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Citation for published version (APA):

Argento, G., de Jonge, N., Söntjens, S. H. M., Oomens, C. W. J., Bouten, C. V. C., & Baaijens, F. P. T. (2015). Modeling the impact of scaffold architecture and mechanical loading on collagen turnover in engineered cardiovascular tissues. *Biomechanics and Modeling in Mechanobiology*, 14(3), 603-613.
<https://doi.org/10.1007/s10237-014-0625-1>

DOI:

[10.1007/s10237-014-0625-1](https://doi.org/10.1007/s10237-014-0625-1)

Document status and date:

Published: 15/06/2015

Document Version:

Publisher's PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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Modeling the impact of scaffold architecture and mechanical loading on collagen turnover in engineered cardiovascular tissues

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Received: 19 February 2014 / Accepted: 3 October 2014 / Published online: 16 October 2014
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Abstract The anisotropic collagen architecture of an engineered cardiovascular tissue has a major impact on its in vivo mechanical performance. This evolving collagen architecture is determined by initial scaffold microstructure and mechanical loading. Here, we developed and validated a theoretical and computational microscale model to quantitatively understand the interplay between scaffold architecture and mechanical loading on collagen synthesis and degradation. Using input from experimental studies, we hypothesize that both the microstructure of the scaffold and the loading conditions influence collagen turnover. The evaluation of the mechanical and topological properties of in vitro engineered constructs reveals that the formation of extracellular matrix layers on top of the scaffold surface influences the mechanical anisotropy on the construct. Results show that the microscale model can successfully capture the collagen arrangement between the fibers of an electrospun scaffold under static and cyclic loading conditions. Contact guidance by the scaffold, and not applied load, dominates the collagen architecture. Therefore, when the collagen grows inside the pores of the scaffold, pronounced scaffold anisotropy guarantees the development of a construct that mimics the mechanical anisotropy of the native cardiovascular tissue.

Keywords Collagen turnover · Scaffold · Microscale · Contact guidance · Anisotropy

1 Introduction

Tissue engineering is a promising technique to produce living replacements for biological tissues, able to repair, grow and remodel under biochemical and mechanical cues ([Langer and Vacanti 1993](#)). Tissue engineering involves the seeding of cells onto a porous degradable scaffold and subsequent conditioning in a bioreactor system. The cells produce and organize the extracellular matrix that defines the load-bearing properties of the tissue once the scaffold has degraded ([Walters and Stegemann 2013](#)). Previous studies on native connective tissues have shown that the morphology of the collagen network is responsible for the mechanical properties in terms of stiffness and load-bearing capability of the tissue ([Billiar and Sacks 2000](#); [Driessen et al. 2005](#)). Analogously, the morphology of neo-formed tissue-engineered collagenous networks is of fundamental importance, since it determines the mechanical properties and functionality of the constructs ([Driessen et al. 2007](#)).

The formation of collagen networks in engineered constructs is a complex process. A number of load-driven remodeling mechanisms influence the reorganization of the collagen network over time. In cardiovascular tissues, the collagen fibers mainly remodel along and in between the principal loading directions ([Driessen et al. 2005](#); [Dahl et al. 2008](#)), and the cells play an active role in the remodeling process ([Soares et al. 2011](#); [Vlimmeren et al. 2012](#)). When fibroblast-like cells are seeded onto a fibrous scaffold and cultured under pulsatile conditions, they align preferentially along the fibers of the scaffold and produce collagen along the same direction ([Hwang et al. 2009](#); [Niklason 2009](#)). The neo-formed collagen fibers stay in close contact with the scaffold ([Eckert et al. 2011](#)), indicating that the contact guidance by the scaffold is a crucial factor in early stages of the devel-

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opment of the collagen network. Next to collagen synthesis, also enzymatic degradation of the collagen fibers takes place. The degradation of collagen fibers happens according to strain-dependent kinetics. When strain is applied to a collagen gel, the collagen fibers that are oriented in the direction of the strain will degrade slower than the collagen fibers in all the other directions (Bhole et al. 2009; Hadi et al. 2012).

Although the phenomena that drive the collagen reorganization in engineered tissues have been studied extensively, it is not known to what extent each of them influences the newly formed tissue. Recently, de Jonge (2013) demonstrated that contact guidance by a microfiber electrospun scaffold dominates over strain-driven collagen formation under static as well as under cyclic loading conditions. In addition, due to collagen turnover, the mechanical properties of the neo-tissue will evolve over time. These findings indicate that the structure of the scaffold can be exploited to obtain anisotropic native-like load-bearing tissues. However, a systematic characterization of the morphology and the mechanics of the engineered constructs resulting from scaffolds with different initial morphologies and subjected to different loading conditions (static and cyclic) is missing. The aim of the present paper is to investigate how scaffold design and loading conditions influence engineered tissue mechanics, with the aim of mimicking the mechanical anisotropy that characterizes cardiovascular tissues.

We developed a microscale model describing the interaction between the scaffold architecture and the evolving collagen network, as well as the mechanical properties of the neo-construct in response to dynamic loading conditions. The biaxial mechanical properties of the constructs were analyzed experimentally using both isotropic and anisotropic scaffolds. The scaffolds were composed of a biodegradable elastomer (PCL-bisurea) (Wisse et al. 2006) and were manufactured using electrospinning. The computational model captured the key experimental observations.

2 Materials and methods

2.1 Microscale growth and degradation model

A representative area element (RAE) of a tissue-engineered construct is built. The RAE is composed of a network of scaffold fibers and a network of collagen fibers. For both networks the initial fiber distributions are described by the fiber volume fractions ϕ_i^γ and ϕ_i^γ ($i = c, s$ referring to collagen and scaffold respectively) along each direction γ with the periodic version of the normal distribution function introduced by Gasser et al. (2006) and adapted by Driessen et al. (2008):

Fig. 1 Normal fiber volume fraction described in Eq. 1 (top). Schematic of a representative area element (RAE), composed of a network of scaffold fibers (red) and a network of collagen fibers (light green) embedded in a continuum matrix (dark green) (bottom)

$$\phi_i^\gamma = A_i \exp \left[\frac{\cos \left(2 \left(\frac{\gamma}{\delta_i} - \alpha_i \right) \right) + 1}{\beta_i} \right]. \quad (1)$$

Equation 1 describes the volume fraction distribution of the collagen and scaffold fibers, respectively, as a function of the angle γ . α_i , β_i and δ_i ($i = c, s$) represent the main fiber orientation, the standard deviation and the frequency scaling factor of the collagen and scaffold fibers. The lower β the more anisotropic the fiber distribution will be. The parameters A_i are calculated in order to equal the total volume fractions ϕ_i ($i = c, s$) of the collagen and scaffold fibers (Fig. 1). The fibers are equally spaced in a discrete way with an angular interval $\Delta\theta = 10^\circ$ between -90° and $90^\circ - \Delta\theta$. The initial collagen volume fraction is assumed to be low and isotropically distributed ($\alpha_c = 0^\circ$, $\beta_c = 3e + 4$), as in principle cells can produce collagen in every direction. Over time collagen fibers are developing through a combination of cell-mediated collagen synthesis and enzymatic degradation. Therefore, the turnover rate of the collagen fiber radius $\frac{dr(t)}{dt}$ for a collagen fiber in the direction γ is the difference of the radius growth rate $\mathcal{S}$ and the radius degradation rate $\mathcal{D}$

$$\frac{dr(t)}{dt}(\gamma) = \mathcal{S} - \mathcal{D}. \quad (2)$$

Table 1 Model parameters

Parameter	Value	Source or evaluation method
k_1	$8.3\text{e-}7 \mu\text{m/h}$	^a
k_2	$0.33 [-]$	^a
k_3	$3.78\text{e-}6 \mu\text{m/h}$	Hadi et al. (2012)
k_4	$3.09\text{e-}6 \mu\text{m/h}$	^b
k_5	$2.1\text{e-}2 [-]$	^b
ϕ_{max}	0.15	Van Geemen (2012), Mol (2005)

^a Fit on total collagen content after 2 weeks culture of isotropic and anisotropic statically cultured constructs

^b Fit on total collagen content after 2 weeks culture of isotropic statically cultured construct and anisotropic cyclically cultured construct

The growth of the collagen fibers along the direction γ is ruled by the fiber radius growth rate

$$S = k_1 \cdot \frac{\phi_s(\gamma)}{\phi_{tot,s}} \cdot \frac{(\phi_{max} - \phi_c(t))}{\phi_{max}} \cdot \frac{\exp\left(\frac{k_2}{\beta_s}\right)}{2\pi r(\gamma, t) l(\gamma)} \quad (3)$$

where $\phi_s(\gamma)$ represents the scaffold fiber volume fraction along the direction γ , $\phi_{tot,s}$ the total scaffold volume fraction. The linear dependency of the partial scaffold volume fraction, $\phi_s(\gamma)$, indicates that the contact guidance by the scaffold fibers plays a major role in the distribution of the developing collagen fibers. ϕ_{max} is the maximum collagen volume fraction, which is set 0.15 [estimated as possible collagen volume fraction after 2 weeks of tissue culture from the work of Van Geemen (2012) and Mol (2005)], and ϕ_c is the current total collagen volume fraction. The exponential of β_s indicates that the scaffold anisotropy influences the amount of collagen that is produced (Lee 2005; Teh et al. 2013). Furthermore, the inverse proportionality with the current radius $r(\gamma)$ and the collagen fiber length $l(\gamma)$ indicates that the volume of collagen deposited by the cells in each direction is constant at each time step. The constant value k_1 is intended to represent the mean synthetic activity of the cells.

As long as the collagen fiber has a positive radius, the enzymatic degradation rate along the direction γ is described by means of a strain-based Michaelis–Menten law (Menten and Michaelis 1913), which is often used to describe enzyme kinetics. It is null when the radius becomes null.

$$\mathcal{D} = \begin{cases} k_3 - k_4 \cdot \frac{(\lambda_f(\gamma)-1)}{(k_5+\lambda_f(\gamma)-1)}, & r(\gamma) > 0 \\ 0, & r(\gamma) \leq 0 \end{cases} \quad (4)$$

where $\lambda_f(\gamma)$ represents the collagen fiber stretch along the direction γ , and k_2, k_3 and k_4 are the parameters that rule the degradation kinetics. All the model parameters that are used in the current work to describe collagen synthesis and degradation are reported in Table 1.

The growth and degradation rules described are implemented in a Forward Euler formulation over a total time interval of 2 weeks. At each time step, the growth and degradation rate are calculated and the current radius of the j th collagen fiber along the direction γ is updated

$$r_j(\gamma, t+1) = r_j(\gamma, t) + (dr(\gamma, t))_g - (dr(\gamma, t))_d \quad (5)$$

The volume variation of the j th collagen fiber is

$$\frac{dV_j}{dt}(\gamma) = \pi \cdot (r_j(\gamma, t+1)^2 - r_j(\gamma, t)^2) \cdot l(\gamma) \quad (6)$$

The updated total collagen fiber volume takes into account the volume variation along each direction

$$V_c(t+1) = V_c(t) + \sum_{i=1}^N w_i \cdot dV_j(\gamma, t) \quad (7)$$

where w_i is the number of collagen fibers in the direction γ . Finally, the updated collagen volume fraction

$$\phi_c = \phi_c(t+1) = \frac{V_c(t+1)}{V_{RAE}} \quad (8)$$

is used to calculate the growth rate at the next step.

2.2 Finite element model

The microscale model is made of a RAE composed of two fibrous structures embedded in an isotropic matrix (Fig. 1). In the model one network mimics the fiber distribution that can be observed from a scanning electron microscopy (SEM) image of an electrospun scaffold. The other network represents a collagen network, which is assumed to be initially isotropic, as in principle collagen fibers can assemble in all directions. For both networks, a discrete distribution function is chosen (Eq. 1). An exponential relationship is assumed to relate the microscale collagen and scaffold fiber stress ψ_i and stretch λ_i ($i = c, s$) for positive stretches:

$$\psi_i^\gamma = \begin{cases} h_{1,i} \lambda_i^2 \left[\exp^{h_{2,i}(\lambda_i^2-1)} - 1 \right], & \lambda_i \geq 1 \\ 1, & \lambda_i < 1 \end{cases} \quad (9)$$

where $h_{1,i}$ and $h_{2,i}$ are a stress-like and dimensionless parameter for collagen fibers for $i = c$ and scaffold fibers for $i = s$, respectively.

The nonlinearity of the material law is chosen to account for the undulations in the electrospun fibers and collagen fibers that is otherwise not represented in the model.

As described by Argento et al. (2012), the coupling of the microscale to the macroscale follows averaging theorems proposed by Hill (1963, 1984) and Nemat-Nasser (1999),

and used by Kouznetsova et al. (2001) in the development of a full multi-scale framework. The sum of the resulting external forces at the boundary nodes is averaged over the initial RAE volume to evaluate the macroscopic first Piola–Kirchhoff tensor and the Cauchy stress tensor.

The discrete representation of the electrospun fibrous network was generated with the method previously developed by Argento et al. (2012). First, given the diameter of the scaffold fibers, a MATLAB (The Math-Works, Natick, MA, USA) algorithm evaluates from the fiber volume fraction the number of scaffold fibers in each direction. In each direction, the scaffold fibers are defined at a random distance from the center of the RAE. An interconnection between elements is defined as a node placed at the intersection of the two fibers. A percentage of all the possible collagen fiber intersections defined from the SEM images of the scaffold are randomly picked as fiber interconnections and defined as nodes of the finite element mesh.

A similar method is used to describe the discrete collagen network. In each direction, the same algorithm evaluates the amount of collagen fibers in each direction, and the collagen fibers are placed along the scaffold fibers. The possible remaining collagen fibers in one direction are placed with a random distance from the others in the considered direction. All the microstructural properties of scaffold and collagen network (diameter, volume fraction, interconnectivity, orientation) can be varied. Connections between the elements of the network are set and introduced as nodes at the intersection of the elements, to enable transmission of forces.

In the finite element model, the scaffold and the collagen fibers are modeled by using 2D beam elements where bending plays a minor role. The finite element microscopic problem was solved by using the software Abaqus (Simulia, Providence, RI). A user material (UMAT) was developed to implement the growth and degradation laws described in Sect. 2.1 via an explicit procedure using automatic time incrementation, which performs well to model such a dynamic process. The time dependency was accounted for in the model by updating the diameter of the collagen fibers over time. At each time step, the new fiber diameter is evaluated. The updated diameter is used to evaluate the new fibers area, used in turn to update the element stiffness. To simulate an applied cyclic strain, a static strain of equivalent magnitude to the maximum amplitude of the applied cyclic strain (10 %) was used, and a time integration was performed.

2.3 Engineered constructs

Two different PCL-bisurea (Wisse et al. 2006) scaffolds were produced to perform the model validation. The scaffolds were electrospun in a climate-controlled electrospinning cabinet (IME Technology, Eindhoven, NL) equipped with a 0.6 mm diameter capillary and a distance of 15 cm between the capil-

Fig. 2 Experimental system. The constructs were cultured either statically (*left*) or cyclically (*right*). In the statically cultured samples, the part in the *black square with solid line* was used to measure tissue compaction. In the statically and cyclically cultured samples, the part in the *black square with the dashed line* was used for tissue analysis (Chloramin-T assay and mechanical testing). For the anisotropic scaffolds, the cyclic strain is applied in direction parallel and perpendicular to the scaffold fibers. *Arrows* indicate directions of applied cyclic strain. The *scale bar* indicates 1 cm

lary and the 5 cm diameter rotating target drum. PCL-bisurea of 20 % (wt) was dissolved in chloroform (CHCl_3) and electrospun with a flow rate of 25 $\mu\text{L}/\text{min}$ and a voltage of 18 kV was applied. Scaffold 1 was spun with a rotational speed of 100 rpm (corresponding to a surface speed of 0.52 m/s), scaffold 2 was spun at 2,500 rpm (corresponding to a surface speed of 13 m/s). The thickness of the two scaffolds was 135 μm and 125 μm , respectively. The image processing software ImageJ was used to obtain the average scaffold fiber diameter and the distribution from SEM (FEI, Netherlands) images.

Samples of each of the two scaffolds were cut out of the central part of the scaffolds and glued at their edges onto the flexible membrane of Bioflex well plates (Flexcell International, Hillsborough, NC, USA) using Silastic MDX4-4210 (Dow Corning, Midland, MI, USA) as described by (de Jonge 2013). Squared samples (7 mm $\times$ 7 mm) were used for static culture, and rectangular samples (7 mm $\times$ 35 mm) for cyclic culture. Rectangular constructs were glued also at the center to form a square as reinforcement (Fig. 2).

Primary vascular-derived cells were harvested from the human vena saphena (HVS) magna, acquired from a donor in accordance to Dutch guidelines for secondary use of material. These cells have previously been characterized as myofibroblasts (Mol et al. 2006). The myofibroblasts were isolated following established protocols (Schnell et al. 2001). The cells were cultured in Dulbeccos modified Eagles medium (a-DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS; Greiner Bio-One, Monroe, NC), 1 % GlutaMax (Gibco, Carlsbad, CA, USA) and 1 % penicillin streptomycin (PenStrep; Lomza, Belgium) and passaged every 3 days. Cells of passage 7 were used for culturing the constructs. Myofibroblasts were seeded into the scaffolds using fibrin, to prevent cells to fall through

Table 2 Details of the experimental design

Scaffold type	Strain	Num.	Use of collagen content data	Use of mech. data
Isotropic	Static	2	Fit k_1, k_2, k_4, k_5	Fit $h_{1,c}$ and $h_{2,c}$
Anisotropic	Static	2	Fit k_1 and k_2	Validation
Isotropic	Cyclic	2	Validation	Validation
Anisotropic	Cyclic par.	2	Fit k_4 and k_5	Validation
Anisotropic	Cyclic perp.	2	Validation	Validation

the scaffold. Briefly, bovine fibrinogen (Sigma, St. Louis, MO, USA) and bovine thrombin (Sigma) were combined to produce final construct concentrations of 10 mg/ml fibrinogen, 10 IU/ml thrombin and 15×10^6 cells/ml, based on our methods for cardiovascular tissue engineering (TE) (Mol et al. 2005). Suspensions were incubated for 30 min at 37 °C in a humidified 95/5 % air/CO₂ incubator to allow gelation before culture medium was added. Culture medium consisted of a-DMEM supplemented with 10 % FBS, 1 % GlutaMax, 1 % PenStrep and 0.25 mg/ml l-ascorbic acid 2-phosphate (Sigma). Medium was replaced three times a week. The constructs were cultured for 2 weeks. A biaxial constraint was applied to the isotropic and anisotropic scaffold. Furthermore, biaxial and uniaxial dynamic strain (10 %, 1 Hz) was applied to the isotropic scaffold and to the anisotropic scaffold (with uniaxial strain applied along and perpendicular to the scaffold fiber direction) (Fig. 2; Table 2). Dynamic straining was started 30 min after seeding the cells into the scaffold, so that the cells could feel the strain immediately, before they start producing matrix.

After 2 weeks of culture, the engineered constructs were cut loose from the membranes and left to rest for half an hour. Before detaching the samples from the membrane, a rectangular shape was drawn on each statically cultured sample with a tissue felt pen (Fig. 2). After resting time, the change in size of the rectangle was measured, to quantify tissue compaction. Compaction was only measured for statically cultured tissues, as this allowed to estimate the prestress used in the model. Cyclically cultured tissues tended to fold more when detached from the Flexcell membrane. This made the evaluation of the compaction more difficult, and therefore, those data were considered less reliable and were not used in the work.

Chloramin-T assay (Huszar et al. 1980) was used to quantify the hydroxyproline content, as a measure of the collagen content. The collagen distribution within the constructs was evaluated with a CNA staining technique (Krahn et al. 2006) using a Confocal Laser Scanning Microscope (Carl Zeiss, Oberkochen, Germany). Briefly, samples were labeled with Cell Tracker Orange (CTO; Invitrogen Molecular Probes) and CNA35-OG488 (CNA), to fluorescently stain cell cytoplasm and collagen, respectively. Scans were made from the

bottom layer of the tissue until 40 μ m into the constructs with a 4 μ m interval, through the transparent membrane of the Bioflex culture plates, with the constructs still attached. The scanned area was $250 \times 250 \mu$ m, located at the center of the tissue.

The quantification of the collagen orientation was performed by using a fiber orientation algorithm, based on the work by Frangi et al. (1998). The algorithm calculates of the Hessian at each pixel on the image. The Hessian matrix describes the local structure of the image, and its largest eigenvector indicates the principal direction of the ellipse, and thus the orientation of the collagen fiber. The algorithm was implemented in Mathematica (Wolfram, UK) and was used and validated in previous studies (Jonge et al. 2012).

After culture, one construct for each group was soaked for 10 min in a bleach product (Sodium hypochlorite) to remove the cells and the neo-tissue and then washed for three times in water. After drying, the scaffold samples were imaged with SEM.

2.4 Scaffold and collagen characterization

Scaffold 1 contains fibers with an average diameter of 5.6 μ m, a fiber volume fraction of 27 % and an isotropic fiber distribution ($\beta = 13.7$). Scaffold 2 is composed of fibers with an average diameter of 4.6 μ m, a fiber volume fraction of 33 % and an anisotropic fiber distribution ($\beta = 0.65$) (Fig. 4).

Biaxial tests were conducted on cell-free scaffolds as well as engineered constructs at the end of the 2 weeks culture time using a Biotester (CellScale Biomaterial Testing).

Three samples per scaffold were soaked in ethanol, kept in water in the incubator for 24 h, and tested in wet conditions at 37 °C. The samples were stretched with a biaxial and then uniaxial stretch in both directions at 10–20–30 % (Fig. 3), and the results were averaged for each type of scaffold.

The same protocol was used to test the engineered constructs. For each group, two constructs were tested (Table 2).

To simulate the biaxial tests, the displacements of all the nodes on the RAE boundary were prescribed to either reflect a constrained case or a uniaxial strain.

The microstructural fiber parameters of the anisotropic scaffold $h_{1,s}$ and $h_{2,s}$ (Eq. 9) were identified using a least squares optimization of the stress–strain curves at 30 % biaxial strain, evaluated by using a homogenization procedure (Kouznetsova et al. 2001). The same parameters were used to predict the mechanical behavior of the isotropic scaffold.

The collagen volume fractions calculated by Chloramin-T assay (Huszar et al. 1980), in combination with the collagen orientation data from CNA staining, were used to evaluate the constants that describe the collagen turnover kinetics in Eq. 2, using a least square optimization procedure. Parameters k_1 and k_2 that describe the collagen growth were evalu-

Fig. 3 Strain pattern used for biaxial tests. The strain pattern consists in five cycles of equibiaxial strain and uniaxial strain in the x and the y direction at 10, 20 and 30 % strain. A prestrain of 0.2 % is applied. A strain rate of 100 % strain/min is used

ated on the mechanical data of the statically cultured isotropic and anisotropic constructs. The parameter k_3 refers to the degradation rate in absence of strain evaluated by Hadi et al. (2012) on the basis of the experiments of Bhole et al. (2009). Parameters k_4 and k_5 , describing the collagen degradation, were estimated using the experimental mechanical data of the biaxial isotropic statically loaded constructs and uniaxial cyclically loaded constructs, with strain applied along the scaffold fibers. All the parameters used in the synthesis and degradation model to simulate the described experiments are summarized in Table 1.

The compaction was measured for the constructs cultured with static strain. To account for the compaction, in the finite element framework, a prestress was assigned to each collagen fiber. The collagen prestress represents the stress developed by the cells during culture, which leads to compaction. The collagen fiber prestress evaluated by fitting the experimentally measured compaction in an isotropic construct was used to predict the compaction which occurs in an anisotropic statically cultured construct.

A least square optimization in the isotropic static case was used to determine the parameters $h_{1,c}$ and $h_{2,c}$ (Eq. 9), used to describe the biaxial tensile mechanical behavior of the collagen fibers. The same parameters were used to simulate the tensile mechanical behavior of an anisotropic static and the cyclic constructs. All the microstructural mechanical constants used to simulate scaffold and collagen and the Young moduli E assigned to the linear elastic matrix are reported in Table 3.

3 Results

3.1 Structural characterization of the engineered constructs

The Confocal Laser Scanning Microscopy images taken at the bottom side of the samples reveal that the collagen is deposited mainly along the scaffold fibers, independently

Table 3 Parameters of the scaffold and collagen fibers constitutive behavior and prestress

Parameter	Value	Estimation method
$h_{1,s}$	804.77 kPa/mm ²	a
$h_{2,s}$	2.77 [-]	a
$h_{1,c}$	4027.95 kPa/mm ²	b
$h_{2,s}$	13.54 [-]	b
E	1 kPa	—
$\sigma_{prestress}$	116.84 kPa	c

a Fit on the biaxial mechanical data of the anisotropic scaffold

b Fit on the biaxial mechanical data of the isotropic statically cultured construct

c Fit on compaction of the isotropic statically cultured construct

of the applied strain (Fig. 4). Surprisingly, the Confocal Laser Scanning Microscopy from the top shows the presence of a dense collagen layer on top of the scaffold. The collagen orientation in the neo-tissue on top of the scaffold is always anisotropic, varying its arrangement at different depths (Fig. 5) and not influenced by the underlying scaffold and tissue architecture. For this reason, the collagen orientation in the top layer is globally assumed to be isotropic in the simulations.

The collagen volume fractions calculated with CNA staining for each construct are reported in Table 4. It can be observed that a higher scaffold anisotropy enhances the production of collagen. In the computations, for each construct, one-third of the evaluated collagen volume fraction is considered to belong to the thickness of the scaffold, two-third to the top layer, which is here assumed to be isotropic. Since it is not possible to split the samples to quantify the amount of collagen belonging to the top layer, these values are estimated by fitting the results of the biaxial mechanical tests of the statically cultured construct that was grown on anisotropic scaffold. Images show that the top layer is comparable in thickness and denser than the collagen within the scaffold.

Fig. 4 Representative SEM pictures of the isotropic (a) and anisotropic (b) scaffold, with respective fiber distribution. Representative confocal microscopy images of tissue-engineered constructs cultured in isotropic (c) and anisotropic (d) scaffold, with respective collagen fiber distribution, imaged from the *bottom* of the construct. In figures (c) and (d), the collagen is represented in *green*, and the scaffold fibers are represented in *red*. The scale bar represents 50 μm

The bare scaffolds, imaged with SEM after the culture period and after removal of the cells and the neo-tissue, do not show loss of structural anisotropy.

3.2 Mechanical behavior

The evaluated structural parameters were used to build a microscale model of the two scaffolds. The optimal parameters that characterize the mechanical behavior of the PCL-bisurea scaffold fibers $h_{1,s}$ and $h_{2,s}$ were estimated with reference to the biaxial mechanical behavior of the anisotropic scaffold (Table 3). The values were used to predict with good

accuracy the mechanical properties of the isotropic scaffold (Fig. 6).

The microstructural features of the scaffold and the collagen networks were used to build the computational model of the isotropic and anisotropic tissue-engineered constructs.

In the isotropic statically loaded construct, the model is able to capture a quasi-isotropic compaction (Fig. 7) and evaluates an optimized cell prestress associated to each collagen fiber of 116.84 kPa (Table 3). The optimized cell prestress was used to model compaction in the anisotropic construct. In the anisotropic static construct, compaction is bigger in the direction parallel to the scaffold fiber main orientation (Fig. 7).

Taking into account a top isotropic collagen layer, the simulation at 30 % equibiaxial strain of the isotropic scaffold after compaction describes correctly the isotropic mechanical properties of the construct. This allowed the estimation of the collagen material properties $h_{1,c}$ and $h_{2,c}$ (Table 3). With these parameters, the equibiaxial strain of the anisotropic construct and of the cyclically strained constructs could be predicted. All the constructs show an exponential-like stress-strain curve, and a higher stiffness than the bare scaffolds, implying the formation of tissue. The isotropic statically loaded construct shows isotropic mechanical properties, while the isotropic cyclically loaded construct shows a slightly higher stiffness in the direction of the applied stress, due to strain protection from degradation. All the simulations of constructs cultured in the presence of cyclic strain show a slight underestimation of the stiffness in the strain direction. The cyclically cultured anisotropic tissue-engineered construct with strain perpendicular to the scaffold fibers shows an isotropic mechanical behavior, while the predicted mechanical behavior is more anisotropic, probably due to a dominant orientation in the collagen top layer (Fig. 8).

All the engineered constructs cultured with an anisotropic scaffold show a decrease of the mechanical anisotropy of the initial scaffold and a more isotropic mechanical behavior. This is the consequence of the presence of a dense collagen layer with variable collagen orientation throughout the layer thickness above and below the scaffold. When the contribution of the top layer is neglected in the simulations, the constructs show structural and mechanical anisotropy (Fig. 9).

4 Discussion

The present study investigated the possibility of relating the structural properties of scaffold and engineered constructs to their mechanical behavior. For this, a mathematical model of collagen turnover was formulated. The model was implemented in a FE microscale model of a tissue-engineered construct, accounting for the contribution of the scaffold and of the collagen network. The parameters that define the mechan-

Fig. 5 Pictures of the top collagen layer throughout the thickness of the layer. On the *first row*, a statically cultured anisotropic tissue-engineered construct is represented on the *second row*, a construct cultured in cyclic

conditions with the strain perpendicular to the scaffold fiber direction is represented. The *scale bar* represents 50 μm

Table 4 Collagen volume fraction in the engineered constructs

Construct	Collagen volume fraction (%)
Isotropic static	3.9
Anisotropic static	6
Isotropic cyclic 10 %	5.5
Anisotropic cyclic 10 %	6.9

ical properties of PCL-bisurea scaffold fibers and collagen fibers were estimated. Two scaffolds, one isotropic and one anisotropic, were used to test the approach. The structural properties of the scaffolds and the total volume fraction of the collagen network were used to evaluate the collagen distribution after 2 weeks of culture under different loading conditions. The model was used to simulate the biaxial mechanics of engineered constructs cultured over isotropic and anisotropic scaffold in static and cyclic loading conditions. The formation of top layers of extracellular matrix with variable collagen distribution was taken into account in the simulations. Finally, the model was used to describe the mechanics of a tissue-engineered construct without any top layer. Its mechanical behavior resembles the anisotropy that characterizes cardiovascular tissues, as it is described by [Driessen et al. \(2007\)](#).

The presented model accounts for a number of phenomena that have been described in previous studies, such as

Fig. 6 Fit of the experimental equibiaxial mechanical behavior for the anisotropic scaffold (*left*). Predicted biaxial mechanical behavior of the isotropic scaffold (*right*) by using the optimal material parameters of the anisotropic scaffold

contact guidance by the scaffold fibers ([Niklason 2009](#); [de Jonge 2013](#)), enhanced collagen deposition due to scaffold anisotropy ([Lee 2005](#); [Teh et al. 2013](#)) or use of cyclic load ([Boerboom et al. 2008](#); [Rubbens 2009](#)) and load protection from enzymatic degradation ([Huang and Yannas 1977](#); [Wyatt et al. 2009](#)). It should be noticed that the described phenomena only occur when microfibrillar scaffolds are used. In contrast, cells seeded on nanofibrillar scaffolds have been shown to spread over more scaffold fibers, following the fibrous pattern and responding to the applied strain ([Eriskin 2013](#); [Subramony 2013](#)). In this case, a different response of the cells to contact guidance might also influence the distribution of the neo-formed collagen. At these conditions, the

Fig. 7 Compaction in the statically cultured isotropic and anisotropic engineered construct. In the anisotropic case, the y direction indicates the direction of main scaffold fiber orientation. The experimental data are averaged from two samples

model is able to predict collagen distributions in agreement with experimental findings (Niklasen 2009; de Jonge 2013).

For isotropic and anisotropic constructs with static and cyclic strain, the model successfully describes that the collagen mainly aligns along the scaffold fibers, accounting for the dominant effect of contact guidance. In addition, the model captures the phenomenon that extensional strain protects the collagen fibers against degradation.

However, the collagen layer that grows on top of the scaffold is, for simplicity, described as isotropic. The enhanced formation of the neo-tissue on top of the scaffold is likely due to proliferation of the cells. These cells might be characterized by a high synthetic activity, as they are not already surrounded (or inhibited) by synthetic fibers. (Figs. 4, 5). The developed model does not intend to describe the phenom-

Fig. 9 Predicted biaxial mechanical behavior of an anisotropic static tissue-engineered construct, when it is assumed that the entire collagen volume fraction estimated follows the rules for collagen turnover within the scaffold fibers

ena that drive the collagen turnover in absence of scaffold. Moreover, images through the thickness of the top layer do not show a clear trend in the collagen orientation. Therefore, in this layer, the complex load-driven phenomena that have been shown to drive collagen orientation in 3D constructs (Foolen et al. 2012) are not accounted for. For these reasons, the surface layers are approximately hypothesized to be isotropic. In particular, the lack of strain protection of collagen in this layer might be considered partly responsible for an underestimation of the mechanical performance in the strain direction in all the loaded constructs.

In the presence of a scaffold, contact guidance dominates collagen deposition. This might indicate that, in case of an aligned scaffold, the prevalence of collagen along the main direction of the scaffold fibers should increase the anisotropic mechanical properties of the constructs. Nevertheless, the constructs that are cultured on anisotropic scaffold substrates

Fig. 8 Fit of the experimental equibiaxial mechanical behavior of the isotropic static tissue engineered construct (a). The material parameters estimated in (a) are used to predict the equibiaxial mechanical behavior of tissue engineered constructs characterized by anisotropic scaffold

with static strain applied (b), isotropic scaffold with cyclic strain applied (c) and anisotropic scaffold with cyclic 10% strain applied parallel (d) and perpendicular (e) to scaffold fibers

show a lower anisotropy than their initially bare scaffold. By imaging the scaffold after culture, it was verified that the scaffold does not lose its structural integrity after 2 weeks of culture, thus losing the characteristic anisotropy. Furthermore, the amount of compaction was investigated to verify whether different compaction in the two straining direction might cause a different initial straining point. Nevertheless, the measured compaction, between 5 and 8 %, cannot explain alone the decrease of an initially pronounced anisotropy. Finally, the presence of the top layer with variable collagen orientation and thickness comparable to the scaffold thickness was observed. This was considered to be the main responsible factor for the poorly anisotropic response of the constructs.

In conclusion, the developed model can successfully describe the collagen distribution between the fibers of an electrospun scaffold. Furthermore, it can predict the mechanical properties of the engineered constructs resulting from scaffolds with different architectures and with different loading conditions applied. The dominance of contact guidance over strain suggests that the mechanical anisotropy that characterizes cardiovascular tissue can be mimicked when an anisotropic scaffold is used. However, it is necessary to verify that cells penetrate well into the scaffold and avoid the formation of superficial collagen layers, whose architecture might decrease the mechanical anisotropy of the construct.

Acknowledgments This research forms part of the Project P1.01 iValve of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs. The financial contribution of the Nederlandse Hartstichting is gratefully acknowledged. The authors gratefully thank Marc Simonet for the electrospinning of the scaffolds.

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