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Non-invasive Assessment of Elastic Modulus of Arterial Constructs during Cell Culture using Ultrasound Elasticity Imaging

Debaditya Dutta^{1,+}, Kee-Won Lee^{2,+}, Robert A. Allen², Yadong Wang^{2,3,4}, John C. Bringham^{2,5}, and Kang Kim^{1,2,4,*}

¹Center for Ultrasound Molecular Imaging and Therapeutics – Department of Medicine and Heart and Vascular Institute, University of Pittsburgh and UPMC, Pittsburgh, Pennsylvania 15261, USA

²Department of Bioengineering, University of Pittsburgh and UPMC, Pittsburgh, Pennsylvania 15261, USA

³Department of Surgery, University of Pittsburgh and UPMC, Pittsburgh, Pennsylvania 15261, USA

⁴McGowan Institute for Regenerative Medicine, University of Pittsburgh and UPMC, Pittsburgh, Pennsylvania 15261, USA

⁵Department of Civil and Environmental Engineering, University of Pittsburgh and UPMC, Pittsburgh, Pennsylvania 15261, USA

Abstract

Mechanical strength is a key design factor for engineered arteries. Most existing techniques assess the mechanical property of arterial constructs destructively, leading to a large number of animal sacrifices. We propose an ultrasound-based non-invasive mechanical strength assessment technique for engineered arterial constructs. Tubular scaffolds made from a biodegradable elastomer and seeded with vascular fibroblasts and smooth muscle cells were cultured in a pulsatile-flow bioreactor. Scaffold distension was computed from ultrasound radiofrequency signals of the pulsating scaffold via two-dimensional phase-sensitive speckle tracking. The Young's modulus was then calculated by solving inverse problem from the distension and the recorded pulse pressure. Stiffness thus computed from ultrasound correlated well with direct mechanical testing results. As the scaffolds matured in culture, ultrasound measurements showed increased Young's modulus and histology confirmed the growth of cells and collagen fibrils in the constructs. The results show that ultrasound elastography non-invasively assesses and monitors the mechanical properties of arterial constructs.

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*Corresponding author: Kang Kim. Center for Ultrasound Molecular Imaging and Therapeutics, Department of Medicine and Heart and Vascular Institute, University of Pittsburgh and UPMC. 3550 Terrace Street, Scaife Hall 950, Pittsburgh, PA 15261, USA. Tel: +1 412 624 5092; Fax: +1 412 624 2264; kangkim@upmc.edu.

[†]These authors contributed equally to this work.

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Keywords

Ultrasound Elasticity Imaging; Non-invasive Assessment; Young's Modulus; Inverse Problem; Tissue Engineering; Arterial Construct

Introduction

More than 1.2 million people in US suffer from cardiovascular diseases each year (Roger *et al.* 2012). Because of their high incidences, which is anticipated to further increase, demand for arterial substitutes has grown. Current grafts such as autologous vessels and synthetic grafts have made significant progress for bypassing or replacing diseased and damaged arteries. Autologous vessels, such as the saphenous vein, are known as the standard for small-diameter constructs, but have several limitations including by scarcity of donors, donor site morbidity, and the need for additional surgery (Isenberg *et al.* 2006). Synthetic grafts have widely been used to bypass or replace large-diameter arteries because they complement the limitations of autologous vessels. However, little evidence supported their applications to small-diameter (< 6mm) arteries due to thrombosis in early stages leading to graft failure in most cases (Wang *et al.* 2007). To overcome these limitations, tissue engineering approaches have been developed to engineer small-diameter arterial constructs with structural and mechanical properties similar to native arteries (L'heureux *et al.* 1998; Niklason *et al.* 1999; Weinberg and Bell 1986; Wu *et al.* 2012).

Mechanical strength, especially the tensile strength represented by Young's modulus, is one of the important design factors for engineered arterial grafts to ensure high burst pressure while offering compliance similar to native arteries. To date, most mechanical property assessment methods for arterial constructs require removal of the engineered tissues from culture (Dahl *et al.* 2007; Konig *et al.* 2009; Seliktar *et al.* 2000). Therefore, a large number of sample destructions and animal sacrifices are necessary to correlate mechanical property change with progressive cell growth. Due to variations in preparation and measurement conditions, current destructive methods also have large variations among samples, and require complicated efforts to correlate mechanical properties with culture time and conditions.

Toward this end, non-destructive imaging techniques to monitor the mechanical properties of cell-free scaffolds or cell-seeded constructs have been developed by various research groups. A few of them studied the mechanical responses of tubular scaffolds by measuring the change in outer diameter of the scaffold using optical instruments in response to varying luminal fluid pressure in a pulsatile circulatory system e.g. Inoguchi *et al.* (2006) used laser sensor, Webb *et al.* (2007) used laser micrometer, Syedain *et al.* (2011) used digital image capture. Others monitored the growth of biological tissue to indirectly infer the scaffold stiffness, also using optical methods. Levitz *et al.* (2010) showed non-destructive label-free monitoring of collagen gel remodeling by SMC using optical coherence tomography (OCT). Niklason *et al.* (2010) monitored collagen fiber deposition and orientation in SMC-seeded scaffolds cultured in a bioreactor using nonlinear optical microscopic imaging.

All of the above non-destructive techniques are optics-based. Until now, few studies have reported mechanical strength monitoring of tissue-engineered vascular constructs using ultrasound (US). US elasticity imaging (UEI) has been proved as a useful modality for imaging the stiffness of tissues and organs in pre-clinical and clinical studies (de Korte *et al.* 2011; Lubinski *et al.* 1999; McCormick *et al.* 2012; Ophir *et al.* 1999). Our previous studies have also monitored the elastic strain in abdominal scaffolds *in vivo* without cell seeding (Kim *et al.* 2008; Tripathy *et al.* 2010). In this study, UEI and inverse elastography are

applied to monitor the mechanical strength (Young's modulus) of cell-seeded arterial constructs in the course of cell development for three weeks.

Poly(glycerol sebacate) (PGS) is a tough, biodegradable elastomer and has good mechanical properties and biocompatibility to be applied to tissue engineering (Wang *et al.* 2002). We previously reported that PGS supports the proliferation and phenotypic protein expression of primary baboon arterial SMCs (Gao *et al.* 2007). Furthermore, we demonstrated that PGS scaffolds seeded with primary baboon SMCs co-expressed collagen and elastin (Gao *et al.* 2008) and produced a physiological compliance (Rice *et al.* 2009) and a significant mature elastin (Lee *et al.* 2011) in a pulsatile flow bioreactor culture. Recently, we designed a fast-degrading, cell-free PGS graft reinforced with electrospun polycaprolactone (PCL) fibers to enable rapid host remodeling and constructed neoarteries similar to native arteries from 3-month grafting in a rat abdominal aorta (Wu *et al.* 2012).

The objective of this study is to monitor the mechanical strength of tubular PGS arterial constructs during culture in a pulsatile flow bioreactor using non-invasive UEI. Tubular PGS scaffolds were fabricated and cultured with primary arterial fibroblasts and SMCs in the pulsatile flow bioreactor for 3 weeks. US radio frequency (RF) frames were captured at different time points and the distension due to pulsatile flow was estimated using a widely adopted two-dimensional phase-sensitive speckle tracking algorithm (2DST) (Lubinski *et al.* 1999). The stiffness of the construct was calculated from the distension estimated as above and the recorded pulse pressure using a parametric finite element (FE) based elastography inverse problem (Baldewsing *et al.* 2008; Barbone and Gokhale 2004; Brigham and Aquino 2007; Oberai *et al.* 2003; Richards *et al.* 2008). Mechanical strengths of harvested constructs were then measured using destructive testing and were compared with UEI results. Cell ingrowth and collagen deposition were examined by histology and immunofluorescence staining of the harvested constructs.

Materials and Methods

Fabrication of tubular scaffolds

Tubular PGS scaffolds were fabricated using a modified solvent casting/salt leaching method (Lee and Wang 2011). Ground salt particles with the size of 25-32 μm diameter were used as porogens. After PGS curing at 150°C for 24 h in vacuum oven, tubular scaffolds were released from glass molds by adding 1 mL of deionized water to their inner walls to dissolve hyaluronic acid. Released scaffolds were transferred to 15 mL tubes, frozen in a -80°C freezer for 30 min, and lyophilized for 24 h. The outer surface of tubular scaffolds were reinforced with PCL fibers by electrospinning a precursor solution of 14 w/v % PCL ($M_n = 80$ kDa, Sigma-Aldrich, St. Louis, MO) dissolved in trifluoroethanol (Fisher Scientific, Fair Lawn, NJ)/water (5:1) mixture around the outer surface of lyophilized scaffolds attached to rotating mandrel. Electrospinning conditions were: 10 kV (+) and 12 kV (-) applied voltage, 22 gauge needle size, distance between needle and aluminum collector = 20 cm, infusion rate = 25 $\mu\text{L}/\text{min}$, and mandrel rotational speed = 300 rpm. After electrospinning, scaffolds were dried in a vacuum chamber for 24 h, put into a series of two water baths (first bath for 24 h and second bath for 48 h) to leach out residual salts, and then lyophilized. The dimensions of final scaffolds are 30 mm long, 6 mm outer diameter, and 0.5 mm wall thickness. Scaffold structure was examined by using a field emission scanning electron microscope (6330F, JEOL, Tokyo, Japan). The final tubular scaffolds and their surface morphologies are shown in Fig.1.

Cell isolation and culture

Primary arterial fibroblasts and SMCs were isolated from common carotid arteries of 3-year-old male baboons (*Papio Anubis*) and characterized in 2D culture before passing and seeding (Gao *et al.* 2007). All protocols of animal tissue handling were approved by the Research Ethical Committee of the University of Pittsburgh. Fibroblasts and SMCs were expanded using MCDB 131 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Lonza, Walkersville, MD), 1% L-glutamine (Mediatech), 50 µg/mL ascorbic acid (Fisher Scientific), and 1% antibiotic-antimycotic solution (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B; Mediatech).

A pulsatile flow bioreactor was designed for co-culturing fibroblasts and SMCs on tubular scaffolds *in vitro* (Lee and Wang 2011). Each bioreactor chamber was made with polycarbonate square tubing (dimension = 19.05 mm × 19.05 mm, wall thickness = 1.6 mm) for compatibility with US scanning. Pulsatile flow was applied with a commercial pulsatile blood pump (Model 1405, Harvard Apparatus, Holliston, MA). To prepare cell seeding, scaffolds were attached to the luminal tubing of their bioreactor chambers using a Teflon thread sealant tape and sterilized by ethylene oxide gas for 24 h. They were rinsed subsequently with 70% ethanol and phosphate-buffered saline (PBS; Mediatech) for 4 h and pre-wetted with culture medium for 24 h at 2.0 mL/min in the flow loop. After detaching each chamber from the flow loop, fibroblasts (passage 4-6) were seeded into the lumen of scaffolds with a density of 1.5×10^6 cells/cm² and all chambers were horizontally rotated in a hybridization chamber at 37 °C for 4 h for uniform distribution. After rotation, each chamber was reattached to the flow loop and through-wall perfusion was resumed at 2.0 mL/min for 15 min followed by the luminal flow. The flow rate and pressure were increased gradually from 2.0 mL/min and 20 mmHg on day 1 to 8.0 mL/min and 80 mmHg on day 3 using the peristaltic pump. From day 4, mechanical conditioning was provided using the pulsatile blood pump with 1 mL of stroke volume, 1 Hz of stroke frequency, and 80-120 mmHg of pressure until day 6.

For co-culture, all chambers were detached from the flow loop on day 7 and SMCs (passage 4-6) were seeded into the lumen of scaffolds with a density of 2.0×10^6 cells/cm². All chambers were horizontally rotated in a hybridization chamber at 37 °C for 4 h. After through-wall perfusion for 15 min, the flow rate and pressure were maintained as 2.0 mL/min and 20 mmHg until day 10 and increased gradually to 8.0 mL/min and 80 mmHg on day 13 using the peristaltic pump. Medium was changed on day 14 and the same mechanical conditioning as fibroblast culture was applied with the pulsatile blood pump until day 21.

UEI via 2DST

In total, six PGS scaffolds were cultured with cells for three weeks. On each of the days 6, 13 and 21, two constructs were tested using US. After US measurements, those two constructs were harvested and sent for direct mechanical testing and histology. US testing were also done on two separate PGS scaffolds without any cells seeded for the day 0 time point. In total, five independent short-axis US scans were performed on each of the days 0, 6 and 13 and four independent scans were performed on day 21.

A 21 MHz US linear probe (MS 250, Visualsonics, Toronto, ON, Canada) connected to a high frequency US scanner (Vevo2100, Visualsonics) was used to collect IQ data while the construct distended due to the flow generated by the pulsatile pump. The RF data were later reconstructed from the stored IQ data using standard procedure codes provided by the manufacturer of the US scanner (Visualsonics). US gel was applied to the surface of an ultrasonically transparent polycarbonate bioreactor. The US probe was held by hand, mildly pressing against the rigid bioreactor tube for proper contact and stability during scanning.

Since the US data acquisition time was less than 1 s, any hand motion artifact was neglected. Three independent scans were done on three different regions of each construct – one each near the top, middle and bottom section of the construct. For each section, the RF frames were acquired at a frame-rate of 20 Hz while the pump pulsed at 1 Hz. The pressure measured at upstream of the constructs using a pressure transducer (PM-4, Living Systems Instrumentation, St. Albans, VT) was synchronized with the US data acquisition. A schematic diagram of experimental setup and US data acquisition is shown in Fig.2.

The constructs were scanned in the transverse direction relative to the construct axis. 2DST was applied to the RF frames to obtain the frame-to-frame distensions, which were later accumulated over the desired frames of interest. The compliance (S) of the construct, which is a measure of the elastic deformation of the construct in response to luminal pressure, was calculated according to the ANSI guidelines (De Valence *et al.* 2011):

$$S [\%/100mmHg] = \frac{u_r(a)/a}{\Delta p [mmHg]} \times 10^4 \quad (1)$$

where a is the inner radius of the construct in the first US RF frame, $u_r(a)$ is the outward radial displacement of a point located on the inner boundary of the construct and is tracked by 2DST, and p is the pressure difference between the frames of interest in mmHg. Eq. (1) assumes that the geometry of the construct is perfectly annular and all the deformations are infinitesimal. These limitations are addressed in the following elastography inverse problem section. Compliance (S) in Eq. (1) can be converted to Young's modulus using the method described below.

The radial displacement (u_r) of a point in the construct that is a distance r away from the center is given by (Timoshenko 1970; Kim *et al.* 2004):

$$u_r(r) = -\frac{A}{r} + Br \quad (2)$$

where A and B are constants related to the geometry, material characteristics, and boundary conditions. In the derivation of Eq. (2), the construct is assumed to be homogeneous, isotropic, linear elastic (with Young's modulus, E and Poisson's ratio ν) and with an annular cross-section. A and B are given by:

$$\begin{aligned} A &= \frac{-\Delta p(1+\nu)}{E\left(\frac{1}{a^2} - \frac{1}{b^2}\right)} \\ B &= \frac{-\Delta pa^2(1+\nu)(1-2\nu)}{E(a^2-b^2)} \end{aligned} \quad (3)$$

where b is the outer radius of the construct. Now, substituting Eq. (2) in Eq. (1), we have:

$$S = \frac{\left(-\frac{A}{a^2} + B\right)}{\Delta p} \quad (4)$$

Using Eq. (3) and (4), the Young's modulus (E) can be obtained from the compliance (S) as:

$$E = \frac{(1+\nu)(b^2+a^2)(1-2\nu)}{S(b^2-a^2)} \quad (5)$$

Using $a = 2.5$ mm, $b = 3$ mm (from dimensions of the empty scaffold) and $\nu = 0.3$ (Poisson's ratio of the empty scaffold) the following equation gives the transfer function between E and S :

$$E [kPa] = \frac{5.44 \times 133 \times 10^4}{S [\% / 100mmHg] \times 10^3} \quad (6)$$

Elastography inverse problem formulation

To address the limitations related to geometrical imperfection and finite deformation, an elastography inverse problem was formulated, which when solved gave the Young's modulus distribution in the construct for which a quantitative estimate of the displacement field provided by a mechanical model of the system (in our case a quasi-static FE model) most accurately matches the measured displacement field tracked by US 2DST. The formulation of the elastography inverse problem in this paper is based on the previous work by Richards *et al.* (2008), which was adopted from more sophisticated approaches (Baldewsing *et al.*, 2008; Barbone and Gokhale 2004; Brigham and Aquino 2007; Oberai *et al.* 2003). The inverse problem was solved only for an exemplary data point (day 21 construct).

US B-scan image of a construct section on day 21 was obtained from the RF data *via* standard envelope computation algorithm using Hilbert transform. The construct boundaries were delineated using Sobel edge detection filter on the B-scan image (Gonzalez *et al.* 2003). This 2D section geometry of the construct is then imported into the ABAQUS commercial finite element analysis platform (Dassault Systèmes, Waltham, MA). A parametric quasi-static 2D plane strain FE model of the construct was created with its material property (Young's modulus, E) as an unknown parameter that was initially considered constant with respect to cross-sectional position (i.e., homogeneous). The Poisson's ratio was assumed to be 0.3 throughout the entire cross-section. The displacement boundary conditions was prescribed such that the displacement at the inner and outer boundaries of the construct matches the measured displacement by US 2DST. The deformation of the construct under experimental pressure conditions was simulated using ABAQUS for different values of the parameter E (Fig. 3 (a)). A genetic algorithm (GA) based global optimizer is used to find the optimal value of E for which the deformation predicted by FE simulation matches most closely to the experimentally observed construct distension obtained *via* US 2DST (Brigham and Aquino 2007). The objective function (f) for the optimization problem is defined as:

$$f(E) = \sum_{i=1}^N (u_{FE}(E) - u_{obs})^2 + R(E) \quad (7)$$

where u_{FE} is the FE displacement at the i^{th} point in the construct, u_{obs} is the experimentally observed (*via* US 2DST) displacement at that point, and N is the total number of reference points against which the optimal solution is sought. R is a regularization term that penalizes un-physically large spatial variations of E . Note that the regularization term is applicable only when a spatially varying solution of E is sought. Otherwise, $R(E)$ is set to zero. To compute f in Eq. (7), we picked three points that were observed to have relatively unique and sensitive behavior from the construct displacement field (i.e. $N = 3$ in Eq. (7)) (Brigham and Aquino 2007).

Once an optimal solution for E was obtained from the inverse solution strategy, it was compared with the Young's modulus estimated using the method described in the previous section (i.e. using Eq. (6)). The FE displacement field was also plotted for this optimal Young's modulus estimate to compare with the experimentally observed displacement field.

To address the issue of non-uniform cell growth in the construct, another inverse problem similar to the first one is now formulated in which the construct geometry was segmented into two roughly equal by area regions – inner (closer to the lumen) and outer (away from the lumen) – and the parametric FE model now had two different Young's moduli parameters (Fig. 3 (b)). GA based optimization algorithm was applied again to find optimal solutions for the three Young's moduli (E_i , $i = 1, 2$). Three reference points from each region i.e. a total of six reference points were considered against which the objective function was minimized (i.e. $N = 6$ in Eq. (7)).

Direct mechanical measurements

After US measurements were taken on each of the days 6, 13, and 21, the construct was harvested and cut into pieces – each piece contained the section from which US measurements were taken. Each piece was again cut into two – one was used for direct mechanical testing, the other was used for histology. To ensure consistency, we marked the positions of bioreactor chambers containing the scaffolds and performed the pressure-diameter tests around the same positions where US scans were done.

Compliance of construct segments was directly measured by pressure-diameter (P-D) testing. The segments (1.5 cm long) were strained longitudinally to 10% and pressurized by infusing with PBS using a syringe pump (NE-1000, New Era Pump Systems, Farmingdale, NY). Constructs were pre-conditioned between 0 and 12.5 mmHg until pressure vs. diameter curves were reproducible. Compliance was calculated using the last cycle. Pressure and diameter data were measured using a pressure transducer (PX309 Omegadyne, Sunbury, OH) and optical laser micrometer (LS7070, Keyence, Itasca, IL) respectively, and were synchronously recorded with a data acquisition system (PowerLab 8/30, ADInstruments, Colorado Springs, CO). Compliance and Young's modulus were calculated from the pressure-diameter curves according to Eq. (1)-(6).

Histology and immunofluorescence staining

Construct segments harvested on days 6, 13, and 21 were cut into 5 mm long ring segments, embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek Inc., Torrance, CA) and snap-frozen in liquid nitrogen. After cryosectioning, 8 μ m thick cross-sections were stained with hematoxylin and eosin (H&E) and picosirus red.

The presence of cells and collagen was examined by immunofluorescence staining. Cryosections (8- μ m thick) of constructs were dried, fixed in cold acetone for 15 min, air-dried, and rinsed twice with PBS. Tissue sites of each slide were incubated with 5 % normal goat serum (Sigma) for 1 h at 37 °C, incubated with primary antibodies in 1 % goat serum for 45 min at 37 °C, rinsed twice with PBS, incubated with secondary antibodies in 1 % goat serum for 45 min at 37 °C, and rinsed twice with PBS and once with dH₂O. Fibroblast staining used mouse monoclonal anti-vimentin (1:100, Abcam, Cambridge, MA) primary antibody and Alexa Fluor 594 goat anti-mouse IgG (1:200, Invitrogen, Carlsbad, CA) secondary antibody. SMC staining used FITC-conjugated mouse monoclonal anti- α -smooth muscle actin (SMA) (1:500, Sigma) primary antibody. Collagen staining used rabbit polyclonal anti-collagen I (1:40, Millipore, Billerica, MA) and anti-collagen III (1:100, Abcam) primary antibodies and Alexa Fluor 488 goat anti-rabbit IgG (1:200, Invitrogen) secondary antibody. All primary antibodies used had a cross reactivity with monkey, so there were no problems for using them to characterize baboon vascular fibroblasts and SMCs. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were captured with an inverted microscope (Eclipse Ti-E, Nikon Instruments Inc., Melville, NY).

Statistical analysis

All experimental data were reported as mean $\pm$ standard deviation for $n = 3$ or greater. One way analysis of variance followed by Tukey's HSD *post-hoc* test was performed to assess statistically significant differences ($p < 0.05$) among groups using KaleidaGraph software (Synergy Software, Reading, PA).

Results

US B-scan and displacement field estimated by 2DST

The US B-scan images at days 0, 6, 13 and 21 are shown in Fig. 4 (a). The axial displacement (i.e. displacement in the direction of US propagation) fields due to the pulsatile flow were estimated from 2DST and normalized with respect to the corresponding pressure differential before being plotted in Fig. 4 (b). All the displacements were computed at the same phase of the pulse period *viz.* total distension between trough and peak pressure. All the scans were done within similar pressure range of around 60-100 mmHg. For the sake of completeness, and to see the effect of slight differences in pressure between experiments, the distension of each scaffold for a whole pulsated period is shown in the Appendix.

Construct stiffness with cell growth

Young's moduli increased with culture, demonstrating that the constructs became stiffer as they matured (Fig. 5 (a)). No statistically significant differences existed among day 0, 6, and 13. However, Young's moduli at day 21 were significantly different with those at day 0 (with p-value of 1.42×10^{-5} calculated using a sample size of 5 for day 0 and 4 for day 21). Stiffness obtained from non-invasive UEI was similar to that from P-D testing at each time point (Fig. 5 (b)) and both results demonstrated a significant linear correlation ($R^2 = 0.9015$, sample size = 10, null hypothesis of no correlation rejected with a p-value of 0.001) (Fig. 5 (c)).

Elastography inverse problem solution

The optimal Young's modulus found by the GA based single parameter (E) inverse elastography was 173.7 kPa (Fig. 6 (a)). Note that the Young's modulus of the same construct section estimated by Eq. (6) was 184.2 kPa (ref. Fig. 5 (a)). The result of the two-parameter (E_i , $i = 1, 2$) inverse problem is shown in Fig. 6 (b). The optimal Young's moduli for the inner and outer segments were found to be 193.1 kPa and 125.5 kPa, respectively.

The displacement field of the day 21 construct obtained *via* FE simulation using $E = 173.7$ kPa is plotted (normalized with respect to pressure) in Fig. 6 (c). The value of the objective function (Eq. (7)) for this optimal solution was 0.43×10^{-4} . The FE displacement field of the construct using $E_1 = 193.1$ kPa and $E_2 = 125.5$ kPa is plotted in Fig. 6 (d) and the corresponding value of the objective function was 0.40×10^{-4} .

Histology and immunofluorescence staining

Histological analysis shows that cells are present throughout the wall of the construct, with the majority of cells and ECM concentrated on the luminal surface. More collagen fibrils were observed in the luminal surface of the construct with increasing culture time (Fig. 7 (a)). Immunofluorescence staining demonstrates that fibroblasts (vimentin), SMCs (SMA), collagen I and III are roughly equally distributed in the tissue on the luminal surface of the construct by day 21 (Fig. 7 (b)).

Discussion

The results demonstrate the feasibility of using UEI to monitor the mechanical strength of tissue engineered arterial constructs non-invasively. As tissue matures with time, there was a marked decrease in the distension of the construct per unit rise in luminal pressure (Fig. 4 (b)). This decreased axial displacement provides the qualitative evidence that the construct stiffened with cell growth, and the rate of stiffening could be observed in Fig. 5 (b). According to Fig. 5 (b), the stiffness increased between day 0 and day 6, and then between 13 and 21. However, there was hardly any change in the overall stiffness of the construct between days 6 and 13. There was a high variance between the stiffness of different sections on days 6 and 13 compared to days 0 and 21. We surmise that the tissue forms non-uniformly across the construct during the maturing period, but given enough time (21 days), uniformity sets in.

Significant linear correlation between non-invasive UEI estimate of construct stiffness and direct mechanical measurement of construct stiffness were observed in Fig. 5 (c). This is the key finding of the study and suggests that non-invasive UEI can be a feasible alternative to invasive testing for monitoring the mechanical properties of tissue engineered constructs during cell growth. A few other studies used ultrasound as a non-invasive method to evaluate tissue formation and identify a correlation between ultrasound results and mechanical and biochemical properties of neotissues in cell-seeded constructs. Rice *et al.* reported a real-time online assessment of cartilaginous matrix evolution in chondrocyte-encapsulated poly(ethylene glycol) hydrogels using ultrasound (Rice *et al.* 2009). Their results demonstrated an increased trend of sound (SoS) and slope of attenuation (SoA) from the high frequency transducer corresponding to *in vitro* culture time. However, no quantified, significant correlation between the parameters, compressive moduli and total collagen was reported. Recently, Kreitz *et al.* proposed non-destructive method to evaluate the collagen content of 2D fibrin-gels encapsulated with myofibroblasts *via* ultrasound (Kreitz *et al.* 2011). They calculated the mean gray-scale value of an area corresponding to the central third of each gel and demonstrated gradually increased gray-scale values with culture time up to 18 days and a strong correlation with hydroxyproline contents. However, we could not find any direct non-invasive assessment of mechanical properties in 3D cell-seeded constructs cultured in dynamic conditions. Taken together, our results provide a new non-invasive approach to monitor mechanical properties and tissue development over time in 3D engineered constructs under dynamic culture conditions.

In the derivation of Young's modulus in Eq. (6), the Poisson's ratio was assumed to be same throughout the 21 days of culture ($\nu = 0.3$) although it is expected that the Poisson's ratio changes during the course of cell growth. However, it may be noted that the factor in the numerator only changes between a minimum of 4.90 and a maximum of 5.60 for all admissible values of ν (i.e. between $\nu = 0$ and $\nu = 0.5$), and we admit this much of maximum uncertainty in the Young's modulus estimate (the factor we used in Eq. (6) was 5.44).

A simplifying assumption for the derivations of Eq. (1)-(6) was that the geometries of the constructs were annular and all the deformations were infinitesimal. The constructs, however, were not perfectly annular, and the distension of 10-20% of the original inner diameter should be considered finite deformation. Therefore, the Young's modulus estimated using Eq. (6) was probably not the best representative value for the construct's stiffness. A more realistic estimate of the Young's modulus was possibly obtained through FE modeling and elastography inverse problem – a technique that is not affected by imperfect geometry or large deformations.

The Young's modulus of the day 21 construct section obtained through single parameter inverse elastography was smaller than the Young's modulus obtained through Eq. (6) (173.7 kPa vs. 184.2 kPa). It is well known that in large deformation theory (>10%, as is the case in this study), nonlinear geometric factors cannot be ignored and the simplified stiffness obtained using Eq. (6) is an overestimate (see for e.g. Gurtin 1981). The two-parameter inverse problem resulted in a slightly closer match to the experimentally observed displacement field than the single parameter inverse problem in the sense that a lower value of the objective function was attainable by GA. If we discount the sources of error arising from GA optimization, the above observation suggests that there is indeed a variation of stiffness across the construct cross-section. This two-layered stiffness distributions shown in Fig. 6 (b) was in agreement with results of histological analyses and immunofluorescence staining (Fig. 7), which demonstrated more cells and collagens at the luminal surface of the constructs. Note that it is the two-parameter model that justifies the use of GA; a line search would have been sufficiently economical for the single parameter model.

Since US 2DST provides the entire displacement field in the tissue, N can be relatively large, where it is only limited by the US resolution that determines the number of pixels inside the construct, or by the mesh size in the FE model. However, excessive reference points, particularly when many are relatively insensitive to changes in the material properties, may lead to a higher degree of ill-posedness and the solution of the inverse problem could become more non-unique and/or unstable. Hence, only three points those were observed to have relatively unique and sensitive behavior from the construct displacement field were considered.

We used a 2D plane strain model to represent the construct distension problem in FE. We verified that the problem can be regarded as plane strain near the middle section of the construct, not only from the observation that the length of the construct (30 mm) is about five times the external diameter (6 mm), but also by solving a 3D FE simulation (not reported in this paper) and comparing the solution with the result from 2D plane strain simulation and ensuring that they are similar near the mid-section of the construct which is where the experimental data were collected. The 2D modeling allowed computational economy which is especially significant during the inverse problem solution where the forward problem has to be solved many times in an iterative manner.

Although the focus of this paper is on *in vitro* cell culture, the eventual goal is to translate this technique to *in vivo* applications. The resolution of the optics-based methods ($\sim 1\ \mu\text{m}$ for OCT) are superior to that of UEI ($\sim 100\ \mu\text{m}$), however, US has higher penetration and can image the entire vessel wall as opposed to only surface or shallow sub-surface images provided by optics-based techniques. Due to higher penetration power of US, UEI also has a high potential for non-invasive *in vivo* mechanical strength monitoring of engineered grafts in the future. Compared to other potential modalities for mechanical property monitoring such as magnetic resonance elastography (MRE), US is non-ionizing as well as relatively inexpensive. For monitoring engineered constructs in humans, this non-invasive UEI technique may be relatively easily integrated to commercial US transducers and translated into clinics. To test the feasibility of *in vivo* arterial strength monitoring using our ultrasound system, we applied UEI to native (i.e. non-grafted) rat abdominal aorta and measured Young's modulus, under the animal protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The Young's modulus of the native artery was found to be 264 kPa (using Eq. (1)-(6)), which is in the range of stiffness of abdominal aorta previously reported in the literature (Duck 1990). Detailed methods and the preliminary results are presented in the Appendix.

The elasticity inverse technique presented in this paper has some limitations for *in vivo* application. These include the inherent challenges of *in-vivo* application of elastography in blood vessels which includes the lack of knowledge of the exact blood pressure at the scan section and imperfect knowledge of the boundary conditions *in vivo* (Reneman et al. 2005). One approach to overcome the above limitations would be to compare the stiffness/compliance of the engineered arterial constructs with nearby native artery that experiences similar blood pressure levels and boundary conditions. Also, devices for pressure measurements, e.g. either invasive catheter-based pressure meter or non-invasive tonometry, can be adopted to get an absolute value of pulse pressure.

Since the technique presented in this paper relies on measuring tissue displacement accurately, a note about any effect of hand motion during US scanning in the generation of displacement artifacts is not out of place here. From actual experiments on static phantoms it was observed that the maximum displacement in the US image due to hand motion within 1 s was 30 μm . The effect of such small displacement artifact was neglected since the distension of the constructs reported in this paper is around 0.5 mm (Refer to Figure 4. Note that the images show pressure normalized distension. Since the pressure difference is about 40 mmHg, the actual distension is around $40 \times 12 \mu\text{m}$ i.e. 480 μm).

Conclusions

We demonstrated the feasibility of UEI based non-invasive mechanical strength monitoring of arterial constructs through *in vitro* experiments. Stiffness increase with the growth of fibroblasts and SMC was observed by UEI and the UEI findings matched with direct mechanical measurements and histology. The role of inverse problem formulation to capture local elasticity information was also demonstrated. UEI combined with inverse elastography may serve as a non-invasive tool for quantitative assessments of the mechanical property changes of arterial constructs in *in vivo* animal study, thereby reducing the number of animals significantly and providing accurate information with less variance between subjects. Experiments assessing the performance of the above techniques in *in vivo* animals are underway in order to address some of the potential limitations of UEI to blood vessel applications. In addition, we are hopeful that the current paper will inspire other candidate techniques for non-invasive compliance estimation such as pulse wave velocity, ultrasound shear wave imaging, etc. to be considered for engineered artery applications.

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Appendix

Scaffold distension during a whole pulsated period

While all the scans were done within a pressure range 60-100 mmHg, there were some variations in the pressure from experiment to experiment. Since the material properties of soft tissue depend on the pressure induced stress, it was deemed useful to plot the distension of an entire pulsated period for each scan. It was observed that a stiffer construct after 21 days showed consistently smaller distension during the entire pulsated period compared to the constructs after 0, 6 and 13 days (Fig. A1). The plotted displacement values in Fig. A1 are normalized by the pressure difference from trough to peak which was roughly 40 mmHg in each case.

UEI of a rat abdominal aorta

Under the animal protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee, UEI of abdominal aorta of rats were performed non-invasively using a 21 MHz US linear probe (MS 250, Vevo 2100, Visualsonics) (Fig. A2 (a)). Aortic pressure was measured by inserting a pressure manometer into the aortic arch. The pressure normalized axial displacement field is overlaid on the B-scan image in Fig. A2 (b).

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Figure 1. Tubular scaffolds and their surface morphologies. Final tubular poly(glycerol sebacate) (PGS) scaffolds reinforced the outer surface with electrospun polycaprolactone (PCL) fibers (top, left). Scanning electron micrographs of the scaffold. Top, right: cross-section, bottom: luminal (left) and outer surfaces (right). Scale bar = 1 mm (top), 10 μ m (bottom).

Figure 2. Experimental setup for ultrasound (US) radiofrequency data acquisition in the presence of flow generated by a pulsatile pump. Inset: scanning of the scaffold with handheld US imaging transducer. During imaging, US imaging transducer was held by hand, applying mild pressure for contact against the bioreactor chamber containing the scaffold inside the incubator.

Figure 3.

Finite element (FE) modeling of scaffold. (a) The scaffold boundaries were obtained from the B-scan image via automatic edge detection and the geometry was imported to ABAQUS where other attributes of the model were defined. The stiffness (E) was the unknown parameter for which the inverse problem was solved. (b) For the two segment inverse problem, the scaffold geometry was manually segmented into two parts. A two parameter FE model was then created with E_1 and E_2 as the unknown parameters. The boundary conditions remained the same as the single parameter FE model.

Figure 4.

Ultrasound image of each scaffold at different time points. (a) B-scan images of the mid-sections of the scaffolds at different points in time (6.5 mm vertical $\times$ 8 mm horizontal). (b) Axial displacement maps (normalized with respect to the luminal pressure) obtained using two-dimensional phase-sensitive speckle tracking (2DST) algorithm. The displacement maps are superimposed on B-scan images. The normalized axial displacement is a measure of the scaffold compliance.

Figure 5.

Change in the construct stiffness with cell growth. (a) Young's moduli (E) of the constructs computed from ultrasound (US) two-dimensional phase-sensitive speckle tracking (2DST) displacement using Eq. (1)-(6). Each point and bar represent the stiffness of scaffold obtained from independently obtained US scans and its mean value, respectively. * represents $p < 0.05$. (b) Comparison of Young's moduli of the constructs measured from non-invasive UEI and direct mechanical testing. There were no day 0 data from pressure-diameter (P-D) testing because porous scaffolds were not watertight. (c) Scatter plots of Young's moduli measured via non-invasive ultrasound elasticity imaging (UEI) and direct P-D testing on excised samples. Dotted lines represent the linear correlation between two measurements.

Figure 6.

Elastography inverse problem solutions of the construct on day 21. (a) Scaffold stiffness found from single layer inverse problem ($E = 173.7$ kPa). (b) Scaffold stiffness found from two layer inverse problem (inner layer: $E_1 = 193.1$ kPa, outer layer: $E_2 = 125.5$ kPa). (c) and (d) FE simulation of the construct's distension under experimentally recorded luminal pressure. The Young's moduli used were the solutions of the corresponding inverse problems.

Figure 7.

Presence and distribution of cells and collagens in the constructs. (a) Hematoxylin and eosin (H&E) (top row) and picosirus red (bottom row) staining. Scale bar = 500 μ m (full image), 100 μ m (partial image). (b) Immunofluorescence staining for fibroblasts (vimentin), smooth muscle cells (SMCs) (α -smooth muscle actin (SMA)), and collagen I and III. Scale bar = 100 μ m. * represent lumen of the constructs.

Figure A1.
Displacement fields of the scaffolds on each scanning day for a whole pulsated period (trough to peak pressure from left to right).

Figure A2.

Ultrasound Elasticity Imaging (UEI) of in vivo small animal experiment. (a) Non-invasive trans-cutaneous in vivo ultrasound imaging of abdominal aorta in a rat. Only the chest of the rat was cut open to obtain pressure data invasively from the aorta. (b) Pressure normalized axial displacement fields obtained using non-invasive ultrasound two-dimensional speckle tracking (2DST) superimposed on short-axis B-scan images of rat abdominal aorta.