Published in final edited form as:

Biomaterials. 2011 January ; 32(3): 714–722. doi:10.1016/j.biomaterials.2010.09.019.

Implantable arterial grafts from human fibroblasts and fibrin using a multi-graft pulsed flow-stretch bioreactor with noninvasive strength monitoring

Zeeshan H. Syedain¹, Lee A. Meier¹, Jason W. Bjork¹, Ann Lee¹, and Robert T. Tranquillo^{1,2}

¹ Department of Biomedical Engineering, University of Minnesota

² Department of Chemical Engineering & Materials Science, University of Minnesota

Abstract

Tissue-engineered arteries based on entrapment of human dermal fibroblasts in fibrin gel yield completely biological vascular grafts that possess circumferential alignment characteristic of native arteries and essential to their mechanical properties. A bioreactor was developed to condition six grafts in the same culture medium while being subjected to similar cyclic distension and transmural flow resulting from pulsed flow distributed among the graft lumens via a manifold. The luminal pressure and circumferential stretch were noninvasively monitored and used to calculate stiffness in the range of 80-120 mmHg and then to successfully predict graft burst strength. The length of the graft was incrementally shortened during bioreactor culture to maintain circumferential alignment and achieve mechanical anisotropy comparable to native arteries. After 7-9 weeks of bioreactor culture, the fibrin-based grafts were extensively remodeled by the fibroblasts into circumferentially-aligned tubes of collagen and other extracellular matrix with burst pressures in the range of 1400-1600 mmHg and compliance comparable to native arteries. The tissue suture retention force was also suitable for implantation in the rat model and, with poly(lactic acid) sewing rings entrapped at both ends of the graft, also in the ovine model. The strength achieved with a biological scaffold in such a short duration is unprecedented for an engineered artery.

1. Introduction

Cardiovascular disease is the leading cause of mortality in the world: approximately one million surgical procedures are performed annually in the US alone [1]. Vascular grafts made from synthetic polyesters have shown success in replacement of large-diameter vessels such as the thoracic and abdominal aortas, aortic arch vessels, as well as the iliac and femoral arteries [2]. However, they have generally proven inadequate as small-diameter (<6 mm) arterial grafts. This is primarily a result of acute graft thrombogenicity, anastomotic intimal hyperplasia, aneurysm formation, infection, and atherosclerotic disease progression[3]. Autologous arteries and veins remain the standard of care. However, a

Robert T. Tranquillo(corresponding author), Department of Biomedical Engineering, 7-114 BSBE, 312 Church St SE, University of Minnesota, Minneapolis, MN 55455, Tel: 612-625-6868, Fax: 612-626-6583, tranquillo@cems.umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

significant fraction of patients do not have a suitable vessel that can be harvested as a replacement [3].

Tissue engineering provides a viable alternative to create arterial grafts that can maintain vascular function comparable to native vessels [4]. One approach that involves the rolling of “cell sheets” to form a tubular construct has been most successful in developing grafts for human use [5,6]. The major drawback of this technique is the long culture period required that exceeds 6 months. Developing engineered grafts from cell-seeded scaffolds, synthetic or biological, might enable a shorter incubation as desired characteristics can be achieved by prescribing the initial properties such as thickness, cellularity, and degradation rate [4]. Arterial grafts developed from synthetic polymers have been shown to achieve sufficient strength [7], but acidification resulting from the polymer hydrolysis and the resulting host responses are potential limitations [8]. Fibrin is a suitable scaffold for creation of completely biological tissue-engineered grafts. A strategy has been proposed whereby fibrin and cells are harvested from the patient's blood and a skin biopsy or autologous stem cell source, respectively [4,9]. In previous work, Swartz *et al.*, have shown good viability and remodeling of fibrin-based engineered grafts in the low-pressure venous circulation [10]. However, all grafts based on biological scaffolds have failed to achieve adequate mechanical strength for reliable arterial implantation [10-13].

The strength of the grafts depends on the ability of the entrapped cells to deposit extracellular matrix (ECM), primarily collagen fibers, which confers tissue with tensile strength. Along with high collagen content, the organization of the cell-produced collagen fibers is also of critical importance. It should mimic the predominant circumferential alignment of native arteries to provide burst strength and confer the natural anisotropic mechanical behavior [14,15]. Herein, fibrin-based engineered tissue is used to achieve circumferential alignment by the unique mechanism of cell traction induced alignment of a fibrin gel with the mechanical constraint of a non-adhesive mandrel [16], which by contact guidance induces similar alignment of the cells and the cell-deposited collagen [13]. Previously, applied cyclic stretching was shown to improve the ECM production and mechanical properties of fibrin-based grafts remodeled by neonatal human dermal fibroblasts (nhDF) [17]. The use of steady transmural flow during bioreactor culture of such grafts has also been investigated [18].

For this study, a bioreactor system was designed to allow for culture of multiple grafts under similar conditions via a single flow loop that applies both cyclic distention and cyclic transmural flow for stimulating tissue growth. This bioreactor system has the unique advantage of applying all four parameters that were previously found to be beneficial for the development of mechanically robust engineered arteries: cyclic stretching [17,19,20], luminal flow [12,21], abluminal flow [7,22], and transmural flow [18,23]. Following 2 weeks of static incubation, grafts of 2 mm and 4 mm internal diameter were subjected to pulsed flow-stretch for varying times and analyzed for cell and collagen content, tissue alignment, histology, and mechanical properties under tension and pressure. The potential benefit of periodic shortening of the graft was investigated, and the effects of transmural flow on nutrient transport were assessed. Further, a noninvasive and nondestructive method was developed to monitor maturation of the grafts in terms of predicted burst pressure via *in situ* stiffness measurement.

2. Materials and Methods

2.1. Cell Culture

nhDF (Clonetics) were maintained in a 50/50 mixture of Dulbecco's Modification of Eagle's Medium and Ham's F12 cell culture medium (DMEM/F12, Cellgro) supplemented with 15%

fetal bovine serum (FBS, Thermo-Fisher Scientific), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37°C in 100% humidity and 5% CO₂, passaged at confluency, and harvested for use at passage 9.

2.2. Vascular Graft Preparation and Culture

A fibroblast-seeded fibrin gel was formed by adding thrombin (Sigma) and calcium chloride to a suspension of cells in fibrinogen (Sigma) in 20 mM HEPES-buffered saline. All components were kept on ice prior to mixing. The final component concentrations of the cell suspension were as follows: 4 mg/ml fibrinogen, 1.1 U/ml thrombin, 5.0 mM Ca⁺⁺, and 1 million cells/ml. Cell suspensions were mixed and injected into a tubular mold. The mold had a cylindrical glass mandrel pretreated with 5% Pluronic F-127 solution for 1 hr and a concentric glass casing. The outer diameter of the mandrel was either 2 mm or 4 mm. In both cases, the width of the resulting tubular cavity of the mold was 3.5 mm, and the length of the mold was 70 mm. Poly(lactic acid) (PLA, Concordia medical) sewing cuffs 5 mm long were embedded at the ends of the 4 mm grafts.

After injection of the cell/fibrinogen suspension, the molds were placed vertically in a humidified incubator and maintained at 37°C, 5% CO₂ for 15 minutes to allow gelation. Subsequently, the casing was removed, and the grafts were placed horizontally in culture medium, comprised of DMEM supplemented with 13% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 µg/ml insulin, 50 µg/ml ascorbic acid and 1× nonessential amino acids (Cellgro). Medium was changed 3 times per week. Grafts were cultured for 2 weeks on their mandrels with gentle rocking (Fig. 1a), after which they were transferred to the pulsed flow-stretch bioreactor (Fig. 1b&c). The 2-week static culture allowed for sufficient stiffening and strengthening of the vascular grafts to withstand handling during mounting in the bioreactor.

2.3. Pulsed Flow-Stretch Bioreactor

A schematic of the bioreactor with mounted grafts is shown in Figure 1b. The grafts are mounted on a custom manifold, with a maximum of 6 grafts in this implementation. The manifold is placed inside a jar with 300 mL of cell culture medium, which also acts as the reservoir with a filtered cap for gas exchange. A reciprocating syringe pump connects to the manifold through a 3-way valve. During forward motion, a stroke volume of culture medium flows through the manifold and is distributed to each graft; consequently, each mounted graft experiences a pressure wave with resultant graft distension and transmural flow. Due to the manifold symmetry, each mounted graft is subjected to a similar pressure wave although variations occur since the graft properties at the time of mounting are not identical. The graft distension is measured using digital image capture and used to prescribe a stroke volume that corresponds to the desired peak distension. During reverse motion, the syringe withdraws medium from the jar. An inline pressure transducer (Harvard Apparatus) placed between the manifold and the 3-way valve allows for noninvasive measurement of the pressure profile. Video recording of the bioreactor was used to measure strain amplitude, as detailed below. The 2 and 4 mm grafts were cultured up to 9 weeks with a pulse frequency of 0.5 Hz and time-average distension amplitude (i.e. peak circumferential strain) of 7.3±1.9%.

2.4. Noninvasive Monitoring of Graft Tissue Growth

During conditioning in the bioreactor, graft images and pressures were recorded, the images being used to measure graft distension. Further, to predict evolving strength of the grafts during culture, they were pressurized between 80 and 120 mmHg and the corresponding graft distensions measured. Using true strain defined by Equation 1, where D_0 is the graft

diameter at 0 mmHg and D is the diameter at a designated pressure, stiffness (S) was calculated using Equation 2:

$$\varepsilon = \ln\left(\frac{D}{D_0}\right) \quad \text{Eq. 1}$$

$$S = \frac{\Delta P}{\Delta \varepsilon} \quad \text{Eq. 2}$$

The stiffness based on $P = 80$ and 120 mmHg was plotted versus burst pressure (see below) for grafts of different culture durations. A correlation from linear regression of these data was then used to predict burst pressure of subsequent samples by monitoring *in situ* via the pressure-distention measurements.

2.5. Burst Pressure, Compliance, and Suture Retention Testing

Grafts were removed from the bioreactor and mounted in a system designed for pressurizing individual grafts to failure. The graft was cannulated at one end and the other end tied with suture to a stainless steel cannula of matched diameter that was connected to controlled syringe pump (Harvard Apparatus) and an in-line pressure transducer (Omega). The graft was placed such that a laser micrometer (Mitutoyo) measured the diameter in real time. Using a custom LabView™ program, both pressure and diameter data were simultaneously recorded during injection of phosphate-buffered saline (PBS) into the graft lumen. The PBS was injected at a constant rate of 4 ml/min until graft failure. A measure of compliance (C) was calculated in the pressure range of 80 - 120 mmHg as defined by Equation 3 (represented as %/100 mmHg) [24]. The pressure within each graft at failure was recorded as the burst pressure.

$$C = \frac{\frac{D_{120} - D_{80}}{D_{80}}}{\Delta P} \times 10^4 \quad \text{Eq. 3}$$

Suture retention of the grafts was measured as described by König *et al.* [25]. In brief, a graft section approximately 15 mm long was cut and one end was gripped on one arm of an Instron MicroBionix (Instron Systems). Using 7.0 prolene suture, a loop around 1 mm of tissue from the edge was placed on the free end and looped around a force transducer on the second arm of the MicroBionix. The arm of the Instron attached to the suture was extended at 3 mm/min until failure. The test was repeated on the same sample by placing second suture loop at the opposite end from the first location.

2.6. Uniaxial Tensile Testing

Tissue strips cut from each graft of dimension 2 mm $\times$ 10 mm were tested for tensile properties in both the circumferential and axial directions. The thickness of each strip was measured using a 50 g-force probe attached to a displacement transducer. Tissue strips were placed in compressive grips, attached to the actuator arm and load cell of an Instron MicroBionix (Instron Systems) and straightened with an applied load of 0.005 N. This position was used as the reference length of the strip. Following 6 cycles of 0 - 10% strain conditioning at 2 mm/min, strips were stretched to failure at the same rate. True strain was calculated based on the change in length of the tissue over time divided by the initial strip

length. The stress was calculated as force divided by the initial cross-sectional area. The tangent modulus (E) was determined as the slope of the linear region of the stress-strain curve prior to failure.

2.7. Histology and Fiber Alignment Imaging

Vascular graft rings of 3 mm length were fixed in 4% paraformaldehyde, embedded in OCT (Tissue-Tek), and frozen in liquid nitrogen. Sections of 9 μ m thickness were stained with Lillie's trichrome and picrosirius red stains. Images were taken at 10 $\times$ magnification using a color CCD camera. For picrosirius red-stained sections, images were taken with the sections placed between crossed-plane polarizers. Fiber alignment was measured using a polarized light imaging method [26].

2.8. Collagen, Elastin, and Cell Quantification

The collagen content was quantified using a hydroxyproline assay previously described, assuming 7.46 mg of collagen per 1 mg of hydroxyproline [27]. The insoluble elastin content was quantified using a ninhydrin-based assay as previously described [28]. The sample volume was calculated using the measured length, width, and thickness of the strips (as described above in uniaxial testing). Collagen and elastin concentrations were calculated as the mass per unit volume in each tissue sample. The cell content was quantified with a modified Hoechst assay for DNA assuming 7.7 pg of DNA per cell [29]. Cell concentrations were calculated as the number of cells per unit volume using the dimensions of the strip.

2.9. Transmural Flow and Transport Assessment

Flow with radial velocity v_r through the tissue of thickness L was described with Darcy's law ($v_r = L_p \Delta P$), with hydraulic conductivity, L_p , measured as previously described [18]. Briefly, L_p was determined by placing a tissue taken from a construct after the 2-week static culture in a membrane filter housing (Millipore) with a custom insert to hold the tissue. PBS was forced through the tissue while monitoring pressure and flow rate to estimate L_p based on the Darcy's law. Using the time-averaged value of P ($\langle P \rangle$) during the pulsed flow-stretch operation of the bioreactor, $\langle v_r \rangle$ was then calculated from Darcy's law.

2.10. Statistics

For all experiments, statistical significance of differences between groups was determined using t-test for two treatments and one-way ANOVA for more than two treatments with the Tukey post hoc test in GraphPad Prism® software for Windows. Any reference to a difference in the Results and Discussion sections implies statistical significance at the level $p < 0.05$. In all cases, where the difference was significant, symbols are used to indicate the difference and explained in figure captions.

Burst pressure versus stiffness was plotted in GraphPad Prism, and regression analysis was performed to assess linear correlation and the R^2 value. 95% confidence intervals were plotted based on standard deviation with respect to the linear correlation.

3. Results

3.1. Noninvasive monitoring of stiffness and burst pressure prediction

During the course of 5-7 weeks of culture in the bioreactor, with occasional adjustment in stroke volume (see below), the time-average circumferential strain amplitude during distention was $7.3 \pm 1.8\%$ ($\pm 25.3\%$) based on measuring two grafts closest to the incubator door that could be imaged periodically within the bioreactor for the duration of incubation. Within a single bioreactor, the standard deviation in strain amplitude among the six grafts in

a set when mounted was 10.6%, which can be attributed to variation in graft mechanical properties prior to mounting since the luminal pressures should be equal using the symmetrical manifold mounting. Averaged pressure-strain data for two sets of grafts with substantially different burst pressures because of differences in incubation time (2 wk vs. 7 wk) is shown in Figure 2a. At up to 10% distension, however, no difference in the graft stiffness (defined as S in Eq. 2) existed within the toe region of the stress-strain curve (Fig. 2b). For the applied stroke volumes used, resulting in peak strain of less than 10% (approximately 7% as noted above), a drop in distension only occurred after at least two weeks of bioreactor culture; therefore, frequent increases in stroke volume were not required in order to maintain the average 7% stretch amplitude. Beyond 15%, the slope of the pressure-strain curve (S) did differ for grafts of different maturity, with stiffer samples correlating with higher burst pressures. Based on this observation, a linear correlation ($R^2 = 0.96$) was found between the stiffness of the grafts measured between the physiologic pressures of 80-120 mmHg (defined as Equation 1) and the burst pressure, as shown in Figure 2c. This correlation was subsequently used to predict burst pressures prior to experimental determination (Fig. 2d). The predicted and measured burst pressures were not statistically different. This indicates that during growth of the engineered grafts in the bioreactor described herein, pressurization between 80 and 120 mmHg along with corresponding distension data to calculate graft stiffness was sufficient to accurately predict burst strength during graft development.

3.2. Pulsed flow-stretch grafts compared to constant flow grafts

Preliminary experiments with the bioreactor had two control groups. The first group was cultured statically on the glass mandrel as previously described [30]. The second control group had constant flow in which a peristaltic pump was connected to the bioreactor manifold in place of the syringe pump in order to apply nearly constant flow (no pulsations were evident at the inlet manifold presumably because of the compliance of the Tygon tubing). The total culture medium in all groups was kept constant to allow for equal nutrient availability. The constant flow group had an average upstream luminal flow rate of 3.33 mL/min/graft, while the pulsed flow group had an average upstream luminal flow rate of 2.5-5 mL/min/graft, depending on the stroke volume. In the constant flow group, the luminal pressure sufficed to prevent graft necking due to traction forces exerted by the fibroblasts, without causing graft distension. The mechanical and biochemical properties of the static culture control group were worse than those of the constant flow control group, with burst pressures less than 200 mmHg (data not shown). The thickness of the constant flow and pulsed flow grafts was comparable after 3 weeks in the bioreactor, corresponding to an overall culture period of 5 weeks (Fig. 3a). The cells in the pulsed flow grafts produced 150% more collagen (0.95 ± 0.31 ng/cell) compared to constant flow grafts (0.38 ± 0.12 ng/cell) (Fig. 3b). Burst pressures were also 73% higher for pulsed flow grafts (596 ± 28 mmHg) compared to constant flow grafts (345 ± 37 mmHg) (Fig. 3c).

3.3. Nutrient transport from transmural flow during pulsed flow

The time-averaged pressure during a cycle was measured to be $\langle P \rangle = 430$ Pa. Using the measured tissue hydraulic conductivity $L_p = 4.97 \times 10^{-6}$ cm/Pa/s, the time-averaged transmural flow velocity $\langle v_r \rangle$ was determined to be $\langle v_r \rangle = 2.14 \times 10^{-3}$ cm/s. Corresponding values were determined for the radial Peclet number ($Pe_r = v_r L/D_i$, where D_i is the effective diffusion coefficient in the tissue of species i). For dissolved oxygen (DO), $\langle Pe_r \rangle = 6.0$ using the reported value for the diffusivity of DO in aortic valve leaflets $D_{DO} = 1.06 \times 10^{-5}$ cm²/s. For comparison, $\langle Pe_r \rangle = 42.0$ and 50.4 for IGF-I and EGF, respectively, using the reported values of $D_{IGF} = 1.59 \times 10^{-6}$ cm²/s and $D_{EGF} = 1.34 \times 10^{-6}$ cm²/s [31].

3.4. Controlled graft shortening and graft alignment / mechanical anisotropy

Alignment of the grafts was circumferential when mounted following the 2-week static incubation on-mandrel (not shown), consistent with previous reports [13]. In the initial studies, the length of the vascular grafts was kept constant during culture in the bioreactor. Alignment became axial after 3 weeks of culture in the bioreactor (Fig. 4a). It was hypothesized that axial tension increased during culture in the bioreactor due to cell traction forces and the fixed length mounting, leading to axial fiber alignment [32]. To test this hypothesis, in subsequent experiments 60% shortening was applied incrementally over the first three weeks of culture in the bioreactor by reducing the spacing between the manifolds. 60% shortening was similar to that occurring for the static control grafts incubated on the glass mandrels treated to be non-adhesive. The tangent modulus (E) was greater in the axial direction in grafts of fixed length, but greater in the circumferential direction for grafts that were incrementally shortened (Fig. 4b). Without shortening, alignment was primarily axial, with a circumferential to axial modulus ratio (anisotropy index) of 0.35, but with shortening, the circumferential direction was much stiffer with an anisotropy index of 3.0 (Fig. 4c). This was comparable to a value of 3.4 measured for the ovine femoral artery. Graft shortening during bioreactor culture also improved collagen concentration and burst strength. The ultimate tensile strength (UTS) in the circumferential direction improved 5-fold, from 350 ± 106 kPa for fixed-length grafts to 1761 ± 206 kPa with shortened grafts (Fig. 4d). The corresponding collagen concentration also increased by 230%, from 17 ± 2 mg/ml to 39 ± 3 mg/ml. After shortening, the volume of the graft was reduced by $46 \pm 6\%$.

3.5. Graft mechanical properties after 5-7 weeks of pulsed flow-stretch

Longer term studies were performed with 2 mm and 4 mm engineered grafts using the same pulsed flow-stretch conditions with incremental shortening of 60% in the first 3 weeks of bioreactor conditioning. Grafts were harvested after 5 weeks of bioreactor culture for the 2 mm grafts, and after 7 weeks of bioreactor culture for the 4 mm grafts, which possessed final lengths of ~3cm. The thicknesses of the grafts were less than those found for the sheep femoral artery (a model implant site) by 64% for 2 mm grafts and 43% for 4 mm grafts (Fig. 5a). The burst strength was 1366 ± 177 mmHg for the 2 mm grafts, and 1542 ± 188 mmHg for the 4 mm vascular grafts (Fig. 5c). Compared to the ovine femoral artery, the grafts had 60% and 67% of native burst pressure (2297 ± 207 mmHg) for the 2 mm and 4 mm grafts, respectively. The suture retention force was similar between the 2 mm grafts (0.19 ± 0.05 N) and the rat abdominal aorta (0.25 ± 0.09 N). The 4 mm grafts with entrapped PLA cuffs had a suture retention force of 1.32 ± 0.58 N, equal to the human internal mammary artery (1.35 ± 0.49 N) [25]. The collagen concentrations were 50% and 61% of the ovine femoral artery value for the 2 mm and for 4 mm grafts, respectively (Fig. 5b). The compliance of the vascular grafts between 80-120 mmHg was also comparable to the native artery (Fig. 5d).

Histological comparison of the 2 mm and 4 mm grafts with the ovine femoral artery is shown in Figure 6. Lillie's trichrome staining indicates minor residual fibrin (red) in the 2 mm grafts, whereas none is evident in the 4 mm grafts. This could reflect the 2 week longer bioreactor culture period or remodeling differences in the different sized grafts. The nhDF were evenly distributed across the thickness of the grafts. In the native artery, collagen banding (green) along with muscle fibers (red) in the media layer are visible. Collagen fiber alignment is visible from the picrosirius red staining, with similar circumferential alignment for both the grafts and the native artery.

4. Discussion

Based on previous work isolating the effects of cyclic stretching and transmural flow [17,18], this bioreactor was developed to impart both via pulsed flow of medium through the

lumens of manifolded grafts sharing a common reservoir. This design provides the advantage over single graft bioreactors by allowing for a similar growth environment (shared distension pressure and conditioned medium) thereby eliminating large variability between the matured grafts.

A 7% time-averaged circumferential stretch amplitude with frequency of 0.5 Hz was applied. These values were based on a previous study showing improvement in the mechanical properties and collagen concentration for nhDF-remodeled fibrin with cyclic stretching between 5-15% strain amplitude at 0.5 Hz [17]. As the applied stretch amplitude here was kept under 10%, it was found that vascular grafts with different tangent moduli and burst pressures have comparable stiffness under 10% strain (Fig. 2b). Consequently, frequent changes in stroke volume to maintain constant stretch amplitude were not required. Further, it was found that the stiffness of the graft, as measured in the bioreactor between 80-120 mmHg, had a linear correlation with graft burst pressure. This enabled noninvasive prediction of graft burst pressure from an *in situ* stiffness measurement. To test the correlation, a set of vascular grafts were monitored for stiffness between 80-120 mmHg then tested for burst pressure. The correlation was found to accurately predict the burst pressures. Such noninvasive, nondestructive monitoring of burst strength will facilitate future studies by indicating when during culture a target burst pressure has been attained.

Almost all of the previously-reported engineered graft bioreactors were primarily designed to expose the grafts to culture medium on either the luminal or abluminal surfaces [7,12,18,21,22]. However, this pulsed flow-stretch bioreactor provides for transmural flow along with exposure to tissue culture medium on both surfaces, both of which allow for a more homogeneous distribution of nutrients within the tissue. The symmetrical bioreactor design also provides a similar nutrient environment to all grafts within the bioreactor. The bioreactor operation was not controlled so as to differentiate between stretching (direct mechanical stimulation) and transmural flow (enhanced nutrient transport) as the cause for the improvements found. As the tissue matures, its hydraulic conductivity decreases [18]. By increasing the stroke volume (and associated pressure) to maintain the 7% circumferential stretch amplitude as the tissue stiffens and its modulus increases, these effects (decreasing L_p and increasing P with time), while offsetting to some degree, result in a variable level of transmural flow during the bioreactor conditioning.

The transmural flow was estimated to yield a time-averaged radial Peclet number $\langle Pe_r \rangle = 6.0$ for DO, indicating a significant role for convective transport of DO to the cells. Since all other nutrients typically considered limiting have larger molecular weights and therefore smaller diffusion coefficients than DO, there would be even greater benefits for enhanced radial transport (resulting in increased nutrient levels) in those cases. For example, we estimated $\langle Pe_r \rangle = 42.0$ and 50.4 for IGF-I and EGF, respectively. However, these conclusions must be qualified since the value of L_p used to calculate $\langle Pe_r \rangle$ was based on tissue when mounted in the bioreactor, and the value of L_p and hence Pe_r will almost certainly decrease during the bioreactor conditioning as collagen and other extracellular matrix is deposited. Clearly, more rigorous assessment of the transmural flow and transport is warranted. To further elucidate the effect of cyclic stretching with pulsed flow, a control group was incubated with constant flow through the lumen. The cyclic stretching led to 150% increase in collagen deposition (Fig.3b) with corresponding increase in the burst strength (Fig. 3c). These results confirm the important role of cyclic stretching in the development of the vascular grafts and are consistent with previous findings [17,33].

In order to achieve requisite mechanical strength and to attain mechanical behavior for the engineered grafts that is similar to native arteries, the structural and mechanical anisotropy of cell-produced tissue is critical. The strategy of incremental shortening during bioreactor

culture, counteracting the axial-aligning effects of cell traction forces at fixed length, was implemented to maintain circumferential alignment in the tissue. A mechanical anisotropy comparable to the ovine artery was obtained, consistent with previous reports [34,35]. The circumferential alignment likely improved the mechanical properties through two distinct mechanisms. First, the circumferential alignment yielded increased circumferential strength and burst pressure, as would be expected [11]. Second, the incremental shortening used to achieve the alignment reduced the total graft volume by 46%, thereby concentrating the deposited collagen fibers and improving the mechanical properties. It was observed that a 2.3 fold increase in collagen concentration occurred along with a 5-fold increase in the circumferential UTS, indicating that the increase in the UTS was potentially a combination of both increases in collagen concentration as well as increased fiber reorganization.

For *in vivo* evaluation, grafts were developed with internal diameters of 2 mm for a small animal model (rat) and 4 mm for a large animal model (ovine). The 4 mm diameter is also relevant for clinical applications. For the 2 mm graft, suture retention strength was comparable to the rat abdominal aorta. For the 4 mm graft, PLA cuffs entrapped in the fibrin at both ends of the graft provided suture retention strength comparable to the human internal mammary artery, although in this case the graft was not completely biological; further optimization of the tissue growth may preclude the need for the PLA rings. Notwithstanding, the burst strengths achieved with both the 2 mm and 4 mm vascular grafts over approximately 2 months are the highest values reported for engineered grafts made from biopolymer scaffolds and are sufficient for implantation.

5. Conclusions

This pulsed flow-stretch bioreactor provides a method to grow multiple 4 mm vascular grafts with over 1500 mmHg burst pressure in just 9 weeks, and multiple 2 mm vascular grafts with nearly 1400 mmHg burst pressure in just 7 weeks. The 2 mm grafts possess suture retention strength comparable to the native rat aorta as well as physiological compliance and are thus considered implantable. The stiffness of the grafts, as measured from distension in the bioreactor between 80-120 mmHg, successfully predicts their burst pressure. Cyclic stretching of the grafts associated with the pulsed flow along with incremental shortening of the grafts during bioreactor culture are essential to the increases in collagen concentration and circumferential tissue alignment that correlate with graft strengthening. This bioreactor can be used to culture engineered grafts fabricated by any method and can facilitate development of grafts for clinical use.

Acknowledgments

The authors acknowledge Naomi Ferguson for technical assistance. Funding was provided by NIH R01-HL083880 to R.T.T.

References

1. Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, et al. Heart disease and stroke statistics--2009 update: a report from the American Heart Association statistics committee and stroke statistics subcommittee. *Circulation* 2009;119(3):480-486. [PubMed: 19171871]
2. Kannan RY, Salacinski HJ, Butler PE, Hamilton G, Seifalian AM. Current status of prosthetic bypass grafts: a review. *J Biomed Mater Res B Appl Biomater* 2005;74(1):570-581. [PubMed: 15889440]
3. Conte MS. The ideal small arterial substitute: a search for the holy grail? *Faseb J* 1998;12(1):43-45. [PubMed: 9438409]

4. Isenberg BC, Williams C, Tranquillo RT. Small-diameter artificial arteries engineered in vitro. *Circ Res* 2006;98(1):25–35. [PubMed: 16397155]
5. 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]
6. L'Heureux N, Dusserre N, Konig G, Victor B, Keire P, Wight TN, et al. Human tissue-engineered blood vessels for adult arterial revascularization. *Nat Med* 2006;12(3):361–365. [PubMed: 16491087]
7. Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R, et al. Functional arteries grown in vitro. *Science* 1999;284(5413):489–493. [PubMed: 10205057]
8. Sung HJ, Meredith C, Johnson C, Galis ZS. The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis. *Biomaterials* 2004;25(26):5735–5742. [PubMed: 15147819]
9. Ye Q, Zund G, Benedikt P, Jockenhoevel S, Hoerstrup SP, Sakyama S, et al. Fibrin gel as a three dimensional matrix in cardiovascular tissue engineering. *Eur J Cardiothorac Surg* 2000;17(5):587–591. [PubMed: 10814924]
10. Swartz DD, Russell JA, Andreadis ST. Engineering of fibrin-based functional and implantable small-diameter blood vessels. *Am J Physiol Heart Circ Physiol* 2005;288(3):H1451–1460. [PubMed: 15486037]
11. Yao L, Liu J, Andreadis ST. Composite fibrin scaffolds increase mechanical strength and preserve contractility of tissue engineered blood vessels. *Pharm Res* 2008;25(5):1212–1221. [PubMed: 18092140]
12. Tschoeke B, Flanagan TC, Koch S, Harwoko MS, Deichmann T, Ella V, et al. Tissue-engineered small-caliber vascular graft based on a novel biodegradable composite fibrin-poly lactide scaffold. *Tissue Eng Part A* 2009;15(8):1909–1918. [PubMed: 19125650]
13. Grassl ED, Oegema TR, Tranquillo RT. A fibrin-based arterial media equivalent. *J Biomed Mater Res A* 2003;66(3):550–561. [PubMed: 12918038]
14. Gleason RL, Wilson E, Humphrey JD. Biaxial biomechanical adaptations of mouse carotid arteries cultured at altered axial extension. *J Biomech* 2007;40(4):766–776. [PubMed: 16750537]
15. Guo H, Humphrey JD, Davis MJ. Effects of biaxial stretch on arteriolar function in vitro. *Am J Physiol Heart Circ Physiol* 2007;292(5):H2378–2386. [PubMed: 17209009]
16. Barocas VH, Girtan TS, Tranquillo RT. Engineered alignment in media equivalents: magnetic prealignment and mandrel compaction. *J Biomech Eng* 1998;120(5):660–666. [PubMed: 10412446]
17. Syedain ZH, Weinberg JS, Tranquillo RT. Cyclic distension of fibrin-based tissue constructs: evidence of adaptation during growth of engineered connective tissue. *Proc Natl Acad Sci U S A* 2008;105(18):6537–6542. [PubMed: 18436647]
18. Bjork JW, Tranquillo RT. Transmural flow bioreactor for vascular tissue engineering. *Biotechnol Bioeng* 2009;104(6):1197–1206. [PubMed: 19603425]
19. Isenberg BC, Tranquillo RT. Long-term cyclic distention enhances the mechanical properties of collagen-based media-equivalents. *Ann Biomed Eng* 2003;31(8):937–949. [PubMed: 12918909]
20. Seliktar D, Nerem RM, Galis ZS. Mechanical strain-stimulated remodeling of tissue-engineered blood vessel constructs. *Tissue Eng* 2003;9(4):657–666. [PubMed: 13678444]
21. Hoerstrup SP, Zund G, Sodian R, Schnell AM, Grunenfelder J, Turina MI. Tissue engineering of small caliber vascular grafts. *Eur J Cardiothorac Surg* 2001;20(1):164–169. [PubMed: 11423291]
22. Seliktar D, Black RA, Vito RP, Nerem RM. Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. *Ann Biomed Eng* 2000;28(4):351–362. [PubMed: 10870892]
23. Kitagawa T, Yamaoka T, Iwase R, Murakami A. Three-dimensional cell seeding and growth in radial-flow perfusion bioreactor for in vitro tissue reconstruction. *Biotechnol Bioeng* 2006;93(5):947–954. [PubMed: 16329144]
24. Dahl SL, Rhim C, Song YC, Niklason LE. Mechanical properties and compositions of tissue engineered and native arteries. *Ann Biomed Eng* 2007;35(3):348–355. [PubMed: 17206488]

25. Konig G, McAllister TN, Dusserre N, Garrido SA, Iyican C, Marini A, et al. Mechanical properties of completely autologous human tissue engineered blood vessels compared to human saphenous vein and mammary artery. *Biomaterials* 2009;30(8):1542–1550. [PubMed: 19111338]
26. Tower TT, Neidert MR, Tranquillo RT. Fiber alignment imaging during mechanical testing of soft tissues. *Ann Biomed Eng* 2002;30(10):1221–1233. [PubMed: 12540198]
27. Stegemann H, Stalder K. Determination of hydroxyproline. *Clin Chim Acta* 1967;18(2):267–273. [PubMed: 4864804]
28. Starcher BC, Galione MJ. Purification and comparison of elastins from different animal species. *Anal Biochem* 1976;74(2):441–447. [PubMed: 822746]
29. Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal Biochem* 1988;174(1):168–176. [PubMed: 2464289]
30. Isenberg BC, Williams C, Tranquillo RT. Endothelialization and flow conditioning of fibrin-based media-equivalents. *Ann Biomed Eng* 2006;34(6):971–85. [PubMed: 16783653]
31. Nauman JV, Campbell PG, Lanni F, Anderson JL. Diffusion of insulin-like growth factor-I and ribonuclease through fibrin gels. *Biophys J* 2007;92(12):4444–4450. [PubMed: 17400703]
32. Barocas VH, Tranquillo RT. An anisotropic biphasic theory of tissue-equivalent mechanics: the interplay among cell traction, fibrillar network deformation, fibril alignment, and cell contact guidance. *J Biomech Eng* 1997;119(2):137–145. [PubMed: 9168388]
33. Solan A, Mitchell S, Moses M, Niklason L. Effect of pulse rate on collagen deposition in the tissue-engineered blood vessel. *Tissue Eng* 2003;9(4):579–586. [PubMed: 13678437]
34. Cox RH. Anisotropic properties of the canine carotid artery in vitro. *J Biomech* 1975;8(5):293–300. [PubMed: 1184600]
35. Weizsacker HW, Lambert H, Pascale K. Analysis of the passive mechanical properties of rat carotid arteries. *J Biomech* 1983;16(9):703–715. [PubMed: 6643542]

Figure 1. Pulsed flow-stretch bioreactor. a. 2 mm grafts on glass mandrel during static culture of 2 weeks, b. 2 mm grafts mounted in the bioreactor. c. Schematic of bioreactor with syringe mounted on reciprocating pump causing pulsed flow into the upper manifold, with medium flowing transmural through the tissue and through the lumens collecting on the abluminal side of the grafts and reinjected into the upper manifold (images and schematic are not to the same scale).

Figure 2.

Noninvasive monitoring of graft burst strength. a. Average pressure-strain curve of the grafts of different maturation (harvested at 2 wk and 7 wk), exhibiting different burst pressures, b. Average pressure-strain curve with data for 0–15% strain, showing no difference in pressure-strain behavior, c. Correlation of stiffness between 80–120mmHg with burst pressure; solid line is linear regression of the data points ($R^2 = 0.97$) with 95% confidence intervals shown by dotted lines, d. Predicted and measured burst pressure for grafts after 1 and 3 week of bioreactor culture. Stiffness was calculated as defined in Eqn. 2.

Figure 3. Comparison of grafts cultured under pulsed and constant flow. a. Thickness, b. Collagen/Cell, and c. Burst pressure of grafts conditioned with pulsed flow (stretch amplitude of 7%, average flow of 3.75 mL/min/graft) versus constant flow (3.33 mL/min/graft). Significant difference between groups is shown by paired symbols (*).

Figure 4. Comparison of constant length mounting and axial shortening. a. Fiber alignment maps, b. Modulus, c. Anisotropy index (the ratio of modulus in circumferential direction to axial direction) with comparison to ovine femoral artery (Native), and d. UTS with or without incremental shortening of the grafts. Significant difference between circumferential and axial graft properties is shown by paired symbols (* or #).

Figure 5.

Properties of 2 mm and 4 mm grafts. a. Thickness, b. Collagen concentration, c. Burst pressure, and d. Compliance of 2 mm and 4 mm grafts in comparison to the ovine femoral artery. The grafts were cultured in the bioreactor for 5 and 7 weeks for 2 mm and 4 mm grafts. Compliance was defined and calculated as Eqn. 3. Significant difference between the 2 mm and 4 mm grafts is shown by paired (*) symbol, while (#) indicates a significant difference of the native artery from both the 2 mm and 4 mm grafts.

Figure 6.

Histology of 2 mm and 4 mm engineered vascular grafts. Lillie's trichrome and picosirius red stained sections of grafts and a sheep femoral artery. The green color indicates collagen, with residual fibrin (and/or other proteins) in red visible in the 2 mm graft. Muscle fibers in native tissue also stain red. The cells were evenly distributed through the thickness of the grafts. Picosirius red stain under cross-polarized light shows red bands in the circumferential direction. The red intensity was comparable between samples.