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ELECTROSPINNING COLLAGEN AND ELASTIN: PRELIMINARY VASCULAR TISSUE ENGINEERING

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TABLE OF CONTENTS

1. ABSTRACT

Significant challenges must be overcome before the true benefit and economic impact of vascular tissue engineering can be fully realized. Toward that end, we have pioneered the electrospinning of micro- and nano-fibrous scaffoldings from the natural polymers collagen and elastin and applied these to development of biomimicking vascular tissue engineered constructs. The vascular wall composition and structure is highly intricate and imparts unique biomechanical properties that challenge the development of a living tissue engineered vascular replacement that can withstand the high pressure and pulsatile environment of the bloodstream. The potential of the novel scaffold presented here for the development of a viable vascular prosthetic meets these stringent requirements in that it can replicate the complex architecture of the blood vessel wall. This replication potential creates an "ideal" environment for subsequent *in vitro* development of a vascular replacement. The research presented herein provides preliminary data toward the development of electrospun collagen and elastin tissue engineering scaffolds for the development of a three layer vascular construct.

2. INTRODUCTION

According to recent American Heart Association statistics, cardiovascular disease accounted for 39.4% (2.4 million people) of all deaths in 2000 in the United States alone (1). Over half of these were due to coronary artery disease primarily caused by arteriosclerosis. Arteriosclerosis is a vascular disease that is characterized by thickening of the arterial wall and subsequent decrease in the arterial lumen leading to eventual decrease or loss of circulation distal to the disease site. This disease resulted in an estimated 519,000 coronary bypass procedures in 2000 (1). Peripheral vascular disease also affects upwards of 12 million Americans with significant associated mortality. Once blood flow is compromised, vascular bypass is often the only option to restore blood flow to tissues distal to the restriction or blockage.

The blood vessels of the human body make up a complex organ system whose primary function includes the delivery of oxygen and nutrients to the tissues and organs of the body and removal of their respective metabolites for clearance or re-oxygenation. The vascular wall architecture is highly intricate and imparts unique biomechanical properties that put forth tremendous engineering challenges for the development of a "living" tissue engineered vascular replacement that can withstand the pulsatile nature, high pressure, and high flow rate of the bloodstream. The arteries are composed of three distinct layers or tunics which surround a hollow core or lumen through which blood flows. The innermost layer is called the intima and is composed of a single layer of endothelial cells lining an underlying basement membrane rich in collagen type IV and elastin; the integrity of the intima is crucial to avoidance of platelet activation, as this layer provides a smooth surface over which blood flows. The middle layer or media is usually the thickest of the three

layers and is composed of multiple layers of smooth muscle cells (SMCs) within a surrounding extracellular matrix (ECM) composed of collagen types I and III, elastin fibers (approximate ratio: 44% Type I, 44% Type III, and 12% elastin) (2-4), and various proteoglycans. The SMC and ECM networks are arranged in concentric layers at specific pitches within the arterial system to form a complex composite structure (2). The outermost layer is the adventitia and is composed of fibroblasts and randomly arranged collagen (essentially 100% type I) ECM. The collagens impart tensile strength to the vessel wall, while elastin contributes to its elastic properties.

Current bypass grafts fall into two categories: biological and synthetic. Typical biological grafts are autologous saphenous vein, autologous internal mammary artery, venous homografts, and arterial xenografts. Synthetic polymeric vascular grafts were initially developed to address three weaknesses of biological grafts: 1) inability to bypass or replace large diameter vessels, 2) unsatisfactory performance of non-autologous grafts (xeno- and homografts), and 3) unavailability of autologous grafts (5). Of the countless synthetic materials evaluated over the years, expanded polytetrafluoroethylene (e-PTFE) and woven or knitted polyethylene terephthalate (PET) fibers (Dacron) have proven to be satisfactory in terms of medium (6-10 mm internal diameter (ID)) and large (> 10 mm ID) vascular prosthetics, respectively (6). Unfortunately, these or any other synthetic materials have not proven successful as small diameter vascular prosthetics. Thus, the pursuit for the "ideal" small diameter (< 6 mm ID) vascular prosthetic continues.

The search for the "ideal" small diameter vascular prosthetic has been compared to the "search for the Holy Grail" (7). The ideal vascular prosthetic must have the following performance characteristics: ease of handling, suture retention, ease of suture placement, flexibility with kink resistance, biocompatibility (non-thrombogenic), durability even after implantation and association with surrounding connective tissue, compliance matching that of the native artery, and resistance to aneurysm formation (8). In addition, the graft must be easily manufactured, economical, easily stored, and available in a variety of sizes (lengths and diameters) (8).

The concept for the synthetic vascular prosthesis used in bypass procedures is credited to Voorhees, Jaretski, and Blakemore, who presented the first published hypothesis on synthetic tubes as replacements for natural blood vessel deficits in 1952 (9). The first clinical applications of this hypothesis were published in 1954 and showed that the prosthetic tubes could be used in the arterial setting (10). This publication initiated a race to develop the best material for use as a prosthetic vascular graft. Little progress has been made since that time.

Malcolm Herring introduced the first generation of tissue engineered small diameter vascular prosthetics in 1978 (11). Briefly, this effort involved the seeding of autologous endothelial cells (ECs) on the luminal surface of a polymeric vascular prosthetic. Dr. Herring's hypothesis was simple: by initiating the establishment of an autologous EC lining at the blood-synthetic material interface of a vascular prosthesis, a normal endothelium-lined surface (neointima) would subsequently evolve over the surface, disguising the synthetic material (11). Unfortunately, though this generation of grafts was not successful due to non-confluent EC coverage, the potential of this therapy was apparent. As a result of these initial efforts, researchers have used various techniques, including many variations on Dr. Herring's original ideas, to develop a complete luminal EC lining in pursuance of a clinically acceptable small diameter vascular prosthesis. These efforts can be grouped into three categories: gravitational settling (11-15), hydrostatic seeding (16-17), and electrostatic seeding (18-23). To date, no attempts have reached the goal. Lack of an appropriate ECM and other sub-endothelial components likely led to the failure of these endothelial cell seeding techniques.

The second generation of tissue engineered small diameter vascular prosthetics was even more of a hybrid construct (24-30). These grafts consisted of a variety of natural materials (ECM, collagen, SMCs, ECs) with a synthetic material incorporated as a structural support. The simplest of these models used the synthetic material as the adventitial layer as the main structural support. The medial component lining the synthetic support was composed of a collagen gel lattice (type I) impregnated with SMCs. Finally, an EC monolayer was added as a neointima lining the medial layer (24).

The current state of the art and third generation of tissue engineered small diameter vascular prosthetics consist completely of natural materials (collagen, fibroblasts (FBs), ECs, and SMCs) (24, 31-32). The first of these completely tissue engineered vascular constructs was introduced in 1986 by Crispin Weinberg and Eugene Bell (24). This construct was developed around an annular mold or cast incorporating collagen, culture media, and vascular cell lines (SMCs, FBs, and ECs). The first models did not incorporate any synthetic material for structural support between the medial and adventitial layers and were highly distensible and failed during burst strength testing at low intraluminal pressures (< 10 mm Hg) (24).

L'Heureux *et al.* introduced their first completely tissue engineered vascular construct in 1993 (31). In this model, the investigators used a collagen gel containing SMCs around a glass mandrel support to create the medial layer of the construct. The adventitia was then created on the outside of the medial layer in the same fashion except with FBs instead of SMCs. The intima was developed by subsequent EC transplantation on the luminal surface of the media. This construct resembled a native artery in terms of composition, but it had poor mechanical properties and could not withstand arterial intraluminal pressures. L'Heureux *et al.* introduced the latest version of a tissue engineered vascular construct in 1998 (32). For this model, SMCs were grown to confluence over a 30 day culture period in the presence of sodium ascorbate, which allowed for excessive collagen production. The resulting SMC sheets were peeled from the culture flask surface and

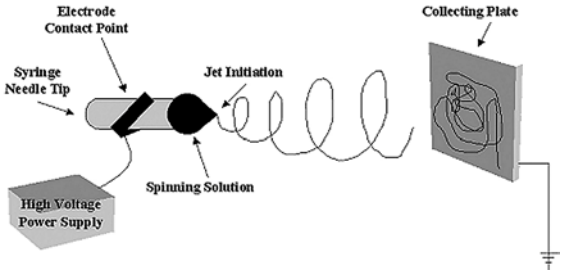

Figure 1. A schematic of an electrospinning system for the production of micro- to nano-scale fibers from a solution and subsequent tissue engineering scaffolds.

wrapped around a tubular support mandrel to form the concentric layered cylinders of the media. The adventitia was formed in a similar manner with FBs. Again, the intima was formed by EC transplantation within the construct lumen. This construct was a success in that it was the first to withstand intraluminal pressures greater than physiologic pressures. Both models presented by L'Heureux *et al.* present structurally supportive collagen scaffolding with random 3-D orientation that does not mimic the native arterial structure. In other words, the cellular components contained within this matrix must reorganize the ECM to accommodate the forces encountered upon implantation. Thus, questions of long-term structural integrity are raised due to collagenase activity (ECM remodeling) (24,32).

Others have tried to incorporate biodegradable polymers such as poly(glycolic acid) (PGA)/poly(lactic acid) (PLA) hybrids as a scaffolding to engineer vascular constructs (33-37). However, there are two main complications associated with utilizing biodegradable polymers in this model. First, as these materials degrade, the major by-products are glycolic acid and lactic acid. These substances are produced naturally in the body and are, therefore, assumed to be safe and nontoxic. However, there is concern about high local concentrations in the microenvironment of the degrading scaffold. If levels are too high, pH in the local environment decreases; this is toxic to the cells and can induce an inflammatory response. Thus, care must be taken to ensure proper by-product removal from the tissue-engineered construct when using biodegradable materials. The second possible complication of vascular prostheses created with biodegradable materials is aneurismal formation as the bioabsorbable structural materials are degraded. This aneurismal formation is due to an improper balance between polymer degradation and native tissue remodeling. Overall, the degradation (loss of strength) of the biodegradable polymer acting as the structural support degrades too quickly prior to the development of a fully developed ECM capable of maintaining arterial pressure leading to the aneurysm.

To date, investigators have developed materials such as collagen, PLA, and PGA for use in scaffolding or synthetic matrix construction with improved, yet frequently unacceptable, clinical results. Thus, the race for the "Holy Grail" has entered its fifth decade without success. Recently, a revisited polymer processing technique called electrospinning has entered the race and shows great promise. In theory, the method of electrospinning of natural polymers provides a simple method to achieve the necessary properties of a successful tissue engineered vascular prosthesis (38).

Briefly, electrospinning is accomplished by inducing a large electric potential on the order of 15 to 30 kilovolts DC in a polymer solution or melt. By then placing an oppositely charged target some distance from the charging polymer solution, a static electric field is created. As the field strength grows, the charge separation between the two overcomes the surface tension of the solution and a thin jet is ejected from the polymer solution reservoir. This jet whips around in space as it travels toward the target. This whipping action causes further thinning of the jet and evaporation of the solvent. By the time the jet reaches the target, a dry fiber is collected in the form of a non-woven mat.

The basic elements of a laboratory electrospinning system are a high voltage power supply, ground electrode that acts as a collector plate or target, source electrode, and polymer spinning solution. The collector can be a flat plate, wire mesh, or, in more sophisticated modifications, a rotating metal drum or plate on which the produced polymer fiber is wound. The sample can be confined in any material formed into a nozzle with various tip bore diameters such as a syringe needle. A schematic of the electrospinning apparatus is shown in Figure 1.

With these methods, collagen and elastin have been electrospun to produce nano- to micro- diameter fibers that mimic the molecular and structural properties of the ECM (38,39). Here, we describe the development of a three-layered vascular construct consisting of a separate media and adventitia composed of electrospun collagen and elastin seeded with SMC and FBs that are combined to form the structural component of which the lumen is seeded with ECs to form a complete three-layered vascular construct. This biomimicking graft could revolutionize vascular tissue engineering. The interaction between cells and the ECM play an important role in multiple biological processes, including, but not limited to, cell motility, proliferation, metabolism, morphology, differentiation, and matrix deposition/remodeling (40). The scaffold architecture presented here has the potential to allow these interactions and consequent natural tissue development to proceed uninterrupted with minimal additional scaffold regeneration/reorganization requirements compared to alternative scaffold models presented in the literature. This could result in a viable tissue engineered vascular prosthetic mimicking the complexity of the native arterial structure.

3. METHODS

3.1. Electrospinning

For this study, collagen (Type I calf skin and Type III human placenta) and elastin from ligamentum nuchae were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol

Figure 2. Representative photograph of 2 mm and 4 mm ID collagen scaffolds with an assembled composite structure shown in the insert at the lower left (photographs taken before crosslinking and cell seeding).

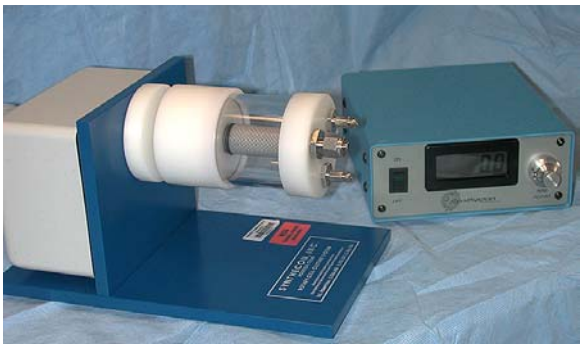

Figure 3. Photograph of the general rotary cell culture system (RCCS), "bioreactor", (Slow Turning Lateral Vessel (STLV); Synthecon, Inc.) used in preliminary studies to create the microgravity culture environment. The bioreactor (left) is placed in a standard incubator during culture, while the controller (right) is maintained outside the incubator.

(HFP). All components were obtained from Sigma-Aldrich Chemical Company. Specific concentrations will be noted in the cases below. The polymer solutions were then loaded into a 5.0 ml syringe and placed in a syringe pump for metered dispensing at a rate between 2-8 ml/hr. The positive output lead of a high voltage supply (Spellman CZE1000R; Spellman High Voltage Electronics Corp.) set to 20-25 kV was attached to a blunt 18 gauge needle on the syringe as depicted diagrammatically in Figure 1. Scaffolds were electrospun as flat mats for structural characterization and as tubes to serve as components of the three-layered construct. For mats utilized for characterization, fibers were electrospun onto a stainless steel grounded target (1" Wide × 4" Long × 1/8" Thick) that was placed 10 cm from the needle tip. For three layered construct fabrication as depicted in Figure 2, 2 and 4 mm diameter x 12 cm long stainless steel mandrels were used to create seamless tubes with 2 and 4 mm internal diameters (ID). The targets were rotated at approximately 500 revolutions per minute (RPM) to ensure an even coating; this rate, however, is not high enough to impart a large degree of fiber alignment to the resulting constructs.

3.2. Scaffold Characterization

Following flat scaffold fabrication, samples were cut from each mat for characterization. A representative sample from each was sputter-coated with gold (Model 550; Electron Microscope Sciences) for scanning electron microscopy (SEM) (JSM-820 JE Electron Microscope; JEOL) and photographed at 400 to 8,000X magnification. The images were digitized with a flat bed scanner (Scanjet 6200C; Hewlett-Packard) and analyzed with ImageTool 3.0 (shareware provided by the University of Texas Health Sciences Center at San Antonio) to determine the average fiber diameters and pore areas in the various scaffolds. For fiber diameter and pore area, the average and standard deviation were calculated from 60 measurements per micrograph. All measurements were calibrated using the scale on the micrograph as a reference to avoid errors in calculating the magnification after digitization. For pore area, a subjective approximation of surface pore area was completed from the SEM micrographs by tracing the outline of the pores (41).

3.3. Cell Lines and Cell Culture

Human cell lines (human umbilical vein endothelial cells (ECs), aortic smooth muscle cells (SMCs), and dermal fibroblasts (FBs)) were purchased from Clonetics Corporation (San Diego, CA). The cells were cultured in a 37°C incubator at 5% CO_2 and constant humidity utilizing standard cell culture protocol and culture media. The culture media was composed of 67% Dulbecco's Modified Eagle Medium (DMEM; high glucose with L-glutamine, sodium pyruvate and pyridoxine hydrochloride), 22% F-12 Nutrient Mixture, 10% fetal bovine serum, and 1% Penicillin-Streptomycin (10,000 Units/ml each). All media components were purchased from Gibco BRL Life Technologies. All cell culture procedures were performed under aseptic conditions.

3.4. Scaffolding Crosslinking, Disinfection, Seeding, and Culture

After scaffolding fabrication, the electrospun constructs were crosslinked in glutaraldehyde vapor for 15 min at room temperature and then rinsed through three changes of sterile phosphate buffered saline. The crosslinked scaffoldings were then disinfected by soaking in 70% isopropyl alcohol for 15 minutes followed by three 5-minute rinses in agitating room temperature sterile water. The scaffoldings were seeded in a seeding chamber which consisted of a hollow stainless steel cylinder with an inside diameter of 6.4 mm and a total length of 7 cm (total volume: 3.5 ml) with the open ends sealed with caps. Within the chamber, the scaffold was fully immersed in cell media (10% serum) containing a predetermined cell type and density. The seeding chamber was placed in an apparatus that rotated the chamber at a constant 1/8 RPM. To maintain the proper temperature during the seeding procedure, the rotating chamber was placed in an incubator at 37°C and 5% CO_2.

Immediately after seeding, the scaffolds were transferred to a 55 ml capacity slow turning lateral vessel (STLV) on a rotating cell culture system (RCCS) as shown in Figure 3. These devices are designed to maintain cells

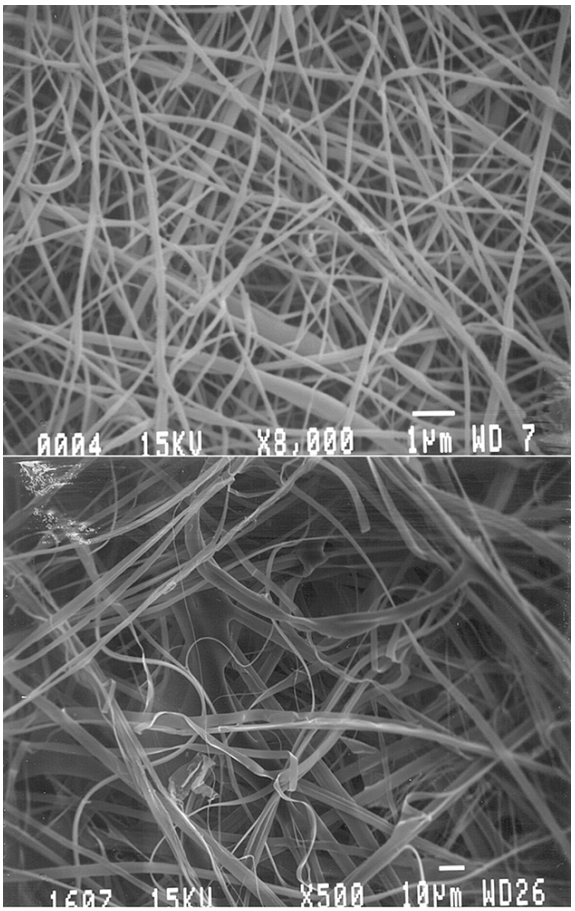

Figure 4. (Top). Electrospun type I collagen (calf skin) illustrating 100 ± 40 nm fiber diameter (Magnification 8,000X with the inserted scale bar at 1.0 micron), (Bottom). Electrospun type I collagen (calf skin) illustrating 4.62 ± 2.09 µm fiber diameter (Magnification 500X with the inserted scale bar at 10.0 microns).

and tissues in continuous free fall, simulating microgravity while providing a low shear environment that also allows high mass transfer of nutrients (42). The buoyant environment created by these devices promotes three-dimensional cell infiltration, thereby fostering cell-cell and cell-matrix contacts and the resulting formation of large cell masses. The proper rates of rotation to provide the optimal environment, neutral buoyancy (structure suspension in "free fall"), were determined experimentally. Over the culture period, cell proliferation increases the cell density within the scaffold (increased tissue density), thereby requiring a greater chamber rotation rate to maintain the tissue in "free fall". During culture in the RCCS, the media was refreshed every third day via access ports on the reactor. The chamber was periodically degassed to prevent bubbles from placing shear stress on the cells.

3.5. Preliminary Fabrication of a Three Layered Vascular Construct

In an attempt to closely mimic the physiological protein distribution of a small diameter blood vessel ECM (2,3), a solution of 80:20 collagen type I:elastin was produced at a concentration of 0.083 g/ml in HFP and electrospun onto a 4 mm ID tubular mandrel. As in a native vessel wall, this layer functions as the adventitial layer of the prosthetic to be seeded with FBs and SMCs. The tube was tied closed at both ends with silk suture, its outer surface was seeded with FBs ($5x10^5$ cells/ml), and it was then placed in the RCCS. After four days in culture, the tube was removed and untied at one end. A solution of suspended SMCs ($5x10^5$ cells/ml) was injected into the tube lumen. The tube was then retied with suture and placed in culture for an additional 4 days. While the first tube was in culture, a second tube consisting of 30% collagen type I and 70% elastin was electrospun onto a mandrel with a 2 mm ID. After fixation, the 2 mm ID tube was inserted into the seeded 4 mm ID tube still tied at one end. The lumen was then filled with a SMC suspension ($5x10^5$ cells/ml) with the open end again tied off to contain the SMC suspension. This combined structure was placed in the RCCS for 3 days to allow SMC migration into the smaller tube, media equivalent. After 3 days in culture, one end of the structure was untied and a suspension of HUVECs ($5x10^5$ cells/ml) was injected into the lumen. The construct end was then resealed and allowed to cultured for 2 more days. The resulting prosthetic was then removed from culture and fixed in 10% formalin for histology.

3.6. Histology

The formalin fixed specimens were used for basic histological staining with Hematoxylin and Eosin (H&E) and Masson's Trichrome. Standard paraffin-embedding and sectioning procedures were followed for the preparation of glass slides for histological review. This procedure included dehydration with increasing concentrations of ethanol, infiltration with xylene, embedding in paraffin, and thin sectioning with a microtome.

4. RESULTS

4.1. Electrospinning Collagen

To successfully electrospin collagen, we first had to identify a solvent that suitably dissolved collagen at sufficient concentrations to effect electrospinning. An additional requirement was that the solvent be relatively volatile to ensure rapid drying of electrospun mats. With detailed research, we noted that 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) is sufficiently volatile (Boiling Point 61^{o}C) and has been used as a solvent in which proteins and simpler amino acid sequences have been suspended for various conformational analysis studies (43). HFP has been shown to induce an alpha-helix conformation in amino acid sequences that possess a natural tendency to form an alpha-helix (44).

First attempts to electrospin collagen were performed utilizing acid soluble type I collagen (calf skin; 30 – 100 mg/ml) dissolved in HFP. Electrospun mats possessed substantial structural integrity, allowing for careful removal from the mandrel and handling for evaluation. Via SEM investigations, it was found that the electrospun type I collagen scaffolds were comprised of

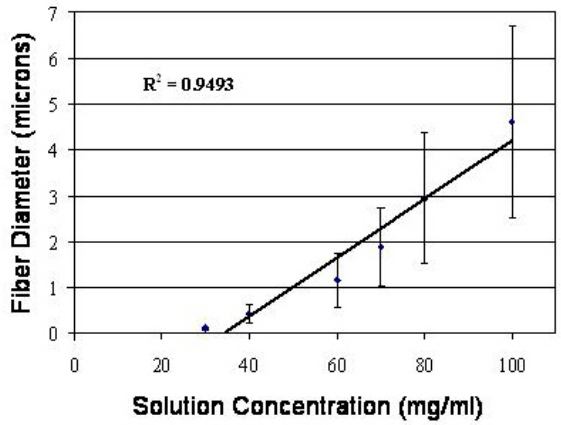

Figure 5. Graph of electrospun type I collagen illustrating the dependence of fiber diameter on electrospinning concentration.

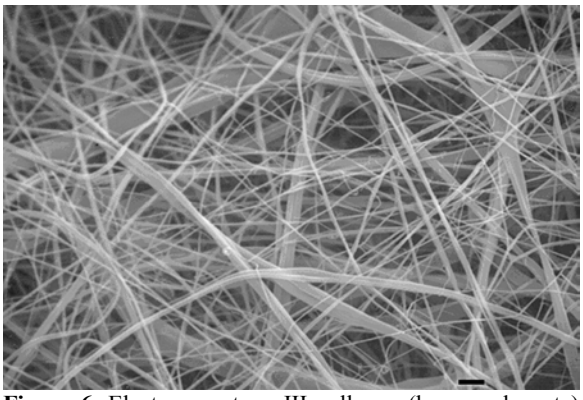

Figure 6. Electrospun type III collagen (human placenta) illustrating 250 ± 150 nm fiber diameter with random fiber orientation (Magnification 4,300X with the inserted scale bar at 1.0 micron).

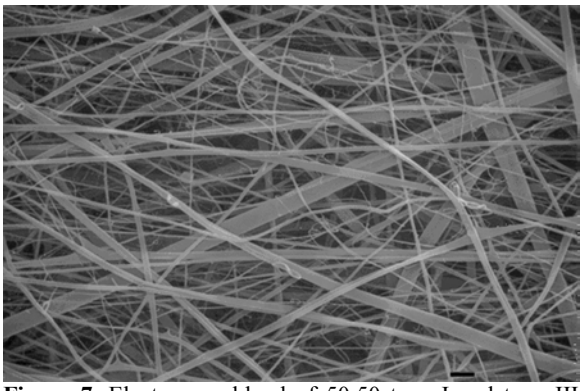

Figure 7. Electrospun blend of 50:50 type I and type III collagen (human placenta) illustrating 390 ± 290 nm fiber diameter with random fiber orientation (Magnification 4,300X with the inserted scale bar at 1.0 micron).

polymerized collagen fibers with average fiber diameters ranging from 0.10 ± 0.04 μm to 4.6 ± 2.1 μm (Figures 4 and 5). These small diameters approach the physiological fiber minimum of 50 nm for type I collagen fibril formation (38). Transmission electron microscopy (TEM) evaluation of the 100 nm type I collagen fibers revealed presence of the typical 67 nm banded appearance that is characteristic of the native collagen fiber ultrastructure. These evaluations confirm the potential of utilizing electrospinning in the successful creation of a truly biomimicking fiber and scaffold.

As has been seen with other polymers, collagen has a linear relationship between solution concentration and resulting fiber diameter; it is thought that the increase in viscosity (increased chain entanglements) that comes with concentration increase causes this effect. This is clearly shown in Figure 5, in which fiber diameters resulting from electrospinning out of solutions of varying concentration are plotted. It is clear in this Figure and the others included in this manuscript that a wide range of fibrous structures in terms of morphology, fiber diameter, and subsequent properties is possible with electrospinning. This illustrates the potential variability of the scaffolds that can be produced by controlled alteration of the electroprocessing conditions.

After electrospinning collagen type I, the next goal was the electrospinning of collagen type III. To determine whether this material could be electrospun, a sample of collagen type III (human placenta; 0.083 g/ml) was dissolved in HFP and electrospun. A micrograph of the electrospun fibers is illustrated in Figure 6. The fiber formation during electrospinning of type III collagen resulted in fiber dimensions of 250 ± 150 nm with random orientation, as obtained by analysis of SEM micrographs. TEM analysis was not performed on the collagen type III samples. The relationship between solution concentration and fiber diameter has yet to be determined for collagen type III.

Once electrospinning potential of each collagen component was explored, a 1:1 blend of types I and III collagen from human placenta was electrospun. The rationale behind use of this particular blend is that type I and III collagen are often found together in some combination in many tissues including the blood vessel ECM. Type I and type III collagens were dissolved and mixed together in HFP with the final combined collagen concentration at 0.062 g/ml. The analysis of the resulting type I and III collagen mat following electrospinning revealed a fiber diameter of 390 ± 290 nm with random fiber orientation throughout, as illustrated in Figure 7. This is not the optimal result but demonstrates the potential for electrospinning of collagen blends. Future detailed studies will examine collagen type distribution along the fibers throughout the scaffold to determine the actual degree of blending of the two collagen types, as well as the relationship between collagen concentration and fiber diameter produced.

4.2. Electrospinning Elastin

The next milestone for creating vascular tissue engineering scaffoldings was the electrospinning of elastin. The elastic properties of many tissues are due to the presence of elastin in the ECM. This elastin fiber component also often alternates among collagen fibers.

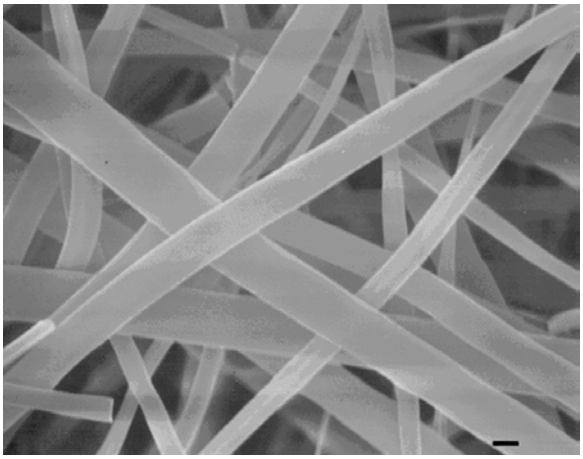

Figure 8. Electrospun elastin (bovine ligamentum nuchae) illustrating 1.1 ± 0.7 μm fiber diameters produced (Magnification 4,300X with the inserted scale bar at 1 micron).

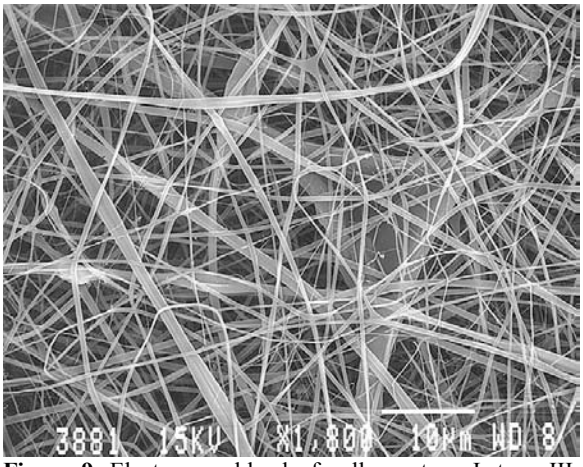

Figure 9. Electrospun blend of collagen type I, type III, and elastin (40:40:20) illustrating 0.49 ± 0.22 μm fiber diameter with random fiber orientation (Magnification 1,800X with the inserted scale bar at 10 microns).

Pure elastin (bovine ligamentum nuchae) was electrospun at both 20% and 30% (w/v) from HFP. While both elastin concentrations could be spun into fibers, the 30% concentration of elastin displayed optimal fiber formation properties. Analysis via SEM micrographs revealed an average fiber diameter of 1.1 ± 0.7 μm. Results are shown in Figure 8.

4.3. Electrospinning Collagen/Elastin Blend

Since elastin alternates among collagen fibers in many tissues, the next goal was to electrospin a blend of collagen and elastin. For this demonstration, a blend of 40% type I, 40% type III (both human placenta collagen), and 20% elastin (bovine ligamentum nuchae) was electrospun from HFP at a concentration of 0.083 g/ml. Structural analysis under SEM revealed a fibrous mat with random fiber alignment due to use of a low mandrel rotation rate during spinning. Fiber diameters produced were 0.49 ± 0.22 μm (Figure 9). This success was very promising, as it shows the feasibility of this idea for a potential scaffold for elastic tissues. Preliminary physical data using atomic force microscopy (AFM) have shown homopolymeric fibers suggesting that actual composition along each fiber is an even distribution of the collagens and elastin in the original blend solution. Further detailed studies are underway to confirm this finding.

4.4. Preliminary Fabrication of a Three Layered Vascular Construct

Upon histological examination, the scaffold cross-sections revealed a three-layered construct with complete cellular infiltration as shown in Figure 10. The intima is completely covered with morphologically mature ECs. Immediately beneath the EC layer, a basal lamina is clearly defined. Smooth muscle cells are found throughout the media. Overall, the cell distribution is equal; however, it appears that a dense layer of SMCs had formed immediately beneath the basal lamina (Figure 10). These SMCs were beginning to align in a circumferential fashion around the tube axis. Conversely, FBs and SMCs in the adventitia formed a dense population throughout the collagen/elastin outer scaffolding. As with the ECs in the intima, the SMCs were actively proliferating at the time of fixation and had not fully established a defined cellular orientation. Future studies will analyze the SMC phenotype during the culture period to indicate whether the SMCs are contractile (for construct strength) or synthetic (producing ECM) phenotype.

5. DISCUSSION

Many investigators believe that cell differentiation and normal behavior may be more readily observed when cells are given an ideal physiologic ECM – "Ideal" scaffolding. As demonstrated in this study, the ability to construct an alternating polymer layer scaffold shows the potential for the engineering of a biomimicking vascular prosthetic with an ECM and cell composition identical to the section of vasculature that needs replacement. This is important for optimal prosthetic performance, as the vasculature exhibits a very dynamic composition throughout the body. While the focus of this investigation was a small diameter (4 mm ID) blood vessel, the use of electrospinning and these methodologies in the design of larger caliber vessels is a viable option for even better large caliber replacements and should be seriously considered. The future success of an electrospun biomimicking vascular graft certainly has a place, as can be predicted by current clinical failures.

Failure of both the natural and synthetic vascular bypass grafts is due to acute thrombus formation (< 30 days post-operation) (45-47) and chronic intimal or anastomotic hyperplasia (> 30 days post-operation) (48-51). The exact mechanism behind initiation of these failures of vascular prosthetics is not clearly understood. It is thought that the thrombosis and intimal hyperplasia processes may, at least in part, originate from aberrant EC and SMC interactions. To date, EC seeding onto e-PTFE vascular grafts has been shown to reduce the rate of anastomotic hyperplasia but not halt it. Due to inefficiency of historic EC seeding techniques, it is clear that this approach is going down the

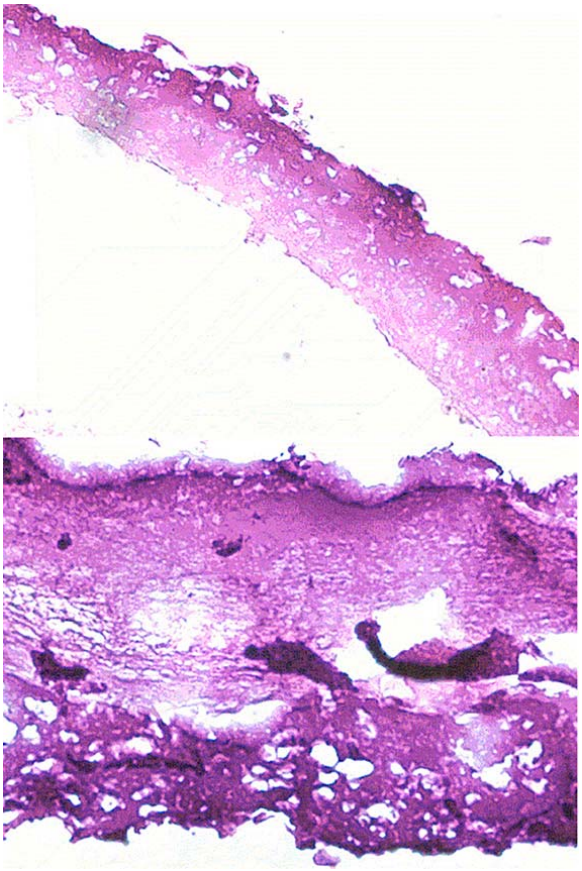

Figure 10. (Top). Three layered vessel wall demonstrating defined intimal (dark purple stripe, left), medial (light purple, center), and adventitial (dark purple, right) layers. Sample fragmentation is an artifact of histological processing (some fragments seen in bottom left of micrograph) (Bottom). Three layered prosthetic wall demonstrating defined intimal (top, thin dark purple strip), medial (middle), and adventitial (bottom, thick dark purple layer) layers. The fibrous material located above the intimal lining is a direct effect of tubular collapse during histological processing. Longer culture times will increase wall strength and decrease the chance of such collapse. This is a normal complication associated with the histology of blood vessels in particular the delaminating of the intima.

right path but does not yet solve the problem. A confluent EC monolayer is necessary for success (48). A biomimicking tissue engineered vascular prosthetic, composed of the natural arterial components such as ECs, SMCs, and the proper ECM (collagen and elastin) will take us further down that path and likely promote natural interactions that are beneficial for three reasons. First, the EC lining (neointima) will provide a natural barrier between the ECM (vascular prosthetic) and the bloodstream, creating a truly non-thrombogenic vascular prosthetic. Second, intimate EC-SMC interactions will progress normally and prevent or reduce intimal hyperplasia. Third, as speculated by the authors, the media composition (SMCs, elastin, and collagen) in direct contact with the adjacent native artery along with the confluent EC lining may prevent anastomotic hyperplasia due to there no longer being a cell-synthetic material interaction occurring to stimulate the initiation and propagation of hyperplasia.

Proper development of this cellular and ECM organization is important for function of the implanted prosthetic. Paramount for function is sufficient burst strength. Previous studies have demonstrated that the application of forces of stretch, torsion, and pulsatile flow to the construct prior to implantation can alter cellular composition and morphology, ECM composition and organization, and overall construct mechanical properties (36,52). A possible less invasive alternative to direct forces for preconditioning uses magnetic fields to manipulate collagen orientation *in vitro* (53). While this investigation only focused on manipulation during fibrillogensis, use of this method should be considered during the remodeling phase of the construct as well. A third approach is to exploit the duration of cell culture and maturation periods to control wall strength and thickness. Burst pressure of cellular constructs was found to be directly dependent on culture time (32). Therefore, by making use of these three approaches accordingly, precise cellular and matrix arrangements can be obtained.

Additionally, the immediate incorporation of elastin in electrospun collagen scaffolds provides an advantage over the previously studied collagen gels which did not incorporate elastin. The presence of fibrous matrix proteins provides a head start in vascular wall development and remodeling prior to and during any preconditioning modality. Elastin is an integral component of the matrix and, when co-spun with collagen as part of the matrix, is available for immediate metabolism by the cells. In previous tissue engineered constructs, production of fibrillar elastin can take months. The presence of elastin in the scaffolding at the initial time point of cell attachment is likely to decrease the demand and period of cellular remodeling associated with the development of the final vascular construct.

Beyond composition, the orientation of the matrix must match the native tissue in order for it to properly direct the remodeling phase, with or without mechanical preconditioning. Rotating the collecting mandrel at a particular rate can induce this orientation. At a mandrel rotation rate of 500 RPM, the collagen fibers in the final mat are largely randomly oriented (Figure 11). However, using the same mandrel under identical collagen electrospinning conditions except an increased mandrel rotation of 4,500 RPM, extremely aligned collagen fiber scaffolds are produced (Figure 11). This ability to control fiber orientation, and therefore tailor the scaffold ECM organization, is important in the fabrication of an "ideal" tissue engineered scaffold. Future work is underway to redesign an electrospinning device that can further control order and increase the speed and efficiency of scaffold production.

Some of the factors of interest in the large-scale production of the "ideal" tissue engineering scaffold are overall construct parameters (composition, dimensions, and

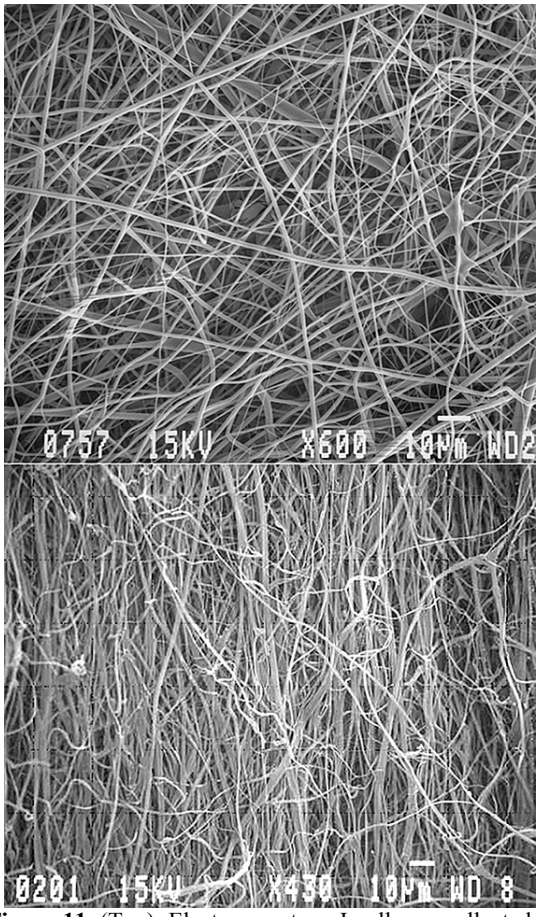

Figure 11. (Top). Electrospun type I collagen collected on a mandrel revolving at 500 RPM (Magnification 600X with the inserted scale bar at 10 microns), (Bottom). Electrospun type I collagen collected on a mandrel revolving at 4,500 RPM (Magnification 430X with the inserted scale bar at 10 microns).

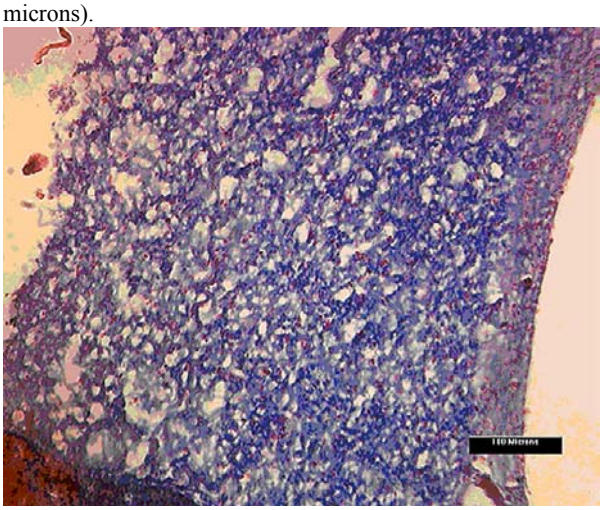

Figure 12. Electrospun collagen/elastin scaffolding with an average pore diameter of 3.7 microns after 21 days in culture, showing extensive smooth muscle cell infiltration into the collagen nano-structured matrix with an even, highly dense distribution of the cells across the entire cross-section of nanostructured scaffold.

orientations) and efficient material usage. For example, one can fabricate a 4 mm diameter tube scaffold with a length of 6 cm and a wall thickness of 200 microns on a metal mandrel in about 10 minutes utilizing a very simple electrospinning apparatus. The amount of material required is approximately 0.21 g of calf skin collagen type I and 2.5 ml of HFP. Almost all of this material is directed towards and collected on the grounded mandrel by the strong electric field, resulting in no loss of material. Thus, it is clear that, in terms of time of production, materials, and flexibility, electrospinning is a very efficient and attractive method for the production of a variety of tissue engineering scaffolds. Again, this example is for a very basic electrospinning apparatus, and, with future design modification (easily scaleable), production time per scaffold will be reduced to just a few minutes or less and still be re-producible (Quality control). Basically, this process historically has been a wonderful research tool that now is being transitioned into mass production where this method will still work and be successful in that environment—this is not just another lab dream.

With all the promise of electrospinning vascular prostheses, some basic questions remain to be answered. Histological evaluation shows clear migration of SMCs (Figure 12) and FBs into the scaffolds; however, this observation seems to contradict a widely held paradigm that cells will not migrate through pores that are less than 10 microns in diameter. Pore sizes in the scaffolds presented here start below 10 microns and approach 200 nm. To explain this, it is possible either that fibers are degrading during cell migration or that cells are pushing fibers out of the way during migration to fit through the pores. If the former modality is correct, appropriate seeding density may be inversely proportional to structural integrity during the migration phase. If the latter is correct, the degree of crosslinking may be critical to promote or prevent migration. Preliminary work in our laboratory has shown an inverse relationship between degree of crosslinking and degradation rate. Regardless, more work is needed to elucidate the mechanisms behind cell migration. The right balance of degradation and mechanical integrity is critical for success. Overall, the success of electrospinning to this point offers guarded optimism that a truly biomimicking small diameter tissue engineering vascular prosthesis is possible.

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Disclosure: The authors have several patents pending concerning technology presented in this manuscript and this technology has been licensed to NanoMatrix, Inc. of which several authors have a financial interest.

Key Words: Electrospinning, Tissue Engineering, Scaffold, Vascular Prosthesis

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