A Novel Cylindrical Biaxial Computer-Controlled Bioreactor and Biomechanical Testing Device for Vascular Tissue Engineering

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It is becoming evident that tissue-engineered constructs adapt to altered mechanical loading, and that specific combinations of multidirectional loads appear to have a synergistic effect on the remodeling. However, most studies of mechanical stimulation of engineered vascular tissue engineering employ only uniaxial stimulation. Here we present a novel computer-controlled bioreactor and biomechanical testing device designed to precisely and simultaneously control mean and cyclic values of transmural pressure (at rates up to 1 Hz and ranges of 40 mmHg), luminal flow rate, and axial length (or load) applied to gel-derived, scaffold-derived, and self-assembly-derived tissue-engineered blood vessels during culture, while monitoring vessel geometry with a resolution of 6.6 μm . Intermittent monitoring of the extracellular matrix and cells is accomplished on live tissues using multi-photon confocal microscopy under unloaded and loaded conditions at multiple time-points in culture (on the same vessel) to quantify changes in cell and extracellular matrix content and organization. This same device is capable of performing intermittent cylindrical biaxial biomechanical testing at multiple time-points in culture (on the same vessel) to quantify changes in the mechanical behavior during culture. Here we demonstrate the capabilities of this new device on self-assembly-derived and collagen-gel-derived tissue-engineered blood vessels.

Introduction

SINCE THE FIRST TISSUE-ENGINEERED BLOOD VESSEL (TEBV) was reported by Weinberg and Bell in 1986,¹ tremendous advances have been made toward the development of a clinically useful small-diameter TEBV, including short-term patency *in vivo*.²⁻⁴ These advances have relied on tools and techniques from a wide variety of disciplines, ranging from molecular and cell biology and biochemistry to material science and biomechanics. With regard to the latter, biomechanical stimuli have been shown to stimulate remodeling of TEBVs to greatly improve their mechanical behavior.^{2,5} These (as well as other) studies demonstrate the central role that mechanical cues play in the growth and remodeling of TEBVs; there is a pressing need, however, to develop theoretical and experimental frameworks that can be used to characterize these mechanisms. Such frameworks can be used to motivate strategies to improve mechanical and functional

properties and reduce requisite culture times for TEBV development.

In native blood vessels, remodeling mechanisms appear to be aimed toward maintaining the local, three-dimensional (3D) mechanical environment (i.e., the local stresses or strains). Specifically, experimental perturbations in mean or cyclic circumferential^{6,7} and axial⁸⁻¹¹ stresses (or strains) as well as wall shear stress¹² induce changes in vessel geometry and material properties that appear to be aimed at restoring these stresses to target levels. Tissue-engineered constructs also adapt to altered mechanical loading. Seliktar *et al.*⁵ demonstrated collagen fiber remodeling and increased mechanical strength in response to cyclic pressure stimulation, showing highly organized collagen lamellar assemblies and circumferential smooth muscle cell (SMC) alignment throughout the thickness of the wall in dynamically conditioned constructs compared to statically conditioned constructs; circumferential alignment of SMCs has been shown to significantly increase functional

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properties in response to histamine, a known vasodilator.¹³ Ng and Swartz¹⁴ have shown cellular and extracellular matrix reorganization in response to fluid flow, with human dermal fibroblast and collagen fiber realigning perpendicular to the direction of flow in collagen gels. Whereas less is known about the role of axial extension on TEBV remodeling, axial stretch-induced remodeling in native arteries has been reported at rates that were unprecedented compared to pressure- and flow-induced remodeling.^{8,15,16} In addition to these individual loading scenarios, it is now becoming evident that specific combinations of multidirectional loads appear to have a synergistic effect on the remodeling and tissue formation.¹⁷ Thus, to truly characterize mechanically mediated remodeling in TEBVs, one must consider multiaxial loading.

Many groups have developed bioreactors capable of studying the individual and combined mechanical effects on a number of tissue types. Most bioreactors are designed to mimic *in vivo* conditions allowing engineering of functional tissue *in vitro*.¹⁸ For example, Engelmayer *et al.* reported synergism for multidirectional loading that was performed on a custom device capable of precisely controlling cyclic flexure, stretch, and flow, mechanical stimuli commonly experienced by heart valve tissue.¹⁹ Multiple bioreactors have been reported in the literature to culture vascular tissue under altered luminal flow and circumferential distension,^{5,20,21} but fewer have developed one capable of precisely controlling axial distension.^{22–24} Similar systems such as the LumeGen bioreactor from Tissue Growth Technologies and the ElectroForce system from Bose are now commercially available. None of these devices, however, offer the combined capabilities of (i) simultaneous independent control of pulsatile pressure, luminal flow, and axial load (or stretch) over long-term culture while monitoring vessel geometry, (ii) performing intermittent cylindrical biaxial mechanical testing on the same vessel at multiple time-points in culture, and (iii) performing intermittent multiphoton microscopy on a live vessels mounted within the bioreactor at multiple time-points in culture and under multiple loading scenarios. Toward this end, we have built and tested a novel bioreactor capable of long-term culture of TEBV subjected to individual or combined external mechanical stimuli: mean and cyclic pressure, axial load (or length), and luminal flow, with monitoring vascular geometry throughout culture. In addition, this device is designed to perform intermittent cylindrical biaxial biomechanical tests and multi-photon confocal microscopy to quantify changes in the mechanical behavior and microstructural organization of the TEBVs, at multiple time-points in culture. Here we present the motivation for this experimental design and demonstrate the capabilities of the device to precisely and independently control the multiaxial mechanical loading during culture and perform intermittent mechanical testing and microscopy.

Theoretical Motivation

Mechanically mediated remodeling occurs as cells sense and respond to changes in their local mechanical environments and can be thought of as an iterative process wherein local perturbations in mechanical environment induce local changes in mechanically sensitive biological responses that, in turn, regulate geometry and vessel wall mechanical behavior (Fig. 1). Fundamental to assessing results and guiding

future experiments in mechanically mediated remodeling of TEBV is the quantification of the local mechanical environment under specific loading conditions at any time, that is, determining the distribution of stresses and strains of a vessel under pressure, axial extension, and flow. Whereas universal solutions (e.g., Laplace's law) relate the mean stresses with geometry and applied loads, knowledge of the constitutive behavior of the vessel wall is required to predict how the local stresses vary throughout the wall for a vessel of known geometry and applied loads. Utilizing equilibrium (i.e., the linear momentum balances, assuming no body forces and quasistatic motion, $\nabla t = 0$), the transmural pressure, P , and axial force, f , are related to the Cauchy stress, t , as

$$P = \int_{r_i}^{r_o} (t_{\theta\theta} - t_{rr}) \frac{1}{r} dr \quad \text{and} \quad f = \pi \int_{r_i}^{r_o} (2t_{zz} - t_{rr} - t_{\theta\theta}) r dr, \quad (1)$$

where the subscripts, rr , $\theta\theta$, and zz represent the components of the Cauchy stress tensor in the radial, circumferential, and axial directions, respectively; r_i and r_o represent the inner and outer radii of the vessel, respectively. P and f are two measurable quantities during testing. In the framework of finite elasticity, for example, the Cauchy stress tensor is given as

$$\mathbf{t} = -p\mathbf{I} + 2\mathbf{F} \cdot \frac{dW}{d\mathbf{C}} \cdot \mathbf{F}^T, \quad (2)$$

where p is the Lagrange multiplier (due to the incompressibility constraint), $\mathbf{I}$ is the identity tensor, $\mathbf{F}$ is the deformation gradient is the gradient of the mapping of points from a stress-free (reference) configuration to the current (loaded) configuration, $\mathbf{C}$ is the right Cauchy-Green tensor, and W is the strain energy density function; p may be solved in terms of W and $\mathbf{F}$ via equilibrium. Therefore, by knowing the reference and current configurations at any

FIG. 1. Illustration of overall approach to modeling growth and remodeling in tissue-engineered blood vessel (TEBV). For a known geometry and constitutive equation with material properties, one can apply equilibrium to calculate the local stresses. Given the local stress, kinetic equations describe mechanobiological responses (e.g., rate of stress-mediated collagen production). Given these kinetic relations, evolution equations can be proposed that describe how biological changes relate to changes in the constitutive equations, material properties, and geometry.

time-point and assuming a strain energy function, one can calculate the local stresses in the vascular wall. Finally, comparing local stresses to the observed kinetics, one can prescribe stress-mediated (or strain-mediated) evolution equations, thus completing the approach to understanding mechanically induced growth and remodeling of TEBV (Fig. 1). Continued iterations will refine experimental parameters and guide future experiments.

The strain energy function can take many functional forms, including purely phenomenological (e.g., Chuong and Fung²⁵) or microstructurally motivated (yet still phenomenological; e.g., Lanir²⁶ and Holzapfel *et al.*²⁷). Microstructurally motivated models can contain aspects of constituent fiber angle, fiber extensibility, active smooth muscle behavior, fiber recruitment, and the like;^{27–30} such microstructurally motivated models are more convenient when quantifying tissue remodeling.³¹ To advance these models, however, quantification of the tissue level mechanical response, along with quantification and incorporation of the measured microstructural data, is necessary.³² Toward this end, we have designed our device to quantify both tissue level mechanical behavior and

microstructural organization on the same vessel at multiple time-points in culture.

Bioreactor and Biomechanical Testing Experimental Setup

To increase throughput of experiments and decrease time requirements for studies, four identical bioreactors are set up in parallel in a dual, water-jacketed CO₂ incubators (NuAir model NU-S8500, Plymouth, MN) and controlled using one computer. A single bioreactor is composed of five subsystems, as shown: (1) the culture chamber, (2) the flow control, (3) the pressure control, (4) axial motion control, and (5) the geometry-monitoring subsystem (Fig. 2).

Culture chamber

The base of the bioreactor is machined from a solid aluminum plate. Two uniaxial spring-actuated stages (Newport model M-423) with appropriate fittings for mounting linear actuators (Newport model LTA-HS, Irvine, CA) are mounted to the base. Atop both uniaxial stages are three axes manually

FIG. 2. Bioreactor/biomechanical testing device for TEBVs with subsystems as noted in the text. Fully assembled system shown in incubator (a). Image of culture chamber showing axial motion controllers as well as mounted biological vascular substitutes (BVS) (b). Detailed image of BVS showing points of attachment to cannula, ultrasound transducer, and load cell (c). Color images available online at www.liebertonline.com/ten.

adjustable stages (Newport model M461) with three manual micrometers to one of allow for alignment of cannula and TEBV. Connected to these stages is a stainless steel rod and custom-machined part to allowing mounting of L-shaped cannula and a load cell (Delta Metrics Inc. model XLU68s, Worthington, OH) to measure axial force. The TEBVs are mounted on the cannula that allow for luminal perfusion. Because this system is capable of being used on a range of vessel sizes, the cannula can range in outer diameter from 1 to 5 mm. The ends of the cannula may be tapered to allow easier mounting of the vessel and given grooves to prevent sliding of the o-rings or sutures that hold the TEBV in place.

A custom polycarbonate top with gas exchange filters is used to prevent contamination during transport and culture. Rubber baffles allow for enclosure of the top around the stainless steel rod while not restricting linear motion. The bath in which the vessel is cultured is made from polycarbonate with a clear cover slip to allow for imaging on an inverted confocal microscope (Zeiss LSM 510, cameo-laser argon laser, Jena, Germany). A second flow loop allows for circulation and medium exchange in the polycarbonate bath.

Flow control

A peristaltic pump (Ismatec model IPC, Glatfbrugg, Switzerland) is used to generate luminal flow with given rate and pattern. The pump communicates with the computer via a data acquisition board allowing a prescribed flow waveform to be generated. The reservoir is a five-port 250-mL Erlenmeyer flask with solid stopper. Medium is pumped out of this flask from a bottom port and travels through the pump before entering the first pressure transducer. Flow continues into the culture chamber and through the vessel before exiting the culture chamber and returning to the second pressure transducer. Once leaving the second transducer, flow reenters the reservoir. The flow loop contains one bypass before the vessel to allow for total removal of air bubbles from the tubing. The bypass is closed during culture to ensure all flow is through the vessel. Measurements of flow rates are obtained using an ultrasonic flow-monitoring system (Transonic model T402, Ithaca, NY) with appropriate probe size. The probe is located on the vessel to directly measure the flow rate through the vessel, but is large enough as to not impose any external traction forces on the vessel. Data were collected using an analog output from the flow-monitoring unit and time-averaged over each second.

Pressure controls

Pressure in the vessel is controlled by regulating static gas pressure in the reservoir. Static pressure in the reservoir is controlled using a custom-designed syringe pump. A syringe is mounted onto a uniaxial stage whose position is controlled via a linear actuator. The syringe plunger is fixed allowing movement of the stage to move the syringe tube relative to the plunger. Tubing and a filter connect the syringe to the reservoir. As the plunger moves into the tube, pressure is increased in the reservoir. Likewise, as the plunger moves out of the tube, pressure is decreased. Because we control static pressure in the reservoir, pressure at the reservoir inlet and outlet are equal, thereby not inducing any pressure gradient nor flow. Pressure in the vessel is monitored using two pressure transducers (Honeywell model FGP, Columbus, OH) located on the bottom rack of the incubator

properly calibrated to account for static pressure head. A feedback control was developed in LabView (National Instruments v7.1, Austin, TX) to deliver periodic pressure curves with adjustable set-points for minimum and maximum pressure, and waveform period. Further development with a more sophisticated control algorithm could use a Fourier series approach to deliver a physiological pressure waveform at physiological rates. Due to the risk of vascular leakage or air leakage in the syringe, a three-way solenoid pinch valve is used to reload the piston once it reaches the end of its range of travel. Reloading of the syringe takes approximately 10 s. Analog inputs from the pressure transducers and load cell, as well as analog outputs to the pump and digital outputs to the pinch valves, are communicated with the computer via a data acquisition board (National Instruments model USB-6259).

Axial motion control

Uniaxial sliding stages on the culture device fit linear actuators that are controlled using a Newport motion controller (model XPS). This motion controller is capable of controlling eight linear actuators simultaneously. Two actuators are used per device to prevent translation of the vessel in the camera focus area. Therefore, one motion controller is capable of controlling all four parallel bioreactors. This motion controller is capable of monitoring actuator movement with 0.035 μm resolution. LabView communicates with the motion controller via an Ethernet cable to precisely control movements of the actuators to prescribed spatial locations; hence, axial extensions are referenced to the unloaded length of the vessel. Additionally, using a feedback loop in LabView, oscillatory axial distensions are capable at frequencies of ~ 1 Hz.

Vascular geometry monitoring

A digital camera (AVT Guppy IEEE 1394, Newburyport, MA) is mounted on an imaging lens (VZM 200i; Edmund Optics, Barrington, NJ) above the vessel and combined with a fiber optic light source (Dolan-Jenner Fiber-Lite, Boxborough, MA) for illumination. Real-time processing of the digital image is accomplished using LabView vision software to measure outer diameter of the vessel. In addition, markers may be attached to the outer surface of the vessel and tracked in real time to quantify the displacements in a local region of the vessel (Fig. 3a), thereby eliminating end effects on the quantification of strain. Wall thickness is measured using a pulse-echo ultrasonic technique. An ultrasonic pulser-receiver (Olympus NDT model 5900PR Waltham, MA) is used in combination with a 25-MHz, 0.25"-diameter, flat focused transducer (Olympus NDT model V-324SM). Measurements were made in a fluid medium bath with a distance of approximately 0.5" between the transducer and the front wall of the vessel. As the ultrasonic traverses the vessel, at each vessel-medium interface, there is a change in acoustic impedance causing a reflection that is captured by the transducer, and peaks are observable on the ultrasonic signal from all four of these interfaces. The signal is acquired via a 250-megasample/s, 8-bit digitizer (National Instruments model PCI-5114). Approximately 20 waveforms are averaged and manipulated using a hilbert transform in combination with convolution to accentuate the peaks over the noise. A cross-correlation algorithm is then used to determine the location of the four peaks corresponding to the

FIG. 3. Image of a BVS during mechanical testing with markers to track local displacement to determine local strains (a). Outer diameter of a BVS cultured under cyclic pressure application (32.5 ± 7.5 mmHg, rate of one cycle per minute) and no axial stretching (b). Data were collected once per minute for 96 h. Scale bar = 500 μm. Color images available online at www.liebertonline.com/ten.

dimensions of the vessel. The specifications of the digitizer allow a theoretical resolution of 3 μm.

Experimental Methods

Preparation, sterilization, and sterile tissue cannulation

Before sterilization, all components are washed in a soap bath and thoroughly rinsed with water. Pressure transducers are rinsed three times with 70% ethanol and allowed to evaporate overnight. Ethylene oxide is used to sterilize the fully assembled device (with chamber cover removed), pressure transducers, and load cell. Sterilization of additional tubing segments and the reservoir is accomplished in an autoclave. The flow loop is created consisting of the reservoir, pressure transducers, pump, and appropriate tubing. Cannulation of the vessel occurs in a biological safety cabinet. Once securely mounted using sterile sutures or o-rings, the chamber cover is attached. The flow loop is connected to the culture chamber, and lastly the linear actuators are secured to the uniaxial stages. Typically, cannulating the vessel and setting up the bioreactor is accomplished in under 1 h.

Culture capabilities of this device are demonstrated using biological vascular substitutes (BVS) created using the self-assembly method as previously described.³³ Vessels were cultured and tested in Dulbecco's modified Eagle's medium (with 4500 mg/L of D-glucose and L-glutamine; Mediatec, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Mediatec, Inc.), antibiotic (P/S; 50 IU/mL penicillin and 50 mg/mL streptomycin; Mediatec, Inc.), and additional L-glutamine (5000 g/L; Mediatec, Inc.).

Biomechanical testing

The custom LabView program allows us to prescribe a variety of loading patterns for mechanical testing (e.g., pressure-diameter, force-length, and stress-relaxation tests). To demonstrate the device capabilities, pressure-diameter and force-length tests were performed. Biomechanical testing may be performed in the bioreactor immediately after culture without any required handling of the TEBV. Before testing, the unloaded length of the vessel was measured. Under quasistatic conditions, pressure was varied from 0 to 120 mmHg at fixed axial stretches of $\lambda_z = 1.0$, 1.15, and 1.25, where $\lambda_z = l/L$, l is the current length and L is the unloaded length, at the time of interest. From these data, pressure-diameter ($P-d$) and axial force-pressure ($f-P$) curves were generated. Additionally, λ_z was cyclically varied up to 1.25 at pressures of 20, 40, and 120 mmHg. Axial force-length ($f-L$) curves were generated from these data.

A second set of mechanical testing data are shown using collagen-gel-based TEBVs. The collagen-gel-derived TEBVs were developed following the methods of Stegemann and Nerem.³⁴ Briefly, SMCs were isolated from rat aortas and combined with reconstituted bovine type I collagen. Controlling the environmental conditions of the collagen, gelation was induced around glass mandrels to form the TEBVs. After 2-day static culture, the TEBVs were mounted on this device and cyclically inflated at $\lambda_z = 1.00$ from pressures of 0–10 mmHg for five cycles, then 0–20 mmHg for five cycle, then 0–30 mmHg for five cycles, and so forth until these freshly gelled vessels ruptured; pressure, diameter, and axial force were monitored at a fixed axial length.

Confocal imaging

Decellularized BVS were used to image collagen content and organization via multi-photon microscopy. A Zeiss LSM 510 META inverted confocal microscope with Chameleon Ultra Ti:sapphire femtosecond laser (Coherent) was tuned to 800 nm and used to excite and detect collagen. The laser reached the sample through either a 40×/1.3NA or 63×/1.4NA oil immersion objectives (Zeiss). The fields of view were 225×225 μm and 143×143 μm, respectively. The META module of the microscope was configured as a 380–420 bandpass filter to detect backward scattering second harmonic signal from collagen.³⁵ Imaris software (Bitplane) was used for 3D reconstruction of images.

Results

Vascular geometry monitoring

Camera calibration was performed using a glass slide with etched lines spaced at 500 μm. The imaging lens was focused to achieve 20 μm per pixel, representing a field view of

8500×11,400 μm on the digital camera. A representative image of a BVS and the outer diameter are shown for a vessel cultured at an axial extension $\lambda_z^* = l/L(0)$, where $L(0)$ is the unloaded axial length at the beginning of culture ($t = 0$), with cyclic pressure application (32.5 ± 7.5 mmHg, rate of one cycle per minute), and incremental flow (5 min at 3 mL/min once a day) for nutrient and metabolite exchange (Fig. 3). Notice that we have placed markers on the vessel to allow for measurements of local displacement, in parallel to measurements of diameter and overall vessel length. Data were collected once per minute for 96 h. As seen, there is little variation in the diameter measurements (less than 100 μm) between consecutive data points. The discontinuity occurring around 50 h is due to removal of the culture chamber from the incubator and medium exchange in the reservoir.

Wall thickness measurements are accomplished using ultrasonic techniques. One sample digitized waveform is shown (Fig. 4a). To determine the peaks from this waveform (which represent the interfaces between the vessel and the medium), the waveform is subjected to an algorithm that includes crosscorrelation and a Hilbert transform. After the raw data are processed (Fig. 4b), the peaks can be easily determined using a simple peak search algorithm. Sequential waveforms can be processed in the same manner allowing thickness measurements to be determined throughout the course of the test or culture. The thickness measurements for one pressurization cycle (0–150–0 mmHg) can be seen in Figure 4c.

Loading control

To demonstrate pressure–flow independence, two experiments were performed. The first was a low-pressure/high-flow test where pressure was held constant at 20 mmHg while maintaining a constant flow rate of 350 mL/min. The second was a high-pressure and low-flow demonstrated by holding pressure constant at 100 mmHg and flow at 50 mL/min. There is no variation in both pressure (range 3 mmHg) and flow (range 10 mL/min) during low-pressure/high-flow testing (Fig. 5). Likewise, there is little deviance in both pressure and flow during high-pressure/low-flow conditions.

To illustrate the independence between the four bioreactors, two bioreactors were set with mean pressures of 50 and 100 mmHg and ranges 20 and 40 mmHg, respectively, while two bioreactors were set to hold constant pressures of 20 and 40 mmHg. All four reactors are under constant axial stretch and no flow. Due to the independent design of the pressure control system, all four bioreactors are capable of operating in parallel without adverse effects (Fig. 6).

Biomechanical testing

Loading and unloading curves from statically cultured BVS demonstrate the cylindrical biaxial biomechanical testing capabilities of our device. These results, generated from a single BVS, provide representative data regarding the capabilities of our device. As previously described, a standard testing protocol consists of three pressure cycles from 0–120–0 mmHg at a constant axial stretch. During this cycle, we monitor outer diameter as well as axial force (Fig. 7a and b, respectively). Axial force–length curves, generated at three constant pressures, exemplify a typical viscoelastic response of biological materials as seen by the hysteresis occurring

FIG. 4. Ultrasound measurement results showing the collected waveform (a), manipulated waveform (b), and thickness measurements for one pressurization cycle (0–150–0 mmHg) (c).

between the loading and unloading phases (Fig. 7c). All samples $P - d$ tests were pressurized and depressurized at a rate of 2 mmHg/s. These data combined with ultrasound thickness data can be used to generate mean axial and circumferential stress–strain curves, as well as perform stress analysis; see Equation (1).

FIG. 5. Loading results demonstrating independence of pressure and flow with constant low pressure of 20 mmHg and high flow of 350 mL/min (black lines), constant high pressure of 100 mmHg and low flow of 50 mL/min (gray lines).

Biomechanical tests were also performed on 2-day statically cultured collagen gels. These data were generated from a single collagen construct and provide representative capabilities of the device. At just 2 days after gelling, these vessels are very fragile. Given the fragility of these vessels, it was clear that they would not exhibit high burst strength at these early culture times. Thus, we conducted a testing protocol that gradually increases the cyclic testing pressure from 0–10 mmHg to 0–20 mmHg, and so forth (see Fig. 8a). We were able to successfully mount and mechanically load these vessels and conduct pressure–diameter and axial force–length tests (Fig. 8a and b, respectively). This protocol allows for the collection of several loading and unloading curves for stress analyses before rupture.

Confocal imaging

In multiple loaded configurations, collagen fibers of a BVS were imaged using confocal microscopy. With a single BVS mounted in the bioreactor, two successive images were taken at the same location within the vessel in two different loading scenarios: unloaded (Fig. 9a) and loaded to 20 mmHg (Fig. 9b). Densely arranged large and small bundles of collagen fibers can be seen. Quantification of these fibers at the various loading conditions will provide information relating fiber kinematics to the kinematics of the whole tissue, as well

FIG. 6. Independent pressure loading patterns of four bioreactors operating simultaneously from one control system.

FIG. 7. Loading and unloading pressure–diameter (a) and axial force–pressure (b) curves at three axial stretches generated during biomechanical testing on a statically cultured BVS. Loading and unloading axial force–length results at three constant pressures (c). Note the hysteresis typical of viscoelastic materials.

as information on the evolution of fiber content, orientation, and kinematics with tissue growth and remodeling to more accurately generate microstructurally motivated biomechanical and growth and remodeling models.

While Figure 9 simply demonstrates the ability of our system to image a vessel under various loading conditions, a representative sample of the microstructure would have to be determined using multiple images of the same vessel at random locations. When the imaged length (255 μm) is compared with the total unloaded length (30–40 mm), there is sufficient vascular length to attain multiple samples from different locations on the same vessel. Multiple algorithms have been developed to detect fiber angle from images for use across a wide variety of mediums.^{36,37} Additionally, these algorithms have been used to quantify structural information from biologic.^{32,38–40} These algorithms will be

FIG. 8. Cyclic biomechanical testing performed on cell-seeded collagen gel TEBV showing the loading history (a), pressure–diameter (b), and axial force–pressure data (c).

sufficient to extract the quantitative structural information required for microstructurally motivated biomechanical models.

Discussion

Many techniques have been developed for constructing cellularized TEBVs, including gel-derived, polymer-scaffold-derived, and self-assembly-derived approaches. Common to all of these techniques is the opportunity to subject these vessels to mechanical loads. It has been shown that gel- and polymer-based approaches respond to cyclic circumferential stimulation in a manner that increases their mechanical strength.^{2,5,21} Our goal was to develop a system that is capable of TEBV culture under multiaxial mechanical stimulation with the ability to monitor geometry, structural organization, and vascular responses throughout the course of culture.

Many bioreactors have been designed to study the role of mechanical stimulation on TEBVs. Perhaps the most popular

FIG. 9. Collagen fibers on a statically cultured BVS imaged using multiphoton confocal microscopy in the bioreactor. Two distinct loading scenarios, unloaded (a) and 20 mmHg (b), demonstrate the versatility of the bioreactor. Distinct fibers are formed during culture. Scale bar = 20 μm.

approach is to culture the TEBV on a distensible tube, which allows for cyclic circumferential distension to be applied to the construct.^{2,5,21} The advantage of such devices is the capability to deliver precise cyclic circumferential displacements. In addition, given that the distensible tube is impermeable to fluid, this tube also prevents leakage, a particularly significant challenge for polymeric scaffold- and gel-derived constructs at very early time-points in culture.

There are, however, several disadvantages with these devices as it relates to quantifying mechanically mediated remodeling of TEBVs. First, whereas the change in circumferential distension (i.e., change in inner diameter) is known, the true circumferential strain is not known and likely changes throughout culture. Consider the circumferential component of Green strain, $E_{\theta\theta} = (\lambda_\theta^2 - 1)/2$, where $\lambda_\theta(r) = \pi r/(\Theta_0 R)$ (in 3D), where r and R are radial locations in the current and stress-free configurations, respectively, and Θ_0 is the opening angle (see Chuong and Fung²⁵) or $\lambda_\theta = d/D$ (in 2D) if we neglect variations across the vessel wall. In both the 3D and the 2D case, even though this device carefully controls d , since the stress-free diameter D of the vessel can change during culture, via growth and plasticity, the circumferential strain can change. In 3D, these changes are more pronounced, for example, as the vessel thickens or compacts, or as the opening angle changes with growth and remodeling. Second, although the pressure applied to the distensible sleeve is known, the transmural pressure applied across the TEBV wall is not known; thus, the circumferential stress cannot be calculated. Third, in most cases the vessels are free to retract axially, and given the distensible sleeve, wall shear stress cannot be applied along the luminal surface; thus, multiaxial stimulation is not possible. Another bioreactor that has multiaxial loading capabilities was designed by McCulloch *et al.*⁴¹ This design allows for vascular pressurization as well as axial strain induction; however, this device is not capable of monitoring vascular geometry during culture, which restricts the ability to perform stress analyses or quantify growth kinetics. In addition to McCulloch's device, it should be noted that there are several multiaxial organ culture devices that offer some of the same capabilities^{11,22,42} and cylindrical biaxial mechanical testing devices for TEBV.⁴³ Whereas these devices offer the capability to quantify the tissue level biomechanical response, none of these devices have the ability to perform multiphoton confocal microscopy on the same (live) vessel at multiple time-points in culture under multiple loading scenarios.

One issue not addressed in this report is functional testing capabilities. Due to the noncontractile phenotype of cells used in the vascular equivalents, functional testing was not performed. However, as shown by Gleason *et al.*,²³ the design of this system permits functional tests. As previously described, the structural hierarchy of a blood vessel controls its mechanical properties. Grenier *et al.*¹³ has shown significantly improved contractility of tissue-engineered vascular medium when human vascular SMCs were aligned circumferentially. Because these vessels were created using the self-assembly method, alignment of vascular SMCs was accomplished by induced strain on 2D tissue cultures before tissue-engineered vascular medium formation. The nature of the self-assembly method lends itself to prealignment, while other approaches such as collagen gel or polymer scaffold will require remodeling of the 3D structure. Our device will allow for quantification of functionality intermittently during culture. Additionally, because our device allows for cellular imaging, we will be able to quantitatively relate structural organization to functional response.

In summary, we have developed a novel computer-controlled bioreactor capable of TEBV culture subjected to external mechanical stimulation, intermittent cylindrical biaxial mechanical testing, functional testing, and confocal imaging of small-diameter vascular tissue-engineered grafts.

This experimental device promises to provide new insights toward mechanically induced growth and remodeling of TEBVs. Using experimental results, theoretical models can be developed to mathematically describe these complex processes. Additionally, intermittent biomechanical testing in combination with microstructural imaging will allow us to gain further insights into both microstructure influence on tissue-level biomechanics as well as constituent production, removal, and reorganization in response to mechanical stimuli. Further, these theoretical models will guide future experiments in an effort to optimize mechanical culture conditions to develop TEBV with adequate mechanical strength and functionality, which can ultimately be used as an *in vivo* vascular graft. We have described capabilities for the system and shown performance data. This system allows users to gain further insights into mechanically induced growth and remodeling of TEBV.

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Disclosure Statement

No competing financial interests exist.

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