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Nondestructive mechanical characterization of developing biological tissues using inflation testing

P.J.A. Oomen\textsuperscript{a,b,*}, M.A.J. van Kelle\textsuperscript{a,b}, C.W.J. Oomens\textsuperscript{a}, C.V.C. Bouten\textsuperscript{a,b}, S. Loerakker\textsuperscript{a,b}

\textsuperscript{a}Department of Biomedical Engineering, Eindhoven University of Technology, 5600MB
Eindhoven, Netherlands
\textsuperscript{b}Institute for Complex Molecular Systems, Eindhoven University of Technology, 5600MB
Eindhoven, Netherlands

Abstract

One of the hallmarks of biological soft tissues is their capacity to grow and remodel in response to changes in their environment. Although it is well-accepted that these processes occur at least partly to maintain a mechanical homeostasis, it remains unclear which mechanical constituent(s) determine(s) mechanical homeostasis. In the current study a nondestructive mechanical test and a two-step inverse analysis method were developed and validated to nondestructively estimate the mechanical properties of biological tissue during tissue culture. Nondestructive mechanical testing was achieved by performing an inflation test on tissues that were cultured inside a bioreactor, while the tissue displacement and thickness were nondestructively measured using ultrasound. The material parameters were estimated by an inverse finite element scheme, which was preceded by an analytical estimation step to rapidly obtain an initial estimate that already approximated the final solution. The efficiency and accuracy of the two-step inverse method was demonstrated on virtual experiments of several material types with known parameters. PDMS samples were used to demonstrate the method’s feasibility, where it was shown that the proposed method yielded similar results to tensile testing. Finally, the method was applied to estimate the material properties of tissue-engineered constructs. Via this method, the evolution of mechanical properties during tissue growth and remodeling can now be monitored in a well-controlled system. The outcomes can be used to determine various mechanical constituents and to assess their contribution to mechanical homeostasis.

Keywords: soft tissues, growth and remodeling, mechanical characterization, inverse analysis, ultrasound

*Corresponding Author: P.J.A. Oomen, Department of Biomedical Engineering, Eindhoven University of Technology, P.O. Box 513, 5600MB Eindhoven, Netherlands; E-mail, P.J.A.Oomen@tue.nl; Phone, +31402475415

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1. Introduction

Biological tissues have an intriguing capacity to adapt in response to changes in their mechanical environment through growth and remodelling. While the underlying mechanisms that govern tissue growth and remodelling are not fully understood, it is well accepted that they occur at least partly to maintain a certain mechanical homeostasis [1, 2, 3]. An improved knowledge of this process is of key importance to understand the functioning of human tissues, during both health and disease. To date, it remains unknown what mechanical quantity determines tissue homeostasis; in fact, different measures have been associated to play important roles in this process, such as stress [4, 5], strain [6, 7], strain rate [6, 8] and strain energy [8]. Moreover, mechanical homeostasis is not necessarily determined by the same mechanical parameters across different tissue types.

Identification of the main determinant(s) of mechanical homeostasis is far from straightforward, as the mechanical constituents relate through constitutive behavior, making it challenging to study their independent effects. To this end, the mechanical properties of a tissue need to be followed during its development, for which a nondestructive mechanical test is required. As the commonly used mechanical testing methods, such as tensile and indentation tests, typically require sacrificing the test specimen, we developed a bioreactor that allows for culturing planar soft tissues for a prolonged time, while being subject to dynamical loading to induce growth and remodelling [9]. This bioreactor is designed to perform mechanical testing during culture by means of a classical inflation test. Ultrasound (US) imaging to track the tissue displacement allowed for nondestructive mechanical testing, and therefore assessment of the temporal changes in the tissue’s mechanical state and mechanical properties due to growth and remodelling.

Previously, inflation, or bulge, tests have been developed to measure the properties of isotropic thin films that undergo small deformations. Due to these conditions, bending effects can be neglected and an in-plane stress situation can be assumed. The stresses and strains in these tests can be calculated from a set of well-prescribed equations, using the pressure and out-of-plane sample deflection [10, 11]. Furthermore, analytical solutions have been developed that relate the pressure to the apex displacement by assuming small displacements, in order to estimate linear mechanical properties in terms of the Young’s modulus and Poisson’s ratio [12].

However, when characterizing biological soft materials using bulge testing, these analytical solutions no longer yield an accurate representation of the mechanical state. This is caused by the intrinsic properties and loading conditions of these tissues. First, they undergo large displacements and typically feature nonlinear material behavior, as a result of which the analytical solution using linear material theory no longer applies. Second, many planar biological tissues, such as skin and arteries, feature an anisotropic fiber structure, resulting in an anisotropic displacement field. Third, passive residual stretches and active cell tractions are typically present in soft tissues [13, 14, 15, 16]. For this reason the unloaded, pressure-free, state cannot be assumed to be stress-free. Neglecting
these residual stresses can lead to an underestimation of material strains, and consequently to an overestimation of the tissue stiffness [17]. Finally, biological planar tissues are not always thin enough to be considered as membranes, leading to bending moments and out-of-plane stresses. Several studies have successfully incorporated tissue nonlinearity and anisotropy in parameter estimation schemes of bulge tests [18, 19, 20, 21, 22, 23]. Yet, no study has, to the best of the authors' knowledge, addressed the effects of tissue thickness, nor has tissue prestretch been accounted for. Moreover, the properties of biological tissues have never been estimated during growth and remodeling.

The goal of the current study was to develop and validate a nondestructive mechanical test and a two-step inverse analysis method to estimate the mechanical properties of biological tissue during in vitro tissue culture. Nondestructive mechanical testing of developing tissues was realized by performing bulge tests inside a novel bioreactor [9] while tracking tissue displacement with US imaging. The mechanical properties were estimated using a full inverse finite element (FE) method, which accounts for tissue geometry and architecture, and implementation of prestretch. To increase robustness and minimize the high computational costs that this method entails, its initial estimate was provided by an analytical estimate. The inverse method was verified by performing virtual experiments using different material models, tissue thicknesses and prestretch magnitudes. Next, the proposed experimental and numerical methods were validated by performing both bulge and tensile tests on polymer samples, and comparing their results. Finally, the method was applied to nondestructively estimate the mechanical properties of living, tissue-engineered constructs.

2. Methods

2.1. Sample preparation

To validate the proposed nondestructive mechanical characterization method, both bulge and tensile tests were performed on polydimethylsiloxane (PDMS: Sylgard 184, Dow-Corning, Midland, MI, USA, using a weight ratio of 10:1 prepolymer to curing agent) samples (n=10) with a thickness of 0.38 ± 0.03 mm. The samples for bulge tests (n=5) were cut in a circular shape with a diameter of 18 mm to fit inside the bioreactor. Tensile test samples (n=5) were cut in rectangles (15.0 × 8.0 mm) and sprayed with graphite to enhance image contrast for digital image correlation.

To demonstrate the method’s feasibility to nondestructively characterize developing tissues, bulge tests were performed on tissue-engineered constructs, previously used in a different study. In brief, human vascular derived cells from the vena saphena magna, previously characterized as contractile, matrix-producing myofibroblasts, were seeded on rapidly degrading nonwoven polyg-lycolic acid (PGA) scaffolds (Biomedical Structures, Warwick, USA, 0.5 mm thickness) [24, 25]. The outer edge was reinforced with a ring of polycaprolactone scaffold (PCL, thickness 0.25 mm, inner diameter 13 mm) to prevent sample rupture at the clamped edge. These constructs were cultured inside the
bioreactor system in supplemented standard culture medium at 37° and 5% CO₂ for a duration of either 3 (n=4) or 4 (n=3) weeks. Additionally, n=6 samples were statically cultured for three weeks to validate the thickness measurements.

2.2. Mechanical testing

2.2.1. Bulge tests

Mechanical bulge tests were performed inside the novel bioreactor system that allows to culture tissues during growth and remodeling [9]. The bioreactor was made of polysulphone and consists, for the sake of sterility during culturing, of two chambers (Figure 1): a tissue chamber and a pressure chamber. In the former, samples can be clamped and cultured, surrounded by culture medium, while the latter was connected to a previously developed dynamic pumping system [26]. During the bulge tests, this pump was replaced by a Harvard pump (PHD2000, Harvard Apparatus, Holliston, MA, USA) to gradually apply a pressure by increasing the fluid volume in the pressure chamber. The two chambers were connected by two silicone membranes to ensure pressure transmission between the pressure and the culture chamber, where the pressure in the culture chamber was measured by a pressure sensor (P10EZ-1, BDSENSORS, Thierstein, Germany). To mechanically test the samples, the volume in the pressure chamber was increased until a pressure of approximately 2 kPa was applied for five cycles, of which the first four were used for pre-conditioning.

To nondestructively measure tissue displacement as a function of pressure, the sample was imaged using a MyLab 70 US system (Esaote, Maastricht, Netherlands), equipped with a 8 or 12 MHz linear transducer. The US transducer was mounted on top of the bioreactor, perpendicular to the sample surface and aligned to coincide with the sample center (Figure 1). In the current study only isotropic samples were tested, yet for anisotropic samples two measurements would be required: one with the US transducer aligned along the main fiber direction $\mathbf{v}_1$, and a second with the US transducer aligned perpendicularly to the main fiber direction, defined as $\mathbf{v}_2$ (Figure 2C).

From the B-mode frames of the US video (frame rate 20 Hz, resolution 21.5 px/mm), the sample profile was tracked using a custom MATLAB (Mathworks, Natick, MA, USA) script (Figure 2A,B). For each frame, the tissue edges were detected using Matlab’s built-in edge detection function, followed by a morphological closing operation. To obtain an analytical representation of the central area of the tissue profile, a circle with radius $R$ was fitted through the central 50% of this edge region (Figure 2B). From this circle, the radius of curvature $\kappa (= 1/R)$ and current sample length $L$ were calculated, followed by the in-plane sample stretch due to bulging $\lambda = L/L_0$, where $L_0$ is the initial tissue length at the pressure-free state. The initial constrained thickness $t_0$ of six additional samples was measured from the raw radio-frequent signals of an ultrasound measurement, as described previously [27], and compared to microscopy measurements of the sample cross-section. The thickness was measured on approximately 15 locations along the tissue cross-section in both measurement techniques.
2.2.2. Tensile tests

To validate the bulge test methodology and analysis, uniaxial tensile tests were performed on a BioTester (CellScale, Waterloo, Canada). The isotropic PDMS samples were stretched to 120% of their original length with a strain rate of 100%/min for five cycles, of which the first four were used for preconditioning. Force data were collected with a sampling frequency of 5 Hz and images were taken with the same frequency by a CDD camera mounted perpendicular to the sample surface. From these images, sample displacements were tracked using a global digital image correlation (GDIC) algorithm, as described previously [28, 29]. From these displacements, the deformation gradient tensor $F$ was computed, from which the right Cauchy-Green deformation tensor $C = F^T F$ was obtained. From this tensor, the two in-plane principal stretches $\lambda_i = \sqrt{C : (e_i \otimes e_i)}$ ($i = 1, 2$) were obtained, where the unit direction vector $e_1$ coincides with the tensile direction, and $e_2$ with the perpendicular in-plane direction. Assuming incompressibility, the thickness $t$ and width $w$ of the sample during stretching could be estimated as a function of the initial thickness $t_0$ and width $w_0$ and the in-plane stretches:

$$t = \frac{t_0}{\lambda_1 \lambda_2}$$  
$$w = w_0 \lambda_2$$  

Finally, the current thickness and width were used to compute the Cauchy stress $\sigma$ from the measured force $F$:

$$\sigma = \frac{F}{t w}$$  

2.3. Tissue prestretch

When performing a bulge test on living soft tissues, the total elastic stretch of the tissue generally does not equal the stretch from the deformation due to mechanical loading. Instead, it is the product of this stretch and both passive residual stretches and active cell tractions that were already present in the unloaded configuration, causing tissue prestretch [13, 14, 15, 16]. Therefore, a multiplicative decomposition was employed, thereby splitting the total elastic deformation $F_e$ into a deformation part due to loading $F$ and a deformation induced by tissue prestretch $F_p$ [17]:

$$F_e = FF_p$$  

In the tensile tests, no prestretch was measured ($F_p = I$) since the samples were released before mounted in the tester. However, the samples in the bioreactor used for bulge testing could feature prestretch. This was measured after the bulge test: a 3x3 rectangular grid of ink markers were placed on the tissue while constrained and imaged using a high-resolution digital microscope (VHX-500, Keyence, Itasca, IL, USA, resolution 100px/mm, magnification 20x). After imaging, the tissues were dissected from the sample holder using a cork borer and immediately imaged again to quantify tissue compaction. The markers
were considered as nodes, spanning a quadratic quadrilateral element. Using the ‘nodal’ displacements, the deformation gradient tensor \( F_p \) accounting for prestretch was obtained, describing the deformation from the unconstrained to the constrained state.

2.4. Constitutive models

Two constitutive models were used to estimate material behavior. For the PDMS samples, a Neo-Hookean model was used, which has been shown to be well capable of describing the mechanical behavior of PDMS materials for stretches up to at least 1.5 [30]. For the biological materials, an exponential fiber-reinforced model was used. The constitutive behavior was modeled as a function of the total elastic deformation \( F_e \).

2.4.1. Neo-Hookean model

The Cauchy stress for the Neo-Hookean model was defined as:

\[
\sigma = \frac{\mu}{J_e} \left( B_e - J_e^{2/3} I \right) + \kappa \ln(J_e) I
\]

where \( \mu \) is the shear modulus, \( \kappa = \frac{2\mu(1+\nu)}{3(1-2\nu)} \) the bulk modulus, \( J_e = \det(F_e) \) the total elastic Jacobian, \( B_e = F_e F_e^T \) the left Cauchy-Green deformation tensor, and \( I \) the second order identity tensor.

2.4.2. Fiber-reinforced model

The fiber-reinforced material was assumed to consist of an isotropic matrix \( (m) \) and a (potentially anisotropic) collagen fiber network \( (f) \). Both material components additively contribute to the total Cauchy stress:

\[
\sigma = (1 - \Phi_f)\sigma_m + \Phi_f \sigma_f
\]

where \( \Phi_f \) is the total fiber volume fraction, set at 0.5 [31] and \( \sigma_m \) and \( \sigma_f \) are the Cauchy stress tensors of the matrix and fibrous parts, respectively. The matrix was modeled by the Neo-Hookean constitutive model (Equation 5), while the fiber part was modeled by an exponential Holzapfel-like model [31, 32]. For the fibrous component, an equidistant angular fiber distribution was assumed of a discrete number of \( N \) fibers that are situated in the plane of the material. Each fiber \( i \) oriented in the direction \( \mathbf{n}_0 \) (in the reference configuration) contributes to the total fiber stress:

\[
\sigma_f = \sum_{i=1}^{N} \phi_f^i \sigma_f^i
\]

Each fiber has a certain volume fraction \( \phi_f^i \), described by a periodic version of the normal probability distribution function [31, 33]:

\[
\phi_f^i = A \exp \left( \frac{\cos(2(\gamma^i - \alpha)) + 1}{\beta} \right)
\]
where \( \gamma^i \) is the fiber angle with respect to the main fiber direction \( \mathbf{v}_1 \), \( \alpha \) the main orientation, \( \beta \) the dispersity and \( A \) a normalisation factor which is defined such that the sum of individual fiber fractions equals 1:

\[
A = \frac{1}{\sum_{i=1}^{N} \exp \left( \frac{\cos(2(\gamma^i-\alpha)) + 1}{\beta} \right)}
\]  

(9)

Due to symmetry, a semi-circular distribution was used with \( N = 60 \) fibers and hence an angular resolution of \( d\gamma = 3^\circ \). Note that \( \beta \) determines the degree of anisotropy: if \( \beta \to \infty \), the material is fully isotropic, whereas \( \beta \to 0 \) indicates a fully anisotropic architecture. The fibers were distributed equidistantly in the plane spanned by the main fiber direction \( \mathbf{v}_1 \) and the cross-fiber direction \( \mathbf{v}_2 \), where the direction of each fiber in the reference configuration is given by a unit vector \( \mathbf{n}_0^i \), defined with respect to \( \mathbf{v}_1 \) and \( \mathbf{v}_2 \):

\[
\mathbf{n}_0^i = \cos(\gamma^i)\mathbf{v}_1 + \sin(\gamma^i)\mathbf{v}_2
\]

(10)

For each individual fiber \( i \), its contribution to the total fiber stress (Equation 7) was defined by an adapted version of the Holzapfel-Gasser-Ogden model [32, 34], where it was assumed that the fibers only resist tension:

\[
\sigma_f^i = k_1 (\lambda_e^i)^2 \left( \exp \left[ k_2 ((\lambda_e^i)^2 - 1) \right] - 1 \right) \mathbf{n}^i \otimes \mathbf{n}^i
\]

(11)

with \( \langle \rangle \) the Macaulay brackets, \( k_1 \) and \( k_2 \) material parameters, \( \lambda_e^i = \sqrt{C_e : (\mathbf{n}_0^i \otimes \mathbf{n}_0^i)} \) the total elastic stretch of fiber \( i \), and \( \mathbf{n}^i \) the unit fiber direction vector in the deformed configuration, related to the stress-free configuration by \( \lambda_e^i \mathbf{n}^i = \mathbf{F}_e \mathbf{n}_0^i \).

2.5. Mechanical characterization

To estimate the material parameters, inverse methods are required for both bulge and tensile tests. For the bulge tests, a combination of analytical and FE methods was used to reduce computational costs and increase robustness and accuracy, whereas for the tensile tests already an analytical method sufficed. The set of material parameters that were to be estimated are indicated by \( \xi \), which for the Neo-Hookean model only comprised one parameter (\( \xi = \mu \)), whereas the fiber-reinforced model required at least three parameters to be estimated (\( \xi = [\mu, k_1, k_2] \)); if the degree of anisotropy \( \beta \) is unknown a priori, it also needed to be estimated.

2.5.1. Bulge tests: initial estimate

The initial material parameter estimates were provided by the classical bulge equations. From the bulge tests (Section 2.2.1), the pressure \( p \) and the radii of curvature \( \kappa_i \) in the direction of \( \mathbf{v}_i \) (\( i = 1, 2 \)) were available. From these data, the stress resultants \( T_i \) were computed according to membrane theory [10, 11]:

\[
T_1 = \frac{p}{2\kappa_2}
\]

(12a)

\[
T_2 = \frac{p}{\kappa_2} \left( 1 - \frac{\kappa_1}{2\kappa_2} \right)
\]

(12b)
These equations were initially derived for thin axisymmetric geometries \([10, 11]\), however it has been demonstrated that they can closely approximate the stress resultants in geometries that do not necessarily deform axisymmetrically due to material anisotropy \([35, 36]\) including anisotropic tissues subjected to inflation testing \([19, 20, 37]\).

To estimate the material properties, the in-plane orthogonal stress resultants were also expressed in terms of the in-plane Cauchy stresses (Equation 5 or 6) in the direction of \(v_i\) \((\sigma_i = \mathbf{\sigma} : (v_i \otimes v_i)\) with \(i = 1, 2\) \([10, 11, 20]\):

\[
T_{1}^{\text{est}} = \int_{0}^{t} \sigma_1 (\lambda_c, \xi) \, dz
\]
\[
T_{2}^{\text{est}} = \int_{0}^{t} \sigma_2 (\lambda_c, \xi) \, dz
\]

with \(t\) the thickness of the deformed tissue (Eq. 1) and \(z\) in the direction normal to the tissue surface, with \(z = 0\) at the bottom and \(z = t\) at the top of the tissue. The parameter set \(\xi\) was to be estimated, whereas the in-plane elastic stretches at the sample top surface \((\lambda^*_{e,1} \text{ and } \lambda^*_{e,2})\) were available from the post-processed US data (Figure 2). Assuming that the curvature change through the thickness is negligible, a linear variation of the in-plane stretch through the tissue thickness was present, according to linear beam theory \([20]\):

\[
\lambda_{c,1}(z) = \lambda^*_{e,1} + \kappa_1 (z - t)
\]
\[
\lambda_{c,2}(z) = \lambda^*_{e,2} + \kappa_2 (z - t)
\]

To estimate \(\xi\), MATLAB’s least-square optimization algorithm (lsqnonlin, trust-region algorithm) was employed, aiming to find the minimum difference between the analytical and estimated stress tensions (Equations 12 and 13, respectively) by varying the parameter set \(\xi\). To this end, the following cost function was used:

\[
E(\xi) = \frac{1}{N_p} \sum_{k=1}^{N_p} \sqrt{\left( T_1(p^k) - T_{1}^{\text{est}}(p^k, \xi) \right)^2 + \left( T_2(p^k) - T_{2}^{\text{est}}(p^k, \xi) \right)^2}
\]

where \(N_p\) is the number of pressure steps.

2.5.2. Bulge tests: inverse finite element analysis

The second step in the inverse analysis employed an inverse FE analysis in order to obtain a more accurate estimate of the material parameter(s) \(\xi\). The experiment was simulated by an FE model in Abaqus FEA (Dassault Systèmes Simulia, Providence, RI, USA). Due to symmetry, the circular sample was modeled as a quarter disk, using quadratic full integration brick elements (Figure 2). Mesh convergence tests showed that at least 10 elements were required through the thickness and 20 along each radius, resulting in a total of 4800 elements. In
line with the experiments, a uniform pressure was gradually applied at the bottom plane of the sample and near-incompressibility was implemented by setting the Poisson ratio to $\nu = 0.498$.

The bulge experiment provided the two principal surface profiles $z_1$ and $z_2$ (coinciding with $v_1$ and $v_2$, respectively) of the sample as a function of the pressure $p$. In this inverse method, the material parameter set $\xi$ was adjusted until the difference between the profile displacements perpendicular to the tissue surface in the initial configuration of the experiment ($\Delta z_1$ and $\Delta z_2$) and simulation ($\Delta z_1^{\text{est}}$ and $\Delta z_2^{\text{est}}$) was minimized using MATLAB’s `lsqnonlin` function. This resulted in the following cost function:

$$E(\xi) = \frac{1}{N_p N_x} \sum_{k=1}^{N_p} \sum_{l=1}^{N_x} \sqrt{\left( \Delta z_1(p^k, x^l) - \Delta z_1^{\text{est}}(p^k, x^l, \xi) \right)^2 + \left( \Delta z_2(p^k, x^l) - \Delta z_2^{\text{est}}(p^k, x^l, \xi) \right)^2}$$

(16)

where $N_p$ is the number of pressure steps and $N_x$ the number of points along each principal surface profile with in-plane position $x$ along $v_1$ or $v_2$. The initial estimate of $\xi$ was provided by the membrane method of the first step of the inverse method (Section 2.5.1).

### 2.5.3. Tensile tests: inverse analysis

The in-plane principal stretches and Cauchy stresses were available from the tensile tests (Section 2.2.2). In order to estimate the material properties from these tests, an estimate for the Cauchy stress along the stretching direction ($\sigma_1^{\text{est}}$) was obtained by varying $\xi$, until the difference with the measured stress $\sigma_1$ was minimized. Once again, this was achieved through MATLAB’s `lsqnonlin` function, using the following cost function:

$$E(\xi) = \frac{1}{N_s} \sum_{k=1}^{N_s} \sqrt{\left( \sigma_1(\lambda_1^k) - \sigma_1^{\text{est}}(\lambda_1^k, \xi) \right)^2}$$

(17)

where $N_s$ is the number of stretch steps.

### 2.5.4. Inverse method validation

To verify the numerical framework, several virtual experiments were performed. FE models were generated as described in Section 2.5.2 with a diameter of 10 mm and different thicknesses (0.10–0.50 mm, no prestretch) and degrees of prestretch (1.0–1.3, thickness of 0.10 mm). Three different material types were tested: Neo-Hookean (NH), isotropic fiber-reinforced (IFR) and anisotropic fiber-reinforced (AFR).

The shear modulus for the Neo-Hookean material was (arbitrarily) chosen to be $\mu = 300$ kPa. The material parameters for the fiber-reinforced material were chosen to resemble native aortic heart valve leaflet tissue [34, 38]: $\mu = 50$ kPa, $k_1 = 0.7$ kPa, $k_2 = 9.9$; with material isotropy and anisotropy enforced by setting the fiber dispersity to $\beta = 100$ and $\beta = 0.5$, respectively.

To assess the reduction of computational costs gained by the first estimation step, 5 inverse FE analyses with random initial parameter estimates were
performed for each material type, for thin samples ($t_0 = 0.10$ mm) and no pre-stretch.

3. Results

3.1. Numerical verification: virtual experiments

To assess the accuracy and performance of the proposed numerical framework, several virtual experiments were performed, in which bulge tests were simulated for three different kinds of known material behavior, at different sample thicknesses and prestretch magnitudes. The NH behavior was estimated closely by the initial estimate, for all thicknesses and prestretch magnitudes (Figure 3A,B). The stiffness of the IFR material was slightly overestimated, particularly in the lower stretch regions, and became less accurate with increasing degrees of prestretch (Figure 3C,D). Similarly, the stiffness of the AFR behavior was overestimated, particularly in the direction perpendicular to the main fiber direction, becoming more severe with higher degrees of prestretch (Figure 3E,F). For both isotropic and anisotropic fiber-reinforced models, the nonlinearity was not captured for simulations with low diameter-thickness ratios and prestretches due to the low stretch intervals present in these cases.

The initial estimates were subsequently used for the inverse FE analyses. For all cases the final estimated material properties correctly described the preset material behavior of the virtual simulations. 5 Iterations were needed to estimate the shear modulus of the NH material, while as few as 2 iterations were enough to estimate the isotropic and anisotropic fiber-reinforced material parameters (Figure 4). In contrast, when using random initial material parameters ($n=5$), $8.2 \pm 3.0$ iterations were required to successfully estimate the NH material’s shear modulus, and $17.8 \pm 13.1$ and $10.6 \pm 3.4$ for the isotropic and anisotropic fiber-reinforced materials, respectively. Some of the estimations with random parameters needed to be restarted because of diverging parameter estimates, or failing FE simulations due to low stiffness estimates.

3.2. Experimental validation: comparison with biaxial tensile tests

The accuracy of the proposed methodology was assessed by performing both bulge and tensile tests on the same PDMS material (considered to behave as a Neo-Hookean material). After the bulge tests, the sample profile was successfully tracked as a function of pressure from the ultrasound images (Figure 2), in order to estimate the tissue’s in-plane tensions and profile displacement, which were respectively used in the initial and full FE parameter estimation steps. The shear modulus found in the initial estimate underestimated material stiffness (Figure 5A,B); this estimate was improved in the inverse FE analysis (Figure 5C,D), estimating a shear modulus of $\mu = 657.5 \pm 67.9$ kPa (Figure 6). The tensile tests resulted in an estimated shear modulus of $\mu = 622.7 \pm 20.4$ kPa, which was similar to the one estimated from the bulge test, yet with a smaller standard deviation (Figure 6).
3.3. Application to tissue-engineered constructs

Seven tissue-engineered constructs were mechanically characterized while being cultured inside the bioreactor system. The average initial thickness (and standard deviation) of the tissue-engineered constructs was determined using both nondestructive ultrasound measurements, and digital microscopy on cross-sections of dissected samples (Figure 9). For each sample, the thickness was determined on approximately 15 locations throughout the tissue. Both methods showed comparable average thicknesses. The prestretch in the tissue-engineered constructs was found to be between 1.03 and 1.05.

Similarly to the PDMS samples, the initial estimate underestimated material stiffness (Figure 7A,B), which was improved in the inverse FE analysis (Figure 7C,D). All samples featured a nonlinear stress-stretch response, as typically seen in soft tissues (Figure 8).

4. Discussion

The goal of the current study was to develop and validate a mechanical characterization method to nondestructively estimate the mechanical properties of soft tissues that can be applied during tissue culture. Mechanical testing was achieved by performing a bulge test on samples inside a novel bioreactor [9] using US and followed by an inverse analysis to estimate the mechanical properties. The efficiency and accuracy of the proposed method was demonstrated on virtual experiments of several material types with known parameters. Furthermore, PDMS samples were used to demonstrate the method’s feasibility by comparing it with tensile testing. Finally, the method was applied to estimate the material properties of tissue-engineered constructs during culture.

4.1. Numerical feasibility

The inverse analysis of the bulge experiments was performed in two steps. The goal of the first step was to provide a rapid, but not necessarily fully accurate, estimate of the mechanical properties. This estimate subsequently functioned as an initial estimate in the second step, where a full inverse FE scheme was used to obtain a more accurate estimate of the mechanical behavior.

In the initial estimation step, Laplace’s law was used to obtain an analytical estimate of the material parameters. When characterizing true membranes, without any residual stretches, this method has been shown to be capable of providing a rapid and accurate estimate of the material parameters of soft tissues, even for nonlinear and anisotropic materials [20, 37]. However, this method’s accuracy decreases when the tissue thickness and prestretch increase, as shown by the virtual simulations performed in this study (Figure 3). The estimate’s accuracy was further decreased by a decreasing stretch range due to the structural material changes: an increasing thickness leads to lower maximum stretch and stress, such that only the toe region of stress-strain curve was obtained for nonlinear materials (Figure 3C,E); whereas increasing prestretch lead to a
higher minimum stretch during bulging, such that only the linear region of the stress-strain curve was obtained for nonlinear materials (Figure 3D,F).

Despite these limitations, the analytical estimate still provided a reasonable and therefore important estimate of the materials’ mechanical behavior, for it considerably reduced the computational costs (Figure 4) and increased robustness of the second step. This reduction was most evident in the fiber-reinforced materials, where more parameters were to be estimated. Without such an initial estimate, an inverse FE analysis with 3D elements is computationally costly, therefore previous studies typically resorted to faster methods such as shell meshes [18, 21, 22, 23, 39, 36] or analytical solutions as used in the initial estimate [20, 37]. These methods can provide excellent estimates for thin membranes, but possibly lead to inaccurate material parameter estimates when the tissues become too thick or feature residual stretches. During the characterization of the PDMS and tissue-engineered samples, the inverse FE method was indeed able to improve on the initial estimate, and closely fit the experimental pressure-displacement behavior (Figures 5 and 7).

4.2. Experimental feasibility

The main advantage of the proposed method is its capability to characterize soft tissues while they are being cultured inside a bioreactor, without the need to sacrifice the tissues. US imaging is key to this setup, as it allows for measurements of the tissue deformation during bulge testing, a test that can now be performed inside the bioreactor. Previous studies have typically measured the tissue deformation during bulging using stereo camera setups [19, 21, 22, 23, 37, 39] or advanced optical rigs [18, 40], which are incompatible with the limited view of samples inside a bioreactor. In contrast, US is able to measure throughout the tissue cross-section, and can therefore be easily mounted on top of a bioreactor. The custom MATLAB script that was developed successfully tracked the tissue profile displacement as a function of the applied pressure. Apart from displacement measurements, the tissue thickness could also be measured inside the bioreactor when using a high-frequent US transducer. The radio frequency data of the 12 MHz transducer that was used in the current study yielded comparable results to those obtained by microscopy of fresh tissue cross-sections, albeit with a higher spread on the measurements (Figure 9).

The experimental validity of this method was demonstrated by mechanically characterizing PDMS samples by both tensile tests and bulge tests with the proposed two-step inverse analysis method. The shear modulus estimated by both tested only differed by 5%, although the spread on the bulge tests was slightly higher in the tensile tests (Figure 6). Following the validation experiments, tissue-engineered constructs were successfully characterized (Figures 7 and 8). The displacement-pressure behavior that was measured could be well-fit by the fiber-reinforced material model, resulting in the nonlinear stress-stretch response that is typically found in most biological soft tissues. Changes to a more compliant material behavior could be observed from week 3 to week 4, although the sample number used in this study was too low to draw any significant conclusions.
The tissue-engineered constructs were found to be relatively stiff, with stress stiffening already occurring from a stretch of 1.05 in the three-week cultured samples. This can be explained by the low pressures, and therefore also low stretches, that were imposed on the tissue during bulging. Biological soft tissues are known to demonstrate significant softening behavior due to the Mullins effect [41, 42]. The mechanical properties can therefore be highly sensitive to the applied stretch magnitude, so when applying higher pressures one might find more compliant mechanical behavior. This is also the reason why only the PDMS samples were used to compare the proposed method to tensile testing, as this material does not exhibit softening within the tested stretch range and therefore their material behavior is not dependent on the applied load.

4.3. Limitations

Although US is key to the nondestructive mechanical testing method, it has a limited spatial resolution, which affects the accuracy of the tissue displacement and thickness measurements. In the current study, the radio frequency data [27] of a 12 MHz transducer was used, which is almost the maximum frequency for clinical US systems. Currently, probes with frequencies as high as 50 MHz are available, that can greatly enhance the spatial resolution, which is linearly proportional to the transducer’s frequency. A limited spatial resolution is also the reason why 3D US, which would result in a full-field deformation measurement, is inadequate for accurately measuring tissue deformation and thickness.

All measurements were carried out without damaging the samples, with the exception of the prestretch, that is developed due to prestretch of newly deposited matrix [15] and cell traction forces [16]. Quantification of the prestretch could only be performed at the end of the experiment by taking the tissue out of the bioreactor, thereby bringing it back to a stress-free state, and measuring the dimensional changes. In order to nondestructively test tissues during culturing, it should be assumed that the prestretch remains constant after the initial tissue development, and thus the prestretch that is measured at the end of the experiment is representative for all time points. Alternatively, some samples could be sacrificed during the experiment to measure the prestretch, where it should be assumed that the prestretch is similar in the samples that were not sacrificed.

Finally, the biaxial stress and stretch states that can be explored within this testing setup are limited. With biaxial tensile testing, any arbitrary biaxial stress and stretch state can be explored, whereas with inflation testing the tissue can only be loaded by a uniform pressure, thus limiting characterization of anisotropic tissues. This leads to characterization of only a limited part of the mechanical behaviour of anisotropic tissues, yet this part is most relevant for thin cardiovascular tissues at physiological loading conditions, which are mimicked by the bioreactor.

4.4. Future directions

In the current study, we employed a combination of experimental and inverse methods to nondestructively characterize the mechanical properties of isotropic
and anisotropic soft tissues, without being limited by the presence of prestretch or excessive tissue thickness. The proposed method was applied to a novel bioreactor system, but can without much effort be applied to other bioreactor systems that are capable of applying pressures to culture tissues. The inverse analysis can be combined with any experimental bulge test setup, as long as pressure and tissue displacement are measured.

The main advantage of this method is that tissues can now be cultured in a bioreactor system and mechanically characterized during their development. Using the mechanical properties, different mechanical constituents can be investigated to assess whether any of these determines the mechanical homeostasis, which is key to better understand how soft tissues function during both health and disease.

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6. References


Figure 1: Schematic representation of the bioreactor system in which the mechanical bulge tests were performed [9].

Figure 2: US images of a tissue-engineered construct before (A) after applying pressure (B). The green dots indicate the top tissue profile, while the continuous red lines show the circle that was fitted to the central 50% of the sample profile. The bulge tests were simulated by a FE model, before (C) and after applying pressure (D), where the red dashed lines in (A) indicate the position of the ultrasound transducer to measure the tissue deformation in the main ($v_1$) and cross-fiber ($v_2$) direction.
Figure 3: The influence of thickness and prestretch on the estimation of the stress-stretch behavior of the virtual experiments using NH (A,B), IFR (C,D) and AFR (E,F) constitutive models. The black markers indicate the true stress-stretch behavior. The colored lines indicate the initial estimate of the stress-stretch behavior for different tissue thicknesses (A,C,E) or prestretch magnitudes (B,D,F). The black dashed lines indicate the material behavior estimated by the full inverse FE method, where the correct result was found for all choices of thickness and prestretch. In the AFR model, the black markers and continuous line indicate the stress in the fiber direction, while the stress in the cross-fiber direction is given by the white markers and dashed line.
Figure 4: The number of iterations required to estimate the material parameters of all three materials was lower when using the two-step method, compared to starting the inverse FE with a random initial estimate (n=5 per material). The number of iterations until convergence varied per random initial estimate.

Figure 5: The material behavior of PDMS samples was estimated in the full inverse FE method by fitting the estimated profile displacement (continuous lines) as a function of pressure to the one measured by ultrasound (dotted markers). This figure shows the results from one representative sample, with the apex displacement as a function of pressure (A,C), and the displacement of the central 50% of the sample as a function of pressure (B,D), where the transition from pressure-free to maximum pressure state is indicated by the color transition from green to red. The initial material estimate (A,B) shows an underestimate of the material stiffness, the final estimate (C,D) closely describes the experimental displacement behavior.
Figure 6: The material behavior of PDMS samples was estimated by biaxial tensile tests (continuous line, circular markers) and bulge tests (dashed lines, square markers). Both methods estimated similar shear moduli, with $\mu = 622.7 \pm 20.4$ kPa for the biaxial tests, and $\mu = 657.5 \pm 67.9$ kPa for the bulge tests.

Figure 7: The material behavior of developing engineered tissues was estimated in the full inverse FE method by fitting the estimated profile displacement (continuous lines) as a function of pressure to the one measured by ultrasound (dotted markers). This figure shows the results from one representative sample, with the apex displacement as a function of pressure (A,C), and the displacement of the central 50% of the sample as a function of pressure (B,D), where the transition from pressure-free to maximum pressure state is indicated by the color transition from green to red. The initial material estimate (A,B) shows an underestimate of the material stiffness, the final estimate (C,D) closely resembles the experimental displacement behavior.
Figure 8: Estimated stress-stretch behavior of the tissue-engineered constructs. All samples showed a nonlinear stress-stretch response, as typically seen in soft tissues. The three-week statically cultured samples (n=4, continuous lines) showed a stiffer response than the four-week statically (n=2, dashed lines) and dynamically (n=1, dotted line) cultured samples.

Figure 9: The thickness of 6 tissue-engineered samples was determined on approximately 15 locations using nondestructive ultrasound measurements, and microscopy measurements on dissected tissue cross-sections. The two methods yielded comparable results, yet the data spread was higher in the ultrasound measurements.