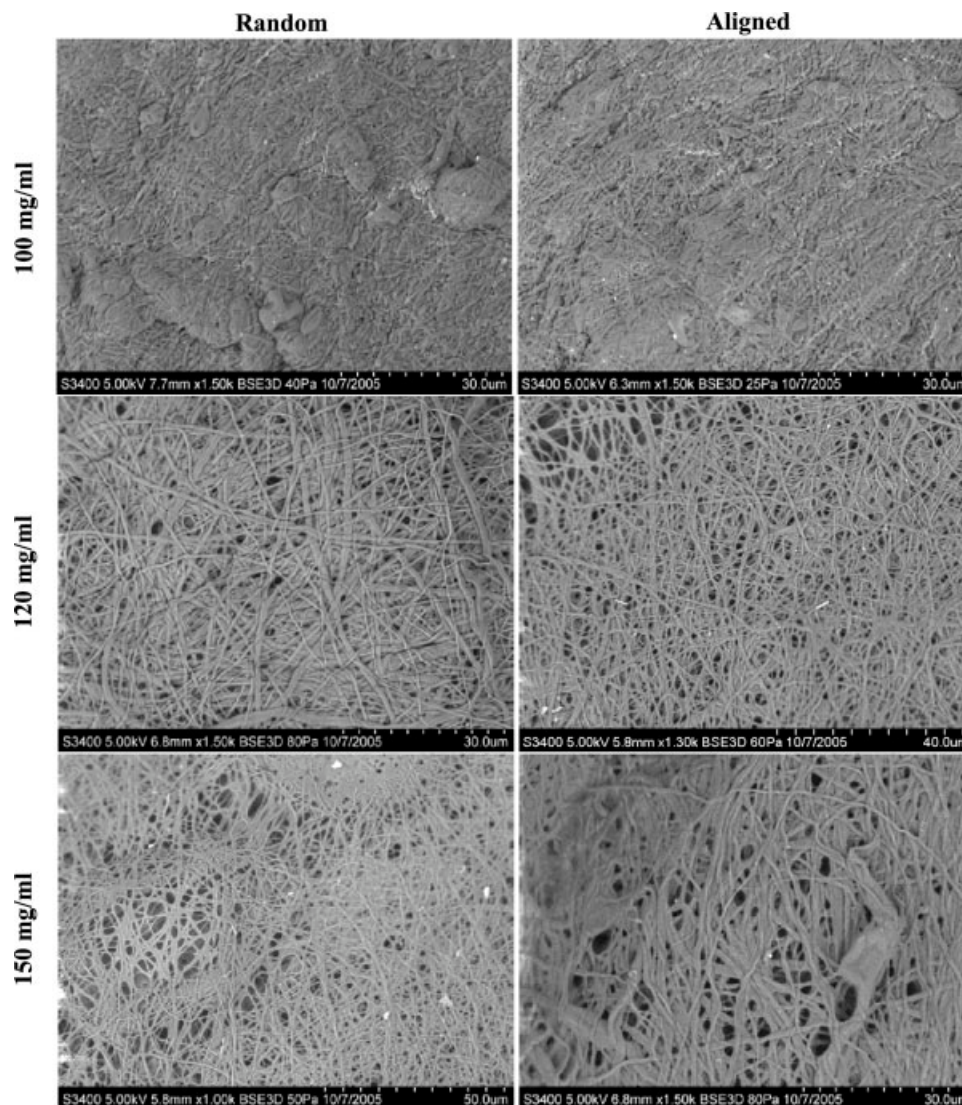


Figure 4. SEMs of electrospun fibrinogen scaffolds hydrated in PBS for 24 h. Micrographs were taken with an environmental microscope under hydrated conditions at magnifications between $\times 1000$ and $\times 1500$. The SEMs show scaffolds composed of both randomly oriented and aligned fibers of three different concentrations of fibrinogen: 100 mg/mL, 120 mg/mL, and 150 mg/mL.

Mean fiber diameters ranged from 0.5 to 1.04 μm and were significantly different between solution concentrations; however, they were not significantly different between fiber orientations, or states of hydration within a single concentration. The major difference between the two sets of scaffolds was in the size of their pores, or void spaces between the fibers. The dry scaffolds contained pores that were clearly visible in the SEMs, while the hydrated scaffolds, particularly the 100 mg/mL concentration, showed very little void space. The hydrated fibers were densely packed leaving little room for pores. Average pore diameters ranged from 0.57 to 3.7 μm , with hydrated pores being significantly smaller than their dry counterparts. All mean fiber and pore diameter data are presented in Table I.

Test of permeability flowmeter efficacy

The results of the flowmeter's test of efficacy clearly showed it to work successfully as designed. All three scaffolds tested (100, 120, and 150 mg/mL) revealed a definite dyed circle in their center, indicative of an area of high fluid flow (Fig. 5). None of the three showed excessive dyeing outside of the desired area of fluid flow, proving the compression applied by the specimen mount to be sufficient to resist leaking around the edges, thereby ensuring more accurate permeability measurement.

Scaffold volume fraction measurement

Prior to permeability testing, the volume fraction of each scaffold was determined (Fig. 6) for use in the

TABLE I
Mean Fiber and Pore Diameters ($\pm$ Standard Deviation) of Electrospun Fibrinogen Scaffolds Determined by Both ImageTool Analysis and Permeability-Based Calculations ($n = 3$)^a

Fibrinogen Concentration	Fiber Orientation	Hydration	Fiber Diameter (ImageTool)	Pore Diameter (ImageTool)	Fiber Diameter (Permeability)	Pore Diameter (Permeability)
100 mg/mL	Random	Dry	0.50 $\pm$ 0.15	1.88 $\pm$ 0.70	0.43 $\pm$ 0.07	0.039 $\pm$ 0.003
100 mg/mL	Aligned	Dry	0.51 $\pm$ 0.18	1.80 $\pm$ 0.60	0.42 $\pm$ 0.09	0.037 $\pm$ 0.009
100 mg/mL	Random	Hydrated	0.54 $\pm$ 0.12	0.61 $\pm$ 0.21	0.29 $\pm$ 0.06	0.017 $\pm$ 0.004
100 mg/mL	Aligned	Hydrated	0.55 $\pm$ 0.11	0.57 $\pm$ 0.22	0.40 $\pm$ 0.02	0.023 $\pm$ 0.001
120 mg/mL	Random	Dry	0.75 $\pm$ 0.22	2.77 $\pm$ 0.94	0.66 $\pm$ 0.06	0.061 $\pm$ 0.006
120 mg/mL	Aligned	Dry	0.71 $\pm$ 0.15	2.81 $\pm$ 1.29	0.71 $\pm$ 0.03	0.059 $\pm$ 0.001
120 mg/mL	Random	Hydrated	0.71 $\pm$ 0.29	1.06 $\pm$ 0.36	0.61 $\pm$ 0.12	0.031 $\pm$ 0.006
120 mg/mL	Aligned	Hydrated	0.70 $\pm$ 0.19	1.13 $\pm$ 0.32	0.61 $\pm$ 0.06	0.035 $\pm$ 0.005
150 mg/mL	Random	Dry	0.97 $\pm$ 0.35	3.48 $\pm$ 1.18	1.34 $\pm$ 0.05	0.101 $\pm$ 0.007
150 mg/mL	Aligned	Dry	1.04 $\pm$ 0.36	3.70 $\pm$ 1.92	1.12 $\pm$ 0.07	0.082 $\pm$ 0.006
150 mg/mL	Random	Hydrated	0.97 $\pm$ 0.22	1.25 $\pm$ 0.36	0.44 $\pm$ 0.03	0.024 $\pm$ 0.002
150 mg/mL	Aligned	Hydrated	1.00 $\pm$ 0.26	1.52 $\pm$ 0.43	0.36 $\pm$ 0.02	0.019 $\pm$ 0.001

^aAll measurements are in micrometers.

fiber diameter and pore size equations. Overall, the dry scaffolds had relatively low volume fractions that ranged from 12.34% to 19.05 %, indicative of scaffolds with large amounts of void space. Both of the dry 150 mg/mL concentration scaffolds had significantly greater volume fractions than the other dry scaffolds, with the exception of the 120 mg/mL aligned dry scaffold. The dry 100 mg/mL concentration scaffolds were not significantly different from each other or the 120 mg/mL random dry scaffold. Hydrated scaffold volume fractions ranged from 33.75% to 38.59%. All hydrated volume fractions were significantly greater than their dry counterparts, but not significantly different from each other. These increased volume fractions corresponded to the SEM images shown previously, which had far reduced pore sizes.

Permeability measurement

Scaffold permeability was recorded every minute for the first 10 min of the permeability test, results of which are shown in Fig. 7. Dry permeabilities exhib-

ited very little change over this time course, the only exception being the randomly oriented 150 mg/ml fibrinogen concentration. This scaffold demonstrated a steady decrease in permeability over the 10-min duration. Dry scaffold permeability behaved as expected, with lower concentrations that have smaller pore sizes being less permeable than higher concentrations containing larger pores. This trend did not translate to the hydrated scaffolds, which showed the 120 mg/mL concentration to have the highest permeability. Surprisingly, the 150 mg/mL concentration had permeabilities that were closely grouped with those of the 100 mg/mL concentration and less than those of the 120 mg/mL concentration. Again, there was little change in permeability over time for the majority of the scaffolds, with the only exception being the 120 mg/mL randomly oriented scaffold, which exhibited an increase in permeability over time.

Upon completion of the 10-min timed test, scaffold permeability was measured additionally at 15 min and 20 min, and finally the total time required for 13 mL to flow through the scaffold was recorded. The permeability was calculated at each time point and an

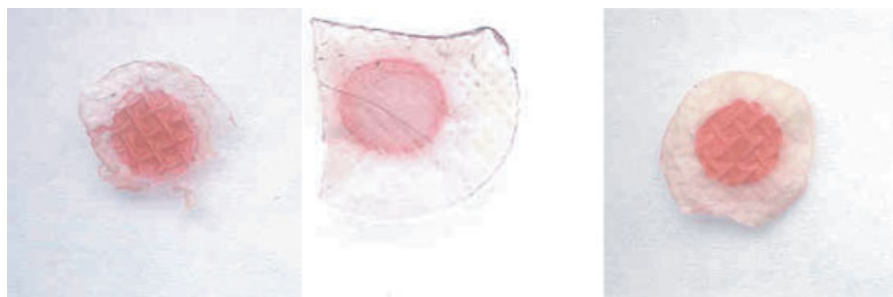


Figure 5. Photographs of electrospun scaffolds of different fibrinogen concentrations (100, 120, and 150 mg/mL from left to right) after 13 mL of dyed PBS had been passed through them to determine the efficacy of the flowmeter's compression-based specimen mount. The faint traces of red dye outside of the desired area of flow were believed to be due to diffusion of dyed PBS after the sample had been removed from the specimen mount. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

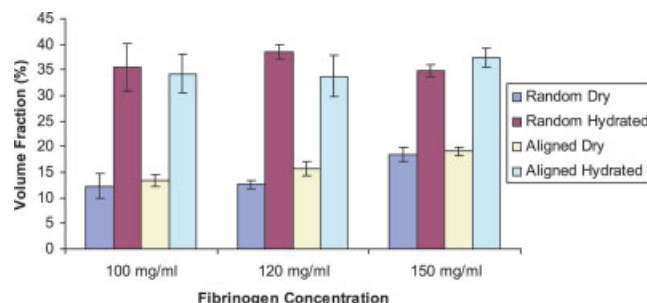


Figure 6. Graph of volume fraction versus fibrinogen solution concentration ($n = 3$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

average permeability was determined (Fig. 8). Mean permeabilities ranged from 0.0013 to 0.0092 D for dry scaffolds, and 0.00029 to 0.0012 D for hydrated scaffolds. While only a small number of different concentrations were tested, the dry scaffolds exhibited a definite trend of increased permeability with increased concentration, while hydrated scaffolds were all closely grouped together with no identifiable trend.

Statistical analysis of average permeability values revealed there to be significant differences between the three fibrinogen concentrations for the dry samples. The 150 mg/mL concentration was significantly larger than the 120 mg/mL concentration, and the 120 mg/mL concentration was significantly larger than the 100 mg/mL concentration. The only difference between fiber orientations was seen with the dry 150 mg/mL concentration where the randomly oriented scaffold had significantly higher permeability than the aligned fiber scaffold. Hydrated scaffolds were not significantly different from one another regardless of concentration or fiber orientation, and were only significantly different from the dry 150 and 120 mg/mL concentrations. Surprisingly, the permeability of the dry 100 mg/mL concentration scaffolds was small enough to be considered insignificantly different from the hydrated samples.

Permeability-based fiber diameter and pore size

Having already determined average fiber diameters and pore sizes through ImageTool analysis, the same quantities were calculated using Eqs. (4) and (5). These results were compared in order to determine the efficacy of the flowmeter as a tool for measuring electrospun scaffold topographical features, in addition to serving as a check of the precision of the ImageTool values. Mean fiber and pore diameter values are presented in Table I. For the most part, average fiber diameter values calculated from scaffold permeability were not statistically different from ImageTool values (Fig. 9). Average fiber diameters of scaffolds of the 100 and 120 mg/mL fibrinogen con-

centrations were not statistically different between the two measurement methods, or within a single concentration for different fiber orientations, and states of hydration. The fiber diameters of the dry 150 mg/mL concentration scaffolds were not statistically different between measurement methods, or fiber orientations. They were also not significantly different from the hydrated fiber diameters determined through ImageTool. However, there were statistical differences between the aforementioned fiber diameters and the hydrated fiber diameters determined by the permeability-based equations. These hydrated fiber diameters were significantly reduced due to the extremely low permeability values of the hydrated 150 mg/mL concentration scaffolds.

Average pore size measurements (Fig. 10) proved to be much more diverse than the average fiber diameter measurements. Regardless of concentration, the pore sizes of hydrated scaffolds were significantly smaller than those of dry scaffolds for both methods of measurement. However, unlike average fiber diameter measurements, the two different methods of measurement yielded significantly different pore sizes. Permeability-based pore sizes were significantly smaller than pore sizes measured with ImageTool for all fibrinogen concentrations, fiber orientations, and states of scaffold hydration.

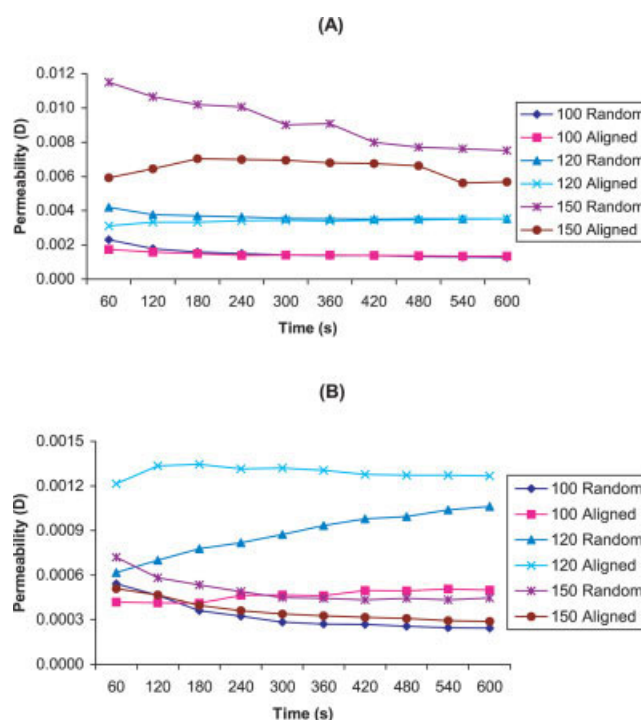


Figure 7. Graphs of average permeability versus time for the first 10 min of permeability testing for both dry (A) and hydrated (B) electrospun fibrinogen samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

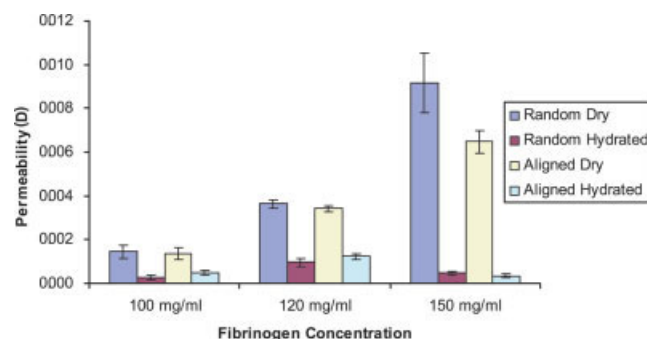


Figure 8. Graph of average scaffold permeability versus fibrinogen concentration ($n = 3$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Scaffold characterization

The trends for both the dry scaffold average fiber and pore diameter values achieved through ImageTool analysis were as anticipated, based upon previously published electrospun data.^{25,26} The results showed both fiber and pore diameters to increase with concentration for the three data points tested. This is behavior that has been seen in all electrospun scaffolding materials used in our laboratory, due to a relationship between increased solution viscosity and polymer chain entanglements.²⁷

As expected, hydrated fibrinogen scaffolds appeared much different than their dry counterparts

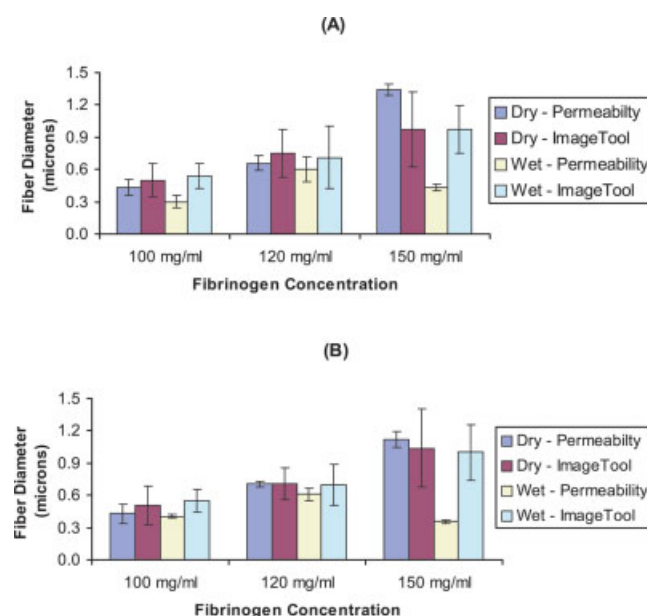


Figure 9. Graph of average fiber diameter versus fibrinogen concentration ($n = 3$) for randomly oriented (A) and aligned (B) scaffolds. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

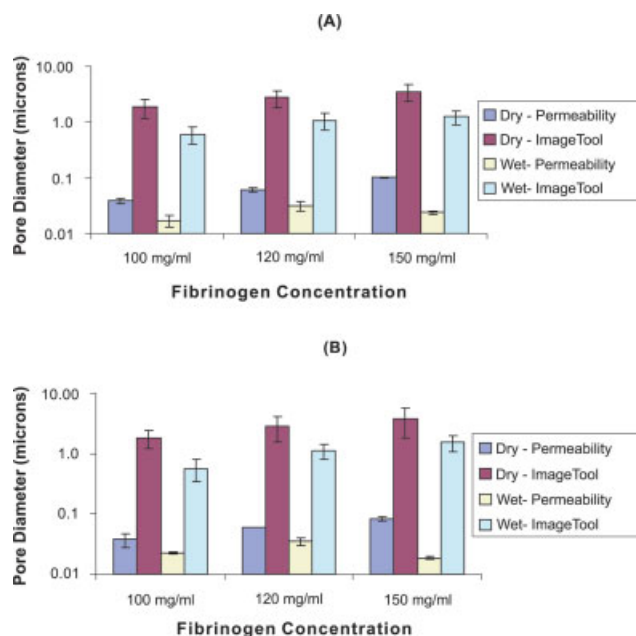


Figure 10. Graph of average pore diameter versus concentration ($n = 3$) for randomly oriented (A) and aligned (B) scaffolds. Note that these graphs are on a log scale, due to the wide discrepancy between dry and hydrated pore diameters. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

under SEM examination. Statistical analysis revealed there to be no difference between the dry and hydrated fiber diameters, with significant differences between dry and hydrated pore sizes. Prior to this study, there was question about the hydrated behavior of electrospun fibrinogen. It had been observed with the naked eye that when dry fibrinogen scaffolds were hydrated they almost immediately shrunk in diameter and curled, although not wetting for several minutes after immersion. The cause of this behavior was not understood. It was not known if the electrospun fibers were hydrophilic and swelling, altering the shape of the scaffold, or if they were hydrophobic and collapsing in upon themselves, or what other phenomenon may have contributed to this behavior.

Scaffold characterization with ImageTool made it obvious that fiber diameters did not change, ruling out hydrophilic fiber swelling. To investigate further, preliminary water droplet contact angle measurement was performed, with data which showed the electrospun fibrinogen scaffolds to be strongly hydrophobic for a period of about 10 min prior to wetting, which corroborated their behavior in this study (unpublished data). This short-term scaffold hydrophobicity may have been due to the sub-micron scale surface roughness of the electrospun scaffolds, with the hydrophilic nature of the protein eventually overcoming the surface tension of water and allowing for complete scaffold wetting.²⁸ Of course there is still the possibility that the thermody-

namic state of the protein is raised by the electrospinning process, and the scaffolds were undergoing some thermodynamic reduction due to increased chain mobility in an aqueous solution. However, it should be noted that a previous transmission electron microscopy (TEM) evaluation of electrospun fibrinogen revealed a scaffold that exhibited a banding pattern similar to that of a native fibrinogen extracellular matrix, indicating that the scaffold was already at a thermodynamically stable level equivalent to its native structure.⁷ Further investigation will be required to determine exactly what happens during scaffold densification.

Scaffold volume fraction measurement

Having seen the results of the SEM image analysis and scaffold characterization, the results of the volume fraction and permeability measurements were somewhat intuitive. As expected, hydrated scaffolds, which visually appeared considerably denser, had significantly higher volume fractions than their dry counterparts. Dry scaffolds had mean volume fractions that ranged from 12.34% to 19.05%, while the hydrated scaffolds had mean volume fractions between 33.75% and 38.59%. This data shows electrospun scaffolds, in this case fibrinogen scaffolds, to have extremely high porosities. It is not beyond reason to extrapolate this data to other electrospun polymer scaffolds, as they all form similar structures. This high porosity bodes well for cell migration and nutrient transport, particularly in an electrospun structure where all void spaces are interconnected.¹² While high porosity typically results in poor mechanical properties, electrospun scaffolds of various polymer compositions have proven to be mechanically durable in laboratory testing.^{6,18,26}

Permeability measurement

The data of permeability versus time for the first 10 min of testing did not correspond to the trends that were hypothesized. Dry scaffold behavior was closest to the hypothesis that higher concentrations would result in increased permeability, with aligned fiber scaffolds being more permeable than randomly oriented scaffolds. It was also hypothesized that dry scaffold permeability would decrease over time, as the dry scaffold became hydrated and contracted. Testing revealed the 150 mg/mL concentrations had higher permeabilities than the 120 mg/mL, and the 120 mg/mL concentrations had higher permeabilities than the 100 mg/mL concentration. However, there was no difference between randomly oriented and aligned scaffolds, with exception of the 150 mg/mL

concentration. In addition, there was no permeability loss over time for the dry scaffolds, with the exception of the dry 150 mg/mL randomly oriented scaffold. This scaffold started with a higher permeability than the dry 150 mg/mL aligned scaffold and became less permeable as time went on. The difference between the 150 mg/mL concentration behavior and the lower two may have been due to the difference in fiber sizes. The larger fibers created by the 150 mg/mL concentration are easier to align than the smaller fibers created by the lesser two concentrations. This may have created a scaffold that was more aligned for the 150 mg/mL concentration, while the smaller 120 mg/mL and 100 mg/mL concentration fibers may have appeared somewhat aligned in SEMs, but may not have been truly aligned throughout the scaffold. These aligned fibers may have created a scaffold of tightly packed fibers, thereby increasing scaffold tortuosity and reducing scaffold permeability. In addition, the aligned scaffold may have had less room for contraction and fiber reorganization, due to a higher packing density, resulting in a constant permeability compared to the gradual reduction in permeability seen in the randomly oriented scaffold. The restraint of the scaffold within the specimen mount may have played a role in maintaining scaffold permeability over time, as it was anticipated that dry scaffolds would become less permeable as they became more hydrated. However, being pinched firmly between the silicone gaskets the dry scaffolds may not have been able to contract, keeping their permeabilities nearly constant.

In general, the hydrated scaffolds exhibited no change over time and no difference between random and aligned fiber orientations during the first 10 min of testing. Both the 150 and 100 mg/mL concentrations were closely grouped and nearly constant over time after a small permeability decrease at the outset of testing. The 120 mg/mL concentration exhibited higher permeabilities than the other two concentrations. This time the aligned scaffold was more permeable than the randomly oriented one, while the randomly oriented scaffold gradually increased in permeability until it reached values equivalent to those of the aligned scaffold. There is no explanation for this behavior at this time, as it is quite the opposite of the behavior seen with the other concentrations. Perhaps there were small tears or holes in these scaffolds that propagated as testing took place. Further investigation will need to be performed to explain what caused this behavior, as it may have simply been an aberration, or may have some underlying principle behind it.

Average permeability measurement resulted in trends that for the most part, could have been anticipated based upon the volume fraction and fiber and pore diameter values recorded earlier. Hydrated

scaffold permeabilities were significantly less than dry scaffold permeabilities for all concentrations, as was expected. There were no statistically significant differences between the hydrated scaffold values, thereby reinforcing what was determined earlier: that the hydrated scaffolds had all reached a maximum volume fraction and could not contract further. This maximum scaffold density prevented fluid flow through the scaffold and resulted in minimal permeability. While this does support the earlier findings, it conflicts with the original hypothesis that permeability would increase as fibrinogen concentration was increased. However, the dry scaffolds supported the original hypothesis as there was a definite trend between scaffold permeability and fibrinogen concentration. A similar relationship was expected for the hydrated scaffolds, with the 150 mg/mL concentration scaffold exhibiting much higher permeability. There were also differences between the average permeability values for the different concentrations with regard to random and aligned fiber orientations: as fibrinogen concentration increased, the random orientation had larger permeability values than the aligned orientation. As explained previously, this may be due to the increasing fiber size of the scaffolds as larger fibers are easier to align.

The densification of fibrinogen scaffolds results in a significant increase in their volume fraction and a significant reduction in their permeability from that of their dry state. As electrospun fibrinogen scaffolds are ultimately intended for use as *in vivo* ECM analogue scaffolds, their wetting is inevitable. This wetting will cause their contraction and densification. As shown through previous studies,¹⁷ this densification does not effect cellular penetration into and migration through the scaffolds. However, this densification will reduce the permeability of the scaffold resulting in decreased nutrient and waste diffusion throughout the scaffold. This lack of proper diffusion may further limit electrospun fibrinogen scaffold thickness and hamper its ability to sustain cellular life.

Permeability-based fiber diameter and pore size

The reasons for calculating fiber and pore diameters using permeability-based equations were two-fold. Firstly, a correlation between the permeability-based fiber diameters and the fiber diameter values obtained through image analysis would confirm the accuracy of the scaffold permeability measurements. Fiber diameters are the easiest scaffold topographical feature to measure accurately through image analysis, and were therefore the gold standard by which the permeability-based values were compared. Electrospun scaffold pore sizes are extremely difficult to measure accurately with an image analysis program; consequently, the values obtained through image

analysis were compared to the permeability-based pore sizes upon confirmation of the efficacy of the permeability measurements.

Average fiber diameters were found to be statistically the same for the 100 and 120 mg/mL concentrations, independent of fiber orientation, state of hydration, or method of measurement. Since there was no statistical difference between fiber diameters measured with image analysis software or calculated with permeability-based equations, one could conclude that the permeability flowmeter worked well in producing accurate permeability measurements. However, the hydrated 150 mg/mL concentration fiber diameters calculated with the permeability-based equations were statistically different from those measured with ImageTool. This would indicate that the permeability values for this concentration were incorrect and lower than they should be, possibly due to some collapse or closing of pores due to applied hydrostatic pressure. Further permeability testing needs to be performed on scaffolds that do not densify in solution in order to more accurately demonstrate the usefulness of permeability-based equations in measuring scaffold fiber diameters.

Results of the average pore size comparison revealed there to be statistically significant differences between all groups, excluding fiber orientation. The pore sizes of hydrated scaffolds were universally significantly smaller than those of their dry counterparts. However, this result was expected due to the contraction evident in the SEM images when electrospun fibrinogen scaffolds were placed in a PBS solution. This decrease in permeability corresponded to the increase in scaffold volume fraction, and was looked upon as a positive result. Pore sizes calculated with the permeability-based equation were up to 52 times smaller than the pore sizes measured through image analysis; however one can only assume that as permeability-based fiber diameter measurements proved accurate there must be some credibility to the permeability-based pore size measurements. Perhaps, as the permeability-based equation relies on fluid taking the path of least resistance through the scaffold, a functional pore size is given. This functional pore size may then be much smaller than the apparent pore size seen through visual inspection of SEM's. Further testing will be required to validate these assumptions.

Pore diameter comparisons brought to light some of the problems with the use of a two-dimensional image analysis program as a pore size measurement device for three-dimensional pores, as measurements made with ImageTool were up to 52 times larger than those calculated from the permeability-based equation. The problem may not be with ImageTool as a program, as it just measures linear distances on a picture, and is perfectly suited to the measurement of surface fiber diameters. The problem may be that electrospun

scaffolds do not contain pores in the true sense of the word, leading to the measurement of unbounded void spaces. Electrospun scaffolds are merely a collection of nanoscale fibers lying loosely amongst each other, forming what appear to be three-dimensional bounded void spaces on two-dimensional SEMs. In fact, these faux-pores may not be bounded on all sides, instead being several unbounded layers on top of each other. For this reason, the use of permeability-based pore size measurement (effective pore-size), which is determined by fluid traveling the path of least resistance through the scaffold, increases the accuracy of pore size reporting.

CONCLUSION

The major outcome of this study was the construction of a permeability flowmeter and specimen mount that could accurately and efficiently measure the permeability of electrospun scaffolds. The use of this flowmeter demonstrated a positive trend between dry scaffold permeability and electrospun solution concentration. Additionally, permeability-based equations were used to calculate average fiber diameters and pore sizes, and were compared to values measured with the ImageTool image analysis software. This comparison demonstrated the efficacy of the permeability-based equations in determining average fiber diameters for both dry and hydrated scaffolds, while also revealing some of the weaknesses of image analysis software in measuring average pore size of electrospun scaffolds. Further work will include the replication of this study on synthetic polymer scaffolds that do not densify and change shape in solution, as well as more intensive research into the hydrophobic nature of electrospun fibrinogen.

References

- Heineken FG, Skalak R. Tissue engineering: A brief overview. *J Biomech Eng* 1991;113:111.
- Palsson BO, Bhatia SN. *Tissue Engineering*. Upper Saddle River, NJ: Pearson Prentice Hall; 2004.
- Agrawal CM, Ray RB. Biodegradable polymeric scaffolds for musculoskeletal tissue engineering. *J Biomed Mater Res* 2001; 55:141–150.
- Matthews JA, Simpson DG, Wnek GE, Bowlin GL. Electrospinning of collagen nanofibers. *Biomacromolecules* 2002;3:232–238.
- Mooney DJ, Mazzoni CL, Breuer C, McNamara K, Hern D, Vacanti JP, Langer R. Stabilized polyglycolic acid fibre-based tubes for tissue engineering. *Biomaterials* 1996;17:115–124.
- Sell SA, McClure MJ, Barnes CP, Knapp DC, Walpoth BH, Simpson DG, Bowlin GL. Electrospun polydioxanone-elastin blends: Potential for bioresorbable vascular grafts. *Biomed Mater* 2006;1:72–80.
- Wnek GE, Carr ME, Simpson DG, Bowlin GL. Electrospinning of nanofiber fibrinogen structures. *Nano Lett* 2003;3:213–216.
- Boland ED, Espy PG, Bowlin GL. Tissue engineering scaffolds. In: Wnek G, Gary Bowlin, editors. *Encyclopedia of Biomaterials and Biomedical Engineering*. New York: Marcel Dekker; 2004. p 1–9.
- Zong X, Bien H, Chung CY, Yin L, Fang D, Hsiao BS, Chu B, Entcheva E. Electrospun fine-textured scaffolds for heart tissue constructs. *Biomaterials* 2005;26:5330–5338.
- Berry CC, Campbell G, Spadicino A, Robertson M, Curtis ASG. The influence of microscale topography on fibroblast attachment and motility. *Biomaterials* 2004;25:5781–5788.
- Levesque SG, Lim RM, Shoichet MS. Macroporous interconnected dextran scaffolds of controlled porosity for tissue-engineering applications. *Biomaterials* 2005;26:7436–7446.
- Telemeco TA, Ayres C, Bowlin GL, Wnek GE, Boland ED, Cohen N, Baumgarten CM, Matthews J, Simpson DG. Regulation of cellular infiltration into tissue engineering scaffolds composed of submicron diameter fibrils produced by electrospinning. *Acta Biomater* 2005;1:377–385.
- Norman JJ, Desai TA. Methods for fabrication of nanoscale topography for tissue engineering scaffolds. *Ann Biomed Eng* 2006;34:89–101.
- Venugopal J, Ramakrishna S. Applications of polymer nanofibers in biomedicine and biotechnology. *Appl Biochem Biotechnol* 2005;125:147–157.
- Karande TS, Ong JL, Agrawal CM. Diffusion in musculoskeletal tissue engineering scaffolds: Design issues related to porosity, permeability, architecture, and nutrient mixing. *Ann Biomed Eng* 2004;32:1728–1743.
- Li S, De Wijn JR, Li J, Layrolle P, De Groot K. Macroporous biphasic calcium phosphate scaffold with high permeability/porosity ratio. *Tissue Eng* 2003;9:535–548.
- McManus M, Boland E, Simpson D, Barnes C, Bowlin G. Electrospun fibrinogen: Feasibility as a tissue engineering scaffold in a rat cell culture model. *J Biomed Mater Res A* 2007;81:299–309.
- McManus MC, Boland ED, Koo HP, Barnes CP, Pawlowski KJ, Wnek GE, Simpson DG, Bowlin GL. Mechanical properties of electrospun fibrinogen structures. *Acta Biomater* 2006; 2:19–28.
- Ma Z, Kotaki M, Inai R, Ramakrishna S. Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue Eng* 2005; 11:101–109.
- Smith LA, Ma PX. Nano-fibrous scaffolds for tissue engineering. *Colloids Surf B Biointerfaces* 2004;39:125–131.
- Wen X, Shi D, Zhang N. Applications of nanotechnology in tissue engineering. In: Nalwa HS, editor. *Handbook of Nanostructured Biomaterials and Their Applications in Nanobiotechnology*. Stevenson Ranch, CA: American Scientific; 2005. p 1–23.
- Carr ME Jr, Hardin CL. Fibrin has larger pores when formed in the presence of erythrocytes. *Am J Physiol* 1987;253: H1069–H1073.
- Carr ME Jr, Shen LL, Hermans J. Mass-length ratio of fibrin fibers from gel permeation and light scattering. *Biopolymers* 1977;16:1–15.
- Wang H, Pieper J, Peters F, van Blitterswijk CA, Lamme EN. Synthetic scaffold morphology controls human dermal connective tissue formation. *J Biomed Mater Res A* 2005;74:523–532.
- Ayres C, Bowlin GL, Henderson SC, Taylor L, Shultz J, Alexander J, Telemeco TA, Simpson DG. Modulation of anisotropy in electrospun tissue-engineering scaffolds: Analysis of fiber alignment by the fast Fourier transform. *Biomaterials* 2006;27:5524–5534.
- Boland ED, Coleman BD, Barnes CP, Simpson DG, Wnek GE, Bowlin GL. Electrospinning polydioxanone for biomedical applications. *Acta Biomater* 2005;1:115–123.
- Shenoy SL, Bates WD, Frisch HL, Wnek GE. Role of chain entanglements on fiber formation during electrospinning of polymer solutions: good solvent, non-specific polymer-polymer interaction limit. *Polymer* 2005;46:3372–3384.
- Alh A, Hazer B, Menciloglu Y, Suzer S. Synthesis, characterization and surface properties of amphiphilic polystyrene-*b*-polypropylene glycol block copolymers. *Eur Polym J* 2006; 42:740–750.