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Modulation of anisotropy in electrospun tissue-engineering scaffolds: Analysis of fiber alignment by the fast Fourier transform

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Abstract

We describe the use of the fast Fourier transform (FFT) in the measurement of anisotropy in electrospun scaffolds of gelatin as a function of the starting conditions. In electrospinning, fiber alignment and overall scaffold anisotropy can be manipulated by controlling the motion of the collecting mandrel with respect to the source electrospinning solution. By using FFT to assign relative alignment values to an electrospun matrix it is possible to systematically evaluate how different processing variables impact the structure and material properties of a scaffold. Gelatin was suspended at varying concentrations (80, 100, 130, 150 mg/ml) and electrospun from 2,2,2 trifluoroethanol onto rotating mandrels (200–7000 RPM). At each starting concentration, fiber diameter remained constant over a wide range of mandrel RPM. Scaffold anisotropy developed as a function of fiber diameter and mandrel RPM. The induction of varying degrees of anisotropy imparted distinctive material properties to the electrospun scaffolds. The FFT is a rapid method for evaluating fiber alignment in tissue-engineering materials.

Keywords

Electrospinning; Anisotropy; Gelatin; ECM; Fast Fourier transform; Material properties

1. Introduction

The native extracellular matrix (ECM) is comprised of a complex network of structural and regulatory proteins that are arrayed into a fibrous matrix [1]. The fibers that makeup the backbone of this elaborate network exhibit distinctive, and often times, regional, variations in identity, cross-sectional diameter and polarity. For example, the ECM of the dermis can be subdivided into two discrete sub-compartments: a deep reticular layer and a superficial papillary layer. The reticular compartment is occupied by large diameter fibers of Type I collagen and elastin that are distributed into large-scale random bundles. Conversely, the

superficial papillary layer of the skin contains small diameter fibers of Type III collagen and elastin that exhibit a modest degree of anisotropy. This fibrous network defines the bulk material properties of the dermis. However, the three dimensional architecture and composition of the dermal ECM also functions to store and communicate phenotypic signals to the cellular compartment of the skin. These signals modulate cell function during normal hemostasis and also play a central role in modulating the remodeling events that transpire during the wound healing process. The multifunctional nature of the native ECM must be recognized and replicated to varying degrees in the design and fabrication of tissue-engineering scaffolds.

We believe that electrospinning can be used to produce tissue-engineering scaffolds that recapitulate key structural features of the native ECM [2]. This non-mechanical processing strategy can be used to selectively fabricate sub-micron to micron diameter fibers from a variety of synthetic polymers [3–6] and natural proteins [2,7–11]. Fiber diameter and pore dimension can be regulated in the electrospinning process by controlling the composition of the electrospinning solvent and the identity, degree of chain entanglements, and concentration of the starting polymer [12]. Fiber alignment can be manipulated by controlling the motion of the collecting mandrel with respect to the source electrospinning suspension [6,10,13] and/or through electric field effects [14]. Conventional morphometric techniques are typically used to characterize the basic physical properties of electrospun scaffolds [2,4,9,13].

We believe that efforts to produce clinically relevant tissue-engineering scaffolds, using the electrospinning process, can benefit from strategies that objectively measure and assign a value to fiber anisotropy. Varying the degree of fiber anisotropy present in an electrospun scaffold can be expected to impart unique material properties to the construct. Fiber anisotropy also may represent a source of guidance cues that can be exploited to regulate cell phenotype, cell distribution and the macroscopic properties of an engineered tissue.

Laser scattering has been used to evaluate fiber alignment in valve leaflets [15,16]. This elegant technique can be used to resolve information at the individual fiber scale. However, intensive data analysis is necessary to map the large-scale structural properties of a sample. We, and other authors, have used representative scanning electron micrographs to demonstrate fiber alignment in electrospun materials [6,9,13,14,17]. Unfortunately, this approach is highly subjective and is restricted to generating data sampled from the superficial surfaces of the constructs. In this study, we use digital brightfield and confocal microscopy to capture images of electrospun scaffolds and demonstrate the use of the fast Fourier transform (FFT) to measure fiber alignment in the scaffolds. Our approach has several advantages; it is rapid, non-destructive and can be used to rapidly integrate alignment data over a relatively large area.

2. Materials and methods

2.1. Electrospinning

Reagents were purchased from Sigma Aldrich (St. Louis, MO) unless noted. Gelatin was suspended in 2,2,2 trifluoroethanol (TFE) for 48 h prior to electrospinning. This time interval was determined empirically. In preliminary experimentation, gelatin was suspended for 12, 24, 48 or 96 h in TFE. The 48 h time point produced the most consistent fiber diameters (not shown). Starting concentrations of 80, 100, 130 and 150 mg/ml TFE were prepared. Electrospinning suspensions were loaded into a 20 ml Becton Davis syringe capped with an 18 gauge blunt-tipped needle. Samples were electrospun at 22 kV onto a stainless steel rectangular mandrel (70mm×10mm×5 mm) across an air gap of 20 cm.

The positive output lead of a high voltage supply (Spellman CZE1000R; Spellman High Voltage Electronics Corporation) was attached by an alligator clip to the blunt end needle. A Harvard perfusion pump was used to meter the delivery of the electrospinning suspensions to

the electric field. The rate of solvent/polymer delivery was set at the maximal rate that did not induce dripping from the tip of the syringe. Starting concentrations of 80, 100, 130 and 150 mg/ml were delivered at rates of 12, 20, 21 and 35 ml/h, respectively.

The target mandrel was regulated to rotate at varying revolutions per minute (RPM) including: 200 (we chose to use a nominal degree of rotation in our controls to facilitate fabrication of scaffolds for materials testing, which assumes a uniform scaffold thickness), 1000, 2000, 3000, 4000, 5000, 6000 and 7000 RPM. A digital stroboscope (Shimpo Instruments DT3-11A) was used to continuously monitor the RPM of the target mandrel. Samples were stored in a desiccation chamber prior to analysis.

2.2. Viscosity measurements

Gelatin/TFE solutions were suspended for 24 and 48 h at varying concentrations. Viscosity measurements were conducted with a Brookfield RVDV-III Ultra programmable rheometer using a small sample adapter and spindle (SC4-21). Prior to all measurements, the rheometer was calibrated using a viscosity standard fluid that was allowed to equilibrate for 30 min before an initial viscosity reading was recorded.

2.3. Scanning electron microscopy (SEM)

Average fiber diameters and average pore areas were determined from samples processed for conventional SEM (Jeol JSM-820). Dry, unfixed electrospun materials were sputter coated and images were captured on Polaroid film, digitized via a flatbed scanner and analyzed with NIH ImageTool (UTHSCSA version 3). All measurements were calibrated from size bars incorporated into the SEM images at the time of capture.

Average fiber diameter was determined from measurements taken perpendicular to the long axis of the fibers (25 measurements per field). Average pore area (a measure of porosity) was determined by measuring the area encompassed by adjacent fibers (at least 25 measurements per field). $N = 3$ from independent experiments conducted at different time points. Data sets consisting of average fiber diameter and average pore area were screened by two-way ANOVA and tested for the effects of the starting concentration and mandrel RPM, $P < 0.01$. A Tukey test was used for post hoc analysis; significance was defined at $P < 0.05$.

2.4. Light microscopy

All imaging and materials testing was conducted with dry, unfixed samples. This approach was selected to allow us to make direct comparisons between the different analytical approaches described in this study. For light microscopy, dry sheets of electrospun gelatin were cut into 10 mm diameter disks using a circular biopsy punch. Samples were imaged with a Nikon TE300 microscope equipped with a Nikon DXM 1200 digital camera. Images (3840×3072 pixels) were captured with a 20×(0.40 n.a.) brightfield objective lens, resulting in pixel dimensions of 0.17×0.18 μm .

2.5. FFT

The FFT was used to characterize fiber alignment as a function of electrospinning conditions. The FFT function converts information present in an original data image from “real” space into mathematically defined “frequency” space [18]. The resulting FFT output image contains grayscale pixels that are distributed in a pattern that reflects the degree of fiber alignment present in the original data image. As subjectively judged from SEM images, a representative FFT analysis of a “random” matrix and an “aligned” matrix are presented in Fig. 1. The FFT of an original data image containing random fibers (Fig. 1A) generates an output image containing pixels distributed in a symmetrical, circular shape (Fig. 1B). This distribution occurs

because the frequency at which specific pixel intensities occur in the data image is theoretically identical in any direction. In contrast, the FFT of a data image containing aligned fibers (Fig. 1D) results in an output image containing pixels distributed in a non-random, elliptical distribution (Fig. 1E). This distribution occurs because the pixel intensities are preferentially distributed with a specific orientation.

A graphical depiction of the FFT frequency distribution can be generated by placing a circular projection on the FFT output image and conducting a radial summation of the pixel intensities for each degree between 0° and 360°, in 1° increments. For example, data corresponding to 0° in Fig. 1C was obtained by summing the pixel intensities encountered along that specific radius in the FFT output image depicted in Fig. 1B. The summed value of the pixel intensity was then plotted as a function of the degree and in this example provided a value of approximately 0.02 at 0°. A similar analysis at 100° in Fig. 1E generates a value of approximately 0.16. In our analysis the pixel intensities were summed along each degree and then plotted between 0 and 180° (the FFT is symmetric about the horizontal axis so a pixel summation to 360° is unnecessary). In all images, the FFT data set has been rotated 90° to correct for the mathematical transformation inherent to this type of analysis, allowing the principal axis of orientation to be directly determined from the position of the peak in the intensity plot. The amount of alignment present in the original data image is reflected by the height and overall shape of the peak present in this plot (Fig. 1C and F).

For our analysis, digitized SEM images and brightfield light microscopic images were converted to 8-bit grayscale TIF files. SEM images were cropped to 1024×1024 pixels and brightfield images were cropped to 2048×2048 pixels. Images were processed with ImageJ software (NIH, <http://rsb.info.nih.gov/ij>) supported by an oval profile plug-in (authored by William O'Connell). All FFT data was normalized to a baseline value of 0 and plotted in arbitrary units, allowing different data sets to be directly compared.

2.6. Confocal laser scanning microscopy

To enhance confocal imaging TFE/gelatin suspensions were supplemented with 1 µl/ml CellTracker CM-DiI (Invitrogen, USA) and electrospun as described ("random" = 80 mg/ml gelatin at 4000 RPM or "aligned" = 130 mg/ml gelatin at 4000 RPM). Confocal images can be captured using auto-fluorescence, however, when working with small diameter fibers this signal is inadequate for image acquisition. Electrospun scaffolds were mounted onto microscope slides; registry marks were incorporated onto the surface of the cover slips. The samples were imaged by brightfield (widefield) microscopy, photographed and processed for FFT analysis of alignment as described above. These same scaffolds were then imaged by confocal microscopy using the registry marks to identify the regions captured by brightfield microscopy. Samples were imaged with a Leica TCS-SP2 AOBS confocal laser-scanning microscope.

The random matrix depicted in Fig. 4D was imaged with a 40× (1.25 n.a.) oil immersion lens. Total surface area measured 185×185 µm and the total depth in the Z-direction was 10 µm sampled at intervals of 0.162 µm. The Voxel dimensions for this image are 0.09×0.09×0.16 µm. The aligned matrix depicted in Fig. 4F was imaged with a 20× (0.70 n.a.) dry objective lens. Total surface area measured 750×750 µm and the total depth in the Z-direction was 25 µm sampled at intervals of 0.36 µm. The Voxel dimensions for this image are 0.36×0.36×0.36 µm. The pinhole was optimized for all images to the Airy disk. The 514 nm laser line was used to illuminate the samples and images were collected with a scan resolution of 2048×2048 pixels and saved as TIF files. Confocal 3-dimensional data sets were compiled as average intensity and maximum intensity projections and processed by FFT. These data were then compared to the FFT alignment values derived from the brightfield (widefield) data sets. Representative

cross-sections in the confocal data sets were calculated and projected along the *X* and *Y* orientations (i.e. *XZ* and *YZ* digital slices).

2.7. Materials testing

Uniaxial materials testing was conducted on dog-bone-shaped samples of electrospun gelatin with a Bionix 200 Mechanical Testing Systems instrument equipped with a 50N load cell (MTS Systems Corp, Eden Prairie, MN). Samples were 2.67mm wide with a gauge length of 11.25mm. Specimen thickness was determined with a Mitutoyo IP54 digital micrometer (Mitutoyo American Corp; Aurora, IL). Dog-bone-shaped samples were used to control for grip effects and the geometry of the samples to be tested. Samples were tested in the parallel (i.e. in the direction of mandrel rotation) and perpendicular orientations (i.e. at 90° to the direction of mandrel rotation) to the point of failure at an extension rate of 10 mm/min. Data sets were screened by one-way ANOVA ($P < 0.01$) to test for the effects of mandrel RPM on material properties. Samples that passed normality and equal variance were subjected to pairwise multiple comparisons (Tukey test, $P < 0.05$).

3. Results

3.1. Fiber diameter and pore area

Average fiber diameters and average pore areas in electrospun samples were determined as a function of starting electrospinning conditions. Under base line conditions (i.e. mandrel RPM = 200) 80 mg/ml suspensions produced scaffolds composed of 0.29 ± 0.1 μm diameter fibers with pore areas of 0.49 ± 0.7 μm^2 ; 100 mg/ml suspensions produced 2.90 ± 1.2 μm diameter fibers with pore areas of 67.60 ± 53.0 μm^2 ; 130 mg/ml suspensions produced 2.80 ± 1.0 μm diameter fibers with pore areas of 95.00 ± 80.0 μm^2 ; and 150 mg/ml suspensions produced 9.10 ± 2.6 μm diameter fibers with pore areas of 301.00 ± 224.0 μm^2 . Fibers produced from the 80, 100 and 130mg/ml suspensions had a rounded cross-sectional profile. The 150 mg/ml suspensions produced fibers with a ribbon-like profile. This conformation is believed to develop from the production of a thin walled, hollow fiber that collapses as solvent evaporates [19,20]. We note that this phenomenon can potentially increase average cross-sectional fiber diameters when samples are measured by SEM.

Screening the morphometric data sets detected three classes of scaffolds composed of distinct fibers and pore areas ($P < 0.001$). These classes consisted of scaffolds produced from: (I) 80 mg/ml suspensions containing fibers with cross-sectional diameters of less than 1 μm and pore areas of less than 1 μm^2 , (II) 100 and 130 mg/ml suspensions containing fibers with cross-sectional diameters between 1 and 5 μm and pore areas ranging between 20 and 100 μm^2 and (III) 150 mg/ml suspensions containing fibers with cross-sectional diameters greater than 5 μm and pore areas greater than 100 μm^2 (Fig. 2A and D).

We were unable to definitively resolve the nature of the relationships that exist between starting concentration, solution viscosity and fiber diameter. Regression analysis examining average fiber diameter as a function of gelatin concentration using a linear model generated a R^2 value of 0.54 and a second-order model gave a R^2 value of 0.56 (Fig. 2B, inset). Linear regression analysis examining how solution viscosity varied as a function of gelatin concentration provided a R^2 value of 0.98, fitting a second-order function to these data produced a R^2 value of 0.99 (Fig. 2C, inset).

3.2. Impact of mandrel rotation

To determine how the rotation of a rectangular grounded mandrel affects scaffold structure, we varied the mandrel speed from 200 to 6000 RPM in 1000 RPM increments. With one exception, fiber diameter was not regulated by mandrel RPM. ANOVA testing and post hoc

analysis of scaffolds produced from the 80 mg/ml suspension sets indicated that fibers deposited onto a grounded target rotating at 2000 RPM were, on average, larger than fibers collected at 200 RPM ($P<0.05$). Mandrel RPM did not alter average fiber diameter in scaffolds produced from suspension concentrations of 100, 130 or 150 mg/ml under any of the conditions assayed (Fig. 2A).

Mandrel RPM had a more pronounced impact on average pore area (Fig. 2D). In scaffolds electrospun from the 80 mg/ml suspensions, average pore area increased when collected at 2000 and 6000 RPM versus those collected at 200 RPM ($P<0.05$). For scaffolds produced from the 100 and 130 mg/ml suspensions, average pore area decreased at all mandrel RPMs with respect to 200 RPM ($P<0.05$). In scaffolds electrospun from 150 mg/ml suspensions, average pore area decreased in the 5000 and 6000 RPM treatment groups with respect to 200 RPM ($P<0.05$). These data suggest that under specific conditions fiber packing is more efficient as mandrel RPM is increased, a result that is consistent with the induction of increased fiber alignment.

3.3. Fiber alignment and scaffold anisotropy as judged by FFT

We used FFT analysis of brightfield images to characterize scaffold anisotropy and assign a numerical value to fiber alignment. For this type of analysis, the degree of fiber alignment in a data image is reported by the height and shape of the peak generated by the FFT plot (Fig. 1). The higher the peak, the more precisely aligned the fibers are along a single axis of orientation. Gelatin electrospun from a starting concentration of 80 mg/ml did not exhibit overt evidence of alignment at any mandrel RPM. The normalized intensity values (or FFT alignment value) of these scaffolds did not exceed 0.05 arbitrary units (Fig. 3A) for any of the conditions assayed and the total area under the curve remained less than 2.10 arbitrary units (Fig. 3E).

Statistical analysis indicates that scaffolds produced from 100 and 130 mg/ml suspensions have similar fiber diameters and pore areas. However, FFT analysis indicates subtle differences may exist to distinguish these two treatment groups. FFT alignment values for scaffolds prepared from the 100 mg/ml suspensions sequentially increased as a function of mandrel RPM (Fig. 3B and E). At 1000 RPM the alignment value was approximately 0.02 units for these scaffolds and peaked at 0.08 units in samples collected at 6000 RPM. In contrast, alignment values assigned to scaffolds produced from the 130 mg/ml suspensions were similar in nature and exceeded 0.08 for samples collected at 1000, 2000, 3000, 4000, and 5000 RPM (Fig. 3C and E). These data are consistent with our conclusions that pore size decreases with respect to controls in these samples, when mandrel RPM is greater than 1000, as a consequence of increased fiber alignment (and packing efficiency, see Fig. 2).

Scaffolds electrospun from starting concentrations of 150 mg/ml produced FFT alignment values of 0.08 units at 200 RPM (Fig. 3D). This FFT alignment value exceeded the values observed for all of the scaffolds prepared from the 80 mg/ml suspensions and most of the 100 mg/ml suspensions. These results may explain why pore size (Fig. 2) in the 150 mg/ml samples was not markedly altered until the mandrel RPM reached 5000 RPM (Fig. 3). These fibers already have a high degree of alignment and pack efficiently even under baseline conditions.

3.4. Effects of optical noise and image magnification on FFT analysis

Images captured by brightfield (widefield) microscopy contain considerable information from outside the image plane (Z direction) and have limited resolution in the XY plane. For example, scaffolds prepared from the 80 mg/ml stock concentrations contain fibers that are at, or below, the nominal limits of resolution for a 20 $\times$, 0.40 n.a. objective (XY resolution = 0.278 μ m). The data images captured with this lens for FFT analysis represent a surface area of 0.35 $\times$ 0.35 mm; in images cropped to 2048 $\times$ 2048 pixels, each pixel represents approximately 1.7 $\times$ 10⁻⁴ mm.

The fibers present in brightfield images of scaffolds prepared from the 80 mg/ml suspensions are approximately 7 pixels in diameter. Using this dimension and the pixel dimensions (1.7×10^{-4} mm) to “measure” these structures yields a fiber diameter of 1.1×10^{-3} mm or about 1 μ m. SEM analysis of this type of scaffold reports an average fiber diameter of 0.29 ± 0.1 μ m. This discrepancy can be attributed to the observation that fibers in a dry electrospun scaffold can act like a diffraction gradient; an effect that explains why many electrospun materials have a white appearance. The “fibers” present in a brightfield image of a scaffold prepared from a suspension concentration of 80 mg/ml actually represent birefringence of sub-resolution fibers.

To characterize how imaging artifacts might affect our FFT analysis we imaged scaffolds by brightfield (widefield) and confocal microscopy. For a representative aligned scaffold (130 mg/ml at 25 kV, 4000 RPM) the brightfield data images produced an FFT alignment value of 0.08 units (Fig. 4A). A confocal average-intensity Z stack projection of this same region gave an alignment value of 0.16 units and a maximum-intensity Z stack projection gave an alignment value of 0.12 units (Fig. 4A). The different FFT values reported by the average-intensity and maximum-intensity Z stack projections develops from the algorithms used to produce the images. An average projection removes noise by summing and averaging pixel values in a Z stack column, “smoothing” the data set. In a maximal projection, noise is retained because the maximum pixel value in a Z stack is used to produce the final image, regardless of its source (signal or noise). We conclude from these experiments that optical (as seen in light scatter in widefield images) and electronic detector noise can degrade the absolute alignment value assigned to a scaffold by FFT analysis. However, the information that is present in a brightfield image allows the FFT approach to discern relative differences in alignment across different samples.

To characterize how image resolution and the number of fibers present in a data set impacts FFT analysis we captured a series of SEM images at different magnifications from a representative aligned scaffold (130 mg/ml at 25 kV, 4000 RPM). Analysis of these data sets indicates that FFT alignment values remain consistent over a wide range of image magnifications (Fig. 4B). We note the frequency plots of these data sets become increasingly noisy above 750 $\times$, an effect that we attribute to surface structures resolved on individual fibers. The confounding effects associated with this information is substantially eliminated when a threshold filter is applied to the image prior to FFT analysis (data not shown).

Information concerning the angle at which electrospun fibers traverse any given optical image plane cannot be discerned from a brightfield, SEM or projected confocal image (which is a 3-dimensional data set collapsed into a 2-dimensional projection). This information can be recovered by sequentially examining the image planes present within a 3-dimensional confocal data set. From this type of analysis we conclude that fibers in random (Fig. 4C and D) and aligned scaffolds (Fig. 4E and F) are not dispersed to any great extent in the Z-direction. The fibers of these scaffolds are deposited into layers by the electrospinning process and are substantially restricted to discrete image planes. Z-distribution and fiber alignment data also is encoded in the shape and relative frequency at which different cross-sectional profiles occur in a confocal data set. The fibers of a random scaffold exhibit a round or oval profile in both the YZ- and XZ-directions (Fig. 4D). In contrast, the fibers of an aligned matrix preferentially exhibit an elongated profile in the YZ-plane (the principle axis of alignment present in this particular image) and a round or oval profile in the XZ-plane (Fig. 4F). This information can theoretically be extracted from confocal images to provide a precise map of overall scaffold anisotropy in three dimensions.

3.5. Materials testing

To scale how FFT alignment values might correlate with scaffold anisotropy we conducted materials testing. Anisotropic materials can be expected to have stress and strain profiles that

change as a function of the angle by which a test load is placed across the material. For an ideal and completely random matrix, stress (and strain) at failure in the parallel and the perpendicular orientations (with respect to the direction of mandrel rotation) should be identical. The ratio of the parallel to the perpendicular stress (or strain) will be equal to 1 for this type of scaffold.

For scaffolds electrospun from a concentration of 80 mg/ml and collected at 200 RPM, the ratio of average stress at failure in the parallel and perpendicular orientations was 0.86; the ratio of the average strain at failure under these conditions was 1.3 (Fig. 5A and B). An analysis of absolute stress at failure for the parallel and perpendicular orientations for scaffolds collected at 2000, 4000, and 6000 RPM failed to detect any statistical differences in the data sets (Fig. 5C and D). This lack of directional bias is consistent with a structure composed of random elements.

For scaffolds electrospun from 100 mg/ml suspensions the ratio of the average stress at failure in the parallel and perpendicular orientations increased modestly and sequentially as a function of mandrel RPM (Fig. 5A). At low RPM (200 and 2000) peak stress was higher in the perpendicular orientation ($P<0.05$). As RPM increased, peak stress became increasingly biased in the parallel orientation ($P<0.02$) (Fig. 5A and C). The ratio of peak strains at failure in the parallel and perpendicular orientations decreased (Fig. 5B). However, statistical analysis of the absolute values for these data sets failed to detect any directional bias in the strain properties of these scaffolds (Fig. 5B and D).

Scaffolds electrospun from the 130 mg/ml suspensions exhibited evidence of anisotropy under all of the conditions that we tested ($P<0.03$, Fig. 5A and C), indicating that a degree of alignment is induced even at nominal mandrel RPM. There was a distinct change in the ratio of the peak stresses (Fig. 5A) and the ratio of the peak strains at failure (Fig. 5B) between 4000 and 6000 RPM.

Scaffolds prepared from the 150 mg/ml suspensions exhibited poor structural integrity under the electrospinning conditions used in this study and were damaged during removal from the mandrel. As a result, the material properties of these scaffolds were not tested.

In summary, the materials testing data suggests that an FFT alignment value of 0.05 units or greater corresponds to the evolution of scaffold anisotropy. Scaffolds that exceed this alignment value exhibit an orientation bias in materials testing. In contrast, scaffolds with FFT values below this threshold appear to be random in nature. The use of a strain extensometer to determine the strain properties of a soft tissue is a less than ideal approach; the weight of the testing apparatus can distort the resulting strain values. Given the results of our study and the potential technical limitations in measuring strain we believe the measured stress properties of an electrospun scaffold more accurately reflect the anisotropic properties of these materials. In our experiments the stress measurements are less susceptible to instrument-induced error.

4. Discussion

4.1. Fiber diameter and pore area

We have examined how starting conditions regulate fiber diameter, pore area and the material properties of electrospun gelatin. Scaffolds were prepared from starting concentrations of 80, 100, 130 and 150 mg gelatin/ml TFE; this represents the range of conditions that produced useful scaffolds. At starting concentrations of less than 80 mg/ml, scaffolds of gelatin are composed of 200–300 nm diameter fibers, exhibit poor structural integrity and are difficult to manually manipulate [2]. Conversely, scaffolds electrospun from stock concentrations containing 150 mg/ml gelatin, or greater, retain solvent and are difficult to recover for analysis.

In our experiments fiber diameter did not vary as a simple linear function of the starting concentration of gelatin (Fig. 2B). There are several potential explanations to account for this result. Theoretically, the viscosity of the starting solution/suspension is the variable most closely linked to fiber cross-sectional diameter in the electrospinning process [19–22]. However, viscosity evolves from properties that are intrinsic to a specific solvent system and the identity, concentration and extent of polymer chain entanglements (and the degree of solubility of a given polymer) present at the onset of electrospinning. Viscosity is further modulated by ambient conditions (temperature, humidity etc.). These fundamental variables are coupled with the rate of polymer delivery to the electric field, the ionic composition of the solution/suspension, and the electrospinning voltage to regulate the stability of the Taylor cone, the trajectory of the charged jet and the dimensions of the developing fiber. We suspect there is a mismatch between one of these coupled electrospinning parameters; multiple charged jets have been observed to develop and episodically collapse when electrospinning from our 100 mg/ml suspensions. Removing either the 100 or 130 mg/ml data sets dramatically improves our regression analysis results when we examine how fiber diameter varies as a function of starting concentration or viscosity. Removing the 100 mg/ml data provides a first-order R^2 value of 0.68 and a second-order R^2 value of 0.86; removing the 130 mg/ml data generates a first-order R^2 value of 0.75. We believe that a re-assessment of data sets generated with other polymer systems may reveal similar discontinuities in the theoretical relationships that exist between fiber diameter and solution viscosity (a variable that has not been commonly reported) [6].

4.2. Mandrel rotation

For each starting concentration, fiber diameter remained constant over a wide range of mandrel RPM (Fig. 2). If the mandrel acted like a spool, and scaffold alignment was induced by fiber winding, average fiber diameter would be expected to decrease as a function of mandrel RPM. This thinning effect has been observed in electrospinning systems where a rotating disk mandrel with a high surface velocity is used as a target [13].

4.3. Fiber alignment and scaffold anisotropy

As judged from this preliminary study, the FFT can be used to predict scaffold anisotropy over a wide range of imaging conditions. It is important to note, however, that because electrospinning produces sub-micron fibers that are often below the resolution of a light microscope objective, images of these fibers are generated through birefringence. Although our data suggests that this type of image produces useful FFT data, the effects of image resolution on FFT output should be calibrated and verified by high-resolution analysis using confocal microscopy and/or SEM (see Fig. 4). SEM images are clearly amenable to analysis by FFT, but this approach must assume a uniform distribution of fibers throughout the cross-sectional diameter of the scaffold. We are continuing to explore the limits of FFT analysis in the characterization of scaffold structure.

Routine microscopic examination of our scaffolds identified evidence of preferential fiber alignment under a variety of different electrospinning conditions (e.g. Fig. 3). FFT alignment values of greater than 0.05 units were correlated with the onset of directional bias in the material properties (Fig. 5). Scaffolds that did not meet this criterion failed to exhibit overt visual evidence of anisotropy or the stress/strain values normally expected of a material composed of aligned elements. The correlation between the materials testing data and the degree of scaffold anisotropy, as determined by FFT, indicate that our optical measurements can be used to predict scaffold alignment on a relatively large scale (i.e. alignment is not just a local phenomena detected by FFT analysis).

5. Conclusion

Anisotropy in an electrospun scaffold develops from a complex set of variables. A surprising degree of anisotropy can be present in an electrospun scaffold composed of relatively large diameter fibers that have been collected at modest rates of mandrel rotation. These results clearly demonstrate that it is necessary to take into account the relative degree of fiber alignment present in an electrospun material when attempting to compare the material properties of constructs composed of different fiber diameters. Our data also underscore the central role that macroscopic architectural features can play in regulating the material properties of the native ECM. Additional experimentation will be necessary to determine if more subtle changes in FFT alignment values can be correlated with the evolution of specific material properties and/or features that can control cell phenotype in an electrospun tissue-engineering scaffold. By tailoring scaffold anisotropy in an electrospun material it may be possible to provide contact guidance cues to cells and more closely mimic the material properties of native structures [23,24]. FFT analysis is a potentially valuable method for quantifying alignment, confirming the reproducibility of nano-manufacturing processes and examining the biological consequences of scaffold structure.

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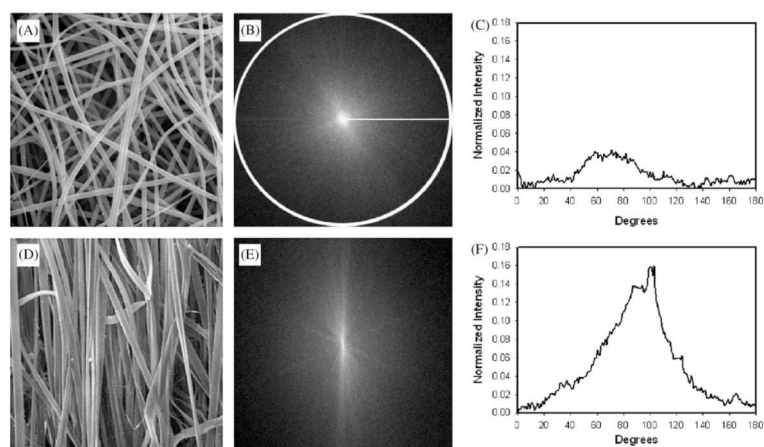
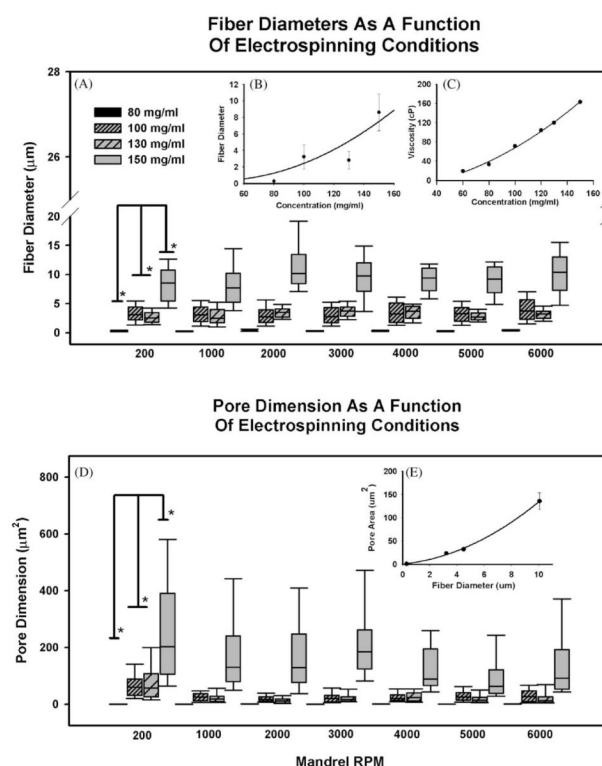
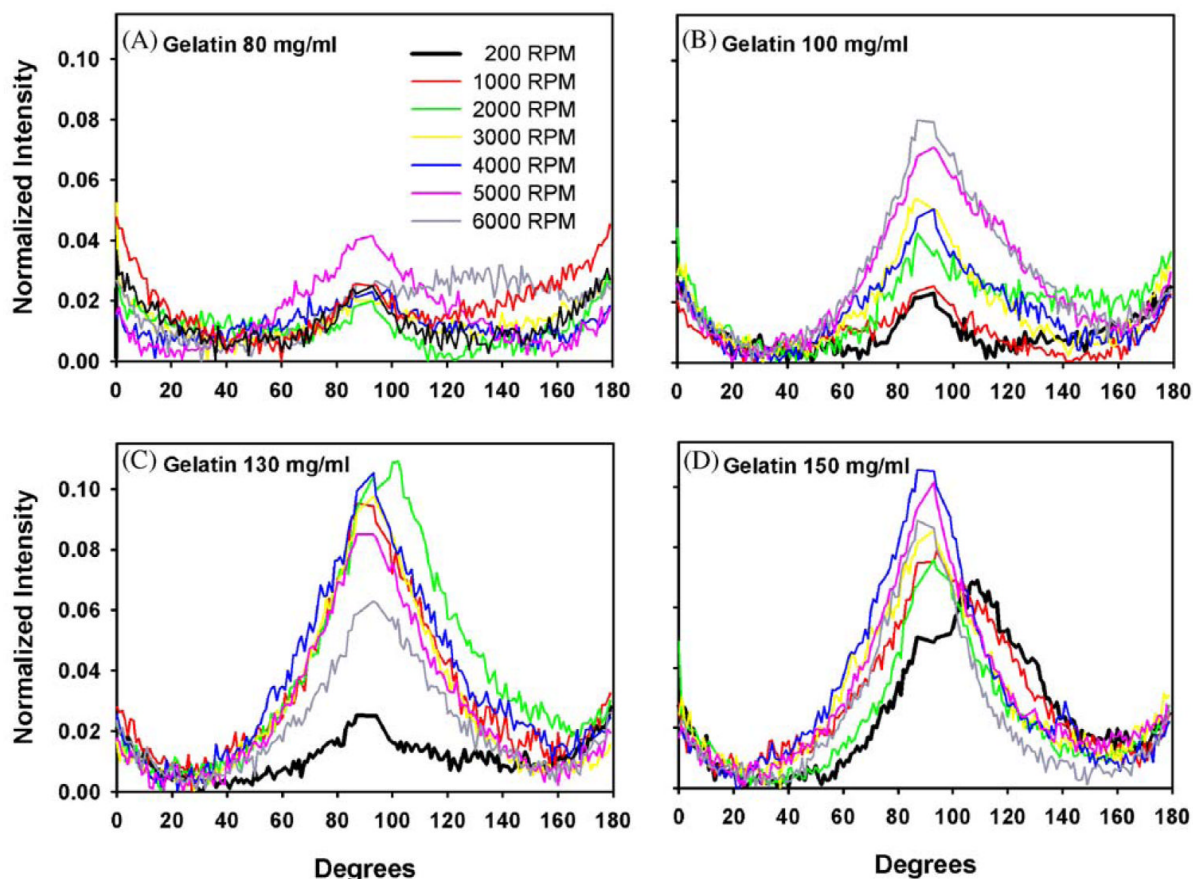


Fig. 1. Representative SEM images of a random (A) and an aligned matrix (D). FFT output images (B and E) and radial projection (B). Pixel intensity plots against the angle of acquisition for a random matrix (C) and an aligned matrix (F). Note the distinctive peak produced by the image containing aligned information (F).

**Fig. 2.**

Fiber diameter (A) and pore area (D) as a function of mandrel RPM. Bars at 200 RPM indicate three classes of fibers (A) and pore areas (D) detected by statistical analysis ($P < 0.001$). Inset (B), second-order modeling of fiber diameter as a function of starting concentration at 200 RPM, $R^2 = 0.56$. Inset (C), regression analysis of the relationship between starting concentration and solution viscosity, first-order $R^2 = 0.98$, second-order $R^2 = 0.99$ (depicted). Inset (E), first-order modeling of pore area as a function of fiber diameter at 200 RPM, $R^2 = 0.33$. Error = bars B and E = $\pm$ standard deviation.

FFT Analysis of Scaffold Structure



(E) Total Area Under the Curves in Frequency Plots

Area Under the Curve				
RPM	80 mg/ml	100 mg/ml	130 mg/ml	150 mg/ml
200	1.06	0.98	1.37	3.64
1000	1.23	1.13	4.93	4.52
2000	0.71	2.38	6.10	3.38
3000	0.96	2.38	4.76	4.45
4000	1.24	2.49	5.65	5.24
5000	2.05	3.95	4.43	4.74
6000	1.63	4.20	3.30	3.73

Fig. 3. FFT analysis of scaffold structure as a function of mandrel RPM. Scaffolds from suspensions of 80 mg/ml (A), 100 mg/ml (B), 130 mg/ml (C) and 150 mg/ml (D). Total area under the curve for frequency plots (E).

FFT Analysis of Scaffold Anisotropy: Effects of Optical Noise and Image Resolution

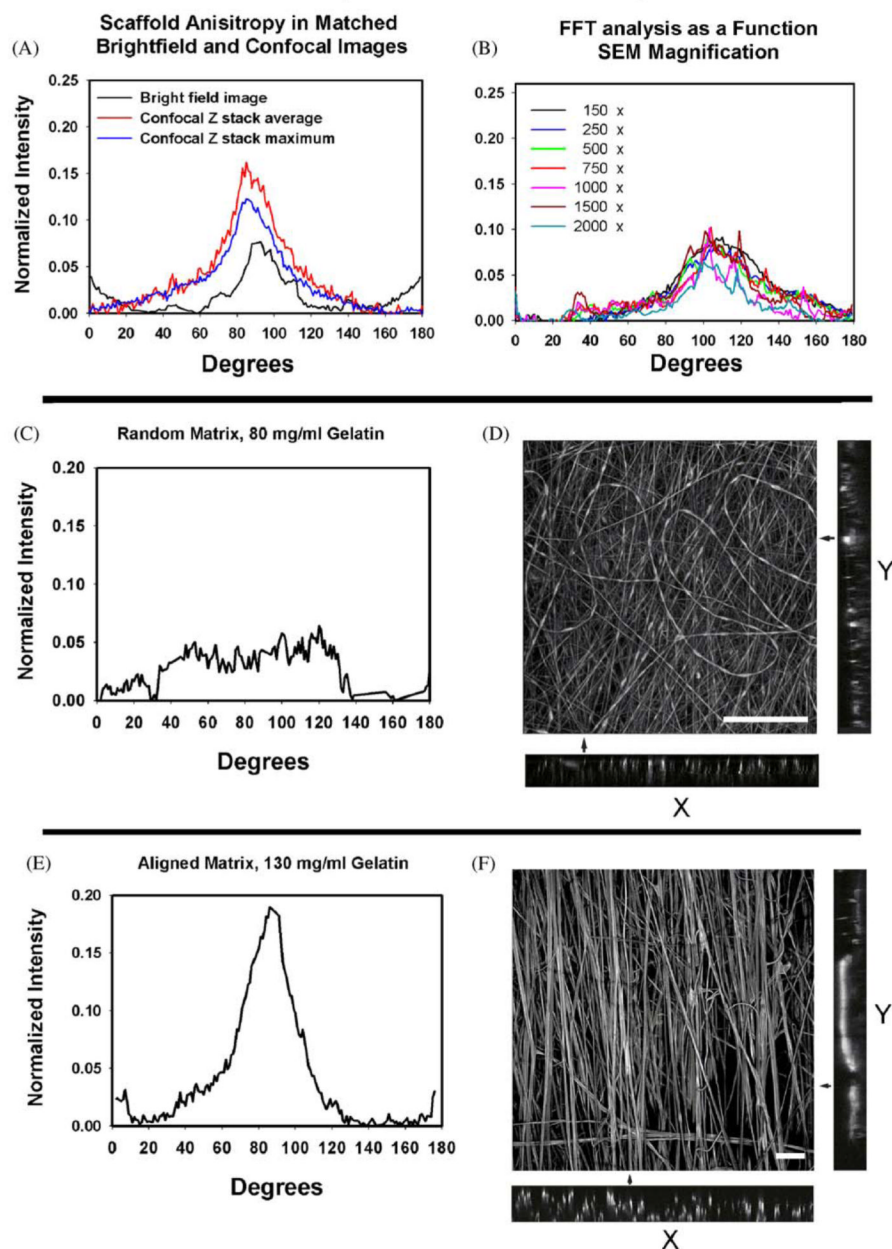


Fig. 4. FFT analysis of brightfield, confocal Z-stack average and confocal Z-stack maximum projections of similar image fields (A). FFT analysis of SEM images as a function of SEM magnification (B). Cross sectional analysis of random (C and D) and aligned (E and F) electrospun scaffolds. Scale Bar D = 5 μ m, F = 10 μ m.

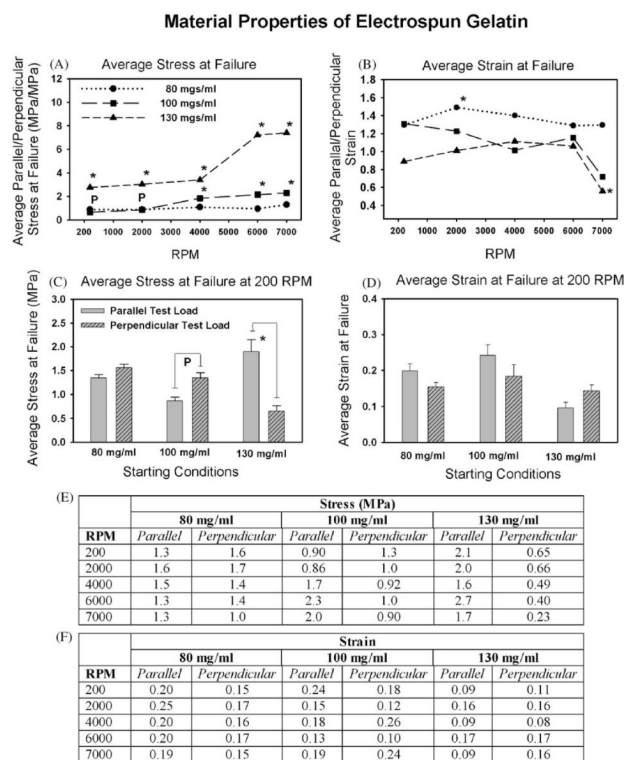


Fig. 5. Material properties as a function of mandrel rotation. Ratio (in the parallel to perpendicular orientations) of stress (A) and strain (B) at failure. Average stress (C) and strain (D) at failure in scaffolds deposited at 200 RPM. * = bias in the parallel orientation, *P* = bias in the perpendicular orientation ($P < 0.05$). Error bars = $\pm$ standard error. Table E = average stress values, F = average strain values as a function of starting concentration and direction of testing.