

Physiologic Pulsatile Flow Bioreactor Conditioning of Poly(ethylene glycol)-based Tissue Engineered Vascular Grafts

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Abstract—Mechanical conditioning represents a potential means to enhance the biochemical and biomechanical properties of tissue engineered vascular grafts (TEVGs). A pulsatile flow bioreactor was developed to allow shear and pulsatile stimulation of TEVGs. Physiological 120 mmHg/80 mmHg peak-to-trough pressure waveforms can be produced at both fetal and adult heart rates. Flow rates of 2 mL/sec, representative of flow through small diameter blood vessels, can be generated, resulting in a mean wall shear stress of ~ 6 dynes/cm² within the 3 mm ID constructs. When combined with non-thrombogenic poly(ethylene glycol) (PEG)-based hydrogels, which have tunable mechanical properties and tailorable biofunctionality, the bioreactor represents a flexible platform for exploring the impact of controlled biochemical and biomechanical stimuli on vascular graft cells. In the present study, the utility of this combined approach for improving TEVG outcome was investigated by encapsulating 10T-1/2 mouse smooth muscle progenitor cells within PEG-based hydrogels containing an adhesive ligand (RGDS) and a collagenase degradable sequence (LGPA). Constructs subjected to 7 weeks of biomechanical conditioning had significantly higher collagen levels and improved moduli relative to those grown under static conditions.

Keywords—Transmural strain, Transmural shear, Hydrogel, Material properties, Medial equivalents.

INTRODUCTION

Blood vessel replacements are frequently necessary in the treatment of advanced atherosclerosis, aneurysmal and peripheral vascular disease, and vascular trauma. In the US alone, roughly 1.4 million patients undergo operations requiring arterial prostheses each year.⁴⁴ Autologous saphenous veins and mammary arteries are currently the preferred graft materials. However, the availability of tissues of appropriate dimensions is limited, with fewer than 90% of patients having suitable autologous tissue,²⁹ and donor site morbidity is a significant complication in these procedures. When autologous tissue is unavailable, synthetic materials (mainly Dacron and polytetrafluoroethylene) are frequently used for the treatment of peripheral vascular disease, but their use is limited to high-flow/low resistance

conditions,^{15,46} i.e., to > 6 mm ID vessels, because of their thrombogenicity, relatively poor elasticity and low compliance.²¹ Tissue engineering represents a potential means to construct functional grafts that could be used in vascular replacement procedures where autologous tissue is unavailable and synthetic materials fail.⁴⁴

Blood vessels consist of three layers: a thin monolayer of endothelial cells, a medial layer composed of smooth muscle cells (SMCs) embedded within a dense network of collagen and elastic fibers, and a loosely organized adventitial layer comprised of fibroblasts. Since the medial layer is the primary load bearing layer of the arterial wall, much of the previous research in tissue engineered vascular grafts (TEVGs) has focused on developing a bioartificial medial layer.^{25,43} While initial results with many of the TEVGs constructed to date are very encouraging, a number of technical hurdles remain before TEVGs can be considered a viable vascular replacement option.⁴¹ The potential for aneurysmal failure is a significant concern, since the mechanical integrity of TEVGs is generally less than that of the arteries they replace and since the mechanical integrity of the engineered grafts may not be maintained with time. In addition, many TEVG studies have been plagued in the *in vivo* setting by thrombosis and intimal hyperplasia.⁴¹

To address the potential for aneurysmal failure, researchers have attempted to create TEVGs with mechanical properties that approach those of native tissue. Since the relatively poor TEVG mechanical properties are considered to be due in large part to differences in graft extracellular matrix composition and microarchitecture relative to that of normal arteries, a range of media additives have been explored to improve TEVG outcome.^{35,38,42} For example, a cocktail of ascorbic acid, TGF- β 1, and insulin has been shown not only to increase net SMC collagen deposition and fibril thickness but also to increase elastin production.³⁵

Another approach that several investigators have taken to improve TEVG mechanical and biochemical outcome involves exploiting the ability of SMCs to sense and respond to mechanical stimuli. Mechanical stretching of SMCs has been shown to have profound effects on cell phenotype,^{2,28} orientation,²⁸ ECM deposition,^{12,32} growth factor release¹¹

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as well as on graft mechanical properties.^{30,42} Important work by Kim *et al.* showed a 15% increase in SMC proliferation and a 49% increase in elastin content for polymeric scaffolds cultured for 10 weeks with cyclic strain at an amplitude of 7% compared to unloaded controls.³⁰ Additionally, the elastic moduli and ultimate tensile strengths (UTS) of these constructs were significantly higher in the mechanically stimulated group. In total, these findings provide support for the inclusion of physiologically relevant mechanical stimulation in the development of tissue engineered constructs containing SMCs.

While the aforementioned mechanical conditioning studies have shown promising results in terms of improving construct stiffness and strength, ECM deposition, and SMC phenotype, none has applied a fully physiological pulsatile wave to the constructs. In addition, these studies have assumed pulsatile distension of the arterial media during the cardiac cycle to be the dominant mechanical stimulus that these SMCs perceive in terms of resultant protein production and behavior. However, SMCs in blood vessels also experience transmural shear stress, as well as bending and torsional stresses induced by cardiac pulsation or by influence of surrounding tissues.

In the present study, a pulsatile flow bioreactor has been designed to allow for both physiological shear and pulsatile conditioning of TEVGs. We examine the effects of these combined stresses on TEVG outcome using poly(ethylene glycol) (PEG) hydrogels as a model scaffold material. PEG has several properties which make it a desirable vessel replacement material. In addition to its biocompatibility, it has been demonstrated to be non-thrombogenic,²³ significantly reducing the potential for hyperplasia and thrombosis that has plagued many other TEVG materials. Diacrylate-derivatized PEG (PEGDA) macromers readily dissolve in aqueous solution, forming an optically transparent, low viscosity mixture that is photopolymerizable in the presence of cells.^{23,45} Thus, seamless, mechanically isotropic¹ cylindrical constructs with homogeneously seeded cells^{4,5,8,9,14,34} can be readily formed by pouring a solution of photoactive PEG macromers, cells, and photoinitiator into an appropriately shaped mold and applying light. The photoactivity of PEGDA combined with its intrinsic resistance to cell and protein adhesion results in a biological “blank slate” which can be modified in a controlled manner to contain bioactive moieties.¹⁸ In addition, PEG hydrogels are highly elastic, which is important in the vasculature where tissues must maintain their form in response to prolonged mechanical stress, and their mechanical properties are tunable, meaning that the strain experienced by the scaffold can be varied independently of an applied pulsatile waveform and shear stress by changing the scaffold composition. Thus, our custom-built reactor combined with PEG-based hydrogels creates a versatile system in which strain amplitude, pulse shape, pulse frequency, and shear can be varied independently and their roles in vascular development examined.

In this study, we demonstrate the flexibility of the system as well as its ability to modulate the matrix deposition of smooth muscle (SM) progenitor cells encapsulated in PEG-based hydrogels.

MATERIALS AND METHODS

Polymer Synthesis

PEGDA was prepared by combining 0.1 mmol/mL dry PEG (MW 3400 Da, 6000 Da, or 10000 Da, Fluka), 0.4 mmol/mL acryloyl chloride, and 0.2 mmol/mL triethylamine in anhydrous dichloromethane (DCM) and stirring under argon overnight. The resulting solution was washed with 2 M K₂CO₃ and separated into aqueous and DCM phases to remove HCl. The DCM phase was subsequently dried with anhydrous MgSO₄, and PEGDA was precipitated in diethyl ether, filtered, and dried under vacuum. Successful acrylation was determined using proton NMR analysis (Avance 400, Bruker).

Synthesis of Acrylate-Derivatized Peptides

The peptide RGDS (American Peptide) was conjugated to an acrylated PEG derivative (3400 Da) by reaction with acryloyl-PEG-*N*-hydroxysuccinimide (ACRL-PEG-NHS, Nektar) at a 1:1 molar ratio for 2 h in 50 mM sodium bicarbonate buffer, pH 8.5. The collagenase sensitive peptide sequence GGLGPAGGK¹⁷ was synthesized using Fmoc solid phase peptide synthesis (Applied Biosystems). Cleavage from the polystyrene resin was effected with 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane. The cleaved peptide was precipitated in ether followed by dialysis. The peptide was then reacted at a 1:2 molar ratio with ACRL-PEG-NHS in 50 mM sodium bicarbonate buffer for 2 h. The acrylate-derivatized product was purified by dialysis, lyophilized, and stored at -20°C until use.

Hydrogel Polymerization

Precursor solutions were prepared by dissolving the desired levels of PEGDA of a given MW in HEPES buffered saline (HBS; 10 mM HEPES, 100 mM NaCl, pH 7.4). 10 μ l of a 300 mg/mL solution of UV photoinitiator 2,2-dimethoxy-2-phenyl-acetophenone (DMPAP) dissolved in *N*-vinylpyrrolidone (NVP) was then added per mL of mixture. The UV transparent precursor solution was poured into a UV transparent cylindrical mold composed of an inner glass mandrel 3 mm in diameter and an outer hollow plastic cylinder of 6.4 mm diameter. The filled molds were then exposed to longwave UV light (UVP model B-100SP, 10 mW/cm², 365 nm) under constant rotation such that each surface point of the mold received equal UV intensity and exposure time. This uniform surface exposure combined with the optical transparency of the PEGDA macromer solution results in uniform polymerization within and between

gels.^{4,5,8,9,14,34} Longwave UV light has been previously used at similar intensities and exposure times to induce rapid polymerization of PEGDA macromers with minimal concomitant cell damage in numerous tissue engineering studies.^{4–6,8,9,14,34,47}

Pulsatile Flow System Design

The system developed for mechanical conditioning of the constructs is shown schematically in Fig. 1A. Briefly, a Masterflex L/S variable speed digital peristaltic pump fitted with an L/S multichannel pump head (Cole-Parmer) operating on STA-Pure platinum-cured silicon tubing (ID 6.4 mm, OD 11.1 mm, wall thickness 2.4 mm; Barnant Company) was used to create the desired base flow rate of media. The flow from the peristaltic pump was passed through a modified 100 mL glass media storage bottle with screw cap (Corning) which served as a compliance chamber to damp oscillations in the flow stream created by the peristaltic pump. The media from the compliance chamber was then passed through a series of CellMax pulsatile pumps (Spectrum Labs), which squeezed the tubing (platinum-cured silicon tubing, ID 6.4 mm, wall thickness 2.5 mm; Cole-Parmer) passing through the pump head to produce the desired pulsation and pressure profiles. Check valves at the inlet and exit of each pulsatile pump prevented backflow of the media in response to pump action. The CellMax pulsatile pumps, which normally operate at 120 beats per min (bpm), were modified to pulse at either 120 bpm or 60 bpm

so that both adult and fetal pulsation conditions could be examined. Peristaltic and pulsatile pumps that perform their work directly on tubing were selected for this system to avoid the contamination issues frequently observed with prolonged culture (> approximately 72 h culture time) in bioreactor systems where the media contacts pump head components (unpublished observations and personal communications).

The flowing liquid was then passed through the constructs fitted on the ends of a custom glass chamber (shown in Fig. 1B). The elasticity of the constructs themselves acted to maintain the constructs on the chamber fittings during pulsation. The pressure profile experienced by the constructs was monitored in parallel to the constructs using a pressure transducer (Maxxim Medical). The media was then passed back to the media chamber. Adjustments to the pressure waveform could be made by altering the number of pulsatile pumps connected in series and/or the degree of tubing occlusion in these pumps. Alterations to mean system pressure were made by adjusting the occlusion in a valve located at the inlet of the media chamber. In this manner, pressure waveforms and flow can be independently varied. Gas exchange in the construct chamber and media reservoir occurred through 0.2 μm filters that were fitted to ports created in the vessels. The CO₂ permeable platinum-cured silicone tubing (Cole-Parmer) interconnecting system components also provided an additional means of gas exchange. System components were sterilized (autoclave or ethylene oxide) prior to assembly in a laminar-flow hood,

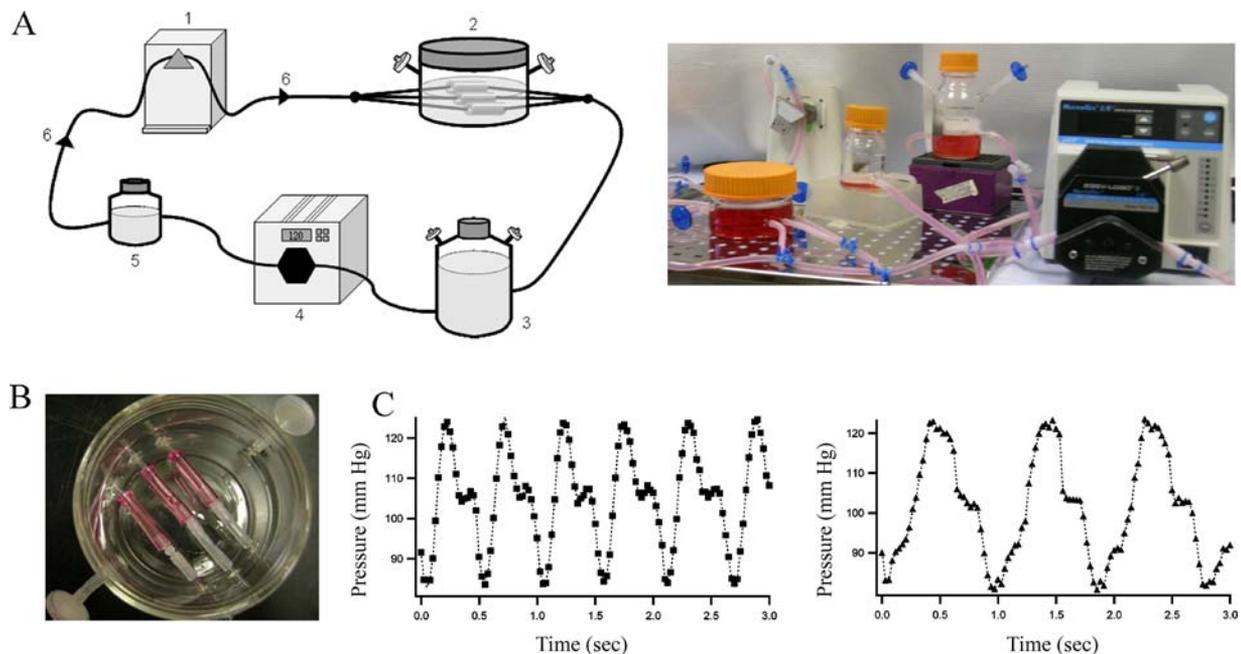


FIGURE 1. Pulsatile flow bioreactor. (A) (*left*) A schematic of system design, which includes (1) one or more pulsatile pumps, (2) custom graft chamber, (3) media reservoir, (4) a peristaltic pump, (5) compliance chamber, (6) check valves. (*right*) An image of the actual bioreactor system. (B) Close up of the graft chamber. (C) Representative pressure waveforms generated using the bioreactor set to either fetal (*left*) or adult (*right*) pulse conditions.

and all components except the peristaltic pump were housed in a 5% CO₂/37°C tissue culture incubator for studies.

The waveforms shown in Fig. 1C resulted from channeling flow at 2 mL/s through two pulsatile pumps in series pumping at either 120 bpm (fetal pulsation) or 60 bpm (adult pulsation), resulting in a mean shear of ~ 6 dynes/cm² at the construct lumens (~ 3 mm ID). Although these conditions mimic mean *in vivo* flow conditions, it is important to note the bioreactor system is not fully biomimetic. In particular, the bioreactor operation does not reflect the fact that the source of the mean flows and shears result, *in vivo*, from both forward and reflected waves.

Transmural Strain Analysis and Correlation with Biomechanical Properties

To examine the dependence of transmural strain on construct composition, the mean radial distensions experienced by the hydrogel formulations shown in Table 1 were analyzed under physiological flow conditions of approximately 120/80 mmHg and 2 mL/s at fetal heart rates. Pressure profiles were continuously monitored with a pressure transducer (Maxxim Medical) connected to a Macintosh computer via an analog-to-digital converter box. To measure radial distension imparted to engineered vessels, a Nikon Coolpix 5000 digital camera was used to image vessels undergoing mechanical pulsation at a capture rate of 15 frames/s. Two independent observers evaluated the maximum and minimum vessel distensions from the recorded images using the Adobe Photoshop measurement tool. Three measurements per gel were made for three different hydrogel samples of each formulation. The strain was then calculated as $(D_{\max} - D_{\min})/D_{\min}$,⁴² where D represents the inner diameter of the vessel. To relate measured strain to biomechanical properties, each construct was sectioned into thin rings and the mechanical properties were assessed as described below. For each gel, the measured strain was validated by comparison with the strain estimated from application of Bernoulli's law to vessel dynamics, namely, $\varepsilon = \frac{\Delta P r_v}{h_v E}$, where ε = strain, ΔP = peak-to-trough pressure rise, E = modulus, r_v = vessel inner radius, and h_v = vessel wall thickness.³⁹

Biomechanical Testing

Biomechanical testing was performed using a modification of the circumferential property testing techniques validated in Johnson *et al.*²⁷ and Hiles *et al.*²² Sections for biomechanical analysis were obtained by placing TEVG constructs on a glass mandrel and subsequently cutting the gels into thin rings using nylon monofilament sutures. Sample dimensions were obtained from digital images (inner and outer diameter) or using calipers (width). Material properties were then measured using an Instron Model 3340 materials testing device equipped with a 10 N load cell (Instron). System control and data analysis were accomplished using Instron Series IX/s software. Custom mounting brackets provided uniaxial strain application at a rate of 6 mm/min for the ring-shaped hydrogels. To calculate stress from the measured force, the area of force application was approximated as two rectangles with sides equal to the width and wall thickness of the ring. The gauge length, l_{gauge} , was taken to be the outer diameter of the ring less the wall thickness and strain was calculated from the measured displacement, Δl , according to the following equation: $\Delta l/l_{\text{gauge}}$. Each sample was run in triplicate and the stress-strain data was used to obtain the UTS and average elastic modulus. The reported UTS for each sample was the maximum of the three measured failure stresses.

Cell Expansion

Mouse embryonic 10T-1/2 SM progenitor cells (ATCC) were expanded in monolayer culture between passages 4–6. Prior to encapsulation within hydrogels, cells were maintained in Eagle's Minimum Essential Medium (MEM, ATCC) supplemented with heat-inactivated fetal bovine serum (FBS, Sigma).

Cell Encapsulation

A precursor solution containing 0.07 g/mL 6000 Da PEGDA, 0.03 g/mL collagenase degradable ACRL-PEG-GGLGPAGGK-PEG-ACRL (P-LGPA-P), and 2.8 $\mu\text{mol/mL}$ ACRL-PEG-RGDS in HBS was prepared and sterilized via filtration (0.22 μm , PES membrane, Millipore). 10T-1/2

TABLE 1. Tunable properties of PEGDA hydrogels.

Formulation	Strain (%)		Modulus (kPa)	UTS (kPa)
	Measured	Bernoulli		
100 mg/mL 3400 Da	6.2 \pm 0.4	7.2	92.1 \pm 2.7	67.0 \pm 6.7
100 mg/mL 6000 Da	6.4 \pm 0.5	9.5	81.2 \pm 1.2	69.8 \pm 8.2
200 mg/mL 6000 Da	2.9 \pm 0.4	4.2	140.4 \pm 5.3	101.7 \pm 12
100 mg/mL 10000 Da	10.9 \pm 1.3	10.9	48.4 \pm 1.7	66.2 \pm 11
200 mg/mL 10000 Da	3.6 \pm 0.4	5.5	76.3 \pm 2.0	69.8 \pm 4.3
70 mg/mL 6000 Da + 30 mg/mL P-LGPA-P ^a	10.4 \pm 0.2	11.3	59.3 \pm 2.5	43.3 \pm 8.4

^aFormulation also contains 2.8 $\mu\text{mol/mL}$ ACRL-PEG-RGDS.

cells at passage 6 were trypsinized and resuspended in the precursor solution at a concentration of 1.3×10^6 cells/mL. 10 μ l of a 300 mg/mL solution of DMAP dissolved in NVP was added per mL of the cell-precursor solution mixture. The low viscosity mixture was gently agitated with a vortex, and the cell suspension rapidly pipetted into the transparent cylindrical molds fitted with inner glass mandrels. Long-wave UV light (10 mW/cm², 365 nm) was applied to the hydrogels under constant rotation of the constructs for an average of ~ 3 min/gel to produce uniform polymerization within and between gels during exposure.

Cyclic Mechanical Conditioning

The polymerized constructs were removed from the molds and allowed to swell in media for 1 h at 5% CO₂/37°C. Ring samples were cut from each hydrogel construct and analyzed biomechanically as described above. The constructs were then transferred to the bioreactor chambers, and maintained under static conditions for one week in MEM (ATCC) supplemented with 10% FBS (Sigma), 10 ng/mL TGF- β 1 (R&D systems), 10 μ g/mL insulin (Sigma), 50 mM L-ascorbic acid (Sigma), 10 μ g/mL ciprofloxacin (Sigma), 100 mU/mL penicillin, 100 mg/L streptomycin, and 0.25 μ g/mL fungizone (Gibco). The cocktail of growth factors and L-ascorbic acid added to the media was selected for two reasons. First, 10T-1/2 SM progenitor cells have been shown to display a SMC phenotype when cultured in the presence of TGF- β 1. In addition, as previously mentioned, SMCs increase both collagen and elastin production relative to basal levels when in the combined presence of L-ascorbic acid, TGF- β 1, and insulin.³⁵ Constructs were maintained at 5% CO₂/37°C, and the media was replaced every two to three days.

After one week of static culture, constructs were randomly assigned to static and dynamic groups. The constructs in the static groups continued to be grown under static conditions during the remaining 7 weeks of culture time. Over the course of the next three days, the flow in each dynamic construct was increased to 120 mL/min in 40 mL/min increments and mean pressures increased to ~ 50 mmHg. At day 4 of flow, the pulsatile pumps were connected and 60/40 mmHg pulsatile waveforms were applied at 120 bpm. At day 5, pressure was increased to physiological levels by adjusting the occlusion valve at the inlet to the media chamber, resulting in a 110/70 mmHg waveform. Wall shear stress was calculated to be ~ 6 dynes/cm², which is similar to, although slightly lower than, physiological baseline wall shear of ~ 10 dynes/cm².³ After 3 weeks of pulsatile flow, samples from both the static and dynamic groups were harvested for mechanical testing as an intermediate assessment of mechanical property alterations induced by the flow system. Pulsatile flow conditions continued to be applied to the remaining

dynamic constructs for an additional four weeks. After 8 weeks total culture time, both static and dynamic constructs were cut into a series of rings for biochemical, biomechanical, and histological analyses. Wet weights of the constructs used for biochemical analyses were recorded and the samples were immediately frozen at -80°C . Biomechanical analyses were carried out as previously described.

Biochemical Analyses

Segments of each vessel, 100–200 mg wet weight, were analyzed for DNA, collagen content, and elastin content. The constructs were hydrolyzed in 700 μ L of 0.1 N NaOH per 200 mg hydrogel wet weight for 36 h at 37°C. The samples were then centrifuged (10,000 $\times g$ for 10 min), and a 10 μ L aliquot was taken from each sample for DNA quantification. Any material pelleted during centrifugation was resuspended by vortexing, and additional hydrolysis was carried out by transferring the samples to a 100°C oven for 90 min. This hydrolysis step served to solubilize collagen but not elastin fibers. Hydrolyzed samples were then cooled to room temperature and centrifuged to pellet elastin (10,000 $\times g$ for 10 min). The elastin pellet was washed three times with distilled water and stored at -80°C . The supernatant was retrieved for collagen quantification.

DNA Analysis

Aliquots of the hydrolyzed samples retrieved for DNA analyses were neutralized. Sample DNA levels were then assessed using the Molecular Probes PicoGreen assay reagents and calf thymus DNA standards that had experienced the same base hydrolysis conditions as the constructs. DNA was translated to cell number using the conversion factor for murine cells of 6.6 pg DNA per cell.²⁰ The conversion from DNA to cell number is commonly used to assess the cellularity of tissue engineered constructs.^{8,19,25,26,36}

Collagen Analysis

The supernatants retrieved for collagen quantification were subjected to acid hydrolysis in 6 N HCl at 110°C for 15–18 h. Equal mass aliquots from each hydrolyzed static sample were pooled and the hydroxyproline (OHP) content of this pooled sample was measured in duplicate by amino acid analysis (AAA Service Laboratory, Inc). Similarly, equal mass aliquots of hydrolyzed dynamic samples were pooled and the OHP content of this pooled sample was measured in duplicate by amino acid analysis. Sample collagen content was calculated from measured OHP levels using a conversion factor of 8 mg collagen per mg OHP³⁷ and was normalized to cell number.

Elastin Content

Elastin levels were determined according to the procedure detailed in Long *et al.*³⁵ Briefly, elastin pellets were digested in 6 N HCl at 100°C for 24 h. Samples were then dried on a rotary evaporator, and the resulting free amino acids were dissolved in 100 μ l of 0.1 M sodium citrate buffer (pH 5.0). Following addition of an equal volume of ninhydrin reagent (Spectrum), samples were boiled for 15 min, cooled, and their absorbance read at 570 nm. Hydrolyzed α -elastin (Elastin Products Company) was used as the standard, and measured elastin levels were normalized to cell number.

Histological Analysis

Segments from each TEVG were harvested at the end of the 8 week study and immediately submerged in freezing media (Triangle Biomedical Sciences). 30 μ m frozen sections were immunostained to detect the presence of the SMC markers calponin and SM α -actin¹⁰ to confirm that the 10T-1/2 precursor cells were displaying a SMC-like phenotype. Briefly, fixation in 10% neutral buffered formalin (VWR Scientific) for 10 min was followed by a two-step blocking procedure (BEAT Block, Zymed) to prevent non-specific interactions. Sections were next incubated with primary antibodies to either calponin (C2687, Sigma), or SM α -actin (A2547, Sigma) for 18 h at 4°C followed by the application of Peroxo-Block for 45 s to quench any endogenous peroxidase activity (Peroxo-Block, Zymed). Positive staining was visualized after successive incubations with a biotinylated secondary antibody for 30 min, an enzyme conjugate for 20 min, and an AEC substrate chromagen mixture for 10 min (Histomouse-SP, Zymed). Nuclei were counterstained with hematoxylin and coverslips mounted using GVA mount (Zymed). Sections not exposed to primary antibodies served as negative controls.

Statistical Analysis

Results are reported as the mean \pm SEM. The statistical significance of biochemical differences between static and dynamic groups and of biomechanical properties within a particular group at various time points was determined using two-tailed student *t*-tests, $p < 0.05$. ANOVA was used to analyze differences in the biochemical property time courses of static versus dynamic constructs, $p < 0.05$.

RESULTS AND DISCUSSION

Transmural Strain Analyses

Table 1 shows the moduli and measured strain for several PEG-based hydrogel formulations. Transmural strains for the materials tested varied from 2.9% for the 200 mg/mL 6000 Da gel to 10.9% for the 100 mg/mL 10000 Da formulation. This range includes the values of 5–10%, which

are representative of *in vivo* arterial strains³¹ and which have been found to induce SMC proliferation and ECM production *in vitro*.³⁰ For each gel formulation, the measured strains correlate closely with the strains predicted by application of Bernoulli's law to the measured pressure profiles and construct moduli. Note that strain can be modulated by altering the concentration of PEG in a construct or by altering the MW composition, and that significantly different strains can result from materials of similar moduli (e.g., 100 mg/mL 6000 Da gels vs 200 mg/mL 10000 Da gels). Furthermore, similar transmural strains can be achieved for the same pulsatile flow conditions using constructs with significantly different moduli (e.g., 100 mg/mL 3400 Da gels vs 100 mg/mL 6000 Da gels). Since cell behavior appears to be significantly altered by network modulus^{5,7} as well as by strain, the ability to separately alter experienced strain and construct modulus should allow for the controlled exploration of the impact of these variables on TEVG outcome.

Due to desirable strain properties at physiological flow profiles (Table 1) and the capacity for microstructural modification in response to cellular enzymes, a hydrogel formulation containing the MMP-degradable peptide sequence LGPA was selected for *in vitro* trials of the bioreactor system. RGDS was covalently immobilized into the construct network to allow for cell attachment to the PEG hydrogel network and to ensure mechanotransduction of the applied forces to the cells.^{30,48}

Biochemical Analyses

After 8 weeks of culture, the degradable constructs exposed to physiological pulsatile flow conditioning were analyzed biochemically and compared to constructs cultured under static conditions. The cellularity in the dynamic constructs was significantly higher than that in the static constructs, as shown in Fig. 2A (dynamic 9.5% higher than static, $p = 0.0335$), indicating that pulsatile conditioning enhanced cell survival/proliferation. This result is consistent with previous studies regarding the effects of pulsation on TEVG cell density at moderate strains.⁴⁸ Comparable amounts of elastin ($\sim 0.35 \times 10^{-4}$ μ g/cell) were measured in the static and dynamic constructs ($p = 0.944 > 0.05$), indicating that mechanical conditioning did not effectively enhance elastin production/retention over the time of culture. Significant variability in terms of the effects of mechanical stimuli on SMC elastin production has been reported in literature,^{38,40} variability which may be due in part to differences in cell source, media additives,³⁵ scaffold,³⁵ and experimental time scale.⁴⁰ This being said, in the present study, the week 8 elastin levels in both the static and dynamic constructs compare well on a per cell basis with elastin levels characteristic of native arteries (roughly 0.8×10^{-4} μ g/cell to 6.7×10^{-4} μ g/cell, depending on the type of artery).^{13,16}

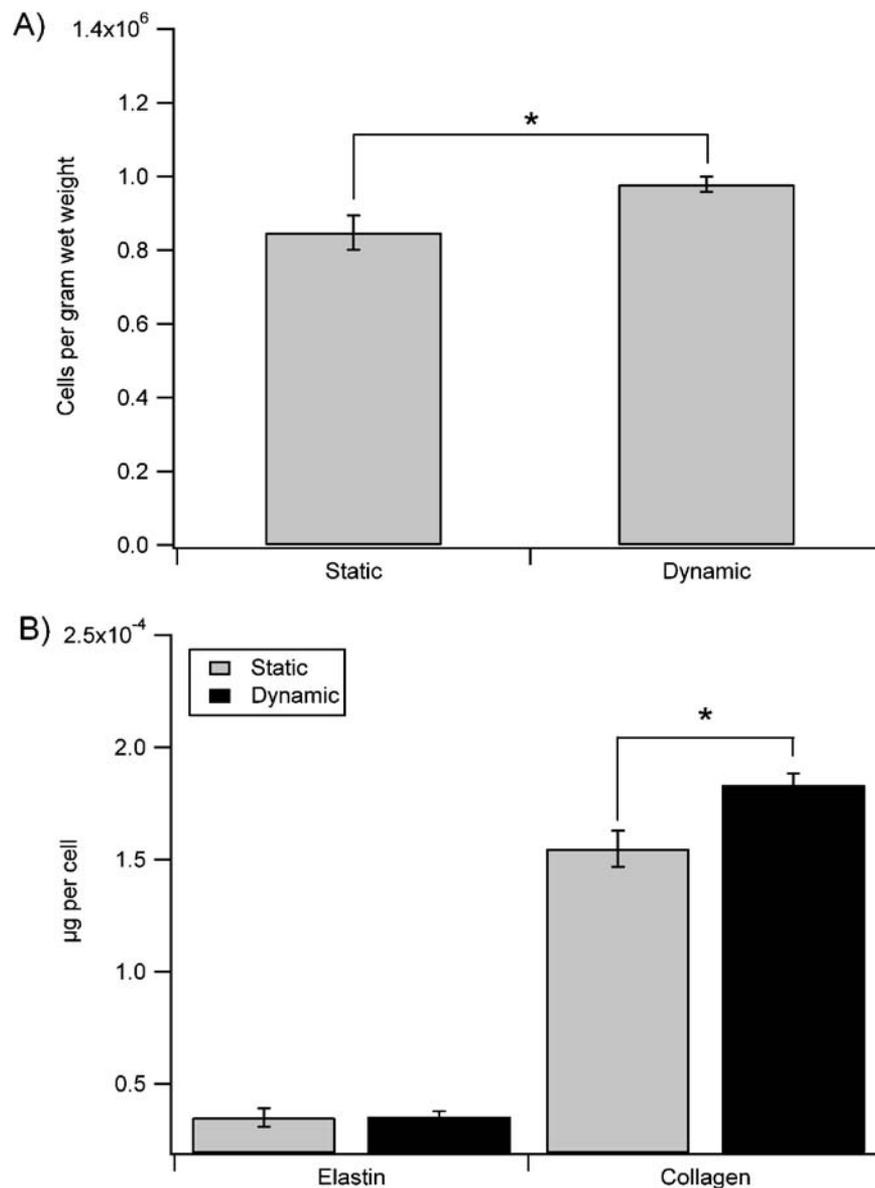


FIGURE 2. Biochemical analyses show an increase in cell number (A) and collagen content (B) in constructs cultured under pulsatile flow conditions at 120 bpm. Similar amounts of elastin were measured in each group. *indicates a statistical difference, cell number: $p=0.0335$, collagen content: 0.0177.

In contrast to the elastin results, the dynamic constructs showed a 18.3% increase in the amount collagen per cell compared to static samples (Fig. 2B, $p=0.0177$). These results are consistent with other studies that have demonstrated enhanced collagen production by vascular cells in response to mechanical stretch. Importantly, the collagen produced on a per cell basis in the dynamic constructs by week 8 ($\sim 1.8 \times 10^{-4}$ µg/cell) compares favorably with the per cell collagen content of native arteries, which ranges from roughly 3.1×10^{-4} µg/cell to 9.7×10^{-4} µg/cell.^{13,38}

Since collagen is a primary structural protein responsible for the mechanical integrity of blood vessels under the high blood pressure and flows experienced *in vivo*, collagen levels approaching native tissue are critical for the success of TEVGs. These combined data indicate that pulsatile flow stimuli increased cell proliferation and improved collagen production and/or retention on a per cell basis, with per cell collagen and elastin values in the dynamic constructs comparing favorably to those of native arteries.

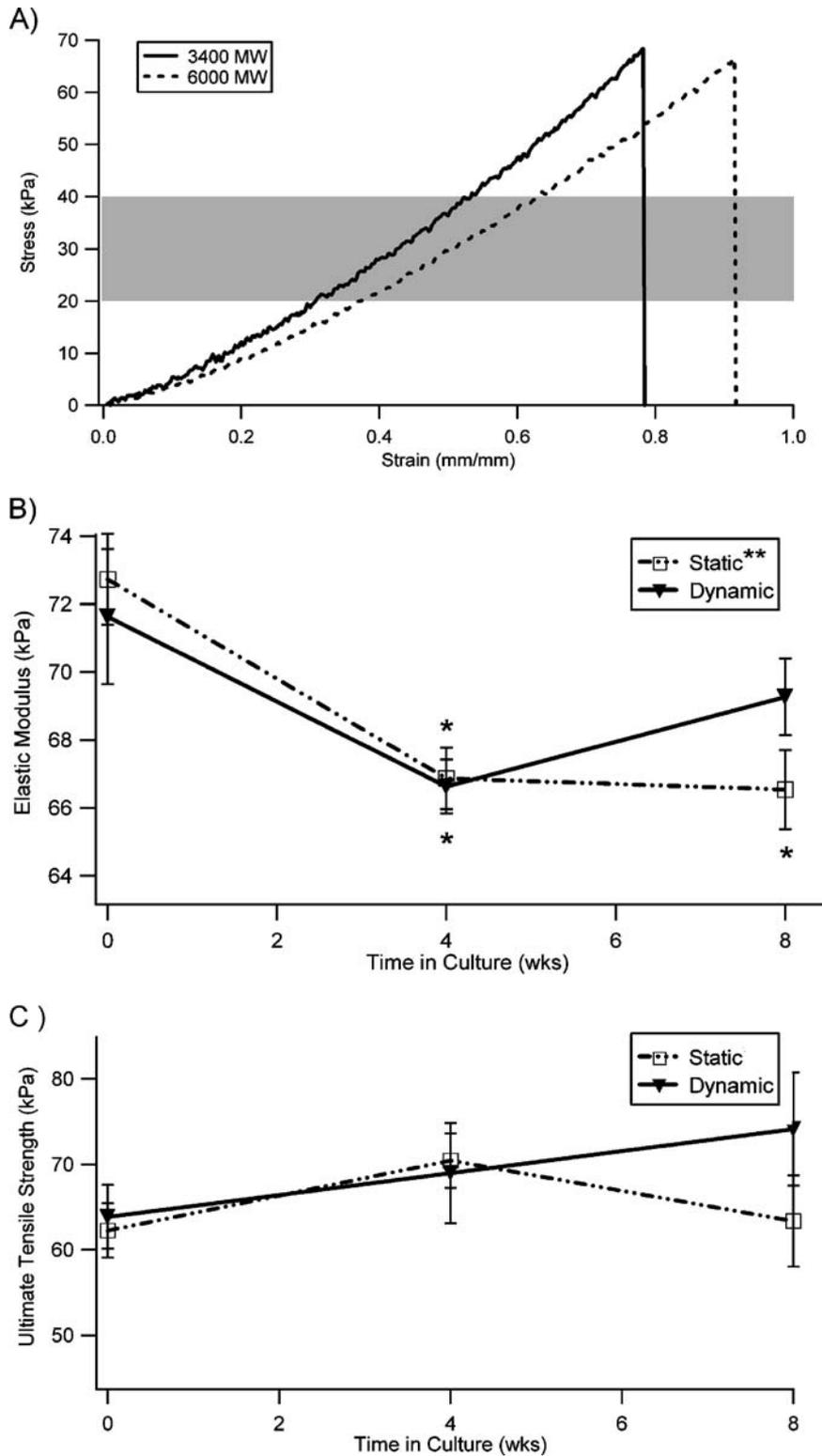


FIGURE 3. Construct mechanical results. (A) Representative stress-strain data for the 3400 and 6000 Da PEGDA hydrogels. The shaded area indicates the linear region of the curve which was used in calculation of the material stiffness. Biomechanical analysis results for elastic modulus (B) and ultimate tensile strength (C) for TEVGs cultured under pulsatile flow conditions at 120 bpm for 8 weeks. Data represent the mean (A) and max (B) \pm standard error of the mean for 5 samples. *indicates a significant difference with the corresponding time zero value for that group (static: $p=0.00467$, dynamic: $p=0.0416$). **indicates a significant decline in the modulus of static constructs over 8 weeks of culture, $p=0.00444$.



FIGURE 4. Immunohistochemical analysis confirms the presence of the smooth muscle cell markers calponin (*left*) and smooth muscle α -actin (*middle*) in sections from TEVGs cultured in this study. These representative samples demonstrate the level of staining that was seen in both the mechanically stimulated and static groups. The red color indicates reaction with the primary antibodies, which were not applied in the negative control (*right*). Scale bars are 10 μ m.

Biomechanical Analyses

To monitor the changes in construct mechanical properties with time, biomechanical analyses were carried out at day 0, week 4, and week 8. For reference, the modulus was defined as the slope of the linear region of the stress-strain curve at a reference stress of 30 kPa (Fig. 3A). As shown in Fig. 3B and C, the mechanical properties of the static and dynamic groups were statistically indistinguishable from each other through week 4 of culture, with the moduli of both groups declining from time zero values ($p = 0.0047$ and $p = 0.0416$, respectively). By week 8, the mean modulus of the static group had continued to decline from its initial value (ANOVA, $p = 0.00442$), whereas mechanical conditioning appeared to halt this decline (ANOVA, $p = 0.326 > 0.05$).

Analysis of these results suggests that the initial decrease in modulus observed from day 0 to week 4 in both the static and dynamic constructs may be due to a reduction in hydrogel crosslinking density resulting from the degradation of the collagenase sensitive peptides linking PEG chains in the constructs. With increasing time in culture, the moduli of the static hydrogels continued to deteriorate, perhaps due to the continued degradation of the hydrogel network not sufficiently matched by SMC protein production. Based on examination of combined biochemical and biomechanical data, the impact of the increased collagen production noted in the dynamic constructs on mechanical outcome is likely minor, and the improved modulus of the dynamic constructs relative to their static counterparts appears to be due primarily to microscale restructuring of the dynamic scaffolds in response to prolonged mechanical stress permitted by the presence of degradable segments in the base hydrogel.

HISTOLOGICAL ANALYSIS

Although TGF- β 1 was added to the cell culture media to induce SMC-like phenotype in the encapsulated 10T-1/2 SM progenitor cells,²⁴ it was still important to verify

that the encapsulated progenitor cells were indeed displaying SMC-like phenotype. Calponin and SM α -actin are two standard markers used to identify cells expressing a SMC phenotype.^{10,33} Immunohistochemical analyses of both static and dynamic constructs revealed comparable expression of calponin and SM α -actin in each (Fig. 4). These results indicate the expression of a SMC-like phenotype under the current culture conditions and validate the use of these progenitor cells for applications in vascular graft regeneration.

CONCLUSIONS

The cellularity, collagen content, and moduli of TEVGs were all positively affected by pulsatile flow stimulation of SM progenitor cells encapsulated in PEG-based constructs. Thus, the combined data support the hypothesis that the custom physiological pulsatile flow bioreactor described herein can be effectively used as a vehicle to improve TEVG quality. Further analysis of engineered constructs using this system may provide valuable insights into the culture conditions which optimize construct outcome. In particular, the separate and combined effects of shear and pulsatile stimuli (both fetal and adult) can be explored with this unique bioreactor system. The use of non-thrombogenic PEG-based hydrogels as constructs supplies several avenues of additional flexibility to the overall bioreactor system. The ability of controlled biochemical moieties, such as peptide RGDS, to be covalently immobilized into PEG hydrogel networks should permit the study of possible synergistic effects between specific biochemical and biomechanical signaling. Moreover, the impact of strain variations on TEVGs can be investigated separately from alterations in pulsatile waveform and shear by tuning the mechanical properties of the base hydrogel via changes in its composition. Thus, by combining the material properties of PEG-based constructs with the physiological bioreactor, a versatile platform is created for the systematic exploration of the impact of controlled biochemical and biomechanical stimuli on TEVGs.

As with other flow systems, this bioreactor can also be used to facilitate graft endothelialization, possibly aided by the use of multi-layered hydrogel constructs to mimic the structural organization of the cells within the native blood vessel. For example, structurally complete TEVGs can be readily fabricated by successive polymerizations of PEG macromer solutions containing cell interaction moieties and/or cell types specific to the intimal, medial and adventitial layers. Furthermore, this bioreactor system provides a platform for simulating growth conditions of native tissues via spatially controlled and/or time dependent presentation of biochemical and biomechanical stimuli to associated cells, a recapitulation of vascular development that may prove beneficial to the advancement TEVGs.

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