Electrospun Fibrinogen-Polydioxanone Composite Matrix: Potential for In Situ Urologic Tissue Engineering

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ABSTRACT

Our objective is to demonstrate an electrospun fibrinogen-PDO (polydioxanone) composite scaffold will retain the superior cellular interaction of fibrinogen while producing a product with the functional strength needed for direct implantation. Fibrinogen-PDO composite scaffolds were electrospun with PDO ratios of 0% (pure fibrinogen), 10%, 20%, 30%, 40%, 50% and 100% (pure PDO) and disinfected using standard methods. Scaffolds were seeded with human BSM (bladder smooth muscle cells) and incubated with twice weekly media changes. Samples were removed at 7, 14 and 21 days for evaluation by collagen assay, scanning electron microscopy and histology. Cell seeding and culture demonstrated human BSM readily migrate throughout and remodel electrospun fibrinogen-PDO composite scaffolds with deposition of native collagen. Cell migration and collagen deposition increased with increasing fibrinogen concentration while scaffold integrity increased with increasing PDO concentration. Electrospun fibrinogen-PDO composite structures promote rapid cellular in-growth by human BSM while maintaining structural integrity. The fibrinogen to PDO ratio can be adjusted to achieve the desired properties required for a specific tissue engineering application. Our ultimate objective is to utilize this innovative biomaterial technology to produce an acellular, bioresorbable product that enables *in situ* tissue regeneration. While there is still much work to be done, these initial findings indicate fibrinogen-PDO composite scaffolds deserve further investigation.

INTRODUCTION

The need for urinary tract reconstruction can result from a large variety of urologic disorders such as congenital hypospadias, stricture disease, cancer and trauma. These disorders may affect the ureters, bladder or urethra, decreasing the availability of adequate autologous tissue. Moreover, complications from inserting autologous tissue, such as enteric segments, into the urinary tract are well known and often manifests metabolic and histopathologic abnormalities. These sequelae have motivated investigators to search for tissue-engineered alternatives.

Ideally, a tissue-engineered scaffold should mimic the structural and functional profile of the native extracellular matrix (ECM). To achieve this objective, the "ideal" scaffold has been described as having the following characteristics: 1) a three-dimensional structure with a surface chemistry that promotes cell attachment, proliferation and differentiation [1], 2) that does not induce adverse immune responses from the surrounding tissues [1], 3) mechanical properties to withstand organ-specific *in vivo* forces while being completely resorbable [2], and 4) a feasible production process that allows for various shapes and sizes [3]. Despite many recent advances, none of the scaffolds in use today meet all of the above mentioned criteria completely.

Fibrinogen is a naturally occurring soluble blood protein that functions as a major structural element in the coagulation cascade, clot formation and wound healing [4, 5]. The reaction of fibrinogen with thrombin produces fibrin. This exposes regions of opposite charge leading to the assembly of fibrous clots and/or other fibrous structures. These fibrous structures function as nature's provisional matrix, on which tissues rebuild and repair themselves, making this type of structure an attractive scaffold for tissue engineering and regeneration [6-8].

Fibrin and fibrinogen have a well established track record in tissue engineering, due to their innate ability to induce cellular interaction and subsequent scaffold remodeling. Fibrinogen based scaffolds have previously been developed in the form of fibrin gels [7-14] and wet extrusion fibronectin-fibrinogen cables [15-17]. These studies demonstrated fibrinogen based scaffolds were easily degradable, nonimunogenic [9] and promoted increased cell migration [15, 17].

Electrospinning is a technology with the potential to fulfill the requirements of an ideal scaffold. Briefly, electrospinning is accomplished by inducing a large electric potential (15 to 30 kilovolts DC) in a polymer solution and separating that polymer from an oppositely charged target. This charge separation creates a static electric field. As the field strength grows, the charge separation overcomes the surface tension of the solution and a thin jet of entangled polymer chains is ejected from the polymer reservoir. As this jet travels toward the target, instabilities within the charged jet define its orientation in space (condition previously described as whipping). By the time the jet reaches the target, the solvent has evaporated and a dry fiber is collected in the form of a non-woven scaffold (*Figure 1*).

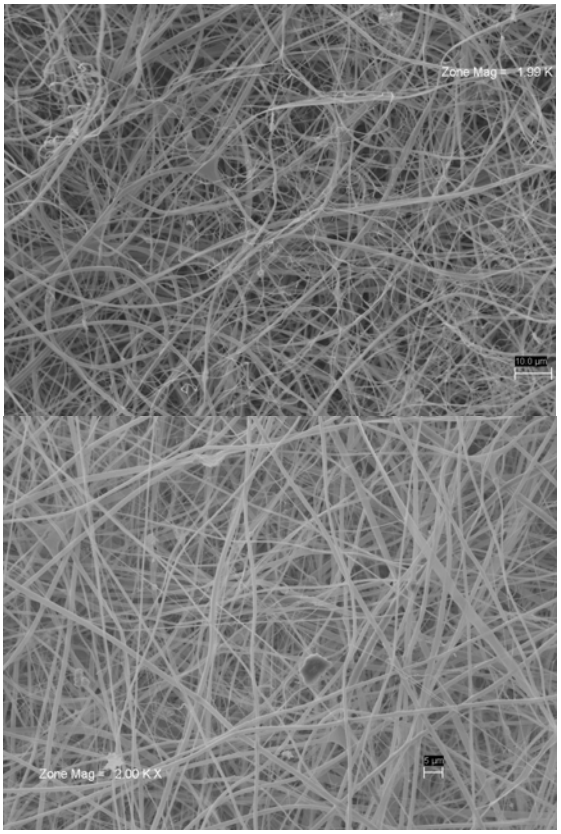

FIGURE 1: Scanning electron micrographs of electrospun fibrinogen-PDO composite scaffolds at 2000x magnification. 10% PDO composite (top) has a scale bar of 10 μm, while 40% PDO composite (bottom) has a scale bar of 5 μm.

METHODS

Electrospinning

For this study, fibrinogen (Bovine soluble fraction 1, Sigma Aldrich Chemical Co.) was dissolved in 9 parts 1,1,1,3,3,3 hexafluoro-2-propanol (HFP, Sigma Aldrich Chemical Co.) and 1 part 10X minimal essential medium (MEM) at a concentration of 110 mg/mL. PDO (Ethicon) was dissolved in HFP at a concentration of 100 mg/mL. These ratios were chosen based on previous work conducted by the authors that demonstrated the best mechanical and cellular interaction characteristics for further investigation. The solutions were then loaded into separate Becton Dickinson 5.0-mL syringes and placed in a KD Scientific syringe pump. The fibrinogen solution was dispensed at a rate of 1.8 mL/hr. The dispensing rate of the PDO solution was varied to produce scaffolds with PDO ratios of 0%, 10%, 20%, 30%, 40%, 50% and 100% by volume. The positive output lead of a high voltage power supply (Spellman CZE1000R; Spellman High Voltage Electronics Corp.), set to 22 kV, was attached to a custom designed 2 input - 1 output nozzle as depicted in *Figure 2*. This nozzle had an estimated component mixing length of 15 mm prior to exiting the nozzle. A grounded target (2.5 cm Wide × 10 cm Long × 0.3 cm Thick; 303 stainless steel) was placed 10 cm from the nozzle tip and rotated at 500 revolutions per minute (RPM) to evenly coat the mandrel, but not impart a large degree of alignment to the deposited fibers.

Mechanical Evaluation

For determining the bulk mechanical properties, samples were incubated at 37ºC for 24 hours in complete culture media containing Dulbecco's Modified Eagle Medium (DMEM)-F12 Nutrient Mixture (F12) (1:1 DMEM:F12) supplemented with 5% Fetal Bovine Serum (FBS) and 1.2% penicillin-streptomycin antibiotic (10,000 I.U. penicillin/mL, 10,000 μg/mL streptomycin, Mediatech, Inc.). Uniaxial tensile testing was performed on a MTS Bionix 200 (MTS Systems Corp.) mechanical testing system incorporating a 50 N load cell with an extension rate of 10 mm/min to failure. A total of six test specimens (n=6) were tested for each study group. Specimens were punched from the scaffolds using a "dog-bone" shaped template to assure uniformity and isolate the failure point away from the grips. The specimens had a width of 2.67 mm and a gauge length of 11.25 mm. Specimen thickness was measured on a Mitutoyo IP54 digital micrometer (Mitutoyo American Corp.) and ranged from 0.03 to 0.20 mm. Tangent modulus (tangential method automatically selected by the MTS TestWorks 4.0 software), peak stress (engineering stress based on the initial cross-sectional area), and strain to failure (also calculated automatically by the software) were determined.

Cell Culture

Experiments were designed to evaluate cell proliferation and cell migration into the scaffolds at

varying fibrinogen and PDO concentrations. Scaffolds were electrospun with PDO as previously described. Seventy-five 15 mm diameter die-cut scaffold specimens, five scaffolds at each concentration at each time point, were placed in separate wells of 24 well culture plates. All scaffolds were sanitized in 100% ethanol for ten minutes then rinsed four times with sterile PBS for three minutes per rinse. Human BSM (Cambrex) were expanded utilizing standard cell culture protocol in complete culture media containing DMEM:F12 supplemented with the Cambrex BulletKit (hEGF, hFGF-B, insulin, gentamicin, amphotericin-B, 5% fetal bovine serum). Scaffolds were seeded with $2x10^4$ cells per scaffold. Culture media was aspirated from the scaffold containing test wells and 500 µl of fresh media was added to each culture well and changed twice per week. Cultures were incubated at 37ºC in 5% CO_2 and removed for testing at 7, 14 and 21 days.

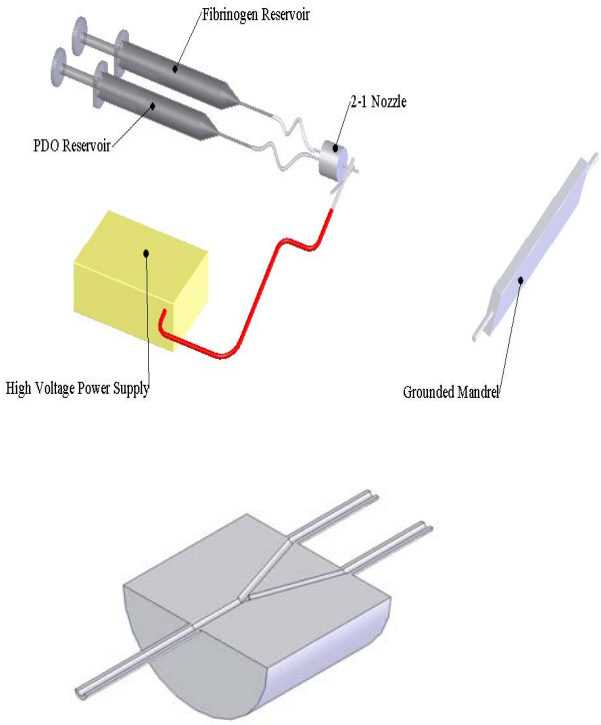

FIGURE 2: Diagrammatic representation of the custom designed nozzle in a typical electrospinning setup, attached to syringes with 18 gauge Tygon® tubing (top) and section view of the 2 input - 1 output nozzle (bottom).

Collagen Assay

Collagen deposition by cultured cells was measured using the Sircol Collagen Assay (Biocolor). This is a colorimetric assay for the quantification of acid-soluble and pepsin-soluble collagens (Types I to IV) extracted from mammalian tissues. The Sircol dye reagent contains Sirius Red, which is an anionic dye with sulphonic acid side chain groups that react with the side chain groups of the basic amino acids present in collagen. The specific affinity of the dye for collagen, under the assay conditions, is due to the elongated dye molecules becoming aligned parallel to the long, rigid structure of native collagens that have intact triple helix organization. Increased collagen content leads to an increase in the amount of Sircol dye bound, which is measured with a spectrophotometer.

After the 7 and 14 day incubation periods, scaffolds were removed from the culture wells and placed in separate 1.5 mL centrifuge tubes. One milliliter of 0.5 M acetic acid solution containing 1 mg of pepsin was added to each centrifuge tube and samples were placed on an orbital mixer overnight. The tubes were gently mixed and 100 µL of solution from each tube was transferred to a new centrifuge tube. To develop a standard curve, four control solutions were prepared by combining 5, 10, 25 and 50 µL (1µg/µL) of reference collagen standard with distilled water to total 100 µL. Four tubes were filled with 100 µL of distilled water to serve as background controls. One milliliter of Sircol dye was added to all tubes, mixed by inversion and then placed on an orbital mixer for thirty minutes. Tubes were then centrifuged at 10,000 times gravity for 10 minutes, the supernatant drained and 1.0 mL alkali reagent added. After mixing, 200 µL of solution from each tube was transferred into wells on a new 96 well microtiter plate where the absorbance of the samples was measured, against the background control, on a microtiter plate reader (SpectraMax Plus, Molecular Devices Corporation) at a wavelength of 540 nm.

Microscopy Evaluations

Scaffolds were fixed in 10% formalin solution for evaluation by scanning electron microscopy (SEM) and histology. Samples to be evaluated by SEM were dehydrated; a representative sample from each scaffold was sputter coated in gold (Electron Microscope Sciences model 550) for SEM (JEOL JSM-820 JE Electron Microscope) and evaluated at 350 to 1500 times magnification. Samples for histologic evaluation were cross-sectioned for slide mounting and hematoxylin and eosin (H&E) staining (Harris Histology Relief Services). H&E slides were evaluated by optical light microscopy (Eclipse TE300, Nikon).

Statistics

All statistical analyses were performed utilizing Sigma Stat (Version 2.03; SPSS, Inc). For the data collected, normality and equal variance tests were set to reject for

$P \leq 0.01$. All samples passed normality and equal variance tests and were evaluated using a one-way analysis of variance (ANOVA) and then subjected to a pair-wise multiple comparison procedure (Tukey Test). The *a priori* alpha value was set at 0.05.

RESULTS

Mechanical Evaluation of Electrospun Scaffolds

Results of mechanical testing of the various electrospun fibrinogen-PDO composite structures are presented in *Figures 3-5*. Properties of pure fibrinogen scaffolds (no PDO) were similar to those previously reported [36]. In general, values for modulus and peak stress increased as PDO concentration increased (*Figure 3*). Values for strain at failure varied from 84% to 130%, but differences were not statistically significant (*Figure 4*).

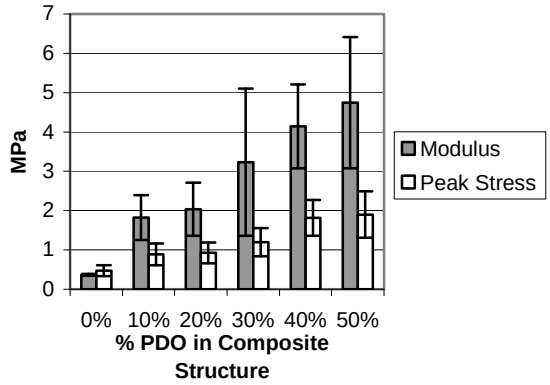

FIGURE 3: Results of mechanical testing of electrospun fibrinogen-PDO composite structures are illustrated in terms of modulus of elasticity (dark bars) and peak stress (white bars) verses the PDO concentrations in the solution from which structures were electrospun.

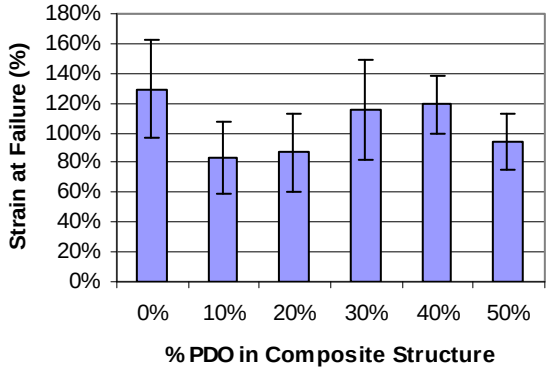

FIGURE 4: Results of mechanical testing of electrospun fibrinogen-PDO composite structures are illustrated in terms of percent strain at failure verses the PDO concentrations in the solution from which structures were electrospun.

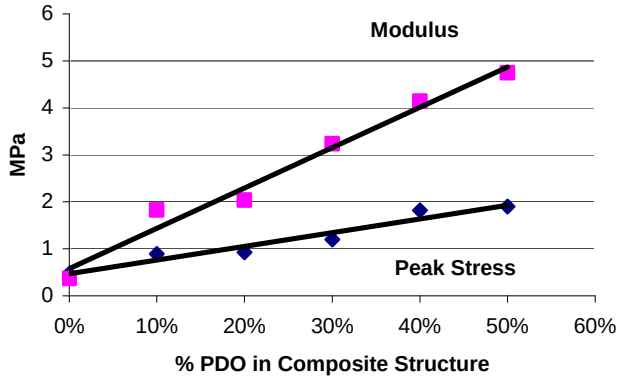

FIGURE 5: Modulus of elasticity and peak stress data demonstrate the linearity of these values from 0-50% PDO concentration. The R^2 value for the linear fit of the curve was 0.977 for modulus and 0.945 for peak stress.

Analysis of modulus of elasticity and peak stress data demonstrate the linearity of these values from 0-50% PDO concentration (*Figure 5*) indicating a predictable increase in modulus of elasticity and peak stress as PDO concentration increases. The R^2 value for the linear fit of the curve was 0.977 for modulus and 0.945 for peak stress. An increase in PDO concentration of 20% and 30% was required to achieve a statistically significant increase in peak stress and modulus, respectively.

Collagen Assay

The controls in *Figure 6* demonstrate the linearity of the assay results from 5 µg to 50 µg. The R^2 value for the linear fit of the standard curve was 0.926 at 7 days and 0.991 at 14 days. Collagen content (*Figure 7*) was calculated from the standard curve in *Figure 6*. Comparison of collagen content at different PDO concentrations demonstrated statistically significant differences at 7 and 14 days. Comparison between individual groups at day 7 only demonstrated statistical significance between the 0% PDO group and the 10% and 100% PDO groups. At 14 days, statistical significance was demonstrated between all individual groups except between the 10% and 100% PDO groups. Comparison of mean collagen content revealed two trends. First, there was a decrease in collagen content as PDO concentration increased up to 40% PDO, with an increase in collagen content in the 100% PDO group. Second, there was a higher collagen content at 7 days than at 14 days. These trends did not hold true for the 0% PDO concentration at 7 days which had a lower than expected collagen content.

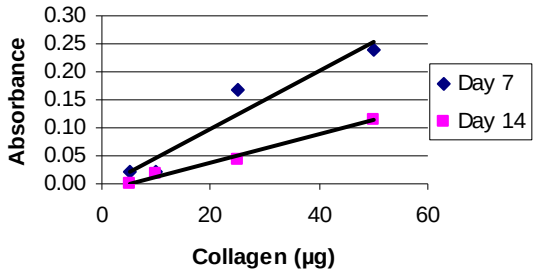

FIGURE 6: Collagen assay controls demonstrating the linearity of the assay results from 5 µg to 50 µg. The R^2 value for the linear fit of the standard curve was 0.926 at 7 days and 0.991 at 14 days.

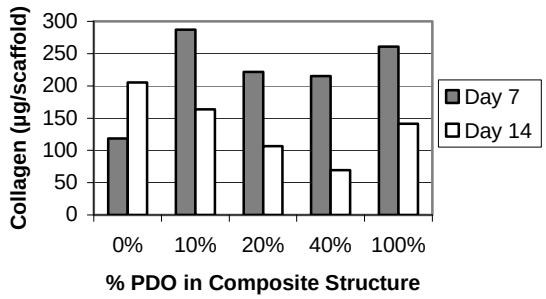

FIGURE 7: Bar graph depicting the collagen assay in terms of collagen content (based on the standard curve) for each scaffold condition.

Microscopy Evaluations

SEM analysis of post-cell culture electrospun scaffolds demonstrated evidence of cellular activity. This evidence is seen as areas of partial scaffold degradation and individual fibers having been pulled out of their expected orientation. Cells were not visible on the surface of the scaffolds.

Samples from the H&E stained histological sections are presented in *Figure 8*. Histological evaluations of internal domains of the scaffolds revealed BSM cells had migrated into, and appear to be evenly distributed throughout, the scaffold thickness in fibrinogen containing groups at 7 days, indicating a substantial degree of cell migration transpired early in the study period. Further comparison of histologic samples revealed two trends. First, there was an increase in the structural integrity of scaffolds as PDO concentrations increased. Second, there was an increase in cell layering and cell spreading as PDO concentrations increased. These trends did not hold true for the 100% PDO samples where no cells were identified on histological examination at any time point.

All PDO containing scaffolds remained structurally intact at 7 days, but areas of broken fibers were observed in the 0% PDO group demonstrating some early loss of structural integrity. Varying degrees of scaffold fragmentation and fusing of fibers with loss of the fibrous architecture were seen in the 0%, 10% and 20% PDO groups at 14 and 21 days. All samples in these groups were significantly fragmented by 21 days. The 40% PDO group maintained its structural integrity and fibrous structure throughout the study period.

Cell layering is an indicator of the extent to which a scaffold is directing cell growth and migration. Some degree of cell layering can be seen in all groups at 7 days, but it is most pronounced in the 40% PDO group. In the 0%, 10% and 20% PDO groups, cell layering is substantially decreased at 14 days and almost nonexistent at 21 days. Cell layering remains consistent throughout the study period in the 40% PDO group.

Cell spreading is an indicator of the degree to which a scaffold supports BSM cells assuming their natural morphology. At 7 days, there is an increase in cell spreading in the PDO containing groups compared to the 0% PDO group. This spreading is most pronounced in the 40% PDO group. There is a significant decrease in the amount of cell spreading in all groups, except the 40% PDO group, at 14 days and 21 days

DISCUSSION

The use of fibrinogen in tissue engineering applications is not new. Fibrinogen hydrogels have received much attention because they possess the benefits of homogeneous cell distribution and collagen deposition [9, 16, 37]. The use of hydrogels for tissue engineering requires mixing cells into the fibrinogen solution prior to forming the hydrogel. Electrospun fibrinogen scaffolds promote rapid cell migration into the scaffolds [35] allowing for cell seeding after scaffold production. However, these structures do not possess the structural integrity needed for direct implantation. Electrospun PDO structures have greater initial structural integrity and take longer to be degraded. The objective of this study was to demonstrate that an electrospun fibrinogen-PDO composite scaffold would retain the superior cellular interaction of fibrinogen while producing a product with functional strength for direct implantation.

In this study, mechanical testing data demonstrated that increasing the PDO concentration in electrospun fibrinogen-PDO composite scaffolds increased strength (peak stress) and stiffness (modulus) in a predictable fashion. All composite scaffolds maintained similar elasticity profiles, indicating composite scaffolds could

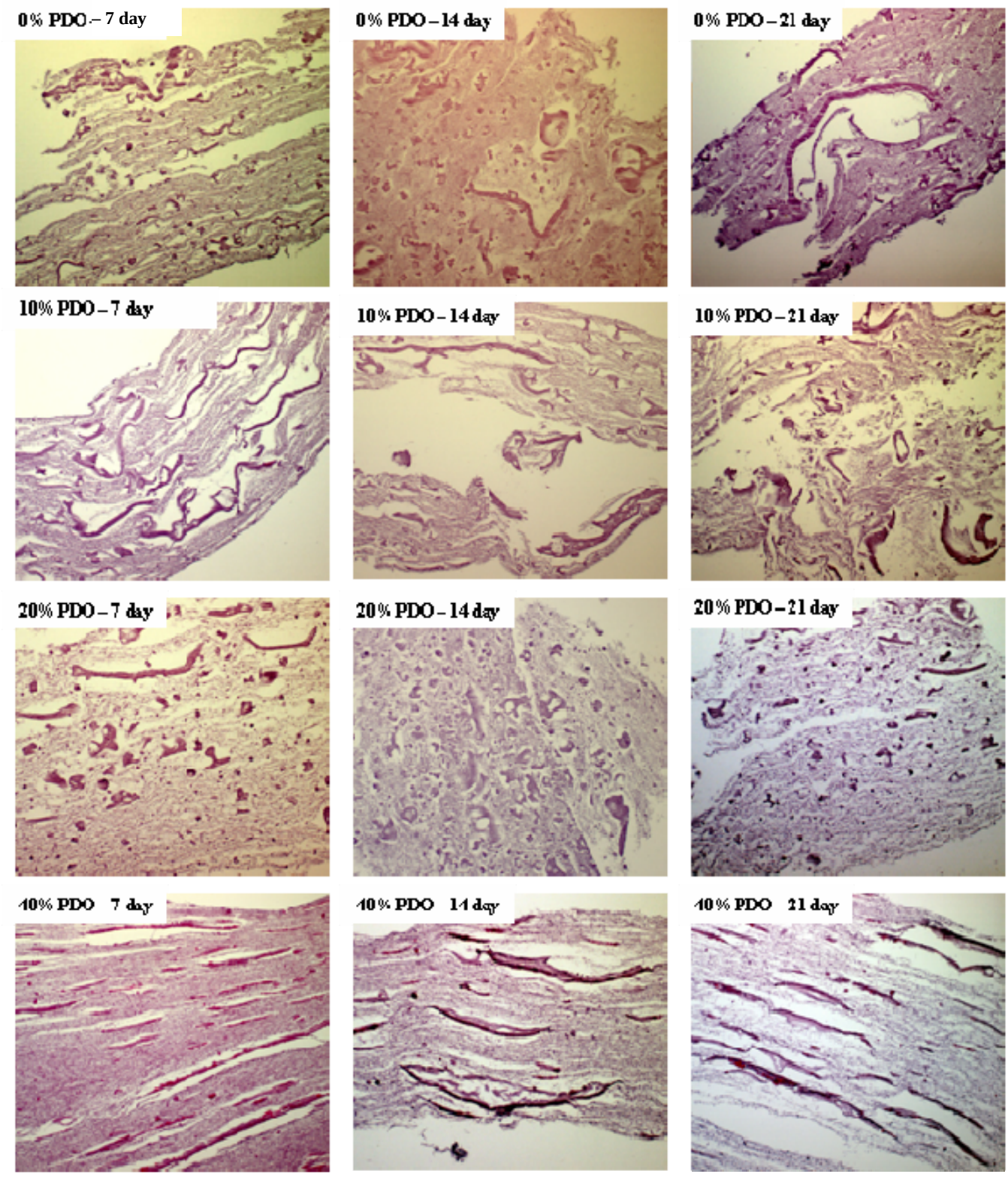

FIGURE 8: Optical micrographs of hematoxylin and eosin (H&E) stained electrospun fibrinogen-PDO composite scaffolds at 20 times magnification. BSM cells migrated through the full thickness of the scaffolds by day 7 (left column). Structural integrity of the scaffolds increased as PDO concentration increased (top to bottom). Cell layering and cell spreading also increased as PDO concentration increased.

be tailored to meet specific tissue engineering mechanical requirements.

The cell culture data demonstrated several trends related to scaffold composition. First, collagen deposition decreased as PDO concentration increased, with the exception of the 100% PDO group. Collagen deposition is an indication of the extent to which cells are remodeling a scaffold [35]. Second, there was increased cell spreading and cell layering at higher PDO concentrations. Cell spreading demonstrates the scaffold supports cells assuming their natural morphology while cell layering within a scaffold demonstrates the scaffold is directing cell growth and migration. Third, higher PDO concentrations produced scaffolds with greater structural integrity and stability. In general, the higher PDO concentrations produced more stable scaffolds that allowed for slower scaffold remodeling and stimulated cells to assume a morphology closer to their native state.

One of the most attractive aspects of the electrospinning process is that scaffolds of various shapes and sizes can be constructed while at the same time precisely controlling fiber orientation, composition (blended fibers), and dimensions. Complex constructs can be fabricated to closely replicate the structural and chemical composition of the native structures. For example, flat sheets for on-lay grafts, seamless tubes, or any three dimensional shape in which a mandrel can be fabricated could be produced. Also, the ability to co-spin polymers with various additives (e.g. growth factors) offers the possibility of tailoring the scaffold to a specific site and application. Thus, with electrospinning, it may be possible to create ideal scaffolding for a wide array of tissue engineering applications.

Previous tissue engineered products for urinary tract reconstruction have been described utilizing decellurized submucosa, natural polymer hydrogels and synthetic polymer scaffolds created from a variety of production techniques [38-40]. Recent work by Atala et al. [41] has even demonstrated the feasibility of bladder augmentation with tissue engineered products using *in vitro* cell culture. This process is labor and resource intensive with multiple procedures required to harvest cells for *in vitro* cell culture followed by later implantation. Electrospun scaffolds produced from other polymers have also been described for non-urologic applications [42, 43], but have failed to promote the rapid cellular in-growth demonstrated in this study.

While an electrospun PDO-elastin composite scaffold has been described [44], this is the first report of an electrospun fibrinogen-PDO scaffold and the first report utilizing an electrospun synthetic and natural polymer composite structure for urologic tissue engineering.

CONCLUSION

This study demonstrates human BSM cells rapidly migrate into and remodel an electrospun fibrinogen-PDO composite scaffold with deposition of native collagen. While there is still much work to be done, these initial findings indicate there is tremendous potential for electrospun fibrinogen-PDO composite structures as urologic tissue engineering scaffolds with the ultimate goal of producing an implantable acellular product that would promote cellular in-growth and *in situ* tissue regeneration.

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