Electrospun Polydioxanone, Elastin, and Collagen Vascular Scaffolds: Uniaxial Cyclic Distension

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ABSTRACT

The development of vascular grafts requires the matching of material and viscoelastic properties to those of native artery. The hypothesis of this study was to subject electrospun tissue engineering scaffolds composed of polydioxanone, elastin, and collagen to cyclic loading in order to quantify the hysteretic properties, uniaxial tensile mechanical properties of conditioned scaffolds, and stress relaxation properties over a period of 400 cycles when compared to ePTFE, one of the most popular vascular prosthetic materials, and decellularized pig artery. In the electrospun graft, polydioxanone would provide a mechanical backbone, providing tensile support and preventing vessel rupture; while the elastin would provide elasticity and collagen would provide bioactivity (promote regeneration *in vitro/in situ*).

INTRODUCTION

Blood vessels are continuously subjected to stress and strain in the circumferential and longitudinal directions. In the circumferential direction, the pulsatile nature of blood pressure induces a cyclic stretch on the arterial wall. Each layer in the wall plays a significant mechanical role to its proper functionality. *In vivo* studies and clinical observations have shown that decreased values of tensile and shear stress in surgically injured vessels are correlated with activation of cell proliferation and extracellular matrix (ECM) production, leading to vessel occlusion [1]. A decrease in tensile properties is not just a change in the circumferential direction, but a multi-directional change. As a consequence, compliance, hoop stress, and overall hysteretic properties can be affected. These changes can affect cell and fiber orientation.

Several studies have demonstrated conformational changes in the properties of smooth muscle cells (SMC) seeded onto synthetic and natural polymeric constructs due to mechanical stimulation [2-4]. Seliktar et al. observed an increase in matrix metalloproteinase-2 (MMP-2) in response to cyclic stretch of a collagen gel seeded with SMCs [5], and that dynamic conditioning of the cell seeded gel resulted in the alignment of cells and fibers [6]. Isenberg and Tranquillo found that long-term cyclic distension of a collagen gel enhanced mechanical properties and increased production of elastin [7], a primary protein responsible for minimal energy loss. Others have looked at the role of growth factors in response to cyclic loading. In particular, transforming growth factor-beta (TGF-β) was observed to increase under mechanical stimulation, which thereby increased ECM production [8]. Fibroblast growth factor-2 (FGF-2), a potent chemokine signal for SMC migration and proliferation, was shown to increase as well due to mechanical stimulus [9]. While collagen gels promote cell adhesion and proliferation, they lack mechanical stability and require a supportive sleeve to undergo cyclic loading.

Synthetic polymers, which have increased mechanical properties when compared to collagen gels, have been studied as well. Webb et al. seeded fibroblasts onto flat polyurethane constructs and cyclically stretched them, finding that fibroblast DNA and mRNA increased compared to static cultures [10]. Kim et al. used polyglycolic acid (PGA) and found SMC alignment and proliferation due to cyclic loading [11]. Therefore, mechanical stimulation of a combination of both synthetic and natural polymers could prove to be beneficial under dynamic conditions.

Electrospun hybrid scaffolds containing synthetics, collagen, and elastin have already been produced and their biocompatibility have been established [12]. Our lab has already demonstrated that blending synthetics and natural polymers is both feasible and beneficial from the mechanical and cellular perspective [13-17]. Interestingly, dynamic compliance testing of polydioxanone (PDO) and elastin blended scaffolds demonstrated positive results comparable to native artery [14, 18]. However, we have yet to observe the amount of energy dissipation, hysteresis, which occurs in these grafts under physiological conditions. Vawter et al. have clearly demonstrated the importance of fully understanding the mechanics of a tissue under different loading conditions including hysteresis [19]. Several other studies have researched the hysteretic behavior of tissues and polymers under uniaxial [20-22], biaxial [23], and computational methods [24]. However, there have been no studies to date, which have addressed the uniaxial cyclic mechanics of an electrospun matrix.

One of the primary goals in vascular tissue engineering of small diameter grafts (< 6 mm), from the mechanical point of view, is to design grafts with material properties nearly identical to those of the host vessel. Only then is there no compliance mismatch at the anastomosis [21]. Therefore, the hypothesis of this study was that the fabrication of an electrospun tissue engineering scaffold composed of PDO, elastin, and collagen would experience a reduced amount of energy loss (less hysteresis) when compared to ePTFE, one of the most popular vascular prosthetic materials.

MATERIALS & METHODS

Collagen Extraction

Collagen type I was extracted from calf skin corium through an acetic acid based process previously described [25]. Briefly, tissue is homogenized, suspended in acetic acid, and subsequently purified via a series of dissolutions, precipitations, and dialyses [26].

Scaffold Preparation

Scaffolds were blended in ratios of 100:0, 70:30, and 50:50, by volume (PDO:elastin), and 50:25:25 and 45:45:10 (PDO:elastin:collagen) and dissolved in 1,1,1,3,3,3 hexafluoro-2-propanol (TCI America). PDO (Ethicon, Inc.) was dissolved at a concentration of 100 mg/ml, soluble elastin from bovine neck ligament (Elastin Products Co., Inc.) at a concentration of 200 mg/ml, and collagen type I at a concentration of 70 mg/ml. These solutions were then inserted into a plastic 5 ml Becton Dickinson syringe with a blunt tip 18 gauge Becton Dickinson PrecisionGlide® needle. The syringe and needle were placed in a KD Scientific syringe pump to be dispensed at a rate of 4 ml/hr. The solutions were electrospun onto a flat rotating mandrel (2.5 cm wide x 10.2 cm long x 0.3 cm thick) to produce a flat sheet with random fiber orientation. Samples were then cross-linked using 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide (EDC, Fluka Biochemika) or genipin (Wako Pure Chemical Industries, Ltd.) [27] for hysteresis and uniaxial tensile testing. Each sample was electrospun with an applied voltage of 22 kV at a distance of 10-13 cm from the needle tip to the mandrel and a rotational speed of 500 revolutions per minute (rpm).

Scanning Electron Microscopy

Scaffold characterization was performed using scanning electron microscopy on small pieces cut from the electrospun mats (SEM, JEOL JSM-820 JE Electron Microscope). SEM images were digitized with a Hewlett-Packard Scanjet 5550c flatbed scanner and analyzed with ImageTool 3.0 software (Shareware provided by UTHSCSA). Characterization included determining the average fiber diameter for the electrospun structure by taking the average of 60 measurements chosen randomly from across the image. For all of the measurements made from the SEM images, calibration of the ImageTool software was done with the scale bar on each image. The sample size for each measurement was n = 60.

Hysteresis and Stress Relaxation

Hysteresis was performed uniaxially on electrospun samples in the direction of rotation, and ePTFE and decellularized pig artery (DPA) in the circumferential direction. All samples were soaked in phosphate buffered saline (PBS) at 37°C for 24 hours prior to testing. "Dog-bone" shaped samples were punched from the electrospun mat (2.75 mm wide at their narrowest point with a gage length of 7.5 mm) and tested on a MTS Bionix 200 testing system with a 50 N load cell (MTS Systems Corp.). Samples were subjected to a preload of 0.1 N prior to undergoing 400 cycles of extension to 5% strain and relaxation back to 3% strain at a rate of 10 mm/min and a frequency of 0.4 Hz. Hysteresis calculations were reported as the total area between the loading and unloading curves divided by the area under the loading curve using UTHSCSA Image Tool version 3.0. Stress relaxation was calculated using the difference in peak force values between the 1st and 400th cycles. The sample size for each specimen was n = 6.

Uniaxial Tensile Testing

Uniaxial tensile testing was performed on all hydrated pre-conditioned and non-conditioned electrospun samples, ePTFE, and DPA. "Dog-bone" shaped samples were punched from the electrospun mat (2.75 mm wide at their narrowest point with a gage length of 7.5 mm) and tested on a MTS Bionix 200 testing system with a 50 N load cell (MTS Systems Corp.) and an extension rate of 10.0 mm/min. Tangential modulus, peak stress, and strain at break were calculated using TestWorks version 4.0. The sample size for each specimen was n = 6.

Statistical Analysis

All statistical analyses were performed utilizing JMP®IN software version 4.0.3 (SAS Institute, Inc.). The data collected was analyzed using a Kruskal-Wallis one-way analysis of variance (ANOVA) and then subjected to a pair-wise multiple comparison procedure (Tukey-Kramer Test). The *a priori* alpha value was set at 0.05.

RESULTS & DISCUSSION

Fiber Diameter

In order to better understand the morphology and characteristics of each scaffold, it is necessary to observe fibrous differences from the micron scale as well. SEM demonstrated that dry PDO, elastin, and collagen scaffolds were successfully electrospun into a random non-woven mats, *Figure 1*. Fiber diameter results of 100:0, 70:30, 50:50, 50:25:25, and 45:45:10 were 1.5 ± 0.4, 0.9 ± 0.4, 1.3 ± 0.5, 1.8 ± 0.9, and 2.6 ± 1.4 μm , respectively.

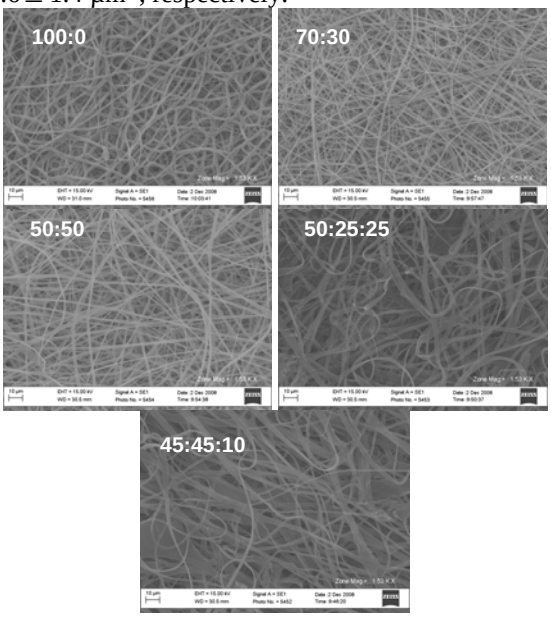

FIGURE 1. SEMs for dry blends of 100:0, 70:30, 50:50, 50:25:25, and 45:45:10 scaffolds. All micrographs are taken at 1500x.

Hysteresis

The majority of artery's mechanical stability is afforded by collagen, which serves as the mechanical backbone of the tissue by providing tensile support and preventing vessel rupture. However, arterial elasticity and the ability to recover from pulsatile deformations is provided by elastin fibers, allowing for energy-efficient transmission of blood flow and damping of excessive pressure fluctuations [28]. The higher the amount of energy loss, the less effective the vascular graft. An effective way of measuring energy loss is to calculate a materials hysteresis.

For our experiment, we used blends of PDO, elastin, and collagen type I and cross-linked them with two different mechanisms known for their low cytotoxicity [29-31]. *Figure 2* displayed an overall low average energy loss, ranging from 1.4 – 4.9%, where ePTFE had the highest energy loss and 70:30 genipin cross-linked had the lowest. The DPA was not significantly different from any material except ePTFE. Only one ratio, 70:30, was significantly different ($p < 0.05$) between cross-linkers, whereby genipin showed a lower percent hysteresis than EDC.

Although not significantly different, 50:50 genipin cross-linked displays a higher average percent hysteresis than 50:50 EDC cross-linking, displaying again a dependence on the cross-linking reagent. Between the blends, 70:30 genipin cross-linked was not significantly different from 50:50 genipin cross-linked, but their EDC counterparts were different ($p < 0.05$). This data indicated that cross-linking with EDC may have had a significantly higher effect on energy loss and elastic recoil than genipin as a higher amount of elastin was added to the scaffold.

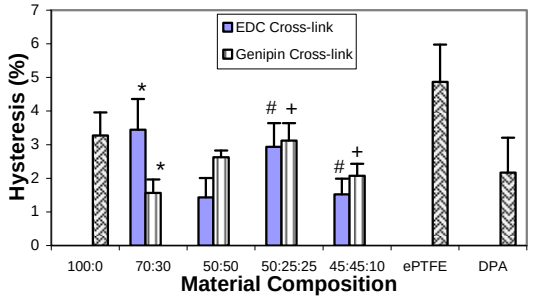

FIGURE 2. Hysteresis percent values for blends of 70:30, 50:50, 50:25:25, 45:45:10 cross-linked with EDC (solid) and genipin (vertical stripe), and 100:0, ePTFE, and DPA (diagonal pattern). The asterisk, number sign, and positive sign indicates that pairwise statistically significant differences exist between 70:30 EDC and genipin, 50:25:25 EDC and 45:45:10 EDC, and 50:25:25 genipin and 45:45:10 genipin, respectively (*p < 0.05).

When collagen was introduced into the blend, there was an increase in energy loss as more collagen was added to the construct. EDC cross-linked scaffolds including 25% collagen and 10% collagen were significantly different ($p < 0.05$) from each other. Therefore, the addition of collagen increased the scaffolds energy loss, suggesting elastin as the dominating factor for smaller percent hysteresis values. Additionally, the scaffold with a higher amount of elastin content (from 25% to 45%) was significantly lower ($p < 0.05$) when cross-linked with EDC. This further indicates that the choice of cross-linking reagent can affect the overall energy loss of the construct. It is evident that PDO and collagen share a mechanical similarity as a controlling factor in the scaffolds energy loss characteristics until a threshold amount of elastin is reached.

Uniaxial Tensile Testing

Uniaxial tensile testing reported peak stress, tangential modulus, and strain to break for all samples tested. Previous data indicated a difference in material properties when using EDC and genipin as cross-linkers [21]. The purpose of this section was to determine if the pre-conditioning experienced by each material changes its ultimate tensile properties over a period of 400 cycles in comparison to their un-conditioned counterparts. These results are displayed in *Figure 3 and Figure 4*.

Uniaxial tensile test results demonstrated an overall decrease in peak stress, tangential modulus, and strain to break as elastin and collagen content are increased, which is consistent with our previous findings.

Conditioning studies involving smooth muscle cells have shown increases in peak stress and scaffold stiffness through increased collagen deposition [8, 32-34]. Therefore, we wanted to see if the scaffold's material properties changed in response to mechanical strains placed on the construct during cyclic loading, i.e. fatigue or material breakdown. The results indicated that peak stress was significantly increased ($p < 0.05$) in 70:30 EDC, tangential modulus was significantly increased ($p < 0.05$) in 100:0 EDC, and strain to break was significantly decreased ($p < 0.05$) in both DPA and ePTFE. This data suggested that conditioning of the scaffolds without cellular interaction does not significantly change the scaffolds overall material properties especially at high elastin content. Similar results were found in *Figure 4*.

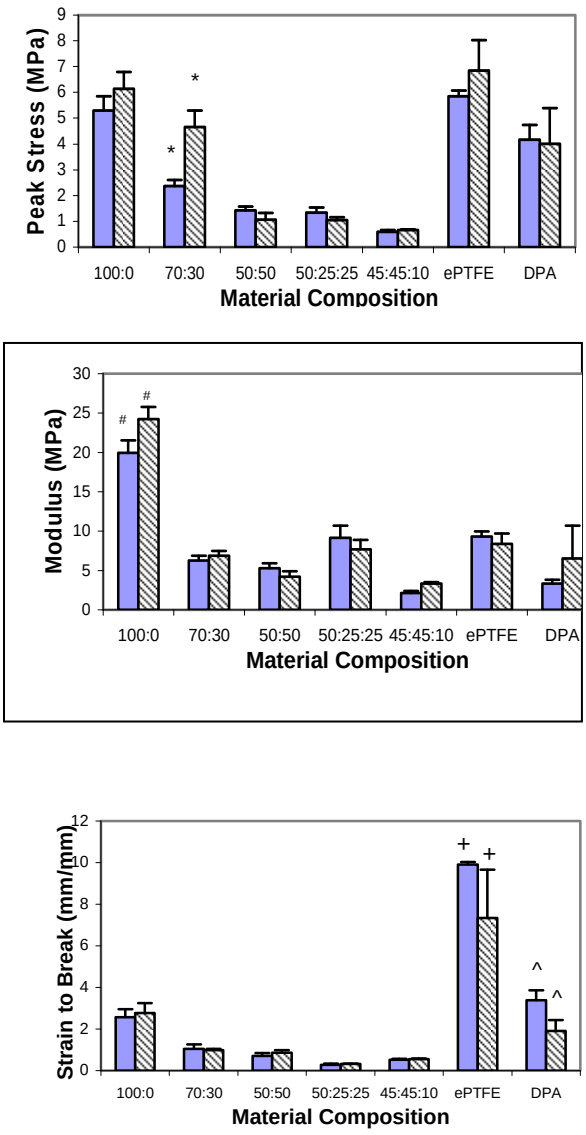

FIGURE 3. EDC cross-linked uniaxial tensile test values for peak stress, tangential modulus, and strain to break for PDO, elastin, and collagen blends, ePTFE, and Pig Artery non-conditioned (solid) and pre-conditioned (striped). The asterisk, number sign, positive sign, and caret indicates that pairwise statistically significant differences exist between 70:30, 100:0, ePTFE, and DPA non-conditioned and pre-conditioned scaffolds, respectively (*p < 0.05).

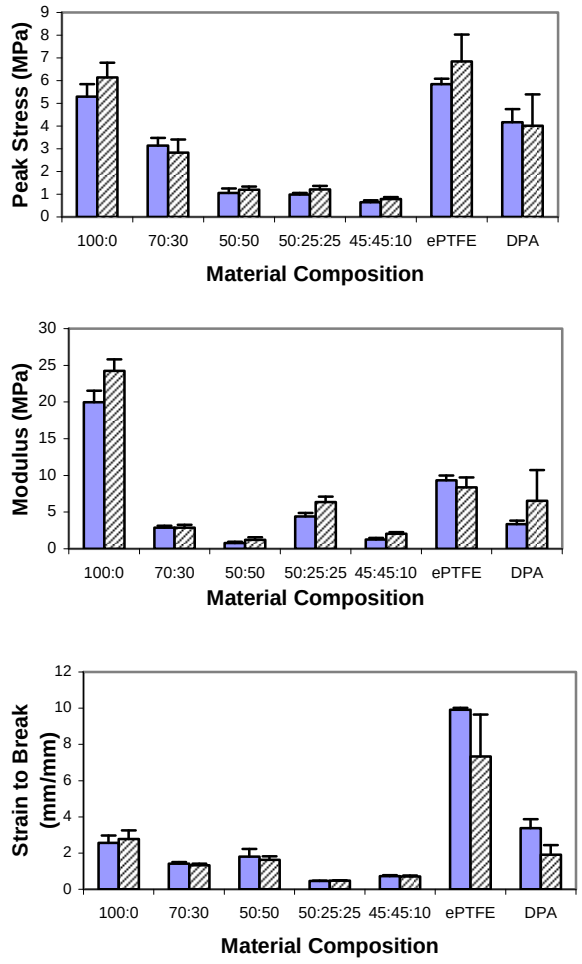

FIGURE 4. Genipin cross-linked uniaxial tensile test values for peak stress, tangential modulus, and strain to break for PDO, elastin, and collagen blends, ePTFE, and Pig Artery non-conditioned (solid) and pre-conditioned (striped). No significant differences existed between non-conditioned and pre-conditioned genipin scaffolds (*p < 0.05).

Genipin cross-linked PDO:elastin:collagen scaffolds showed no significant differences amongst all material properties. This demonstrated that when genipin was used to cross-link elastin the scaffold's integrity remained intact throughout all parameters. Once again suggesting that conditioning of the scaffold did not contribute to a change in material characteristics.

Therefore, pre-conditioned EDC and genipin cross-linked scaffolds displayed no consistent changes in all three uniaxial tensile parameters, which would imply that the choice of cross-linking reagent had no ultimate effect on the material properties. This indicated that the scaffolds would retain their mechanical integrity upon initial implantation *in vivo*, and most changes thereafter would be due to hydrolysis of the PDO and cellular breakdown of the elastin and collagen.

Stress Relaxation

Stress relaxation provides a means to look at the viscoelastic properties of a particular scaffold, whereby the cyclic or static strain imposed on the construct remains constant over a period of time. In this case, as the scaffold undergoes continuous loading and unloading, there is a rapid drop in force followed by levelling to a relatively constant value. The measurement of this relaxation allows us to predict how the scaffold will behave *in vivo* under pulsatile pressures. Stress relaxation values are displayed in *Figure 5*.

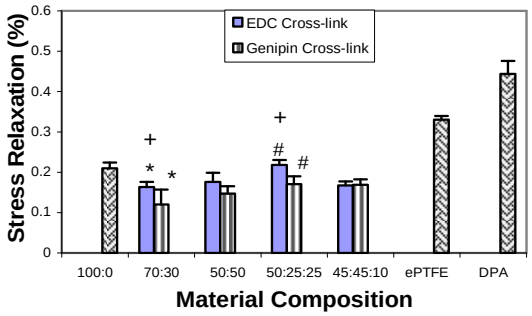

FIGURE 5. Percent stress relaxation of PDO, elastin, collagen scaffolds, ePTFE, and DPA. EDC cross-linked scaffolds (solid), genipin cross-linked scaffolds (vertical stripes), and PDO, ePTFE, and pig artery (diagonal pattern). The asterisk, number sign, and positive sign, and caret indicates that pairwise statistically significant differences exist between 70:30 EDC and genipin, 50:25:25 EDC and genipin, and 70:30 and 50:25:25 EDC scaffolds, respectively (*p < 0.05).

According to the data, DPA had the largest percent stress relaxation followed by ePTFE. Each was significantly different ($p < 0.05$) from all groups. Amongst the blended scaffolds, 50:25:25 EDC and 70:30 EDC were significantly different ($p < 0.05$) from their genipin counterparts. There was also a significant increase ($p < 0.05$) in stress relaxation of EDC and geinpin cross-linked scaffolds as a higher amount of collagen was added to the scaffold, whereby 70:30 is different ($p < 0.05$) from 50:25:25. However, when a higher amount of elastin was added (50:50 and 45:45:10), there was no significant increase in stress relaxation.

The large difference in stress relaxation between the DPA and blended scaffolds partially demonstrated the disparity in viscoelastic properties. This is represented by *Figure 6*, where DPA starts at 0.15 N and ends at 0.08 N and 70:30 genipin starts at 0.19 N and ends at 0.17 N. Although the graph displays the disparity between the blended materials and DPA, it does show a similarity, whereby after 400 cycles both DPA and blended scaffolds reach a constant tension. This represents how the graft would behave after initial implantation *in situ*. However, graft hysteretic properties would change over time due to hydrolytic and proteolytic degradation of the polymer blend.

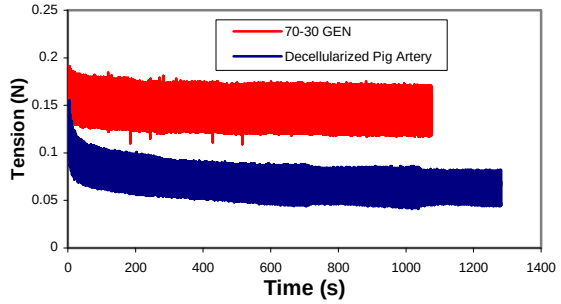

FIGURE 6. Stress relaxation curve over a 400 cycle period. Red represents the 70:30 PDO:elastin cross-linked with genipin. Blue represents DPA.

DPA consisted of natural proteins, which included: collagen I and III, elastin, and proteoglycans. In particular, proteoglycans are known to play active roles in a tissues cellular and viscoelastic response [35]. Additionally, upon DPA extraction from the pig longitudinal tension and pulsatile pressure was no longer acting creating an un-stressed state for the artery. This un-stressed state would highly contribute to the stress relaxation seen here as fibers are elongated back to a stressed state. Protein structure could also play an active role in stress relaxation with crimped collagen fibers and an amorphous elastin structure.

CONCLUSION

The results of this study showed that both the addition of a large amount of elastin and the proper choice of cross-linking reagent was required to create a significant difference in the amount of energy lost from electrospun PDO scaffolds. Uniaxial tensile testing displayed no consistent differences between pre-conditioned and non-conditioned scaffolds, indicating that the material properties of the constructs remains constant. Finally, stress relaxation results showed that the ECM of pig femoral artery, a common model for vascular graft implantation, stress relaxed significantly more than any of the synthetic polymers tested. Previous testing has shown these electrospun scaffolds to be both bioactive and highly compliant, and the result of this study only increases their potential for use as a bioresorbable vascular graft. Future work involves compliance characterization at physiological pressures, seeding with cells, followed by development and pre-conditioning in a bioreactor under simulated physiological conditions.

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