Published in final edited form as:

J Biomed Mater Res A. 2016 September ; 104(9): 2200–2209. doi:10.1002/jbm.a.35753.

Cell layer-electrospun mesh composites for coronary artery bypass grafts

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Abstract

The current work investigates the potential of cell layer-electrospun mesh constructs as coronary artery bypass grafts. These cell-mesh constructs were generated by first culturing a confluent layer of 10T½ smooth muscle progenitor cells on a high strength electrospun mesh with uniaxially aligned fibers. Cell-laden mesh sheets were then wrapped around a cylindrical mandrel such that the mesh fibers were aligned circumferentially. The resulting multi-layered constructs were then cultured for 4 wks in media supplemented with TGF-β1 and ascorbic acid to support 10T½ differentiation toward a smooth muscle cell-like fate as well as to support elastin and collagen production. The underlying hypothesis of this work was that extracellular matrix (ECM) deposited by the cell layers would act as an adhesive agent between the individual mesh layers, providing strength to the construct as well as a source for structural elasticity at low strains. In addition, the structural anisotropy of the mesh would inherently guide desired circumferential cell and ECM alignment. Results demonstrate that the cell-mesh constructs exhibited a J-shaped circumferential stress-strain response similar to that of native coronary artery, while also displaying acceptable tensile strength. Furthermore, associated 10T½ cells and deposited collagen fibers showed a high degree of circumferential alignment.

1. INTRODUCTION

Autologous saphenous veins and mammary arteries are currently the preferred graft materials for small-caliber vessels (< 6 mm ID) such as the coronary artery.^{1,2} However, roughly 10% of patients lack autologous tissue suitable for use in coronary artery bypass graft (CABG) procedures.³ Non-degradable synthetic prostheses – such as expanded polytetrafluoroethylene (ePTFE) conduits – can function as peripheral vessel grafts, but generally fail as small-caliber vessel replacements due to their thrombogenicity and low compliance.¹ Biodegradable scaffolds which promote new tissue formation by associated cells represent a potential means to construct functional CABGs for situations where autologous tissue is unavailable and current non-degradable prostheses fail. A successful biodegradable vascular graft scaffold must have adequate circumferential tensile strength to

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withstand peak physiological stresses upon implantation, while also supporting the neotissue formation necessary for long-term strength. In addition, since compliance mismatch between the graft and native tissue is associated with intimal hyperplasia^{4–6} and aneurysmal failure,^{7,8} CABGs must display appropriate compliance at physiological pressures.

The combined requirement for high strength as well as high compliance (low modulus) under physiological loading conditions has proven challenging to meet with single component scaffolds.^{9–14} For instance, electrospun scaffolds based on nano- or micron-scale fused-fibers have a number of potential advantages in terms of arterial graft applications.^{15–18} In particular, the ability to control fiber alignment by tailoring spinning conditions can allow for circumferentially oriented fibers.^{16,18} Circumferentially aligned fibers have the capacity not only to provide necessary circumferential strength but also to provide contact guidance, inducing cells to take on the circumferential orientation typical of smooth muscle cells within the arterial medial layer.^{19–21} That said, the modulus of electrospun mesh scaffolds at normal arterial stresses is generally significantly greater than the ≈ 200 – 400 kPa circumferential modulus of the coronary artery medial layer at physiological pressures.^{14,16,22} Therefore, a scaffold design that maintains the high tensile strength of an electrospun mesh, while bringing the compliance of the constructs nearer to that of native coronary artery would be desirable.

A multi-component scaffold comprised of interpenetrating layers of a high strength electrospun mesh and a second, lower modulus material may result in a composite graft that approaches the high compliance of native coronary artery at physiological pressures, while retaining necessary tensile strength. In the present work, we investigate the feasibility of this approach through the development and characterization of a composite construct comprised of layers of poly(ester urethane) urea (PEUUR) electrospun mesh bonded together by the extracellular matrix (ECM) deposited by TGF- β 1-stimulated smooth muscle progenitor cells (Figure 1). Our hypothesis is that the ECM deposited by the cell layer will serve as a source for structural elasticity at low strains, while also acting as an adhesive agent between adjacent mesh layers – allowing the graft to harness the strength of the electrospun mesh. In addition, we envision that the alignment of the electrospun mesh fibers will result in the circumferentially aligned cells and ECM needed for long-term graft compliance and strength matching as the mesh degrades.

In the current work, PEUUR elastomers were chosen for the electrospun layers instead of collagen or gelatin due to the greater tunability of PEUUR material properties.^{23–26} Furthermore, 10T $\frac{1}{2}$ progenitor cells were selected for use as a mimic of human smooth muscle progenitor cells,²⁷ which have the potential to avoid the limitations associated with primary vascular smooth muscle cells (SMCs) in terms of ease of harvest and proliferative capacity. To stimulate smooth muscle progenitor cell differentiation as well as the ECM deposition necessary to bond together the electrospun mesh layers, the assembled cell-mesh constructs were cultured in the presence of the growth factor TGF- β 1. TGF- β 1 has been shown to be a potent inducer of progenitor cell progression to a vascular smooth muscle cell phenotype and to stimulate elastin production,^{28,29} an ECM protein critical to long-term graft functionality.^{30,31} Similarly, ascorbic acid was added to the culture media to promote the collagen production necessary for long-term graft strength.²⁹ We demonstrate that the

resulting composite grafts exhibited a “J-shaped” stress-strain response similar to that of native human coronary artery, while also displaying acceptable tensile strength. Furthermore, associated 10T½ cells and deposited collagen fibers showed a high degree of circumferential alignment.

2. MATERIALS AND METHODS

2.1. Electrospun Mesh Fabrication and Characterization

In the current study, the PEUR polymer formulation and electrospinning conditions were selected based on previous literature^{23–26,32–35} to yield an oriented mesh scaffold with a directional modulus appropriate to the linear region of the coronary artery circumferential stress-strain curve. Details regarding this process are provided in the following sections.

2.1.1. Polyurethane Synthesis—A linear, segmented degradable PEUR elastomer, consisting of an alternating poly-ε-caprolactone (PCL) soft segment and a urethane- and urea-containing hard segment, was synthesized using a standard two-step technique in a three-neck, round-bottom flask equipped with argon inlet and outlet, condenser, and stirrer.³² First, the flask was charged with anhydrous dimethyl sulfoxide (DMSO, <50 ppm water; Acros Organics) and 1,6-diisocyanatohexane (HDI; Sigma-Aldrich), immersed in a 75 °C oil bath, purged with argon, and constantly stirred. Next, PCL diol (average M_w 2000 Da, PCL2000; Sigma) that had been dried for 24 h at 80 °C under vacuum (10 mmHg) and dissolved in DMSO was charged into the reactor by means of an addition funnel. The prepolymer content in the reactor was controlled at 14 wt%, and the relative masses of HDI and PCL2000 were selected to achieve a prepolymer NCO:OH equivalent ratio of 2.0:1.0. Dibutyltin dilaurate (Sigma) was added to the flask at 1000 ppm, and the reaction was allowed to proceed for 3 h to produce a HDI.PCL2000.HDI prepolymer. In the second step, a solution of 1,3-propanediol bis(4-aminobenzoate) (Sigma-Aldrich) in DMSO was prepared at 50 °C and added to the resultant prepolymer in the reaction vessel. The NCO:OH equivalent ratio of the polyurethane was controlled at 1.03:1.0, and the polymer concentration was 12 wt%. Dibutyltin dilaurate was added to a concentration of 1000 ppm, and the reaction was allowed to proceed at 80 °C for 20 h. The final polymer, termed PEUR2000, was then precipitated in diethyl ether (Sigma) and dried for 24 h at 80 °C under vacuum.

2.1.2. Electrospinning Procedure—The PEUR2000 polymer was electrospun onto rigid glass supports to form fused-fiber meshes with controlled fiber diameter and fiber alignment as described previously.^{32,35} Briefly, electrospinning was performed with a 10 wt % PEUR2000 solution in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma) using a 22-gauge Teflon tipped needle, a 15 kV potential, a throw distance of 15 cm, and a syringe flow rate of 5 mL/h. A 6-cm-diameter drum rotating at a linear velocity of 8 m/s served as the collector. Meshes were soaked in ethanol for 7 days followed by washing in deionized water for 2 days to remove residual HFIP. The meshes were then dried and stored in a desiccator until use.

2.1.3. Electrospun Mesh Characterization—The mean diameter and angular deviation of fibers within the electrospun mesh sheets were determined by quantitative analysis of scanning electron microscopy (SEM) images. Briefly, electrospun meshes were mounted onto studs and sputter-coated with Pd. Images were acquired using a LEO 1550 Field Emission SEM (Carl Zeiss SMT) operating at 5 kV. Resultant images were imported into ImagePro Plus software (ICube), and fiber diameter and angle of orientation were determined per Bashur et al.³²

2.2. Cell Culture

The 10T½ mouse smooth muscle progenitor cells were thawed and expanded at 37 °C and 5% CO₂. During expansion, cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Hyclone) containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone). Cells were harvested for seeding between passages 10–12.

2.3. Construct Fabrication and Culture

Each construct was fabricated in a three-step process shown schematically in Figure 1. In brief, rectangular segments (2 cm × 3 cm) were cut from the electrospun mesh sheets such that the short edge of the rectangle was parallel to the average direction of fiber orientation. Next, each mesh segment was exposed to DMEM containing 40% serum for 3 h at room temperature to pre-coat the mesh with serum proteins. Each mesh segment was then rinsed with PBS and seeded with 10T½ cells at 10,000 cells/cm². The meshes were then cultured in DMEM supplemented with 10% FBS, 1% PSA (10,000 U/ml penicillin, 10,000 µg/L streptomycin sulfate and 25 µg/ml amphotericin B; Mediatech) for 2 wks to allow cells to form a confluent cell layer with stable ECM-based adhesion to the mesh. To fabricate cylindrical constructs, meshes were wrapped around sterile 3 mm diameter glass rods so that the mesh fibers were aligned circumferentially. The ends of the rolled constructs were secured to the glass rods with suture ties. After forming cylindrical constructs, specimens were cultured in DMEM containing 10% FBS, 1% PSA, 1 ng/ml TGF-β1 (Sigma-Aldrich) and 50 µg/mL ascorbic acid for another 4 wks for a total time in culture of 6 wks.

Tubular cell layers were fabricated as controls for mechanical assessments and were prepared in a similar manner. In brief, cells were seeded at 10,000 cells/cm² onto tissue culture polystyrene petri dishes. The cells were cultured in DMEM supplemented with 10% FBS and 1% PSA for 2 wks to allow cells to form a confluent cell layer and to deposit initial ECM. These cell layers lifted easily from the surface of the polystyrene dishes upon gentle disruption of the boundary cells, and the resulting layer was rolled around a sterile 3 mm diameter glass rod with the ends secured with suture ties. These tubular cell layer controls were then cultured in DMEM containing 10% FBS, 1% PSA, 1 ng/ml TGF-β1, and 50 µg/mL ascorbic acid for another 4 wks for a total time in culture of 6 wks.

2.4. Construct Analyses

After 6 wks of total culture time, the cell-mesh constructs were harvested for mechanical and biochemical analyses. Each construct (n = 4) was cut into 5 ring segments, each ≈5 mm in length using surgical scissors. The two end-segments were discarded to avoid edge-effects in graft evaluation. One segment per construct was then allocated for mechanical testing.

The remaining sections were immersed in formalin overnight, after which they were allocated for histological or biochemical assays. Details of the mechanical, biochemical and histological analyses are given in the following sections.

2.4.1. Mechanical Property Assessment—Allocated construct segments were evaluated mechanically using a modification of the circumferential ring tests validated by Johnson et al.³⁶ A digital micrometer was used to measure the dimensions of each construct segment. Each ring was then mounted onto an Instron 3342 equipped with a 10 N load cell using custom brackets.³⁶ The ring segments were exposed to uniaxial strain at a rate of 6 mm/min until failure. Applied stress was calculated from the measured force by approximating the area of force application as two rectangles, each with sides equal to the width and wall thickness of the ring. The gauge length, l_g , was taken to be the inner diameter, D_v , of the unstretched ring plus the wall thickness, h_v . The tangential modulus, E , of each sample was defined as the slope of the resulting stress–strain curve at a reference stress of ≈ 25 kPa to mimic the circumferential stress experienced under physiological pressures ($\approx 0.5 P_{avg} D_v/h_v$).³⁷ The mechanical properties of the tubular cell layers were assessed in a similar manner.

To permit direct comparison of mesh mechanical properties with those of the tubular cell-mesh composites, rectangular 1.5 cm $\times$ 3 cm mesh strips were cut with the short edge parallel to the direction of fiber alignment. These strips were subsequently wrapped around a 4 mm glass rod so as to give circumferentially-oriented fibers. The seam of the wrapped mesh was temporarily secured with knotted Dacron thread, and 0.3 μ L of 50:50 isopropanol:HFIP was pipetted at ≈ 3 mm intervals along the mesh seam. The solvent locally dissolved the mesh fibers but not the Dacron string. The dissolved mesh fibers reset as the solvent evaporated to form a cohesive tubular mesh. Five ring segments ≈ 5 mm in width were cut from the resultant mesh, after which the segments were wetted in PBS for 24 h. Circumferential mechanical tests were then performed. Tubular cell layers and native sheep coronary artery segments (Lampire Biological Laboratories) were also processed as parallel controls.

2.4.2. Assessment of Cell Alignment—A set of cell-mesh constructs were stained with rhodamine phalloidin and imaged under confocal microscopy. The angle of orientation, θ , of the long axis of individual cells relative to that of the electrospun mesh fibers was determined by analysis of confocal images using ImageJ. The degree of cellular alignment parallel to the mesh fibers was then characterized by the angular standard deviation, σ , for a wrapped normal distribution with a periodicity of π radians:

$$f(\theta) = \frac{1}{\pi} \left(1 + 2 \sum_{i=1}^{\infty} \rho^i \cos(2p(\theta_i - \mu)) \right)$$

where μ is the mean angle and ρ is the mean resultant length.³⁸ These parameters were determined from n measured cell orientations, θ_i , using the following equations:

$$\rho = \frac{1}{n} \sqrt{\left(\sum_{i=1}^{\infty} \cos(2\theta_i) \right)^2 + \left(\sum_{i=1}^{\infty} \sin(2\theta_i) \right)^2}$$

$$\mu = \tan^{-1} \left(\frac{\sum_{i=1}^{\infty} \sin(2\theta_i)}{\sum_{i=1}^{\infty} \cos(2\theta_i)} \right)$$

Finally, the angular standard deviation was calculated from the mean resultant length:

$$\sigma = \frac{1}{2} \sqrt{-2 \ln \rho}$$

2.4.3. Biochemical Analyses—Samples for biochemical analyses were transferred to 2 mL screw-cap microcentrifuge tubes containing 1.5 mL of lysis buffer (PBS containing 1% Triton X-100, 0.5% sodium dodecyl sulfate, and 100 µg/mL phenylmethylsulfonyl fluoride) and 1 mL of 3.2 mm stainless steel beads. Each sample segment was homogenized at 4800 rpm in a Bead-Beater homogenizer (Biospec) in 10 s cycles with 1 min intermediate cooling on ice. Each sample homogenate was centrifuged to separate mesh and cell debris, and the supernatant was collected. Each supernatant was then analyzed using antibodies for collagen I (COL1A1, clone D-13), collagen III (COL3A1; clone S-17), elastin (clone BA-4), and GAPDH (clone V-18) via competitive ELISA. Competitive peptides corresponding to the immunogens for the respective collagen I, collagen III, and GAPDH antibodies were obtained from Santa Cruz Biotechnology (SCBT). For the elastin antibody, bovine α-elastin (Sigma) served as the competitive peptide. Enzyme immunoassay plates were coated with the appropriate peptide at either 500 ng (collagen III peptide) or 2000 ng (collagen I, α-elastin, GAPDH peptides) per well, after which the wells were blocked with bovine serum albumin (BSA). At the time of analysis, samples were incubated with primary antibody for 1 h, following which the mixtures were applied to the coated plates for 1 h. Standards were prepared by similarly incubating primary antibody with varying levels of competitive peptide for 1 h, followed by application to coated plates. For both samples and standards, bound primary antibody was detected using appropriate HRP-conjugated secondary antibody, followed by application of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma) and monitoring of absorbance at 410 nm.

2.4.4. Immunohistological Analyses—Construct segments reserved for histological analyses were fixed in formalin overnight at 4 °C, embedded in OCT media, and cut into 10 µm sections. Cell phenotype and ECM deposition were analyzed using standard immunohistochemical technique. In brief, rehydrated sections were exposed for 30 min to Terminator (Biocare Medical) blocking reagent. Primary antibodies for collagen I, collagen III, elastin, and SM22α were diluted in PBS containing 3% BSA and 0.5% Tween 20 and applied overnight at 4 °C. Bound primary antibody was detected by applying appropriate secondary antibody (Jackson ImmunoResearch) conjugated to either alkaline phosphatase (AP) or horse radish peroxidase (HRP). Sections exposed to AP-conjugated secondary antibody were then treated with the chromogen Ferangi Blue (Biocare Medical), while sections exposed to HRP-conjugated secondary antibody were treated with the chromogen

3,3-diaminobenzidine (Biocare Medical). Stained sections were imaged using a Zeiss Axiovert 200M microscope.

2.4.5. Transmission Electron Microscopy Analyses—To visualize the organization of the collagen fibers deposited by the 10T½ cells, transmission electron microscopy (TEM) imaging was performed on cell-mesh construct sections. In brief, construct segments allotted for TEM imaging were immersed in 5% glutaraldehyde in HEPES buffered saline (HBS) for 20 min, followed by a 6 min vacuum microwave cycle. Following removal of unreacted glutaraldehyde with three HBS washes, an additional 1 min vacuum microwave cycle was performed. The segments were then treated with osmium tetroxide overnight at 4 °C. Segments were subsequently dehydrated using graded methanol baths, with intervening 1 min vacuum microwave cycles through 70% methanol, after which 6 min vacuum microwave cycles were employed. The construct segments were then placed in a graded series of resin-alcohol mixtures, with the resin being composed of 2.8 g Quetol 651, 4.45 g of cycloaliphatic epoxide resin, 12.8 g of nonenyl succinic anhydride, and 0.4 mL benzyldimethylamine. Following embedding and curing at 60 °C for 24 h, ultrathin sections were cut and stained with uranyl acetate and lead citrate. The resulting sections were imaged using a JEOL 1200 EX TEM.

2.5. Statistical Analysis

All results are reported as the mean $\pm$ standard error of the mean. Statistical comparisons of specific aspects of the stress-strain curves of cell-mesh constructs relative to control groups were performed using the Tukey's Post Hoc Test (SPSS software). A p -value < 0.05 was considered statistically significant.

3. RESULTS

As shown schematically in Figure 1, cell-mesh constructs were prepared by first seeding 10T½ smooth muscle progenitor cells onto the surfaces of PEUR electrospun mesh sheets with uniaxially aligned fibers. Following 2 wks of culture to allow for the establishment of a confluent cell layer and initial ECM deposition, each cell-laden electrospun mesh was wrapped around an inner mandrel such that the mesh fibers were circumferentially aligned. Following 4 wks of additional culture, the cell-mesh constructs were analyzed histologically, mechanically, and biochemically.

3.1. Assessment of Cell Alignment

To assess the capacity of the circumferentially aligned fibers of the electrospun mesh to promote desired cell alignment, the average fiber diameter and average fiber orientation within the mesh sheets were first characterized. Quantitative analysis of SEM images of the electrospun mesh fibers taken prior to the experiment outset (Figure 2A) revealed a mean fiber diameter and angular deviation of $1.1 \pm 0.4 \mu\text{m}$ and 21.0° , respectively. A fiber angular deviation of less than 30° is generally associated with a relatively high degree of fiber alignment, and aligned micron-scale fibers have previously been shown to be able to provide contact guidance for cell orientation.³² These results therefore indicate that the fabricated PEUR electrospun mesh sheets may have the capacity to support directional cell

alignment. To confirm this, the average angle of the long-axis of 10T½ cells within the cell-mesh constructs following 6 wks of culture was measured relative to the direction of the average mesh fiber. The average cell angle was 4.8° relative to the average fiber orientation, and the angular deviation of cell alignment was 27.3°. These data indicate a relatively high degree of cell alignment in the direction of the mesh fibers.

3.2. Mechanical Characterization of Cell Layer-Mesh Composites

The mechanical behavior of cell-mesh composites was characterized via circumferential loading following 6 wks of total culture time. The resulting mechanical responses were compared to that of native sheep coronary artery as well as the two, individual components of the cell-mesh composite: 1) the PEUUR electrospun mesh fabricated into a single-layer tubular structure and 2) 10T½ cell layers fabricated into a tubular structure in the absence of a mesh component. In the latter case, confluent 10T½ cell layers were cultured for 2 wks and then rolled (without a mesh) and cultured in the same way as the cell-mesh composites for an additional 4 wks. Figure 2B displays a representative stress-strain curve for sheep coronary artery and highlights the various regions of the stress-strain curve to be discussed in subsequent sections.

The electrospun mesh exhibited a nearly uniform, linear stress-strain relationship, whereas the cell-mesh constructs and tubular cell layer controls displayed J-shaped stress-strain curves (Figure 3A) similar to that of native sheep coronary artery (Figure 2B). Notably, the slope of the “toe” region of the cell-mesh constructs was 29 ± 8 kPa, which was similar to the 25 ± 2 kPa slope of the sheep coronary artery “toe” region ($p = 0.609$) as well as the ≈ 45 kPa slope of the “toe” region of the tubular cell layer control.

In contrast, the slope of the “linear” region of the cell-mesh constructs measured 1125 ± 140 kPa. This value was statistically indistinguishable from the 999 ± 83 kPa slope of the pure electrospun mesh ($p = 0.672$) and the 1291 ± 95 kPa slope of the sheep coronary artery ($p = 0.750$), but over 2-fold greater than the ≈ 450 kPa slope of the “linear” region of the tubular cell layer control. These data suggest that the stress-strain behavior of the cell-mesh constructs in the “toe” region was dominated by cell-cell/cell-ECM interactions of the cell layer component, whereas the stress-strain behavior in the “linear” region was dominated by the electrospun mesh component. Also, these data indicate that the cell-mesh constructs are behaving similarly to a sheep coronary artery with respect to the slopes of the “toe” and “linear” regions of the stress-strain curves.

The multi-component design of the cell-mesh construct also appeared to positively impact the region of upturning between the “toe” and “linear” regions from that of the cell-only controls. Specifically, the region of upturning for the tubular cell layers occurred at circumferential strains between ≈ 0.11 – 0.15 , consistent with previous cell layer work.^{39,40} In contrast, the average circumferential strain at upturning for the cell-mesh constructs was ≈ 0.20 – 0.23 . This strain range is consistent with the average circumferential strain at upturning of ≈ 0.15 – 0.25 observed for human coronary artery,⁸ which is significantly lower than the ≈ 0.55 – 0.65 strain at upturning observed for porcine⁸ and sheep (Figure 2B) coronary artery.

Beyond these parameters, graft compliance at physiological pressures as well as graft circumferential ultimate tensile strength (UTS) are two properties critical to vascular graft performance. As an indirect measure of graft compliance, the modulus of the cell-mesh constructs at physiological pressures (termed the tangential modulus) was evaluated at a reference circumferential stress corresponding to normal physiological pressures (≈ 25 kPa, Figure 3A). The average tangential modulus of the cell-mesh constructs was 493 ± 53 kPa, which was similar to the ≈ 550 kPa tangential modulus of the tubular cell layer and the 426 ± 58 kPa tangential modulus of the sheep coronary artery ($p = 0.455$). The cell-mesh constructs also displayed an average UTS of 0.343 ± 0.071 MPa (Figure 3B), roughly 50% of that of both the 0.700 ± 0.100 MPa UTS of the pure electrospun mesh ($p = 0.016$) and the 0.670 ± 0.100 MPa UTS of the sheep coronary artery ($p = 0.009$). In contrast, the maximum UTS measured for the tubular cell layers was 0.335 MPa.

3.3. ECM Deposition and Cell Phenotype

In the current study, 10T $\frac{1}{2}$ progenitor cells were selected for use as a mimic of human smooth muscle progenitor cells.²⁷ Following construct assembly, TGF- β 1 was added to the cell culture media to promote 10T $\frac{1}{2}$ differentiation into smooth muscle-like cells.^{41,42} In addition, TGF- β 1 was utilized to stimulate the deposition of elastin necessary for appropriate long-term vessel compliance.²⁹ Similarly, ascorbic acid was added to the culture media to promote the collagen production necessary for long-term graft strength.²⁹ To assess the induction of an SMC phenotype in the cell-mesh constructs, immunostaining for the mid-term SMC-marker SM22 α was conducted at the culture endpoint. A representative image of SM22 α staining of a transverse section of a cell-mesh construct is shown in Figure 4A. Positive SM22 α staining was noted for $\approx 95\%$ of cells in every cross-section analyzed, consistent with previous studies indicating that TGF- β 1 treatment induces $>90\%$ of exposed 10T $\frac{1}{2}$ cells to express early- to mid-term SMC markers.⁴³

In assessing 10T $\frac{1}{2}$ collagen deposition, the two primary collagen types associated with the coronary artery wall, namely collagen types I and III, were evaluated by immunostaining. Immunostaining for collagen I revealed dense staining within the cell layers as well as substantial staining within the mesh regions directly underlying the cell layers (Figure 4B). In contrast, positive staining for collagen III was primarily associated with the cell layer and generally did not substantially penetrate into the mesh (Figure 4C). Elastin staining was more spatially variable in intensity than the collagen I and III staining. Specifically, elastin staining was quite intense in some cell layer regions, but was frequently absent in neighboring layers (Figure 4D).

To examine the spatial organization of the deposited ECM, transverse cell-mesh sections were imaged via TEM with a focus on collagen fibrils. Imaging with TEM revealed relatively dense fibers with a high degree of alignment parallel to the average mesh fiber orientation (Figure 5A,B). This implies that the 10T $\frac{1}{2}$ cells were depositing and/or organizing newly synthesized collagen fibrils with an average circumferential orientation, as is observed in native coronary artery.¹⁹ Quantitative assessment of ECM protein deposition was also conducted via competitive ELISA. These analyses revealed that cells within the cell-mesh constructs produced 14.5 ± 6.1 pg/cell of collagen I and approximately 9.1 ± 2.0

pg/cell of collagen III. In contrast, the production of elastin was significantly lower than that of collagen at average of 1.5 ± 1.0 pg/cell (Figure 5C).

4. DISCUSSION

The aim of the present study was to investigate the potential of cell layer–electrospun mesh scaffolds for CABG applications. These composite scaffolds were generated by culturing a confluent layer of cells on PEUUR electrospun mesh sheets with uniaxially aligned fibers. After 2 wks of 2D culture, each PEUUR electrospun mesh sheet was wrapped around sterile 3 mm diameter glass rods, with mesh fibers aligned circumferentially. Following an additional 4 wks of culture, the mechanical properties and ECM production within the tubular cell-mesh constructs were analyzed. The underlying hypothesis of this work is that ECM deposited by the cell layers will provide structural elasticity at low strains, while also acting as an adhesive agent between individual mesh layers, providing strength to the construct.

Consistent with this hypothesis, circumferential mechanical testing of cell-mesh constructs revealed a J-shaped stress–strain curve which was similar to that of native human coronary artery.⁸ This biphasic stress-strain curve was not achieved by electrospun mesh grafts alone (Figure 3). Based on comparison with tubular cell layer controls (no mesh), the low stiffness (“toe”) behavior of the cell-mesh constructs appeared to be dominated by the cell-cell and/or cell-ECM interactions, while the higher stiffness (“linear”) behavior appeared to be dominated by the mesh. In addition, the “toe” and “linear” region slopes of cell-mesh construct stress-strain curves were similar to those of sheep coronary artery controls and to reported values for human coronary artery (“toe”: ≈ 20 – 100 kPa and “linear”: ≈ 1000 kPa).⁸

Due to the association between increased graft-host compliance mismatch and increased risk of long-term graft failure,^{4–8,44,45} the compliance of the cell-mesh constructs was indirectly assessed. Specifically, the tangential modulus of the constructs at physiological stresses was determined and compared to native tissue. The measured tangential modulus of the cell-mesh constructs (493 ± 53 kPa) slightly exceeded the target range of 200 – 400 kPa associated with human coronary artery.⁸ However, this degree of mismatch is similar to that observed between human coronary artery and human internal mammary artery (tangential modulus of 400 ± 200 kPa)³³, the current “gold standard” for coronary artery bypass grafting.² Thus, the compliance of the cell-mesh constructs at physiological stresses appears to be suitable for CABG applications.

In terms of tensile strength, the composite scaffolds exhibited an average circumferential UTS of 0.34 MPa (≈ 2500 mmHg). Importantly, the UTS of our CABG is similar to the ≈ 0.37 MPa UTS of vertebral artery³⁷ and is similar to the ≈ 0.38 MPa UTS of previous CABGs formed from rolled medial-adventitial cell layers.⁴⁰ However, this UTS is significantly lower than the ≈ 0.80 MPa UTS of umbilical cord artery as measured by circumferential ring tests⁴⁰ and is roughly 50% of that of the 0.70 MPa UTS of the pure electrospun mesh component of the graft. Improvement in the agreement between the circumferential UTS of the cell-mesh constructs and that of the mesh alone could potentially be achieved by enhancing the degree of ECM infiltration into the mesh layers. Although

immunostaining indicated significant collagen I penetration into the mesh structure, collagen III and elastin deposition within the mesh network was limited (Figure 4). Increased ECM penetration into the electrospun mesh structure would be anticipated to increase the interfacial strength between the mesh and the intervening cell layers, increasing the stress required to initiate failure of the rolled graft structure.

In addition to providing circumferential strength, it was envisioned that the aligned mesh fibers would inherently support desired circumferential cell and ECM alignment. Such circumferential cell and matrix orientation is generally achieved within CABGs via application of cyclic stretch and/or as a byproduct of cell-mediated scaffold contraction around a cylindrical mandrel.^{46–48} In the present cell-mesh construct design, contact guidance^{19–21,32} was used to induce directional cell elongation and to support directional ECM deposition without the need for external or cell-mediated mechanical loading. Consistent with this hypothesis, the average cell angle relative to the average fiber angle was 4.8° with an angular deviation of ≈27°. Furthermore, TEM imaging of construct sections indicated that deposited collagen fibers also displayed a circumferential orientation, consistent with circumferential alignment of collagen and elastin fibers in the medial layer of native arteries.

At a quantitative level, the amounts of collagen and elastin produced by 10T½ smooth muscle progenitor cells within the cell-mesh constructs were comparable to previously reported values for graft cells cultured under similar culture duration and media conditions. In particular, Sander et al. developed a vascular graft generated from neonatal human dermal fibroblasts cultured within a fibrin gel in the presence of 1 ng/mL TGF-β1 and 50 µg/mL ascorbic acid. Associated fibroblasts produced an average of 75.2 pg/cell total collagen and 2.1 pg/cell elastin over 5 wks of culture.⁴⁹ Similarly, Long et al. demonstrated that adult rat aortic smooth muscle cells cultured in a fibrin hydrogel for 4 wks in the presence of 1 ng/mL TGF-β1 deposited ≈20 pg/cell total collagen and ≈10 pg/cell elastin.²⁹ The combined results indicate that cell-mesh hybrid constructs warrant further investigation as CABGs.

Limitations of the current cell-mesh construct design include the prolonged use of media additives (TGF-β1 and ascorbic acid) and the extended pre-culture time needed for construct assembly. These limitations would need to be addressed to enable to design to be clinically relevant. Furthermore, future studies would need to transition to the use human smooth muscle progenitor cells²⁷ – as opposed to the smooth muscle progenitor cell line used herein - and would need to incorporate a mechanism for graft endothelialization. Beyond these issues, tuning of the PEUR elastomer^{24–26} and/or the mesh fabrication conditions^{50,51} so as to improve cell infiltration and ECM deposition within the mesh while also maintaining initial target mechanical properties will be needed.

Acknowledgments

We would like to acknowledge the NSF DMR CAREER Award 1346807, NIH R01 EB013297, and the NIH R03 EB0152167 for funding. The authors would also like to thank Ms. E. Ann Ellis and Dr. Michael Pendelton of the Texas A&M University Imaging and Microscopy Center for their expert technical assistance in developing protocols for construct TEM imaging.

References

1. Nerem RM, Seliktar D. Vascular tissue engineering. *Annu Rev Biomed Eng.* 2001; 3:225–43. [PubMed: 11447063]
2. Karthik S, Fabri BM. Left internal mammary artery usage in coronary artery bypass grafting: a measure of quality control. *Ann R Coll Surg Engl.* 2006; 88(4):367–9. [PubMed: 16834857]
3. Kempczinski, R, editor. *Vascular Surgery.* Denver: WB Saunders; 2000.
4. Perktold K, Leuprecht A, Prosi M, Berk T, Czerny M, Trubel W, Schima H. Fluid dynamics, wall mechanics, and oxygen transfer in peripheral bypass anastomoses. *Ann Biomed Eng.* 2002; 30(4):447–60. [PubMed: 12085997]
5. Walden R, L'Italien GJ, Megerman J, Abbott WM. Matched elastic properties and successful arterial grafting. *Arch Surg.* 1980; 115(10):1166–9. [PubMed: 6448593]
6. QIU Y, TARBELL JM. Computational Simulation of Flow in the End-to-End Anastomosis of a Rigid Graft and a Compliant Artery. *ASAIO Journal.* 1996; 42(5):M702–709. [PubMed: 8944971]
7. John LC. Biomechanics of coronary artery and bypass graft disease: potential new approaches. *Ann Thorac Surg.* 2009; 87(1):331–8. [PubMed: 19101335]
8. van Andel CJ, Pistecky PV, Borst C. Mechanical properties of porcine and human arteries: implications for coronary anastomotic connectors. *Ann Thorac Surg.* 2003; 76(1):58–64. [PubMed: 12842513]
9. Seliktar D, Black R, Vito R, Nerem R. Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. *Ann Biomed Eng.* 2000; 28:351–62. [PubMed: 10870892]
10. Isenberg B, Tranquillo R. Long-term cyclic distention enhances the mechanical properties of collagen-based media-equivalents. *Annals of Biomedical Engineering.* 2003; 31:937–939. [PubMed: 12918909]
11. Hahn M, McHale M, Wang E, Schmedlen R, West J. Physiologic pulsatile flow bioreactor conditioning of poly(ethylene glycol)-based tissue engineered vascular grafts. *Annals of Biomedical Engineering.* 2007; 35(2):190–200. [PubMed: 17180465]
12. Solan A, Mitchell S, Moses M, Niklason L. Effect of pulse rate on collagen deposition in the tissue-engineered blood vessel. *Tissue Engineering.* 2003; 9(4):579–86. [PubMed: 13678437]
13. Guo FY, Wang N, Wang L, Hou LL, Ma L, Liu J, Chen YE, Fan BB, Zhao Y. An electrospun strong PCL/PU composite vascular graft with mechanical anisotropy and cyclic stability. *Journal of Materials Chemistry A.* 2015; 3(9):4782–4787.
14. Montini-Ballarín F, Calvo D, Caracciolo PC, Rojo F, Frontini PM, Abraham GA, Guinea VG. Mechanical behavior of bilayered small-diameter nanofibrous structures as biomimetic vascular grafts. *Journal of the Mechanical Behavior of Biomedical Materials.* 2016; 60:220–233. [PubMed: 26872337]
15. Ma ZW, Kotaki M, Inai R, Ramakrishna S. Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue Engineering.* 2005; 11(1–2):101–109. [PubMed: 15738665]
16. Wu HJ, Fan JT, Chu CC, Wu J. Electrospinning of small diameter 3-D nanofibrous tubular scaffolds with controllable nanofiber orientations for vascular grafts. *Journal of Materials Science-Materials in Medicine.* 2010; 21(12):3207–3215. [PubMed: 20890639]
17. Rocco KA, Maxfield MW, Best CA, Dean EW, Breuer CK. In Vivo Applications of Electrospun Tissue-Engineered Vascular Grafts: A Review. *Tissue Engineering Part B-Reviews.* 2014; 20(6):628–640. [PubMed: 24786567]
18. Hasan A, Memic A, Annabi N, Hossain M, Paul A, Dokmeci MR, Dehghani F, Khademhosseini A. Electrospun scaffolds for tissue engineering of vascular grafts. *Acta Biomaterialia.* 2014; 10(1):11–25. [PubMed: 23973391]
19. Bashur CA, Dahlgren LA, Goldstein AS. Effect of fiber diameter and orientation on fibroblast morphology and proliferation on electrospun poly(D,L-lactic-co-glycolic acid) meshes. *Biomaterials.* 2006; 27(33):5681–8. [PubMed: 16914196]
20. Ng KW, Hutmacher DW. Reduced contraction of skin equivalent engineered using cell sheets cultured in 3D matrices. *Biomaterials.* 2006; 27(26):4591–8. [PubMed: 16720038]

21. Ahn H, Ju YM, Takahashi H, Williams DF, Yoo JJ, Lee SJ, Okano T, Atala A. Engineered small diameter vascular grafts by combining cell sheet engineering and electrospinning technology. *Acta Biomaterialia*. 2015; 16:14–22. [PubMed: 25641646]
22. Han F, Jia X, Dai D, Yang X, Zhao J, Zhao Y, Fan Y, Yuan X. Performance of a multilayered small-diameter vascular scaffold dual-loaded with VEGF and PDGF. *Biomaterials*. 2013; 34(30):7302–13. [PubMed: 23830580]
23. Guelcher SA. Biodegradable polyurethanes: synthesis and applications in regenerative medicine. *Tissue Eng Part B Rev*. 2008; 14(1):3–17. [PubMed: 18454631]
24. Guelcher SA, Gallagher KM, Didier JE, Klinedinst DB, Doctor JS, Goldstein AS, Wilkes GL, Beckman EJ, Hollinger JO. Synthesis of biocompatible segmented polyurethanes from aliphatic diisocyanates and diurea diol chain extenders. *Acta Biomater*. 2005; 1(4):471–84. [PubMed: 16701828]
25. Guelcher SA, Srinivasan A, Dumas JE, Didier JE, McBride S, Hollinger JO. Synthesis, mechanical properties, biocompatibility, and biodegradation of polyurethane networks from lysine polyisocyanates. *Biomaterials*. 2008; 29(12):1762–75. [PubMed: 18255140]
26. Kavlock KD, Pechar TW, Hollinger JO, Guelcher SA, Goldstein AS. Synthesis and characterization of segmented poly(esterurethane urea) elastomers for bone tissue engineering. *Acta Biomater*. 2007; 3(4):475–84. [PubMed: 17418651]
27. Merkulova-Rainon T, Broqueres-You D, Kubis N, Silvestre JS, Levy BI. Towards the therapeutic use of vascular smooth muscle progenitor cells. *Cardiovascular Research*. 2012; 95(2):205–214. [PubMed: 22354897]
28. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. *Annu Rev Physiol*. 2012; 74:13–40. [PubMed: 22017177]
29. Long JL, Tranquillo RT. Elastic fiber production in cardiovascular tissue-equivalents. *Matrix Biol*. 2003; 22(4):339–50. [PubMed: 12935818]
30. Roach MR, Burton AC. The reason for the shape of the distensibility curves of arteries. *Canadian Journal of Biochemistry and Physiology*. 1957; 35(8):681–690. [PubMed: 13460788]
31. Cleary MA, Geiger E, Grady C, Best C, Naito Y, Breuer C. Vascular tissue engineering: the next generation. *Trends in Molecular Medicine*. 2012; 18(7):394–404. [PubMed: 22695236]
32. Bashur CA, Shaffer RD, Dahlgren LA, Guelcher SA, Goldstein AS. Effect of fiber diameter and alignment of electrospun polyurethane meshes on mesenchymal progenitor cells. *Tissue Eng Part A*. 2009; 15(9):2435–45. [PubMed: 19292650]
33. Soletti L, Hong Y, Guan J, Stankus JJ, El-Kurdi MS, Wagner WR, Vorp DA. A bilayered elastomeric scaffold for tissue engineering of small diameter vascular grafts. *Acta Biomater*. 2010; 6(1):110–22. [PubMed: 19540370]
34. Stylianopoulos T, Bashur CA, Goldstein AS, Guelcher SA, Barocas VH. Computational predictions of the tensile properties of electrospun fibre meshes: effect of fibre diameter and fibre orientation. *J Mech Behav Biomed Mater*. 2008; 1(4):326–35. [PubMed: 19627797]
35. Thayer PS, Dimling AF, Plessl DS, Hahn MR, Guelcher SA, Dahlgren LA, Goldstein AS. Cellularized cylindrical fiber/hydrogel composites for ligament tissue engineering. *Biomacromolecules*. 2014; 15(1):75–83. [PubMed: 24266805]
36. Johnson CP, How T, Scraggs M, West CR, Burns J. A biomechanical study of the human vertebral artery with implications for fatal arterial injury. *Forensic Science International*. 2000; 109(3):169–182. [PubMed: 10725653]
37. Johnson C, How T, Scraggs M, West C, Burns J. A biomechanical study of the human vertebral artery with implications for fatal arterial injury. *Forensic Science International*. 2000; 109:169–182. [PubMed: 10725653]
38. Fisher, NI. Statistical analysis of circular data. Cambridge England ; New York, NY, USA: Cambridge University Press; 1993. xviii277
39. Gauvin R, Ahsan T, Larouche D, Levesque P, Dube J, Auger FA, Nerem RM, Germain L. A novel single-step self-assembly approach for the fabrication of tissue-engineered vascular constructs. *Tissue Eng Part A*. 2010; 16(5):1737–47. [PubMed: 20038201]

40. Gauvin R, Guillemette M, Galbraith T, Bourget JM, Larouche D, Marcoux H, Aube D, Hayward C, Auger FA, Germain L. Mechanical properties of tissue-engineered vascular constructs produced using arterial or venous cells. *Tissue Eng Part A*. 2011; 17(15–16):2049–59. [PubMed: 21457095]
41. Hirschi KK, Lai L, Belaguli NS, Dean DA, Schwartz RJ, Zimmer WE. Transforming growth factor- β induction of smooth muscle cell phenotype requires transcriptional and post-transcriptional control of serum response factor. *Journal of Biological Chemistry*. 2002; 277(8):6287–6295. [PubMed: 11741973]
42. Hirschi KK, Rohovsky SA, D'Amore PA. PDGF, TGF- β , and heterotypic cell–cell interactions mediate endothelial cell–induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *Journal of Cell Biology*. 1998; 141(3):805–814. [PubMed: 9566978]
43. Hirschi KK, Lai L, Belaguli NS, Dean DA, Schwartz RJ, Zimmer WE. Transforming growth factor- β induction of smooth muscle cell phenotype requires transcriptional and post-transcriptional control of serum response factor. *J Biol Chem*. 2002; 277(8):6287–95. [PubMed: 11741973]
44. van Andel CJ, Pistecky PV, Borst C. Mechanical properties of porcine and human arteries: Implications for coronary anastomotic connectors. *Annals of Thoracic Surgery*. 2003; 76(1):58–64. [PubMed: 12842513]
45. John LCH. Biomechanics of Coronary Artery and Bypass Graft Disease: Potential New Approaches. *The Annals of Thoracic Surgery*. 2009; 87(1):331–338. [PubMed: 19101335]
46. Barocas VH, Girtan TS, Tranquillo RT. Engineered alignment in media equivalents: magnetic prealignment and mandrel compaction. *J Biomech Eng*. 1998; 120(5):660–6. [PubMed: 10412446]
47. Grenier G, Remy-Zolghadri M, Larouche D, Gauvin R, Baker K, Bergeron F, Dupuis D, Langelier E, Rancourt D, Auger FA, et al. Tissue reorganization in response to mechanical load increases functionality. *Tissue Eng*. 2005; 11(1–2):90–100. [PubMed: 15738664]
48. L'Heureux N, Paquet S, Labbe R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. *FASEB J*. 1998; 12(1):47–56. [PubMed: 9438410]
49. Sander EA, Barocas VH, Tranquillo RT. Initial fiber alignment pattern alters extracellular matrix synthesis in fibroblast-populated fibrin gel cruciforms and correlates with predicted tension. *Ann Biomed Eng*. 2011; 39(2):714–29. [PubMed: 21046467]
50. Baker BM, Gee AO, Metter RB, Nathan AS, Marklein RL, Burdick JA, Mauck RL. The Potential to Improve Cell Infiltration in Composite Fiber-Aligned Electrospun Scaffolds by the Selective Removal of Sacrificial Fibers. *Biomaterials*. 2008; 29(15):2348–2358. [PubMed: 18313138]
51. Nezarati RM, Eifert MB, Cosgriff-Hernandez E. Effects of humidity and solution viscosity on electrospun fiber morphology. *Tissue Eng Part C Methods*. 2013; 19(10):810–9. [PubMed: 23469941]

Figure 1.

Schematic demonstrating the fabrication of the cell-mesh composite constructs. Step 1: An electrospun mesh sheet was seeded with 10^4 smooth muscle progenitor cells and cultured for 2 wks in DMEM + 10% FBS to allow for the formation of a confluent, stably adherent cell layer on the mesh. Step 2: The cell-laden mesh was then wrapped around a 3 mm diameter glass mandrel to form a multi-layered, tubular cell-mesh composite construct. Step 3: This composite construct was then cultured for 4 wks in the presence of TGF- β 1 and ascorbic acid, for a total of 6 wks of culture time. Constructs were then analyzed mechanically, biochemically, and histologically. Our hypothesis is that the ECM deposited by the cell layer will act as an adhesive agent between adjacent mesh layers and will serve as a source for structural elasticity at low strains. The inset shows a brightfield image of a transverse section through a cell-mesh construct following 6 wks of total culture time. The scale bar in the inset equals 100 μ m. The cell layers between the mesh layers appear brown due to positive staining for smooth muscle 22 α (SM22 α).

Figure 2.

(A) A representative SEM image of the PEUUR electropun mesh used in the current studies. Scale bar = 2 μm (B) A representative circumferential stress-strain curve of a sheep coronary artery. Key regions of the stress-strain curve – the “toe” region, the tangential modulus, and the “linear” region – are labelled for further reference.

Figure 3.

Representative stress-strain responses of the cell-mesh constructs shown alongside representative stress-strain curves for the individual construct components (mesh and cell layer). (A) Representative stress-strain curves prior to failure (B) Representative stress-strain curves through the point of failure.

Figure 4.

(A) A representative image of a transverse section of a cell-mesh construct immunostained for SM22 α . Brown coloration indicates positive staining. Positive stained cells appeared to be primarily isolated to cell-mesh interface, with limited cell migration into the mesh structure observed. (B) A representative image of a transverse section of a cell-mesh construct immunostained for collagen I. Brown coloration indicates positive staining. The intensity of the staining is highest within the cell layer. However, significant levels of brown staining extend into the mesh. (C) A representative image of a transverse section of a cell-mesh construct immunostained for collagen III. Brown coloration indicates positive staining. (D) A representative image of a transverse section of a cell-mesh construct immunostained for elastin. Blue coloration indicates positive staining. The cells on the upper mesh layer show substantial elastin staining, whereas the cells on the underlying mesh layer show no apparent positive staining. Such regional differences were representative of the substantial spatial variability observed for elastin staining. The reasons for these spatial differences in elastin production are unclear. The scale bar in (A) applies to all images and represents 100 μ m. The variable number of cell-mesh layers in images A through D reflects the fact that cryosections of the composite constructs failed to adhere to charged slides. The construct sections therefore experienced significant mechanical stress during the immunostaining process, which tended to result in the separation of individual construct layers during staining.

Figure 5.

(A) A representative TEM image of an ultrathin, transverse cross-section through a cell-mesh construct, scale bar = 500 nm. The arrows in (A) point to fiber structures within the section. The encircled region in (A) is expanded in (B) for enhanced visualization of the fiber structures, scale bar = 500 nm. The fibers in (B) are approximately 20 nm thick, consistent with the thickness of collagen fibrils. Fibers parallel to the arrow in (B) can be said to be circumferentially aligned. (C) ELISA measures of collagen I (Col I), collagen III (Col III), and elastin.