Articles

Electrospinning of Collagen Nanofibers

Jamil A. Matthews,[†] Gary E. Wnek,[‡] David G. Simpson,[§] and Gary L. Bowlin*[,||]

Department of Physiology, Virginia Commonwealth University, Richmond, Virginia 23298-0551; Department of Chemical Engineering, Virginia Commonwealth University, Richmond, Virginia 23298-3028; Department of Anatomy, Virginia Commonwealth University, Richmond, Virginia 23298-0709; and Department of Biomedical Engineering, Virginia Commonwealth University, P.O. Box 980694, Richmond, Virginia 23298-0694

Received March 9, 2001; Revised Manuscript Received June 11, 2001

Electrospinning is a fabrication process that uses an electric field to control the deposition of polymer fibers onto a target substrate. This electrostatic processing strategy can be used to fabricate fibrous polymer mats composed of fiber diameters ranging from several microns down to 100 nm or less. In this study, we describe how electrospinning can be adapted to produce tissue-engineering scaffolds composed of collagen nanofibers. Optimizing conditions for calfskin type I collagen produced a matrix composed of 100 nm fibers that exhibited the 67 nm banding pattern that is characteristic of native collagen. The structural properties of electrospun collagen varied with the tissue of origin (type I from skin vs type I from placenta), the isotype (type I vs type III), and the concentration of the collagen solution used to spin the fibers. Electrospinning is a rapid and efficient process that can be used to selectively deposit polymers in a random fashion or along a predetermined and defined axis. Toward that end, our experiments demonstrate that it is possible to tailor subtle mechanical properties into a matrix by controlling fiber orientation. The inherent properties of the electrospinning process make it possible to fabricate complex, and seamless, three-dimensional shapes. Electrospun collagen promotes cell growth and the penetration of cells into the engineered matrix. The structural, material, and biological properties of electrospun collagen suggest that this material may represent a nearly ideal tissue engineering scaffold.

Introduction

Considerable effort has been invested in the bioengineering community to develop biodegradable polymer scaffolding suitable for tissue engineering applications.[1,2] Ideally, a candidate scaffolding should mimic the structural and functional profile of the materials found in the native extracellular matrix. To achieve this objective, an engineered matrix must be biocompatible and not induce adverse effects in the surrounding tissue.[3] Simultaneously, the scaffolding also must support and define the three-dimensional organization of the tissue-engineered space and maintain the normal state of differentiation within the cellular compartment. It is also highly desirable to use processing methods that are efficient and that can be used to regulate the chemical, biological and material properties of the fabricated matrix.[1,4−6] Finally, we believe that an ideal matrix will lend structural stability to an engineered tissue and withstand manual manipulation yet be completely resorbable.[3] To date several synthetic materials have been used in attempts to meet these criteria, including polymers of lactic acid, glycolic acid, polycaprolactone, and their copolymers. These materials have many favorable properties, although unfortunately these polymers have not performed up to expectations in the clinical setting.[2,3]

In many native tissues, polymers of type I and type III collagen are the principal structural elements of the extracellular matrix.[7] The underlying α chains that form these natural polymers are arranged into a repeating motif that forms a coiled coil structure. At the ultrastructural level, this repeating motif exhibits a 67 nm interval that imparts a characteristic banding pattern to the collagen fibril as seen by transmission electron microscopy. The specific complement of α subunits present within the fibril defines the material properties of the polymer. Type I collagen fibrils are composed of two α1 chains and one α2 chain. In native tissues, polymers of type I collagen are approximately 50 nm in diameter and very uniform in size.[8] Type III fibrils are composed of three α1(III) chains. This isotype of collagen is variable in size, and these fibrils range from 30 to 130 nm in diameter.[8−10] Collagen has been used in a variety of tissue engineering applications. It can be isolated from a

* Corresponding author. Telephone: (804) 828-2592. Fax: (804) 828-4454. E-mail: glbowlin@saturn.vcu.edu.

[†] Department of Physiology, Virginia Commonwealth University.

[‡] Department of Chemical Engineering, Virginia Commonwealth University.

[§] Department of Anatomy, Virginia Commonwealth University.

[||] Department of Biomedical Engineering, Virginia Commonwealth University.

10.1021/bm015533u CCC: $22.00
Published on Web 01/26/2002

variety of sources, is highly conserved, and is relatively nonimmunogenic. However, the procedures used to isolate and reprocess this natural scaffolding into an engineered material compromise many of its biological and structural properties. In this study, we use the novel processing strategy of electrospinning to produce fibrils of type I and type III collagen that mimic the molecular and structural properties of native collagen.

Electrospinning is a process that uses an electric field to control the formation and deposition of polymers. This process is remarkably efficient, rapid, and inexpensive. In a previous report, we have described how electrospinning can be used to fabricate scaffolds of synthetic, biodegradable polymers.[11] In electrospinning, a polymer solution or melt is injected with an electrical potential to create a charge imbalance and placed in proximity to a grounded target. At a critical voltage, the charge imbalance begins to overcome the surface tension of the polymer source, forming an electrically charged jet. The jet within the electric field is directed toward the grounded target, during which time the solvent evaporates and fibers are formed. Electrospinning produces a single continuous filament that collects on the grounded target as a nonwoven fabric.[12] Notably, it is possible to fabricate filaments on the nanometer scale with this technique.[13] In the present study, we describe how we have adapted the electrospinning process to fabricate an engineered matrix composed of collagen fibrils for use in tissue engineering.

Methods

Electrospinning. Acid soluble, lyophilized collagen was used for all experimentation. Unless otherwise noted all reagents were purchased from Sigma Chemical Company (St. Louis, MO). For this study, we used type I collagen from calfskin and type I and type III collagen isolated from human placenta. Collagen was dissolved at various concentrations in 1,1,1,3,3,3 hexafluoro-2-propanol (HFP). Suspensions of collagen were placed into a 1.0 mL syringe mounted in a syringe pump (model 100, KD Scientific Inc., New Hope, PA). The syringe was capped with an 18-gauge blunt end needle. The positive lead from a high voltage supply (Spellman CZE1000R; Spellman High Voltage Electronics Corp.) was attached via an alligator clip to the external surface of the metal syringe needle. A rectangular (0.6 cm W × 0.05 cm H × 4 cm L) grounded target fabricated from 303 stainless steel was mounted 4−6 in. from the tip of the syringe tip. At the onset of electrospinning, the syringe pump was set to deliver the source solution at rates varying from 0 to 25 mL/h. Simultaneously, the high voltage was applied across the source solution and the grounded target mandrel (15−30 kV). The mandrel rotated at approximately 500 rpm, unless otherwise noted. A schematic of the complete electrospinning apparatus is shown in Figure 1. In summary, during the electrospinning process, we examined how the isotype and concentration of collagen, imposed voltages, the air gap distance, and flow rates affected the electrospinning process.

Cell Culture. Type I collagen from calfskin was electrospun onto a 4 mm diameter cylindrical culture platform

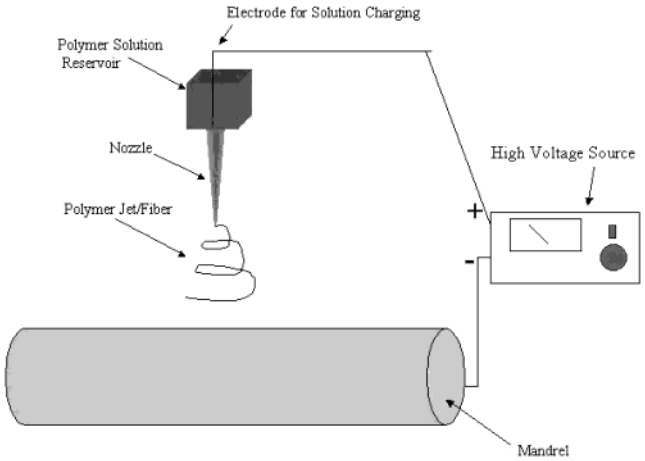

Figure 1. Schematic of the electrospinning apparatus used in the experimentation described in this study. The key components of the electrospinning system including the grounded target (mandrel), high-voltage source, collagen reservoir, and nozzle. Fiber deposition can be regulated by controlling the motion of the target mandrel and source solution with respect to one another. A rotating mandrel was used in our system as a ground target. A syringe mounted in a syringe pump was used as a reservoir for the electrospinning source solution. The polarity of the system depicted in this image is arbitrary and, depending on the material to be processed, can be reversed.

(length = 1 cm). Constructs were cross-linked in glutaraldehyde vapor for 24 h at room temperature and then rinsed through several changes of phosphate-buffered saline. All cell culture experiments were conducted in a rotary cell culture system (RCCS) manufactured by Synthecon, Inc (Houston, TX) that was equipped with a slow turning lateral vessel (STLV). These devices are designed to maintain cells and tissue in continuous free fall, providing a buoyant, low shear environment and high mass transfer of nutrients.[14−17] The buoyant environment of these devices fosters cell−cell and cell−matrix contacts and the formation of large cell masses. Rates of rotation necessary to suspend samples in continuous free fall are determined experimentally. In this study, a rate of 10−11 rpm was sufficient to maintain the cylindrical constructs in free fall.

Cylindrical constructs were placed in a 55-mL STLV vessel with aortic smooth muscle cells (500000 SMC/mL). The culture media was composed of Dulbecco's Modified Eagle Medium (DMEM) and F12 Nutrient Mixture (F12) (2:1 DMEM:F12 with high glucose plus L-glutamine, sodium pyruvate, and pyridoxine hydrochloride) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomyocin (10000 units/mL). Culture media was changed after day two and day five of experimentation; no additional cells were added during the feeding intervals. Constructs were isolated for examination at day seven. All media components were purchased from Gibco BRL Life Technologies.

Microscopic Analysis. For TEM analysis, fabrics of collagen were immersed and fixed overnight at 4 °C in 2% gluteraldehdye prepared in Sorenson's buffer. Samples were rinsed in cacodylate buffer and post-fixed in 1% osmium tetroxide for 1 h. Imbedded samples were thin sectioned and examined with a JEOL-JEM-1200EX instrument (JEOL Inc., Peabody, MA). Fixed and unfixed fabrics were processed for SEM analysis. We determined that collagen fabrics were dry upon deposition on the target mandrel and did not require

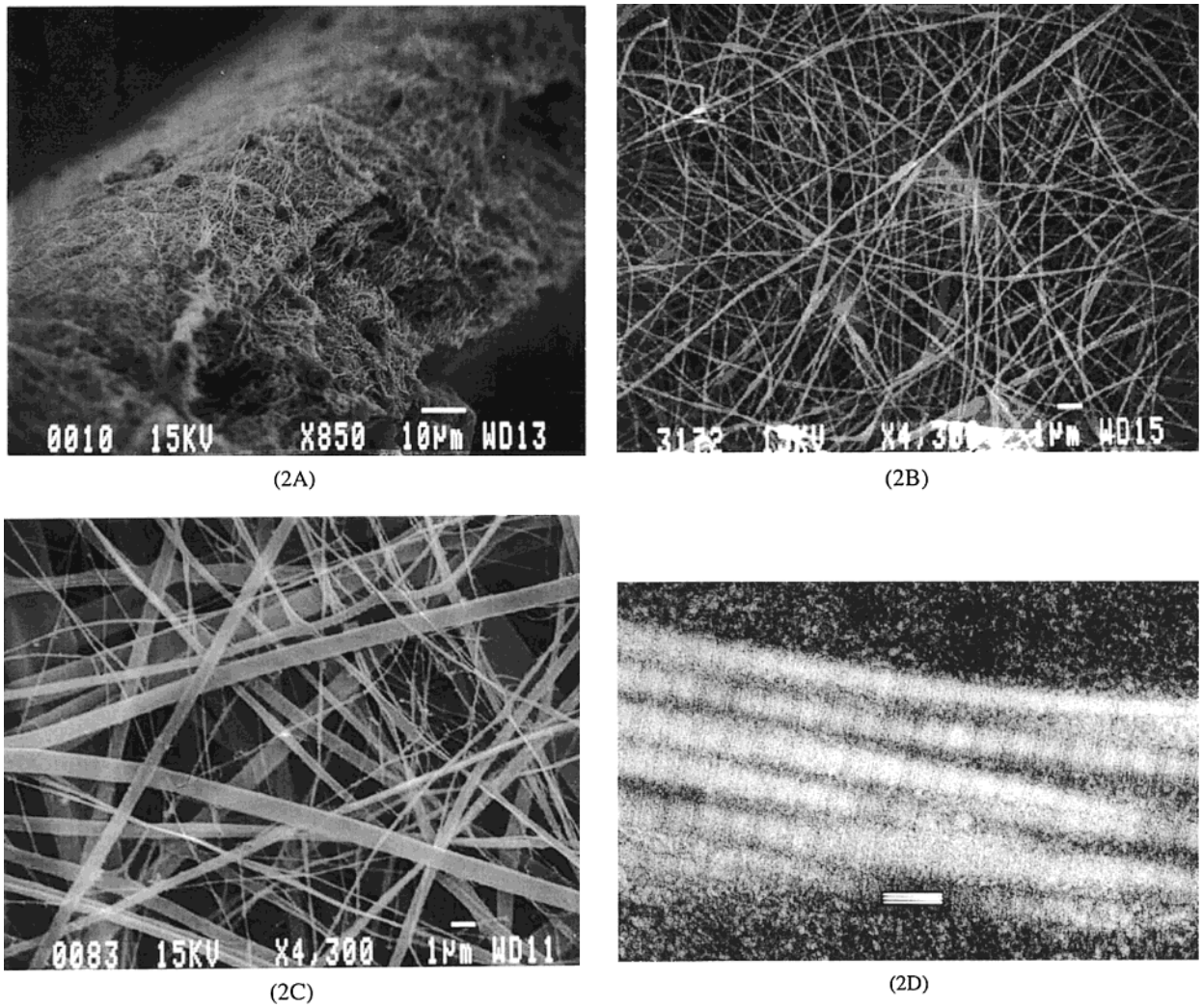

Figure 2. (A) Scanning electron micrograph (SEM) of calfskin type I collagen electrospun onto a static, cylindrical mandrel. Cut edges of the matrix illustrate the porous, three-dimensional nature of the scaffold (magnification 850×). (B). Detail SEM of electrospun calfskin type I collagen. Under optimal conditions electroprocessed collagen collects on the ground target mandrel as a complex, non woven matrix of fibrils. Average filament diameter in this type of matrix is 100 ± 40 nm. Our extensive surveys of this material have not revealed evidence of free ends, this observation is consistent with current views that electrospinning can produce a single, continuous polymer fiber[12] (magnification 4300×). (C) SEM of electrospun type I collagen isolated from human placenta. The source of collagen used in electrospinning dictates the structural properties of the deposited filaments. Collagen fibers electrospun from human placental type I collagen produce filaments that are less uniform in diameter than the filaments produced with calf skin collagen. Filaments range from 100 to 730 nm in diameter (magnification 4300×). (D). TEM of the electrospun type I calfskin collagen. Electroprocessed fibers exhibit the 67 nm banding typical of native collagen (inserted scale bar = 100 nm).

critical point drying during routine processing. Samples were sputter coated and examined directly. Micrographs from SEM analysis were digitized and analyzed with UTHSCSA ImageTool 2.0 to determine the average fiber diameters.

Materials Testing. Fabrics of electrospun type I collagen (calf skin) were subjected to stress−strain analysis using a MTS Bionix 200 materials testing station (MTS Systems Corp.; Eden Praire, MN). Samples were trimmed into a "dog-bone" profile with offset ends to reduce grip effects. Testing was conducted with the tissue grips moving at a rate of 10 mm/min. The data acquisition rate was set to 20.0 Hz. Integration and analysis was completed using the MTS Testworks software (version 4.04A).

Results

Processing Parameters: Preliminary experimentation identified HFP as the solvent of choice for electrospinning collagen. HFP is an organic, volatile solvent with a boiling point of 61 °C. A low boiling point is a desirable characteristic in electrospinning applications because it promotes the evaporation of the solvent under conventional atmospheric conditions. This property promotes the deposition of polymer fibers in essentially a dry state.

The electrospinning of collagen fibers exhibited a concentration dependence on the final fiber diameters produced. A variety of polymers exhibit this property when processed by electrospinning.[18] For example, at a concentration of 0.008 g/1.0 mL acid extracts of type I collagen (calfskin) were readily soluble in HFP. However, at this concentration the collagen did not exhibit any evidence of electrospinning (fiber formation) and, regardless of the input voltage, the polymer solution formed droplets and leaked from the syringe tip. Increasing the concentration of collagen 10-fold to 0.083 g/mL resulted in a cloudy suspension and the formation of fibers during electrospinning. These fibers collected as a

nonwoven mat on the target mandrel (Figure 2, parts A and B). Further increasing the collagen concentration in the source solution did not grossly alter fiber formation. In subsequent experimentation, we have used the gross appearance of fibers within the electrospinning field and the deposition of these filaments on the target mandrel as our criteria to optimize the processing conditions. We recognize these parameters only reflect the bulk characteristics of the spun fibrils. However, we feel these criteria will provide an adequate description of the parameters used to optimize the electrospinning process.

Given these caveats, we next optimized the voltage input parameters. Type I collagen was suspended in HFP at 0.083 g/mL and then subjected to voltages varying from 15 and 30 kV in 2.0 kV increments. Fiber formation was most prominent at 25 kV (electric field magnitude = 2000 V/cm). Varying the air gap distance between the source solution and grounded target at this input voltage markedly affected the electrospinning process. The optimal air gap distance was approximately 125 mm. Collagen fibrils could be spun over substantially shorter air gap distances; however, these fibrils retained considerable solvent and collected on the target in a wet state. When the air gap distance exceeded a critical interval of 250 mm, the spun fibers failed to collect on the target mandrel.

The electric field generated in the electrospinning process is sufficient to draw the collagen source solution from a syringe reservoir. However, we found that it is possible to generate a more uniform collagen mat by metering the rate at which the collagen source solution is delivered to the electrospinning field via a syringe pump. Fiber formation is optimal when the collagen source solution is delivered to the electric field at a rate of approximately 5.0 mL/h. At slower rates of delivery, fiber formation was inconsistent.

As noted in our introduction, we believe that it is desirable to use a processing strategy that can be used to shape and control the tertiary structure of a tissue engineering scaffolding. This characteristic is important for several reasons. First, the distribution and arrangement of the ECM plays a critical role in controlling cell shape, regulating physiological function, and defining organ architecture.[19] In electroprocessing, the shape of the scaffolding and orientation of fibers within an engineered matrix can be regulated to a high degree of precision by controlling the motion of the target mandrel and source solution with respect to one another. We have examined this processing parameter in the production of electrospun collagen by investigating how the rotation of the target mandrel effects the deposition of this natural polymer. Collagen fibrils electrospun onto a mandrel rotating at a rate of less than 500 rpm produced a random matrix of filaments (Figure 2, parts A and B). Increasing the velocity of the mandrel to 4500 rpm (mandrel surface moving at approximately 1.4 m/s) resulted in deposition of fibrils along the axis of rotation (Figure 3). Transmission electron microscopic analysis of aligned and nonaligned fibrils revealed that these filaments all displayed the 67 nm banding that is characteristic of native collagen (Figure 2D).

To function as a tissue engineering scaffolding, a matrix must have sufficient mechanical integrity to withstand

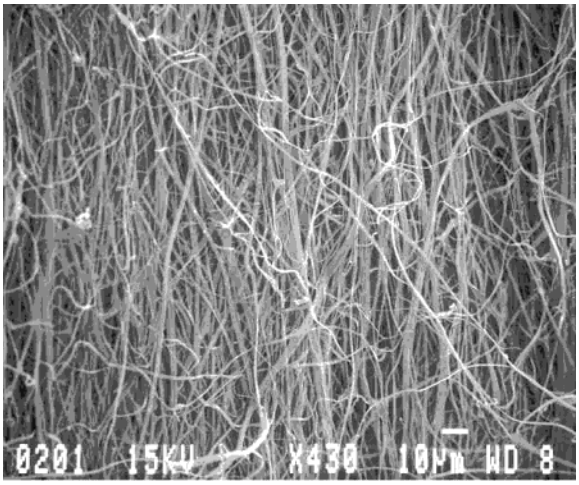

Figure 3. SEM of collagen type I calf skin electrospun onto a rotating mandrel. Rotating the target mandrel at 4500 rpm results in the deposition of collagen fibrils into linear, parallel arrays. Under the conditions used in this experiment, the surface of the mandrel was moving at a velocity of approximately 1.4 m/s. The filaments are deposited along the axis of rotation (magnification 430×).

manual manipulation. To characterize the material properties of electrospun collagen, we measured the stress stain profile of this type of scaffold. Type I collagen from calf skin was electrospun under optimal conditions onto a rectangular target mandrel rotating at 4500 rpm. The scaffolding was removed from the target and fashioned into sheets 25 mm (length) × 25 mm (width). Under the conditions used to fabricate this matrix, the scaffolding averaged 0.187 mm in cross-sectional thickness. Replicate samples were cut into strips that were either parallel or perpendicular to the principal axis of mandrel rotation. This approach allowed us to directly examine how the local fiber direction modulates the material properties of the electrospun matrix. Materials testing of scaffolds in parallel with the principal axis fibril alignment indicated an average load of 1.17 ± 0.34 N at failure with a peak stress of 1.5 ± 0.2 MPa. The average modulus for the longitudinal samples was 52.3 ± 5.2 MPa. In cross fiber orientation, the peak load at failure was 0.75 ± 0.04 N with a peak stress of 0.7 ± 0.1 MPa. The modulus across the fiber long axis was 26.1 ± 4.0 MPa. These data indicate the local orientation of the fibers that compose an electrospun scaffolding directly modulate the material properties of the engineered matrix. Presumably, the incorporation of various degrees of cross-linking into this type of nonwoven matrix can be used to further tailor the material properties of the matrix to specific applications.

We also have examined how the identity and source of collagen can affect the electrospinning process. In these experiments, we first electrospun type I collagen isolated from human placenta using the conditions optimized for type I calfskin collagen. With respect to calfskin collagen, electrospinning this material produced a less uniform matrix of fibers. Individual filaments ranged from 100 to 730 nm in diameter (Figure 2C). We believe this result is a function of a complex interplay of variables. The 0.083 g/mL collagen used in these experiments appears to represent a critical transition concentration for type I collagen when it is isolated from the placenta. Increasing the concentration of human placental collagen present in the source solution (increased

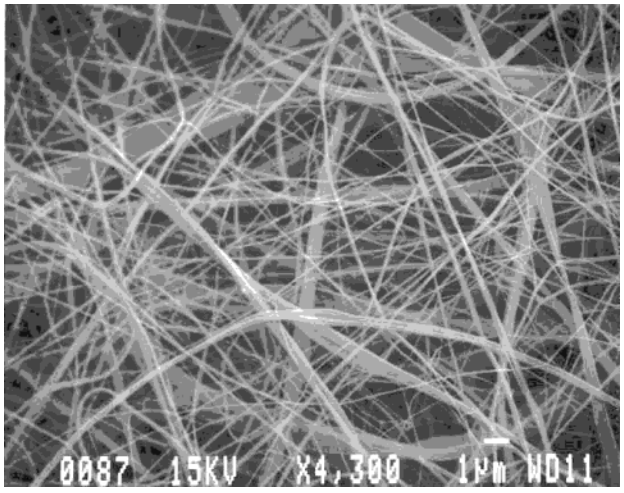

Figure 4. SEM of electrospun type III collagen (human placenta). The isotype (type I vs type III) collagen used in electrospinning also determines the structural properties of the deposited filaments. Under conditions optimized for calfskin type I collagen an electrospun matrix of type III collagen is composed of fibers that average 250 ± 150 nm in diameter (magnification 4300×).

viscosity in electrospinning source solution) and keeping all other variables equal appears to favor the formation of larger diameter fibers. Conversely, decreasing the concentration of the source solution (decreased viscosity in electrospinning source solution) appears to reduce the average filament diameter and produces a matrix composed primarily 100 nm fibers. We have observed this concentration-dependent transition phenomenon in the electroprocessing of poly-(glycolic acid).[20]

Our experiments also indicate the primary sequence of collagen directly effects the formation of this polymer in the electrospinning process. Preliminary attempts to electrospin type III collagen indicated the optimal concentration of this peptide is approximately 0.04 g/mL HFP, a value 50% less than the optimal concentration of type I calfskin. Electrospinning type III collagen at 0.04 g/mL HFP, using all of the other conditions optimized for electrospinning type I calfskin collagen, produced fibers with average diameters of 250 ± 150 nm (Figure 4). The relative ratio of type I to type III collagen within the native ECM plays a critical role in dictating the structural and functional properties of the collagen-based network. Blending optimal concentrations of type I human placental collagen (0.08 g/mL HFP) and type III human placental collagen (0.04 g/mL HFP) at a 50:50 ratio (final collagen concentration 0.06 g/mL) markedly affected the formation of fibrils during the electrospinning process. Under electrospinning conditions optimized for type I calfskin collagen, the blended material formed a scaffold composed of fibers that averaged 390 ± 290 nm in diameter (Figure 5). We were unable to determine from our ultrastructural examination of this material if it is composed of distinct filaments of type I and type III or individual filaments of type I and III blended at the molecular level.

A tissue engineering scaffolding must promote cellular growth and physiological function and maintain normal states of cellular differentiation. We have examined the biological properties of electrospun collagen in tissue culture experiments. Aortic smooth muscle cells were suspended in a RCCS bioreactor and plated out onto different formulations of electrospun collagen. We choose this culture paradigm because the low shear, high nutrient environment afforded by the RCCS bioreactor fosters cell−matrix interactions and the formation of large scale tissue masses in vitro. Microscopic examination of these cultures revealed that the scaffolds were densely populated with the smooth muscle cells, within 7 days (Figure 6B). Cross sectional analysis indicated that electrospun collagen promoted extensive

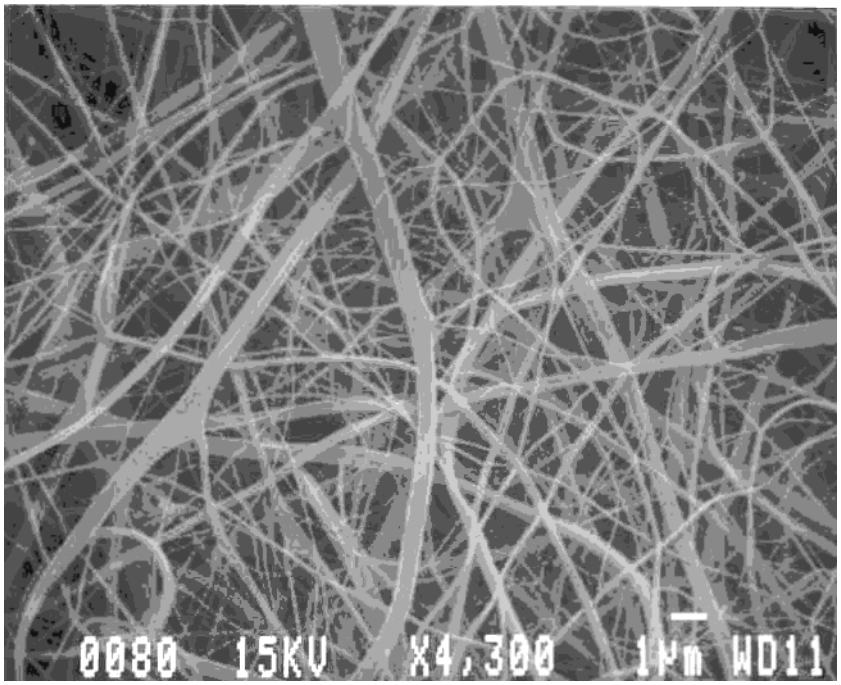

Figure 5. SEM of an electrospun 50:50 blend of collagen type I and III (human placenta). Unique structural properties can be derived by using blends of electrospun material in the source solution. This matrix was electrospun from a single reservoir source of composed of a mixture of type I and type III collagen suspended in HFP (final protein concentration equaled 0.6 g/mL). This heterogeneous network is composed of fibers that average 390 ± 290 nm fiber diameter (magnification 4300×).

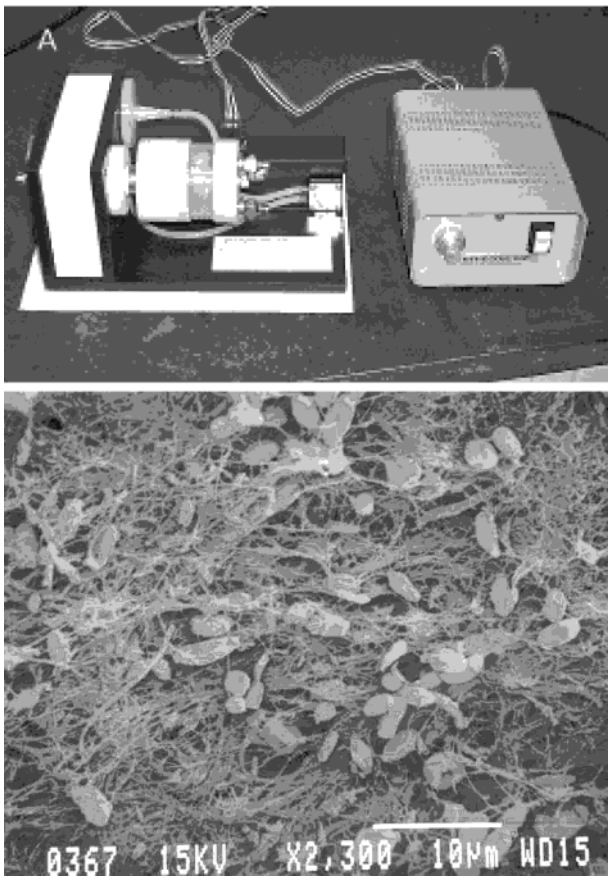

Figure 6. (A) Image of rotary wall bioreactor system (RCCS) (Synthecon Inc.) used to test the biological properties of electrospun collagen. The device used in preliminary testing was equipped with a slow turning lateral vessel (STLV). (B) SEM where smooth muscle cells and cylindrical constructs were placed into the bioreactor for varying lengths of time. Within 7 days of seeding the cells infiltrate the electrospun matrix (calfskin) matrix and are well integrated into the network.

cellular infiltration into the fibrillar network. Smooth muscle cells were observed deep within the matrix and fully enmeshed within the fibrils of the electrospun collagen.

Conclusions

In summary, we have characterized how different concentrations, input voltages, air gap distances, delivery rates, and mandrel motion affect the electrospinning of type I collagen. Each of these parameters impacts the formation and deposition of collagen fibers in the electrospinning process. For example, the source (type I calfskin vs type I placental) and isotype (type I vs type III) of collagen used in the electrospinning process had a direct impact on the structural properties of the resulting polymers. The material properties of electrospun type I fibrils were directly related to the architectural arrangement of the filaments that comprise the final matrix.

We believe the identification of HFP as an electrospinning solvent for collagen represents a critical observation. Type I collagen electrospun from this solvent exhibited the periodic banding pattern observed in the collagen deposited by interstitial fibroblasts. This arrangement is typically not manifested in conventional collagen gels and may limit the structural integrity and biological activity of this type of material. The physical properties of HFP may promote the formation of the in vivo-like structural profile observed in electrospun collagen. This volatile solvent has the unique property of inducing the expression of α-helical domains in proteins with a primary sequence that favors this conformational arrangement.[21,22] This may predispose collagen α chains to assume an alternate structure in solution that favors the subsequent formation of periodic, helical conformations of collagen during electrospinning.

We believe that electrospun collagen holds great promise for a variety of bioengineering applications. In native tissue, the collagen polymer is the principal structural element of the ECM. The electrospinning process has the potential to produce collagen fibrils that closely mimic, and at some point may even fully reproduce, the structural and biological properties of the natural polymer. Our preliminary in-vitro testing suggests that non-cross-linked, electrospun collagen exhibits highly favorable material and biological properties. As a processing strategy, electrospinning is rapid, efficient and can be used to fabricate complex, seamless scaffolds. Simultaneously, it is possible to control the chemical composition and material properties of the engineered scaffolding at several different sites in the electrospinning process. For example, blends or laminates of different materials can be produced with this technique. Electrospinning can even be used to incorporate subtle structural properties into an engineered material by regulating the orientation of fibrils within the fabricated network. These data suggest that electrospun collagen may represent a potentially ideal tissue engineering scaffolding.[23]

Acknowledgment. The authors would like to thank the Whitaker Foundation (RG-98-0465) for the support of this research. We would also like to thank Ms. Judy Williamson for obtaining the SEM and TEM micrographs.

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BM015533U