

Engineering Multiscale, Biomimetic Scaffolds Based on Protein-Based Materials as
Advanced Cardiovascular Implants

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To my amazing parents

List of publications

Dissertation

El Maachi I., Kyriakou S., Rütten S., Kopp A., Köpf M., Jockenhoevel S., Fernández-Colino A., Silk Fibroin as Adjuvant in the Fabrication of Mechanically Stable Fibrin Biocomposites. *Polymers*. 2022; 14(11):2251.

El Maachi I., Loewen A., Acosta S., Rütten S., Rodríguez-Cabello J. C., Jockenhoevel S., Fernández-Colino A., Protein-Engineered Elastin Fibers as Building Blocks for The TextileBased Assembly of Tissue Equivalents. *Adv. Funct. Mater.* 2024, 34, 2313204.

Others

Kyriakou S., Acosta S., El Maachi I., Rütten S., Jockenhoevel S., A DexamethasoneLoaded Polymeric Electrospun Construct as a Tubular Cardiovascular Implant. *Polymers*. 2023; 15(21):4332.

Sallustio F., Schmidt A., El Maachi I., Greenhalgh A., Frydrych M., Jockenhoevel S., Rodríguez-Cabello J. C., Fernández-Colino A., Adaptive Elastin-Like Hydrogel with Unidirectional and Controllable Anisotropic Actuation for Soft Robotics and Tissue Engineering. *Adv. Mater. Technol.* 2025, e00680.

Article

Silk Fibroin as Adjuvant in the Fabrication of Mechanically Stable Fibrin Biocomposites

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Abstract: Fibrin is a very attractive material for the development of tissue-engineered scaffolds due to its exceptional bioactivity, versatility in the fabrication, affinity to cell mediators; and the possibility to isolate it from blood plasma, making it autologous. However, fibrin application is greatly limited due to its low mechanical properties, fast degradation, and strong contraction in the presence of cells. In this study, we present a new strategy to overcome these drawbacks by combining it with another natural polymer: silk fibroin. Specifically, we fabricated biocomposites of fibrin (5 mg/mL) and silk fibroin (0.1, 0.5 and 1% w/w) by using a dual injection system, followed by ethanol annealing. The shear elastic modulus increased from 23 ± 5 Pa from fibrin alone, to 67 ± 22 Pa for fibrin/silk fibroin 0.1%, 241 ± 67 Pa for fibrin/silk fibroin 0.5% and 456 ± 32 Pa for fibrin/silk fibroin 1%. After culturing for 27 days with strong contractile cells (primary human arterial smooth muscle cells), fibrin/silk fibroin 0.5% and fibrin/silk fibroin 1% featured minimal cell-mediated contraction (ca. 15 and 5% respectively) in contrast with the large surface loss of the pure fibrin scaffolds (ca. 95%). Additionally, the composites enabled the formation of a proper endothelial cell layer after culturing with human primary endothelial cells under standard culture conditions. Overall, the fibrin/silk fibroin composites, manufactured within this study by a simple and scalable biofabrication approach, offer a promising avenue to boost the applicability of fibrin in tissue engineering.

Keywords: protein-based polymers; scaffolds; cell-adhesion; bioprocessing; mechanical stability; tissue engineering

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1. Introduction

Tissue engineering (TE) aims to obtain biological substitutes that can restore the functionality of native tissue in vivo. This discipline of biomedical engineering pursues the development of constructs able to interact with cells and to evolve to a native-like tissue, either in vitro (classical TE approach) or in vivo (in situ TE approach). Therefore, the development of advanced materials, capable of mimicking the native extracellular matrix (ECM) and/or promote new tissue formation, is pivotal for the progress of TE [1].

In practice, a single material can hardly ever meet all the requirements needed for full functionality as the target tissue. Indeed, the outstanding performance of many natural systems (including the native ECM) [2–4] responds to a unique combination of materials with complementary or even contradictory properties. This composite strategy, followed by nature [5], should be further exploited in materials science in order to enable synergy

between the different components and to consequently match the requirements for tissue regeneration [6].

Fibrin is described in many studies as an ideal biomaterial for many biomedical applications, owing to its bioactivity, versatility and processability [7,8]. Indeed, the use of fibrin as a biomaterial has been expanded from tissue sealants [9] to a myriad of TE and drug delivery applications [10], including skin wound healing [11], reconstructive maxillofacial and dental surgeries [12], eye surgery [13], cartilage regeneration [14], orthopaedic surgery [15] and cardiovascular implant development [16,17], among others. Fibrin can be isolated directly from the patients' plasma and processed into a scaffold [18]. Therefore, a scaffold made of the patient fibrin and cultivated with the same patient cells, will provide a total autologous construct, which removes any immunological or by-product issues. While blood extraction from the patient may not always be possible (e.g., due to severe injuries or emergencies), fibrin from non-autologous sources have demonstrated great potential. Indeed, commercial fibrin sealants are homologous, i.e., they contain human blood components from either pooled plasma or individual units of plasma. In order to reduce the production costs and increase the availability, heterologous forms of fibrin are emerging as a promising approach [19], and indeed some formulations have reach clinical trials [20].

Fibrin displays a prominent bioactivity, due to the presence of the arginine-glycineaspartic (RGD) sequence in the fibrin network [21]. The RGD motifs together with the cell integrins, create a recognition system for many cell types in the human body, and additionally, they support the attachment of growth factors and essential molecules to the fibrin substrate [22,23]. Additionally, fibrin degradation products are also acknowledged as important modulators of wound healing [24,25].

Despite these advantages, the potential of fibrin in TE is mainly limited by mainly two factors: (i) cell-mediated scaffold contraction, and (ii) insufficient mechanical strength to withstand physiological forces, e.g., hemodynamic ones [26]. Therefore, the identification of a biomaterial able of enhancing the mechanical behavior of fibrin, while still preserving its excellent biological properties, can make an outstanding contribution to the field of TE. In this regard, we hypothesize that blending fibrin with silk fibroin, as natural polymer with superior mechanical strength, could be a promising approach. Silk fibroin is an extraordinary versatile natural material that can be used in a plethora of biomedical applications [27]. Indeed, silk fibroin has been clinically used for decades as a suture line, and there are many examples of silk fibroin-based medical devices approved by the FDA [28–30].

Currently, only a few studies have explored the blending of silk fibroin with fibrin. One study combined fibrin gels with silk fibroin by coating the silk fibroin's porous scaffolds after seeding the cells in order to support the formation of capillary structures [30]. In that approach, the fibrin was used merely as a coating for the silk fibroin scaffold, and therefore, it was not fully integrated in the whole scaffold structure. Another study combined fibrin with a silk fibroin network through an interpenetrating polymeric network (IPN) architecture [31]. The IPN architecture was synthesized with a double enzymatic method, which consisted of the use of thrombin enzyme for the gelation of the fibrin, and horseradish peroxide for the silk fibroin gelation. The study showed promising results regarding the fibrin and silk fibroin blend. However, the method adopted in that study carries numerous disadvantages. The double enzymatic method is an expensive approach, which therefore limits the scalability and consequently the industrialization of these scaffolds. A further limitation is given by the slow gelation using the enzymatic treatment. Although a faster gelation of the silk fibroin can be achieved by increasing the concentration of the horseradish peroxide, a high concentration of this enzyme may be cytotoxic [32]. Additionally, the cellular study was limited to a single cellular type, fibroblasts, which are well-known to be more resilient than other cell types (e.g., endothelial cells).

In this study, we propose a strategy to fabricate composite fibrin/silk fibroin scaffolds in simple and reproducible way. Our motivation was to investigate if a proper balance of mechanical

properties and cell adhesion could be achieved and consequently, if the composite scaffolds could exceed the fibrin ones for their application in TE.

2. Materials and Methods

2.1. Fibrin and Silk Fibroin Preparation

Lyophilized human fibrinogen (Human plasma, Calbiochem, San Diego, CA, USA) was dissolved in purified water (Sartorius, Goettingen, Germany) and dialyzed against Tris-buffered saline (TBS) overnight using Spectra/Por 1 tubing (Fisher Scientific, Schwerte, Germany) with a molecular weight cut-off of 6000–8000 Da. Fibrinogen concentration following sterile filtration was determined by measuring absorbance at 280 nm using a M200 spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland). Fibrinogen was diluted in TBS to a final concentration of 10 mg/mL and 20 mg/mL. The thrombin solution was made by mixing 15% (v/v) of thrombin (bovine plasma, Sigma Aldrich, Saint Louis, MO, USA) at 40 U/mL, 15% (v/v) of Calcium chloride (CaCl₂, Sigma-Aldrich, Saint Louis, MO, USA) at 50 mM and 70% (v/v) of TBS. The TBS buffer was prepared by dissolving Tris HCl, Tris base, NaCl and KCl in purified water and adjusting the pH to 7.4.

Silk fibroin solution was provided by Fibrothelium GmbH and produced according to the PureSilk® technology established by the company. Upon receipt, it was diluted in ultrapure water at a concentration of 2%, 1% and 0.2% (w/v).

2.2. Fabrication of Composite Scaffolds

A double dual injection system was adopted to fabricate the composite scaffolds. The system consisted of syringes containing the fibrinogen, thrombin and silk fibroin solutions placed in a Duploject double syringe holders (Tisseel, Baxter, Deerfield, IL, USA), fitted into the dual injectors and connected to a 3-way connector. To combine the solutions homogeneously, a mixing nozzle (DMG, Hamburg, Germany) was inserted at the end of the system.

The solutions were injected in the mold with the desired shape and left for 20 min to allow the complete polymerization of the fibrin. The samples were then immersed in 100% ethanol (Sigma-Aldrich, Saint Louis, MO, USA) for 2 h in order to promote the β -sheets' formation and, therefore, the crosslinking of silk fibroin. Afterwards, the samples were washed three times with PBS (Thermo Fisher, Waltham, MA, USA) to avoid any ethanol residues. The ratio and the nomenclature of the conditions and controls used in the experiments are stated in Table 1.

Table 1. Different conditions used for the fabrication of the scaffolds.

Nomenclature	Ethanol Bath	[Fibrin]	[Silk Fibroin]
Fib5_NT	✗	5 mg/mL	/
Fib5	✓	5 mg/mL	/
Fib5-Silk 0.1%	✓	5 mg/mL	0.1%
Fib5-Silk 0.5%	✓	5 mg/mL	0.5%
Fib5-Silk 1%	✓	5 mg/mL	1%

2.3. Scanning Electron Microscopy (SEM)

SEM (ESEM XL 30 FEG, Eindhoven, Netherlands) was used to investigate the surface and the internal morphology of the composite scaffolds.

The samples were subjected to freeze-drying in order to make them suitable for SEM visualization. Specifically, the applied process involved three steps: (i) immersion in liquid nitrogen for a few seconds, (ii) fracturing (while immersed) to visualize later on the internal structure and (iii) lyophilization. The scaffolds were lyophilized in an Alpha 2-4-LSCplus freeze dryer (Christ, Osterode am Harz, Germany) at -20°C for the first 12 h, and at 20°C for another 12 h for the final drying.

After lyophilization, the scaffolds were placed in a wellplate, vacuumed and moved to the Electron Microscopy Facility (Institute of Pathology, RWTH Aachen University Hospital, Aachen, Germany). The samples were carefully mounted on an aluminium stub and sputter-coated with 12.5 nm Pd-Au alloy (EM SCD500, Leica, Wentzler, Germany), and then visualized in high vacuum mode.

2.4. Rheological Characterization

For the rheological characterization, scaffolds were fabricated with the composition as stated in Table 1 and with a diameter of 20 mm. To observe the influence of the annealing of silk fibroin within the composites, the composite scaffolds (i.e., Fib5-Silk 0.1%, Fib-Silk 0.5% and Fib5-Silk 1%) without ethanol treatment were also measured.

The scaffolds were measured in the Malvern Kinexus ultraplus rheometer (Malvern, Worcestershire, UK), using a geometry of 20 mm in diameter. The plate was heated at 37 °C and a water trap was placed to avoid the dehydration of the samples. The gap was adjusted to a minimum normal force of 0.01 N to avoid the slippage of the sample. A strain sweep oscillation test was performed on the scaffolds to identify the linear viscoelastic regions (LVE) of the samples. At a fixed frequency of 1 Hz, the storage modulus (G') and the loss modulus (G'') were measured as a function of the shear strain with an amplitude ranging from 0.01 to 100%. Five samples were measured per condition, and the data were expressed as mean and the corresponding standard deviation (SD) of the five replicas.

2.5. Cell Culture Study

2.5.1. Culture of Human Umbilical Vein Endothelial Cells (HUVECs) and Human Umbilical Arterial Smooth Muscle Cells (HUASMCs)

Endothelial cells (HUVECs) and smooth muscle cells (HUASMCs) were isolated from human umbilical cord [33], as previously described [34]. Human umbilical cords were obtained after written consent at the University Hospital Aachen, Aachen, Germany, and were provided by the RWTH Aachen University Centralized Biomaterial Bank (cBMB) according to its regulations, following RWTH Aachen University, Medical Faculty Ethics Committee approval (cBMB project number 323). HUVECs were cultured on 2% gelatine (Sigma-Aldrich, Saint Louis, MO, USA) coated flasks in an incubator at 37 °C with 5% CO₂ and fed with EGM (PromoCell, Heidelberg, Germany). The following supplements were added to the media: basic Fibroblast Growth Factor, Insulin-like Growth Factor, Vascular Endothelial Growth Factor 165, Ascorbic Acid, Heparin and Hydrocortisone. Conversely, HUASMCs were cultured in cell culture flasks with Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher, Waltham, MA, USA) supplemented with FCS and 1% (v/v) ABM, in an incubator of 37 °C and 5% CO₂.

The media of the two cell types were changed every two days and at confluence the cells were trypsinized and centrifuged at 500 g for 5 min. The resulting cell pellets were suspended within the respective media at the desired cell concentration.

2.5.2. Seeding of HUVECs and Evaluation of Cell Adhesion

The composite scaffolds of silk fibroin and fibrin were produced as described in Section 2.2, with three replicas for each condition. Additionally, scaffolds containing only fibrin or containing only silk fibroin were included in the assay as controls.

Two experimental groups were established, i.e., (i) scaffolds pre-treated with Bovine Serum Albumin (BSA, Sigma-Aldrich, Saint Louis, MO, USA) at 10 mg/mL in PBS for 2 h and rinsed three times with PBS, and (ii) scaffolds without BSA treatment (Figure 1a,b). BSA is as a blocking agent, known to impede any unspecific adhesion to the scaffolds [35]. In both cases, HUVECs suspended in media without FCS were added to the samples at a concentration of 1.26×10^5 cells/cm², and incubated at 37 °C with 5% CO₂ for 2 h.

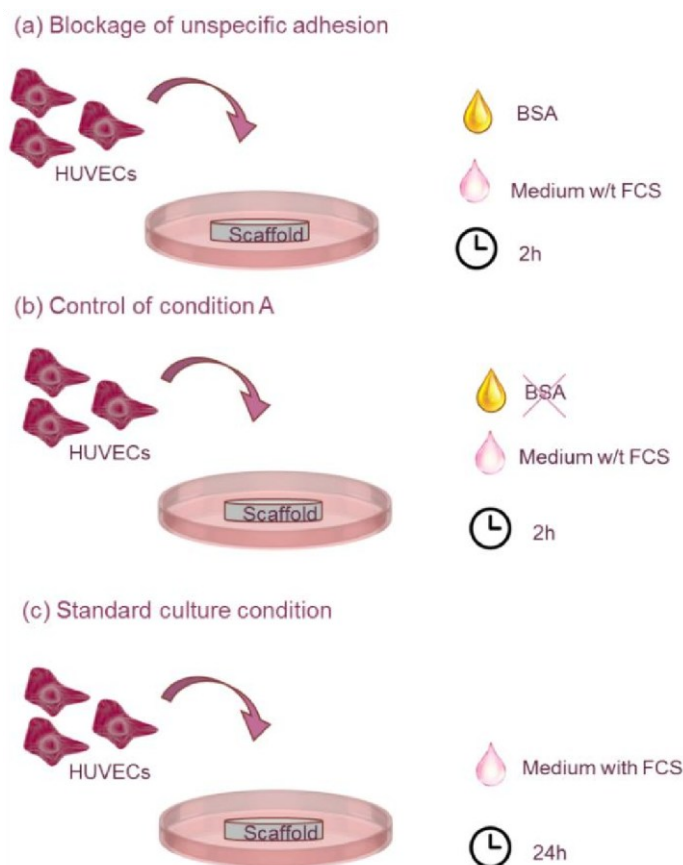


Figure 1. Schematic representation of the experimental groups assessed for adhesion of human umbilical vein endothelial cells (HUVECs) to the composite scaffolds. **(a)** HUVECs suspended in media without FCS and seeded on BSA-treated scaffolds. **(b)** HUVECs suspended in media without FCS and seeded on scaffolds (non BSA-treated). **(c)** HUVECs suspended in media with FCS and seeded on scaffolds without BSA pre-treatment.

Additionally, a third set of scaffolds (non-treated with BSA) was cultured for 24 h with cells suspended in FCS-containing media in order to assess the capability of the scaffolds to support the cell attachment under standard culture conditions (Figure 1c). Each experimental group contained 3 replicas of each type of scaffolds.

2.5.3. Seeding of HUASMCs and Evaluation of Scaffold Contraction

Scaffolds with a disk-shape of 2 cm diameter and 2 mm thickness were prepared as described in Section 2.2, transferred to a 12-wellplate and imaged with a digital camera in order to accurately evaluate the surface at time 0 (before seeding). Afterwards, HUASMC suspension at 1 million cells/mL was seeded on the different scaffolds. Additionally, fibrin scaffolds at 10 mg/mL were included in these experiments, in order to determine whether an increase in the material concentration makes an impact in the stability or contraction of the scaffold. Four replicas of each condition were seeded with cells, and three additional ones were kept unseeded in order to discern whether the contraction of the scaffolds was cell-mediated.

After 24 h of culture, one replica of each condition of the seeded scaffolds was fixed with paraformaldehyde (Carl Roth, Germany) (Section 2.5.4), stained with phalloidin/DAPI and visualized in the confocal microscope. The rest of the scaffolds ($n = 3$ per condition) were cultured for 27 days. Every second day, the medium was changed, and pictures of the scaffolds were acquired in order to monitor changes in the surface, if any. To this end, the images were collected, and then analysed with the software ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.5.4. Fixation and Staining of the Cell-Seeded Scaffolds

After the respective incubation times, the cell-seeded scaffolds were washed one time with PBS and then fixated in 4% paraformaldehyde (methanol-free) diluted in PBS, for 1 h at room temperature. Subsequently, they were rinsed in PBS three times and stored in PBS containing antibiotics/antimycotic (ABM) (Pan Biotech, Aidenbach, Germany) at 4 °C, until staining.

The samples, prior to staining, were permeabilized with the washing buffer (PBS with 0.1% Triton X-100, Sigma-Aldrich, Saint Louis, MO, USA) for 5 min and rinsed three times in PBS. Phalloidin-iFluor 488 conjugate (Cayman chemical, Ann Arbor, MI, USA) with a 1000× dilution in 1% (w/v) BSA in PBS, was added to the samples to stain the actin filaments in the cell's cytoskeleton, with an incubation time of 90 min at room temperature. The samples were rinsed three times in PBS and the nuclei were stained with DAPI (Carl Roth, Karlsruhe, Germany) for 15 min and then washed again in PBS for three times.

2.5.5. Confocal Microscopy Evaluation

The samples were imaged with an inverted Zeiss LSM710 laser scanning confocal commercial microscope at the Confocal Microscopy Facility (IZKF, RWTH Aachen University Hospital, Aachen, Germany). The images were taken with both EC Plan-Neofluar 10× and Plan Apochromat 20× objectives. Images were acquired sequentially by line scanning unidirectionally and saved as czi files. For the visualization of DAPI staining, the samples were excited with a 405 nm laser line, and emission was collected at 410–495 nm, while, for the visualization of the actin-iFluor 488-staining, the excitation was performed with a 488 nm laser line, and emission was collected at 495–630 nm. The software Zen black 2012 (Zeiss, Jena, Germany) was used for image acquisition and ImageJ was used to count the number of the cells.

2.6. Fabrication of Tubular Scaffolds

The silk fibroin and fibrin components were bioprocessed in a similar way, as described in Section 2.2, but this time, they were injected into a custom-made tubular mold. Specifically, the mold had a 1 mm of wall thickness, a 5 cm of length and a diameter of 1.2 cm.

2.7. Burst Strength

The burst strength was measured with an in-house device, which consists of a peristaltic pump (IPC Ismatec, IDEX Health & Science, Carlsbad, CA, USA) that pumps PBS with a rate of 7.5 mL/min inside of a chamber where the samples are positioned. The pressure sensor (Jumo Midas pressure transmitter, JUMO, Fulda, Germany) attached to the chamber, measures the pressure that the PBS applies on the sample. The peak of the pressure measured, at which the samples rupture, is the burst pressure. Since the burst pressure is directly correlated to the thickness of the sample, the scaffolds were prepared in a way to attain a reproducible and equal thickness. i.e., as stated in Section 2.6. The tubular samples were cut longitudinally, and the resulting sheets were laid down on the device chamber. Three measurements were taken for each condition, and the peak of the pressure was noted.

The recorded data are expressed as mean ± SD.

2.8. Statistical Analysis

All data were represented as mean ± SD from at least three experiments. Statistical analysis of the rheological measurements and the HUVECs adhesion tests results were performed with two-way analysis of variance (ANOVA) with a Holm-Šidák test. A one-way ANOVA (Holm-Šidák) was employed in the case of the burst pressure results data. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Fabrication of the Scaffolds

Fibrin/silk fibroin composites with different silk fibroin concentrations (0.1%, 0.5% and

1% w/w) were successfully fabricated by using a dual double injector system (Figure 2a). By increasing the silk fibroin content, a better processing and less deformation on the samples were observed, with an associated change in colour, from an almost transparent behavior of the pure fibrin scaffolds to a whiter (and less transparent) colour on the composites (i.e., fibrin + silk fibroin) (Figure 2b).

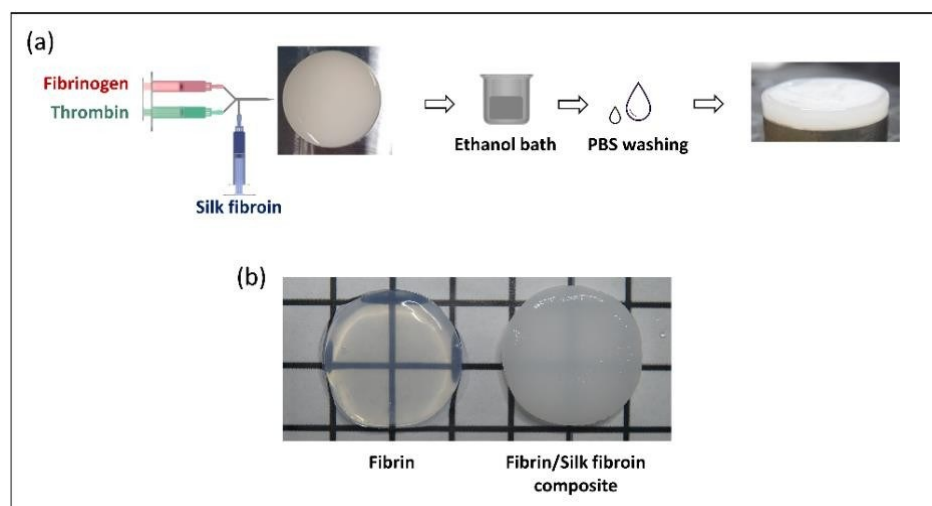


Figure 2. (a) Schematic representation of the fabrication process of the composite scaffolds. (b) Macroscopic picture of a pure fibrin scaffold and a fibrin/silk fibroin scaffold.

The surface and the inner morphology of the samples were characterized by SEM. All the scaffolds presented an open porous structure (Figure 3), and the presence of the silk fibroin in the composites did not hamper the consecution of a sponge-like internal structure.

3.2. Rheological Characterization

Rheological experiments were carried out to evaluate the mechanical behavior of the composite scaffolds and to elucidate (i) whether the incorporation of silk fibroin has an impact on the mechanical performance of the scaffolds and (ii) whether the silk fibroin annealing state affects the mechanical behavior of such composites.

All the samples featured a stable elastic modulus (G^0) regardless of the shear strain for the range between 0.01% and 8% (Figure 4a), which was therefore established as the linear viscoelastic region (LVR) of the scaffolds. For shear strain values higher than 8%, the elastic moduli experienced an evident decrease, especially evident for those samples containing higher percentages of silk fibroin.

For the non-treated scaffolds (i.e., before the crosslinking of the silk fibroin with ethanol; Figure 4b-blue columns), no correlation was observed between the amount of silk fibroin in the composite and the mechanical properties, with all the samples featuring moduli below 100 Pa (Figure 4b-blue columns). This drastically changed once the scaffolds were treated with ethanol (Figure 4b-red columns). In that case, the elastic moduli experienced a clear increase by increasing the silk fibroin content in the scaffold ($p < 0.001$), from a modulus of 67 ± 22 Pa for Fib5-Silk 0.1%, to 241 ± 67 Pa for Fib5-Silk 0.5%, and up to 456 ± 32 Pa for Fib5-Silk 1%. Additionally, the G of the treated scaffolds (Figure 4b-red columns) was significantly higher than for the non-treated counterparts (Figure 4b-blue columns) for Fib5-Silk 0.5% and for Fib5-Silk 1% ($p < 0.001$). Similarly, the ethanol treatment also elicited an increase in the G^{00} values of scaffolds Fib5-Silk 0.5% and Fib5-Silk 1% (Figure 4c).

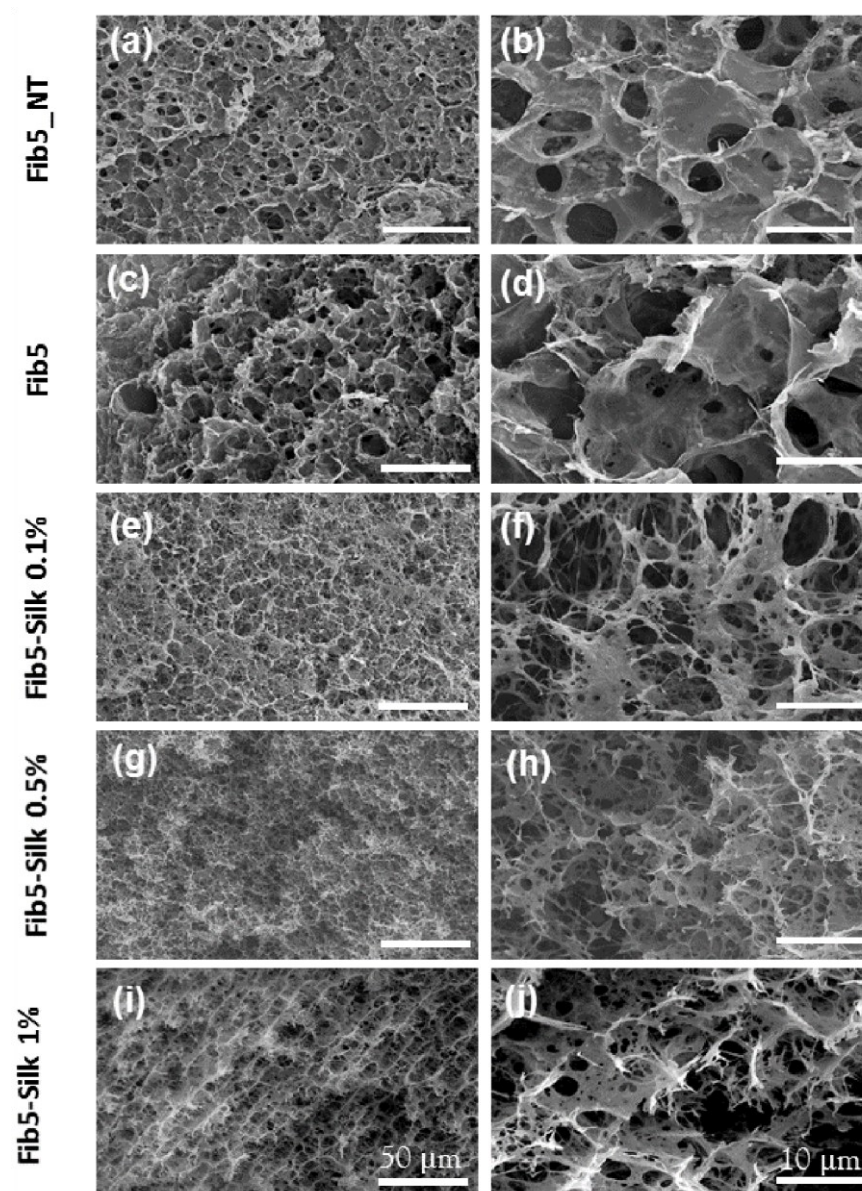


Figure 3. Scanning electron microscopy (SEM) images of fibrin and fibrin/silk fibroin scaffolds. (a,b) Fibrin scaffold at 5 mg/mL (without ethanol treatment). (c,d) Fibrin at 5 mg/mL with ethanol treatment. (e,f) Composite of fibrin (5 mg/mL) and silk fibroin (0.1% w/w). (g,h) Composite of fibrin (5 mg/mL) and silk fibroin (0.5% w/w). (i,j) Composite of fibrin (5 mg/mL) and silk fibroin (1% w/w).

To determine if the scaffolds behave in a more elastic way than in a viscous way, or *vice versa*, the elastic moduli values (G^0) were compared with the viscous ones (G^{00}), as represented in Table 2. The loss factor ($\tan \delta$), that is, the ratio between G^{00} and G^0 , was lower than 0.1 for all the tested scaffolds (see Table 2). In other words, the elastic moduli were more than 10 times higher than the viscous moduli, which indicates a predominantly elastic behavior of the scaffolds.

Table 2. Values of the delta tangent ($\tan \delta = G^{00}/G^0$) for the ethanol-treated scaffolds, expressed as mean $\pm$ SD.

Scaffold	Fib5	Fib5-Silk 0.1%	Fib5-Silk 0.5%	Fib5-Silk 1%
$\tan \delta$	0.078 ± 0.010	0.080 ± 0.003	0.078 ± 0.003	0.074 ± 0.007

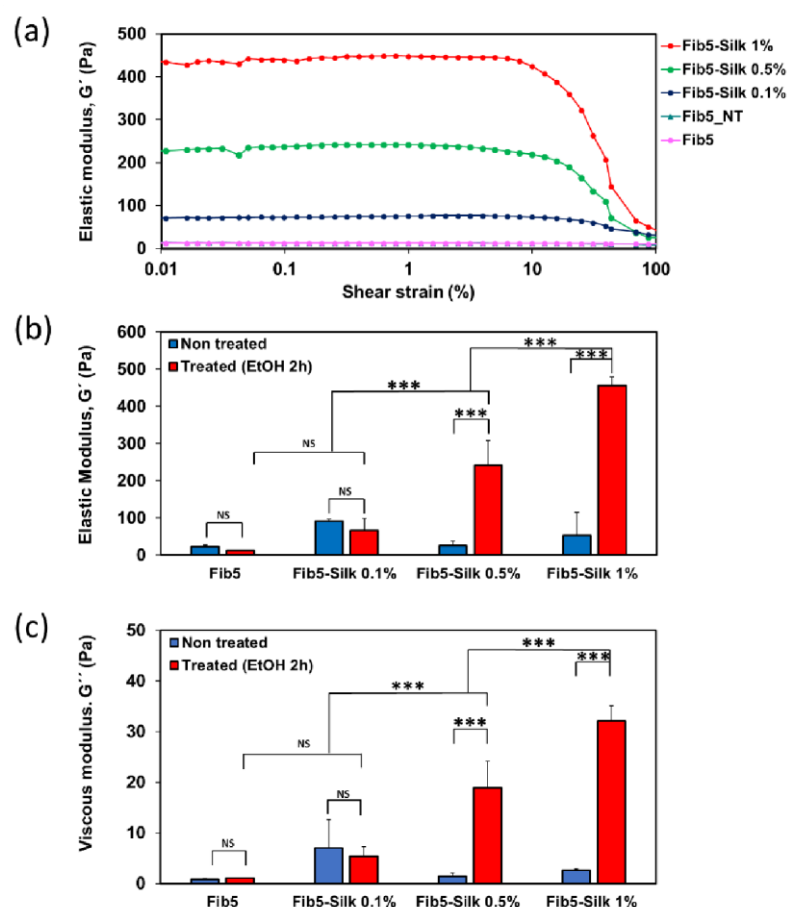


Figure 4. Rheological characterization of fibrin and fibrin/ silk fibroin scaffolds. (a) Dependence of the elastic moduli with the shear strain at 1 Hz. A representative replica for each scaffold is shown. (b) G' and (c) G'' measurements at 0.4% of shear strain and 1 Hz of frequency. Data is expressed as mean $\pm$ SD (***) $p < 0.001$, NS: not significant).

3.3. Cell Adhesion of HUVECs to the Composite Scaffolds

Cell adhesion tests were performed in order to verify if the bioactivity of the fibrin was maintained while combined with the silk fibroin. In order to discern the nature of the cell attachment (i.e., specific or unspecific), two sets of scaffolds were prepared (i) pre-treated with BSA (to block further unspecific cell adhesion) and (ii) non-pre-treated with BSA. In both cases, HUVECs were seeded on top of the scaffolds with media without FCS.

The pure fibrin scaffolds showed a prominent number of cells even when pre-treated with BSA (Figure 5a). In contrast, the composite scaffolds (fibrin + silk fibroin) pre-treated with BSA featured a significant reduction in the number of attached cells, and specifically the composites containing the higher amount of silk fibroin (Fib5-Silk 1%) seemed to contain the lowest number of cells attached (Figure 5a). Almost no cells were observed in the scaffolds Silk 10%, and the small number of cells visualized seemed to be entrapped in the cloud-like structure of the silk fibroin.

The quantitative evaluation of these observations corroborated that the cell number decreased with an increase in the silk fibroin concentration (Figure 5b). This can be explained by the lack of the RGD motif on the sequence of silk fibroin, which is key for specific cell adhesion on the substrate. On the other hand, all the composite scaffolds non-coated with BSA succeeded in supporting HUVECs attachment (Figure 5b-blue bars), and no statistical differences were detected between fibrin and fibrin + silk fibroin under these conditions ($p > 0.05$). Regarding the morphology, the cells presented a predominantly round shape on the composite scaffolds, in

contrast to the extended and cobblestone shape adopted when cultured on the fibrin scaffolds (higher magnification images in Figure 5a).

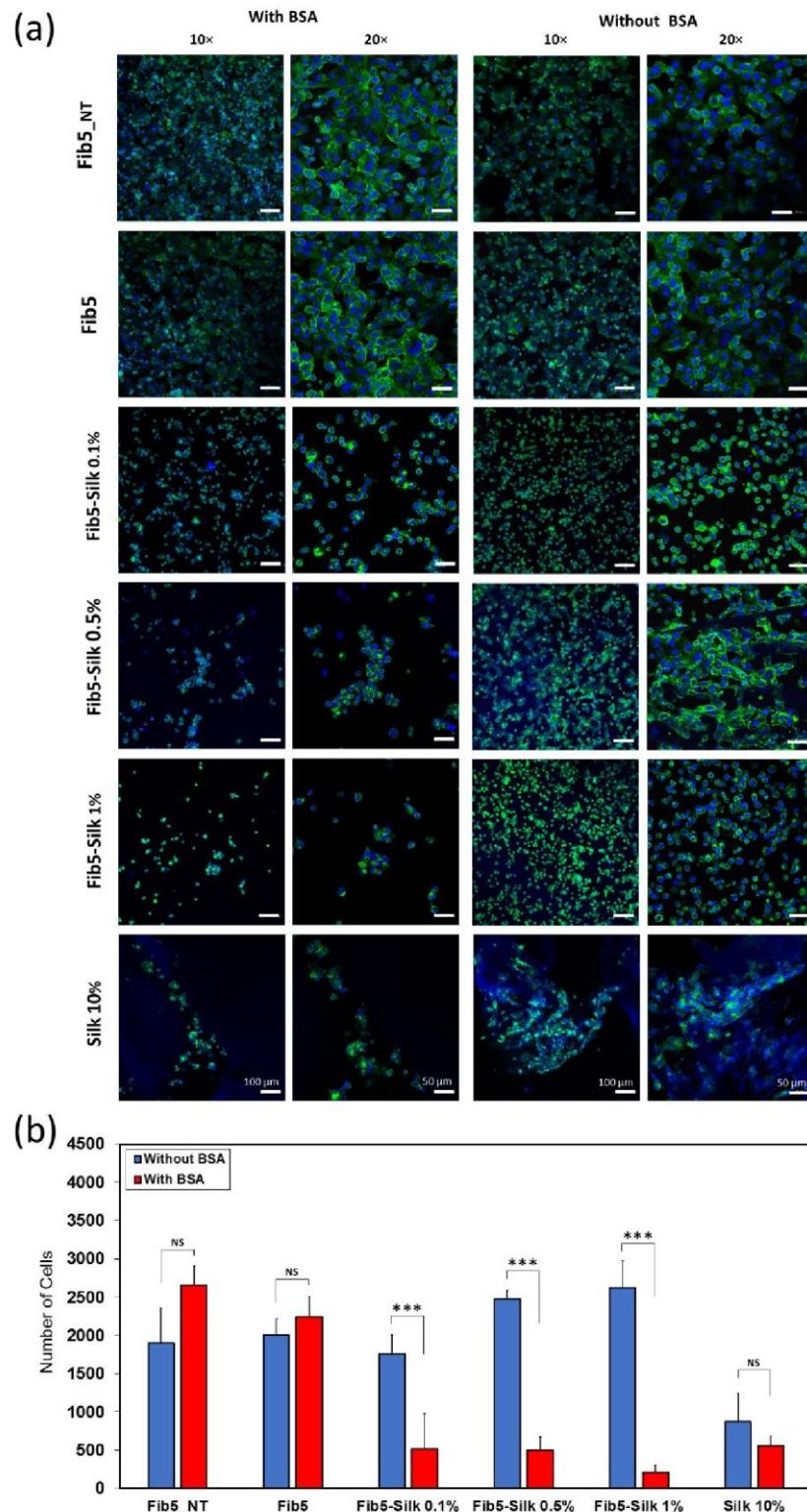


Figure 5. (a) Confocal images of the scaffolds pre-treated with bovine serum albumin (BSA) (first and second column) and without BSA pre-treatment (third and fourth columns), after 2 h in culture with HUVECs. The actin filaments in the cell cytoskeleton are stained with phalloidin (in green) and the nuclei with DAPI (in blue). (b) Number of HUVECs adhered in 840 μm^2 surface of the different types of scaffolds after 2 h. Data

reported as mean $\pm$ SD. For the sake of clarity only the statistical comparison between each BSA-treated scaffold with its counterpart non-treated one is included in the graph. (** $p < 0.001$, NS: not significant).

To check the ability of the scaffolds to support cell attachment under standard culture conditions, HUVECs were also cultured on the scaffolds for 24 h, with medium containing FCS. The cells appeared confluent and with a cobblestone shape in Fib5-Silk 0.1% samples, and less but still confluent in the Fib5-Silk 0.5% and Fib5-Silk 1% scaffolds (Figure 6). The cells displayed a spread and elongated morphology, even for the higher concentration of silk fibroin (Fib5-Silk 1%), which suggests a well cell attachment on the surface. In contrast, the scaffolds Silk 10%, after 24 h of culture, did not show any change concerning the cell shape, which was still round.

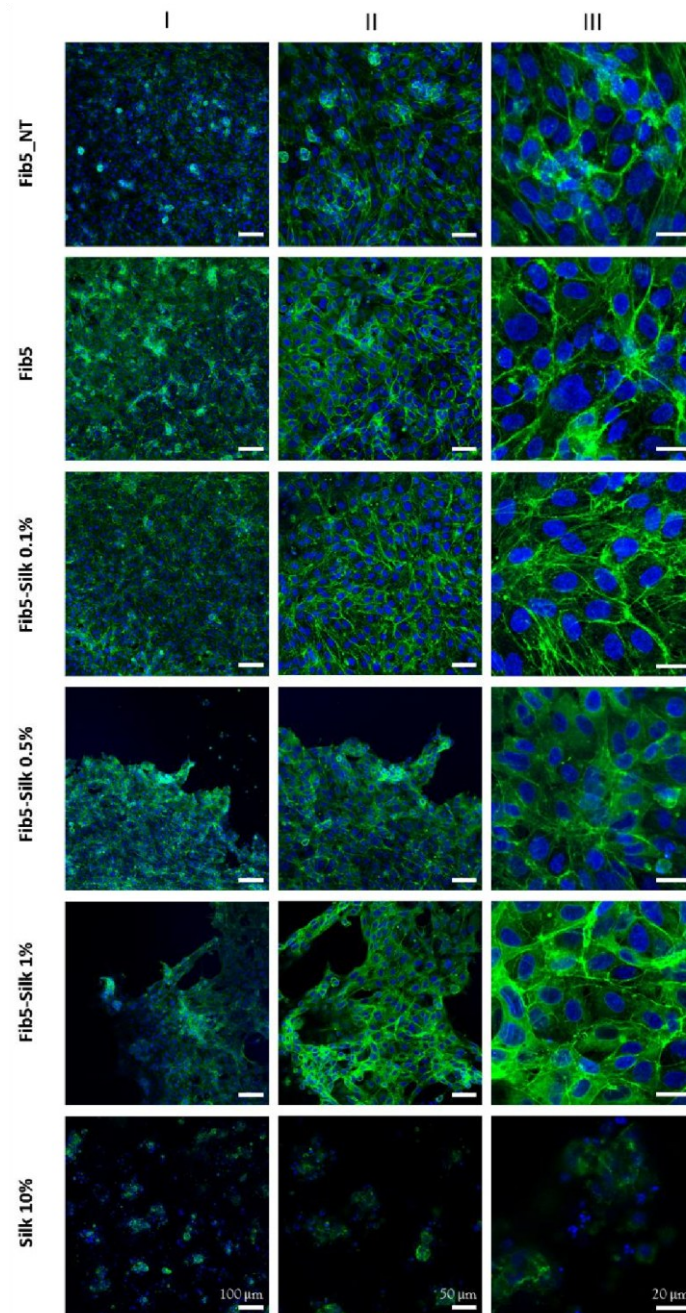


Figure 6. Confocal images of the scaffolds after seeding and culture with HUVECs under standard conditions. The actin filaments in the cell cytoskeleton are stained with phalloidin (in green) and the nuclei with DAPI (in blue). The left column (I) shows an overview, the middle column (II) represents the scaffolds at higher magnification, and the right column (III) shows a closer view of the morphology of the cells.

3.4. Evaluation of the Cell-Mediated Scaffold Contraction

After culturing with HUASMCs, Fib5_NT scaffolds experienced the highest surface contraction over time (Figure 7a), and after 27 days, their size was only 5% of their original one (Figure 7b). In other words, they exhibited a surface contraction of $94.5 \pm 0.4\%$, in relation to their original area. Fib10_NT also experienced a prominent contraction of $77 \pm 8\%$. On the other side, Fib5-Silk 1% and Fib5-Silk 0.5% showed a minimal surface reduction of about 5 and 15%, respectively. Fib5-Silk 0.1% was subjected to a drop in surface of $66 \pm 8\%$.

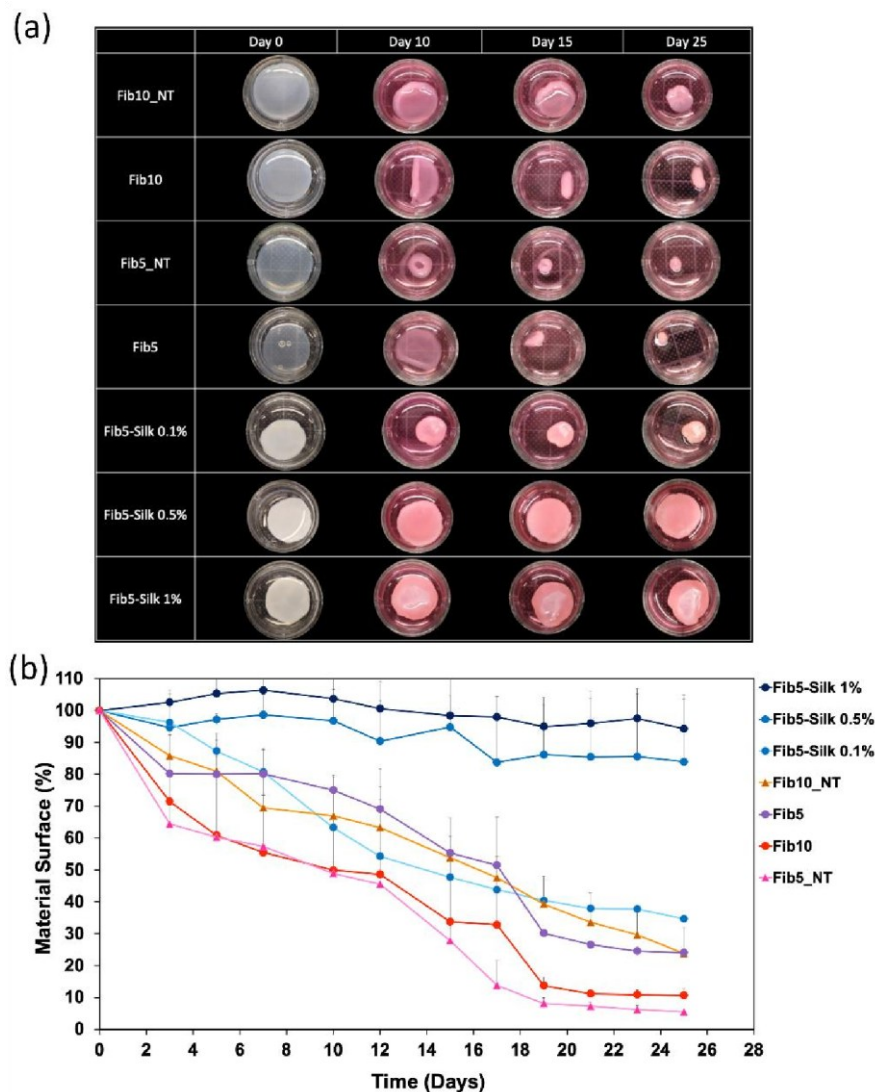


Figure 7. Evaluation of scaffold cell-mediated contraction over time. (a) Image of each scaffold before culture (time 0) and at different time points (10, 15 and 25 days) of culture with human arterial smooth muscle cells (HUASMCs). (b) Evaluation of cell-induced surface contraction of the fibrin/ silk fibroin scaffolds, at different time points of cell culture with HUASMCs at 37°C and $5\%\text{CO}_2$. The data are expressed as percentage of the initial surface. The data are reported as mean $\pm$ SD ($n = 3$).

To investigate if the cells were indeed attached to the composite scaffolds and they are applying their contractile force on them, the samples were stained at two different time-points, i.e., after 1 day and after the end of the culture (at day 27). Indeed, after 1 day of culture, the cells were spread and confluent in all the conditions tested (Figure 8), while at day 27, the number of cells visibly decreased in the controls (i.e., pure fibrin scaffolds), but not in the composites scaffolds. The limited adhesion of the cells to the fibrin scaffolds at this longer time-point could be

related to the acute retraction, which can affect the effective support of the cells and interfere with an adequate mechano-transduction.

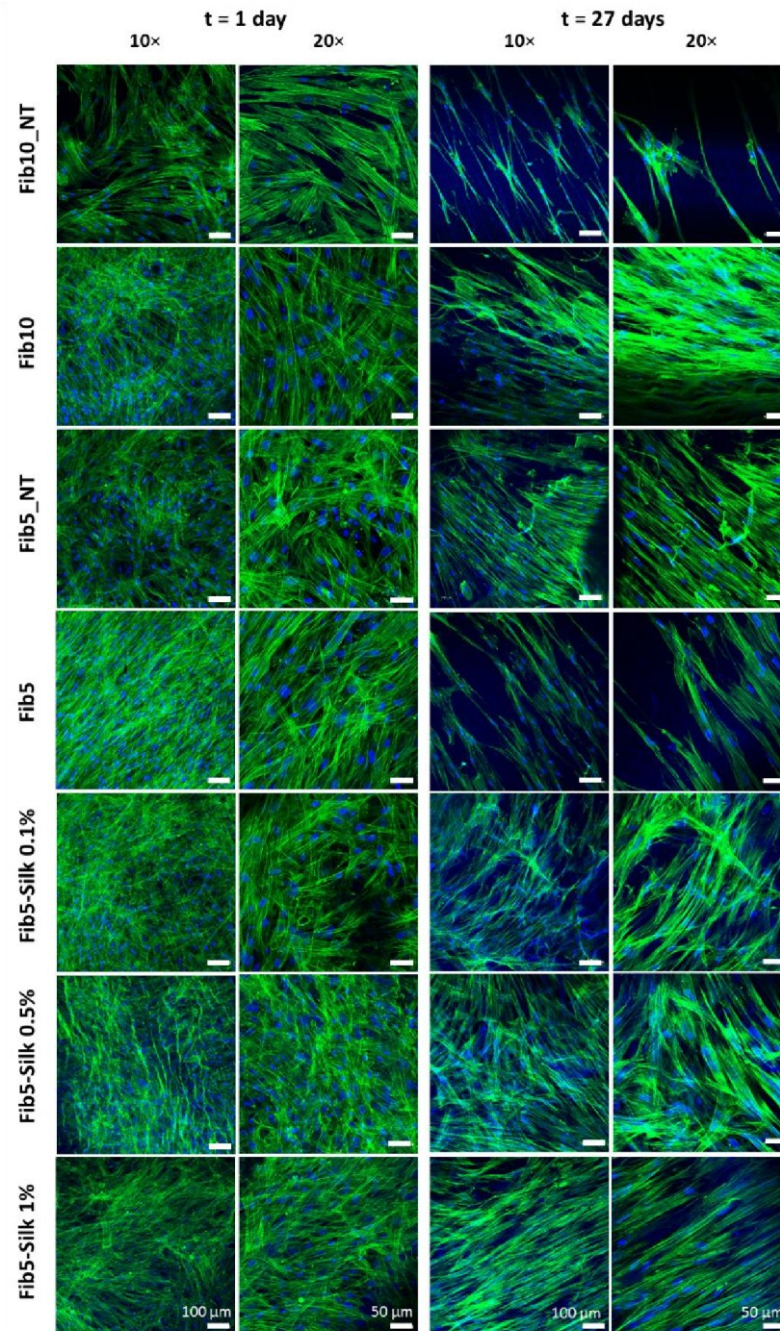


Figure 8. Confocal images of the scaffolds cultured with human arterial smooth muscle cells (HUASMCs) at two different time points: 1 day and 27 days. Actin filaments of the cell cytoskeleton appear in green (phalloidin staining) and nuclei in blue (DAPI staining).

3.5. Fabrication of Composite Scaffolds with Tubular Shape

We explored the feasibility of applying the developed approach to the fabrication of composite scaffolds of a tubular shape (Figure 9a). The composite scaffolds were easy to demold in tubular form, in contrast to the controls of Fib5_NT and Fib5, which could not be demolded without breaking or deforming the tubular shape. The tubular composite scaffolds showed great handleability and outstanding flexibility, and indeed they collapsed under gravity and were able to bend 180° (Figure 9b).

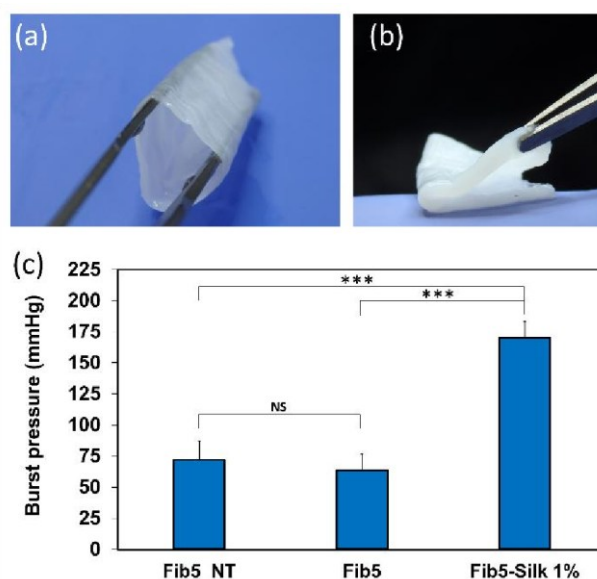


Figure 9. (a) Tubular scaffold of Fib5-Silk 1%. (b) Fib5-Silk 1% tubular scaffold collapsed under gravity, and further bended 180° on the circumferential axis. (c) Burst pressure values of the different scaffolds expressed as mean $\pm$ SD (***) $p < 0.001$; NS: not significant).

The burst pressure of the composite Fib5-Silk 1% (170 ± 19 mmHg) was significantly higher ($p < 0.001$) than those displayed by the scaffolds Fib5_NT and Fib5 (72 ± 15 and 63 ± 13 mmHg, respectively).

4. Discussion

Single polymers often fall short in fulfilling the needed requirements for their successful application. Indeed, materials in nature are rarely used as single polymers, and are rather arranged into composites [36], showing enhanced properties when compared to the single constituents. In this way, complementary or inversely correlated properties (e.g., flexibility and strength) can be combined in the same system. Consequently, the pursuit of advanced scaffolds should focus on the selection of adequate materials as well as on the appropriate fabrication techniques to enable synergy between the different components and to match the needs of the target tissue [37].

In this study, we proposed the combination of silk fibroin (as strong, bioprocessable) and fibrin (flexible, bioactive) materials, in order to obtain composite scaffolds with advanced properties for TE, specifically in regards to resistance to cell-mediated gel contraction, bioactivity and mechanical performance. For their biofabrication, we proposed a strategy that exploits a double dual injector system to mix the fibrin and silk fibroin in a homogenous and reproducible way. Previous studies suggest that an optimal concentration of fibrin ranges between 3 and 5 mg/mL [38]. Therefore, the fibrin concentration was fixed at 5 mg/mL and the silk fibroin content varied (0.1%, 0.5%, 1% w/w) in the composites to determine which composite scaffold attained the best mechanical behavior and resistance against cell-mediated gel contraction, while still featuring an adequate cell attachment.

At both the macro and micro level, the resulting composites showed a homogenous and reproducible architecture characterized by an interconnected porous structure. The open porosity displayed by the composites was not hampered by the increase in content of the silk fibroin. The pore size on the composite scaffolds ranged between 5 μ m and 15 μ m of diameter, which has been reported as suitable for cell infiltration and nutrient diffusion [39–41].

The proposed ethanol-based protocol of crosslinking is suitable for seeding the cells on top but not within the scaffolds. However, the increase in the mechanical properties paves the way for the application of these scaffolds as cell-free systems, able to provide sufficient support and handleability for implantation without any cells or any pre-conditioning steps [42–44]. The

biodegradable nature of both components (fibrin and silk fibroin) together with the open porosity provides a priori a suitable scenario for cell colonization and endogenous remodelling.

Regarding the rheological behavior, the formation of a gel for non-treated scaffolds proves that the silk fibroin did not obstruct the polymerization of the fibrin. The presence of silk fibroin within the composites and subsequent crosslinking (ethanol treatment) contributed greatly to increase the mechanical properties of the fibrin, from an elastic modulus of 23 ± 5 Pa for fibrin alone, to 456 ± 31 Pa for the Fib5-Silk 1%. The β -sheets formation of the silk fibroin is essential to boost the mechanical properties (Figure 4b), as the increase of content of the silk fibroin is only evident when the crosslinking is made with ethanol. The composite with the least in silk fibroin content (Fib5-Silk 0.1%), did not experience an increase in elastic modulus after ethanol treatment (Figure 4a). A possible explanation might be that the silk fibroin concentration in the composite is too low to enable the formation of a silk fibroin network that can support mechanical forces. Both the ethanol-treated and the non-treated fibrin scaffolds (Fib5 and Fib5_NT) showed elastic modulus of just a few pascals, and the ethanol treatment did not elicit an increase in the mechanical properties of the pure fibrin gels. Therefore, the increased mechanical performance observed in the composite scaffolds can be attributed to the ethanol-induced annealing of the silk fibroin contained within them.

A cell adhesion study with HUVECS was carried out in order to check whether the fibrin contained within the hybrid composites was able to provide the specific binding sites, or whether the adhesion was impaired due to the presence of silk fibroin.

Fibrin scaffolds pre-treated with BSA (blocking agent) showed effective cell adhesion, while the composites (fibrin + silk fibroin) showed that, by increasing the silk fibroin content, the number of cells attached decreased by 70% in the case of Fib5-Silk 0.1%, 80% for Fib5-Silk 0.5% and 92% for Fib5-Silk 1% by (Figure 5b). However, the number of cells attached on the composites was significantly higher than for the pure silk fibroin scaffolds. In other words, the inclusion of fibrin within the silk fibroin scaffold improves the specific adhesion of the cells, although such adhesion is reduced when compared to pure fibrin scaffolds, as expected. However, after just 24 h under standard culture conditions (no BSA pre-treatment and media supplemented with FCS), the endothelial cells (HUVECs) seemed to cover most of the composites' surfaces and assume an elongated morphology, which indicates a good adhesion to the substrate. The endothelial cells line the lumen of the vessels and grant hemocompatibility [45]. Additionally, they play an essential role in the dynamic regulation of the vascular hemostasis (e.g., the patency of the blood vessel), by secreting different kinds of vasoregulatory factors [46]. Therefore, the consecution of a confluent and adherent endothelial layer paves the way for the application of these scaffolds in the cardiovascular TE field.

Additionally, the bioactivity of fibrin was not affected by the ethanol-treatment, i.e., regardless of the culture conditions (Figures 5 and 6), Fib5 preserved the same remarkable cell-adhesion as fibrin non-treated with ethanol. This observation agrees with previous studies, in which the ethanol sterilization was shown to not significantly change the mechanical and chemical properties of the fibrin gels [47].

One of the major drawbacks of fibrin as a biomaterial is the cell-mediated retraction that the fibrin-scaffold experiences [48]. Such a retraction can hamper the consecution of implants with a defined shape over time, and therefore, preclude the applicability of fibrin as a fabrication material in TE. However, the composites resulting from blending the fibrin with silk fibroin showed not only an increased in the mechanical properties, but also a significant reduction of cell-mediated scaffold contraction. Figure 7a shows how during 27 days of culture with HUASMCs (strong contractile cells), the surface of the fibrin scaffold changes drastically, while the presence of silk fibroin clearly halts the cell-mediated contraction that the pure fibrin is subject to. The addition of Fib10_NT to the conditions demonstrated that the increase in material content (fibrin concentration) is not the leading factor in the prevention of the fibrin size reduction in culture. Indeed, the Fib10_NT, although in a lower extent than the Fib5_NT, was subjected to cell-mediated scaffold contraction of almost 80% of its original surface area (Figure 7). Moreover, this

high concentration of fibrin in Fib10_NT makes the scaffold more compact and rigid [38], which can negatively impact the proliferation of cells, as shown in the confocal microscope images (Figure 8). In contrast, the addition of silk fibroin to the fibrin in the composites resulted in a confluent layer of HUASMCs.

In a recent study, fibrin and elastin-like recombinamers (ELRs) were combined [49], to obtain better mechanical and biological properties. Despite the promising approach of that study, a final concentration of ELRs of 1% (w/w) did not preserve the shape of the scaffold. While a higher content could maintain the scaffold shape, the increase of ELR concentration resulted in a dense and compact scaffold, which hampered cell proliferation [49]. In contrast, in our approach, the addition of silk fibroin (1% w/w) was sufficient to fully guarantee the stability of the gels and prevent cell-mediated scaffold contraction. Additionally, the HUASMCs were well distributed all over the composite surfaces, with an elongated morphology that indicates a good adhesion on the substrate. Such stability is a target property in many TE applications, including cardiovascular ones. For example, a tissueengineered heart valve must be biofabricated with a resistance to cell-mediated scaffold contraction in order to guarantee a proper opening and closing of the implant. If the leaflets contract, the coaptation is hampered, and therefore also the functionality of the whole implant [50].

By applying the injection molding fabrication approach described in Section 2.6, we demonstrated that it was possible to obtain tubular scaffolds made of fibrin/silk fibroin, without the use of any textile support. The tubular structures are ubiquitous within the human body (e.g., arteries, veins, urine conducts, and trachea). Therefore, the feasibility to fabricate fibrin/silk fibroin composites in a tubular shape paves the way for the application of these composites in several TE applications. In addition, the composite tubular scaffolds displayed great flexibility, especially considering that the tubular scaffolds could be bended without creating any kink (Figure 9b). This flexibility is highly relevant in the cardiovascular field and is a material requisite when engineering vascular grafts and heart valves. For these applications, the material needs to withstand considerable internal pressure without bursting, therefore the burst pressure of the composites was also investigated.

The incorporation of silk fibroin into the scaffolds led to a significant increase in the burst strength of the fibrin scaffolds. As reported in Figure 9c, the burst pressure of the composite Fib5-Silk 1% was two times higher than the respective values of treated and non-treated pure fibrin scaffolds (i.e., Fib5 and Fib5_NT).

Future work is required to acquire the full picture regarding the possible implementation of the composite scaffolds made of fibrin/silk fibroin in the vascular tissue applications, such as testing the compliance of the composites' tubular scaffolds or testing their strength in holding the suture lines in different directions.

5. Conclusions

In conclusion, it was possible to develop a scaffold made of two natural polymers fibrin and silk fibroin, that are able to offer the high mechanical properties of silk fibroin and, at the same time, provide the extraordinary bioactivity of fibrin. The simplicity of the fabrication method, the versatility and flexibility undertaking different shapes, the successful balance between the mechanical properties and the bioactivity meet the requirements for the composite scaffolds to be employed in cardiovascular TE applications.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data associated with this study is available upon request to the corresponding authors.

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Conflicts of Interest: Marius Köpf and Alexander Kopp are affiliated with the Fibrothelium company, which provided the silk fibroin for this study.

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Protein-Engineered Elastin Fibers as Building Blocks for The Textile-Based Assembly of Tissue Equivalents

*Ikram El Maachi, Alexander Loewen, Sergio Acosta, Stephan Rütten, J. Carlos Rodríguez-Cabello, Stefan Jockenhoevel, and Alicia Fernández-Colino**

Native tissues feature unique hierarchical designs, in which fiber units are arranged from the bottom up in anisotropic patterns. The processing of biomaterials into fibers, followed by their textile-like assembly into complex patterns, is therefore a promising avenue to engineer native-like tissue replacements. Here it is shown for the first time the fabrication of meter-long hydrogel fibers prepared from engineered elastin using a microinjection system and exploiting the catalyst-free click chemistry. Given their similarity to native elastin, the fabricated elastin-like fibers achieved excellent stretching (500%) and recoiling performance. Moreover, the fabrication scheme is compatible with the implementation of a salt-leaching gas-foaming approach, resulting in highly porous elastin-like fibers (the first of their kind). From the translation perspective, the fibers can be autoclaved, which allows for sterilization and long-term storage. Human umbilical vein endothelial cells cultured on autoclaved fibers produced a confluent endothelial layer lining the fiber surface, in which the cells became aligned in response to physiological fiber stretching. It is also shown that these functional fibers can be assembled by weaving, braiding and knitting, with various spatial patterns. Overall, the elastin-like fibers can be used as building blocks for the reconstruction of functional tissues using the principles of textile technology.

1. Introduction

Nature builds from the bottom up, converting chemically simple building blocks into sophisticated structures^[1,2] and native tissues are no exception to this construction strategy.

Undoubtedly, nature has demonstrated its excellence as a designer. During billions of years of evolution, nature has shaped and refined the design of biological systems including functional, long-lasting tissues. The outstanding properties of native tissues lie on two different levels: the inherent characteristics of their materials and the arrangement of these materials in controlled hierarchical structures. The hierarchical structure of all tissues has one common element: the fiber. The ubiquity of fibrous proteins (e.g., collagen and elastin) in the native extracellular matrix (ECM) and their arrangement into higher-order net-like structures^[3] means that fibers are the biomimetic building block of choice in tissue equivalents, highlighting the overlap between the fields of tissue engineering (TE) and textiles.^[4,5]

The textile technologies have a history of over 30 000 years, thus encompassing well-established and standardized manufacturing techniques such as weaving, knitting and braiding, that are exploited customized structures spanning multiple scales.^[7]

This requires precise control over various material properties, including the orientation of fibers and the size, shape, arrangement and density of pores, thus approximating the mechanical and functional

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characteristics of real tissues.^[8–10] Thus far, such constructs have been deployed, with relative success, to provide physical support for prolapsed organs and surgical repairs^[11–13] as well as for other load-bearing applications, including tendons as well as cardiovascular medical devices (vascular grafts,^[14] heart valve sewing rings^[15]). However, the implants are still based on synthetic



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for manufacturing daily items (clothes, filters), but also high value products (medical devices).^[6] Textile technologies allow the development of 3D constructs with

materials such as poly(ethylene terephthalate), polylactic acid or polyglycolic acid,^[16] which are strong and versatile but cannot recapitulate the genuine biochemical milieu required for effective tissue regeneration.^[17] This can lead to suboptimal integration or even rejection following implantation, thereby creating a demand for a novel generation of yarns crafted from natural, biologically compatible materials.

Elastin is an essential component of native tissues, contributing significantly to their mechanical and bioactive functions.^[18] For example, the absence of elastin in medical devices such as vascular implants can lead to stenotic and thrombotic events, culminating in device failure.^[19] In contrast, incorporating elastin into a vascular replacement can enhance its performance in vivo due to the ability of elastin-based components to facilitate elastic contraction and expansion, promote rapid endothelialization, and prevent the overproliferation of smooth muscle cells and platelet activation.^[20,21] Artificial elastin, specifically elastin-like recombinamers (ELRs), represents an appealing solution for the production of tissue replacements with enhanced in vivo performance. ELRs are engineered protein polymers that combine the best of both worlds – the reproducibility of synthetic materials and the biocompatibility and bioactivity of biological ones.^[22,23] These protein-engineered polymers can prevent fibrotic tissue formation and favor the proliferation of pro-healing type-2 macrophages over pro-inflammatory type-1 macrophages.^[24,25]

The sequence of ELRs can be tailored to confer specific properties, thereby providing a versatile platform for the fabrication of cell-instructive TE scaffolds.^[19,26] For instance, cell-binding motifs such as RGD, or protease-sensitive sequences (i.e., GTAR or DRIR) can be incorporated into their backbone to modulate cell migration and scaffold integration.^[27] Additionally, ELRs can be chemically derivatized with catalyst-free click cross-linkable groups (e.g., azide groups), thus enabling the fabrication of stable 3D networks using catalyst-free click chemistry. This cutting-edge technology has been used to fabricate protein-engineered scaffolds with exceptional properties for tissue regeneration including the spatiotemporal control of angiogenesis and neurogenesis,^[28] and the modulation of post-ischemic remodeling.^[29] We have also fabricated elastin-based cell-free systems in the form of vascular grafts,^[30] heart valves,^[31] and venous valves,^[32] that withstand hydrodynamic conditions simulated in vitro.

One drawback of the examples described above is their reliance on top-to-bottom fabrication schemes, ultimately failing to fully implement and capitalize on the bottom-up principles inherent to natural tissues.^[33] Here, for the first time, we use these advanced protein-engineered polymers to fabricate fibers as building blocks for further bottom-up assembly according to the principles of textile engineering. The universality of the approach was confirmed by using a library of ELRs with different functionalities, followed by a “collect & click” strategy. Specifically, this encompasses i) the selection of ELRs with desired properties from the library and ii) the use of catalyst-free click chemistry to combine the chosen components. The so-formed elastin-like fibers featured customized porosity and were compatible with terminal sterilization schemes, maintaining their structural and biological integrity. Indeed, the fibers supported the attachment and alignment of human endothelial cells (ECs), and were further assembled in hierarchical patterns,

resulting in a highly attractive and versatile platform that can be used to build up tissue equivalents.

2. Results and Discussion

2.1. Fabrication of Elastin-Like Fibers and Structural Characterization

We fabricated elastin-like fibers using a microinjection molding technique (**Figure 1a,b**). The approach enabled to fabricate fibers with lengths of more than 3 meters, and the diameter of the resulting fibers was easily controllable by changing the diameter of microinjection molding to 500 μm ($\varnothing\text{S}$) and 1000 μm ($\varnothing\text{L}$). Importantly, the production of the fibers did not require toxic reagents or cross-linkers thanks to the reactive groups in each ELR that enabled catalyst-free click chemistry.

We demonstrated the universality of our micro-fabrication approach by using three types of ELRs with varying functionalities. These included protease sensitive (DRIR) and structural (VKV) variants, which were coupled to ELRs containing the RGD sequence for enhanced bioactivity (**Figure 1c** and **Table S1**, Supporting Information).

Analysis of the fibers by confocal microscopy and scanning electron microscopy (SEM) revealed a homogeneous and smooth fiber surface, with sub-micrometer pores (**Figure 2**, first column, and **Figure S1**, Supporting Information).

To increase the versatility of our approach, we also implemented a salt-leaching gas-foaming (SL/GF) step during the micro-fabrication process, allowing us to tune the microstructure of the fibers by incorporating a porogen. Microscopic analysis confirmed the effectiveness of the approach in increasing the porosity when compared to the plain fibers in which no SL/GF was performed (**Figure 2**; **Figure S2**, Supporting Information). The fibers featured a uniform distribution of open pores both in cross sections and along their length, without any clustering, even in the fibers with a small diameter.

Quantitative analysis showed a good correlation between the size range of the sieved porogen particles ($<40\text{ }\mu\text{m}$ and $<100\text{ }\mu\text{m}$) and the average pore size in the resulting fiber (Figure 2b). When the porogen size was $<40\text{ }\mu\text{m}$, the average pore size was $34 \pm 5\text{ }\mu\text{m}$ for the ØS fibers and $42 \pm 10\text{ }\mu\text{m}$ for the ØL fibers. When the porogen size was $<100\text{ }\mu\text{m}$, the average pore size increased to $89 \pm 10\text{ }\mu\text{m}$ for ØS fibers and $108 \pm 10\text{ }\mu\text{m}$ for ØL fibers. This highlights the ability to control the pore size of the fibers, irrespective of the fiber diameter. It is notable that we succeeded in fabricating fibers only $500\text{ }\mu\text{m}$ in diameter with pore diameters of $\approx 100\text{ }\mu\text{m}$, creating thin and highly porous elastin-like fibers, the first of their kind. Previous studies showed the fabrication of tropoelastin-containing yarns composed of densely packed electrospun nanofibers.^[34] Those approaches relied on blending the elastic material (tropoelastin) with a second material (e.g., silk

recombinant methods. Overall, this method allows an unparalleled degree of customization, facilitating the production of fibers varying in molecular composition, length, diameter, and pore size.

2.2. Mechanical Characterization

The mechanical properties of the elastin-like fibers were systematically investigated by tensile testing (Figure 3). The stress–strain curves revealed an excellent stretching performance, with an elongation at break that reached $498 \pm 45\%$ of strain for the plain fibers (Figure 3), thereby indicating their ability to be stretched nearly six times their initial length. This was corroborated by manual stretching up to 500% of the initial fiber length (Figure 3a). The porous fiber showed less but still remarkable elongation of 173

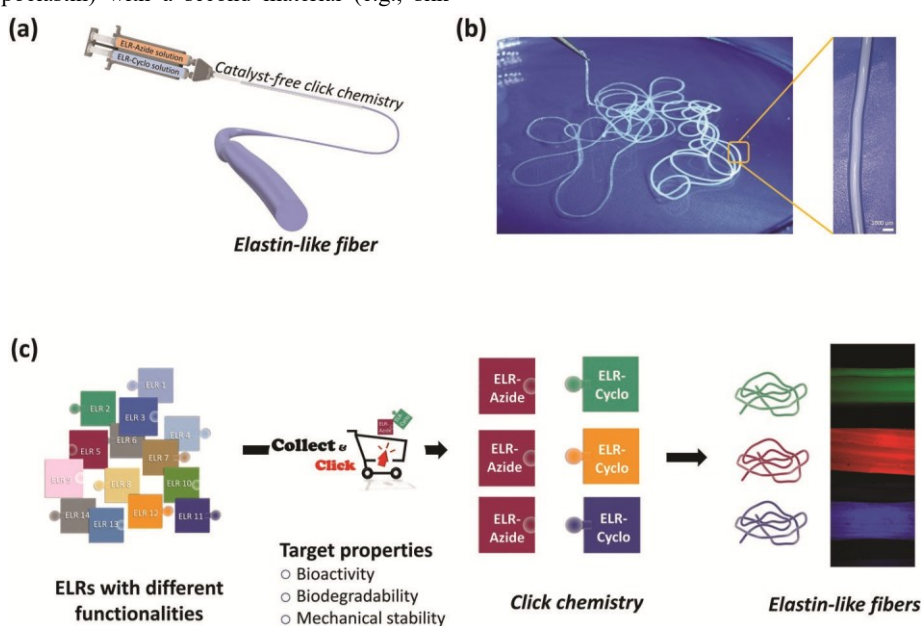


Figure 1. Fabrication of elastin-like fibers. a) Schematic of the fabrication of elastin-like fibers using the microinjection molding technique. b) Image (and close-up) of the resulting elastin-like fiber. c) Outflow of the “collect & click” strategy, which enables a high level of versatility in the molecular composition of the resulting fiber. This consists of first collecting the specific polymers from a library of elastin-like recombinamers (ELRs) with diverse functionalities, and then using click chemistry to fabricate the fibers. To visually demonstrate this concept, we selected three different sets of ELRs from the library. Confocal visualization was enabled by either fiber autofluorescence (excitation wavelength 405 nm, blue fiber) or functionalization of the ELRs with fluorescent labels. Specifically, N-hydroxysuccinimide (NHS)-ester fluorescein (excitation wavelength 488 nm) was used for one set of ELRs (green fiber) and Cyanine5.5 NHS ester (excitation wavelength 561 nm) for another (red fiber).^{[16163028, 2024, 24, Downloaded from https://advanced.onlinelibrary.wiley.com/doi/10.1002/adfm.202313204 by Rwth Aachen University Zentraler Rechnungssgang, Wiley Online Library on [09/07/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License]}

fibroin, PCL), necessary to ensure structural integrity.^[34,35] The lack of a crosslinking method to stabilize the tropoelastin within the composite yarn also resulted in its partial loss.^[35] Our fiber fabrication process offers a practical approach that capitalizes on the benefits of catalyst-free click chemistry to obtain pure elastinlike fibers with customized porosities, with no need for blending materials. The average pore sizes in our study ranged from $34\text{ }\mu\text{m}$ to $108\text{ }\mu\text{m}$, providing ample space for cell infiltration, nutrient diffusion, and tissue ingrowth.^[36–38] We also fabricated macroporous fibers using an alternative ELR combination based on structural DRIR-ELR and bioactive RGD-ELR (Figure S2, Supporting Information). This demonstrates the versatility of the applied strategy, which is not limited to a specific ELR and can be applied to ELRs with different functionalities, tailorable *ad hoc* by

$\pm 11\%$ of strain, which could be attributed to the presence of pores acting as stress concentrators, potentially leading to the premature failure of the material.^[39]

The mechanical performance of the fibers was extensively evaluated by cyclic testing. Specifically, the fibers were subjected to 10 cycles of loading and unloading, with plain fibers stretched to 200% and porous fibers to 60% of their initial length, followed by stretching to break. Notably, no hysteresis loop was observed between the loading and unloading cycles for the plain or porous fibers (Figure 3b). The elastin-like fibers returned to their original shape after deformation, making them suitable for applications that require repetitive loading and unloading (e.g., cardiovascular applications). This matches the intrinsic behavior of natural elastin,

which undergoes high deformation without rupture and returns to its original state once the stress is removed.^[40]

The Young's modulus (the slope of the initial part of the stress–strain curve) of the plain fibers (26 ± 10 kPa) was significantly higher than that of the porous fiber (17 ± 4 kPa). The maximum slope (maximum tangent modulus) of their stress–strain curves differed significantly, with values of 130 ± 69 kPa for the plain fibers and 36 ± 5 kPa for the porous fibers. The plain fibers were therefore much stiffer than the porous ones, particularly at the highest levels of strain. Additionally, the ultimate tensile strength of the plain fibers was 231 ± 101 kPa, whereas that of the porous fibers was 46 ± 6 kPa.

This comparison of mechanical properties provides valuable insights into the influence of porosity on the performance of elastin-like fibers. This will facilitate the design and optimization of ELR-based materials, offering new possibilities for tailoring

confocal microscopy and ii) SEM. The first column shows plain fibers whereas the second and third columns show the microstructure obtained by implementing a SL/GF step during the fabrication process ($\emptyset L$ and $\emptyset S$ refer to fibers fabricated using a microinjection tube of 1000 and 500 μm , respectively). b) Quantitative analysis of the pore sizes. Data are means $\pm$ SD ($n \geq 3$). Statistical significance was determined by two-way ANOVA with Holm–Šidák comparison (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; NS = nonsignificant).

the mechanical properties of these fibers to suit specific requirements.

2.3. Effect of Terminal Sterilization on the Material Properties

For any implant to advance toward clinical use, it is necessary to test and validate aspects such as sterilization. Here, we explored whether sterilization processes (ethanol treatment and autoclaving) had an impact on the mechanical and structural properties of the elastin-like fibers. Microscopic visualization of the porous fibers revealed no structural change (e.g., no collapsing of pores) after either of the sterilization treatments (Figure 4a; Figures S3 and S4, Supporting Information). This finding was supported by the quantitative analysis of porosity (Figure 4b). We observed comparable average pore sizes for non-treated (34 ± 1 μm), ethanol treated (37 ± 2 μm), and autoclaved (39 ± 5 μm) elastin-like fibers prepared using the <40 μm porogen ($p > 0.5$). We also observed comparable average pore sizes for non-treated (105 ± 14 μm), ethanol treated (115 ± 29 μm), and autoclaved (103 ± 8 μm) elastin-like fibers prepared using the <100 μm porogen ($p > 0.5$).

The thermal resistance exhibited by these materials is probably a consequence of their intrinsically disordered nature, enabling the ELRs to respond to and accommodate changes in temperature in a reversible manner. To confirm this hypothesis, we analyzed the ELRs by circular dichroism (CD) spectroscopy before and after autoclaving (Figure S6, Supporting Information). The ELR conformational state is temperature dependent due to their lower critical solution temperature (LCST) phase behavior.^[41] Accordingly, the CD spectra were recorded at different temperatures (5, 37 and 60 °C) in order to determine whether the LCST behaviour persisted after autoclaving (Figure S6, Supporting Information). Typically, protein polymers derived from elastin exhibit a negative band at ≈ 197 nm (indicative of random coils) and a positive shoulder or peak at ≈ 210 nm (correlated to type II β -turns), and the amplitude of those bands is temperature-dependent.^[42] The CD spectra for the ELRs at 5 °C included a positive shoulder at 210 nm and a prominent negative peak ≈ 197 nm, which indicates a predominantly disordered structure.^[42] By increasing the temperature to 37 °C, the signal at 197 nm became weaker, whereas the magnitude of the peak at 210 nm increased, thus indicating an increase of type II β -turns.^[43,44] When increasing the temperature to higher values (60 °C), the peak at 210 remained, while the amplitude of the band at 197 nm further decreased, indicating a concomitant decreased in disordered structures.^[44] The reversible conformational changes correlated with the reversible thermo-responsive behavior of the ELRs (Figure S6, Supporting Information).

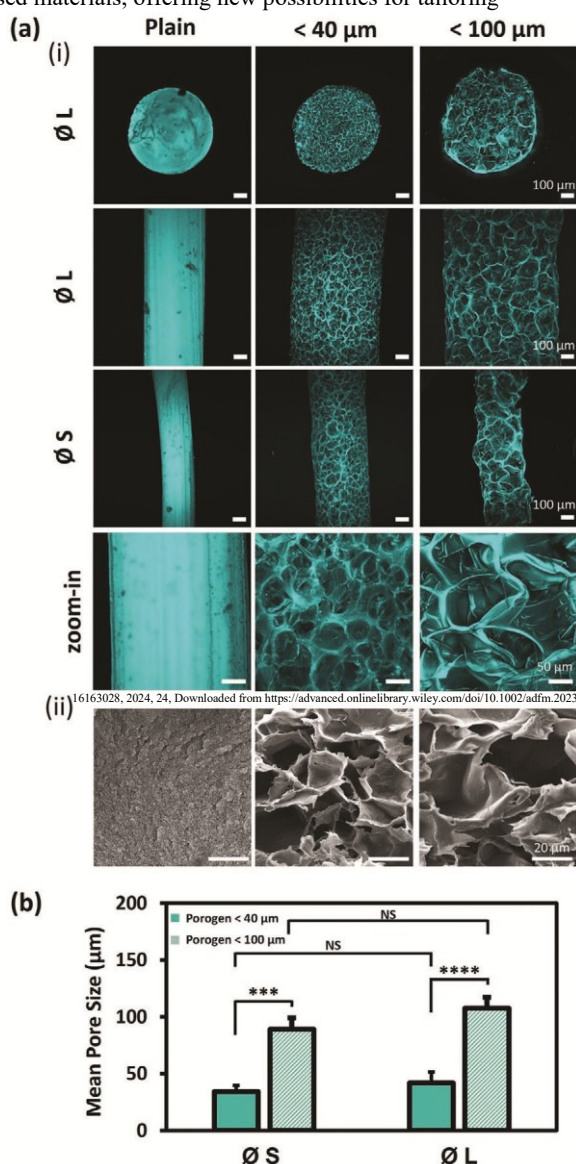


Figure 2. Microstructural characterization of elastin-like fibers prepared from ELR-VKV and ELR-RGD. a) Visualization of the microstructure by i)

The mechanical characterization of the porous fibers before and after sterilization indicated no notable changes in the Young's modulus, ultimate tensile strength, elongation, and maximum tangent modulus (Figure 4d; Figure S5, Supporting Information). This was further evidenced by the overlap of the strain-stress curves (Figure 4c ii). The porous fibers therefore maintained their mechanical properties and integrity even after sterilization by ethanol treatment or autoclaving. For the plain elastin-like fibers, no significant change was observed in the mechanical properties after ethanol treatment, with the exception of a decrease in the Young's modulus, from 21 ± 3 kPa in untreated plain fibers to 13 ± 5 kPa in those treated with ethanol ($p = 0.01$). In contrast, autoclaving the plain fibers resulted in a significant increase in the mechanical properties, as evidenced by the increased slope on the strain-strain curve when compared to non-treated and ethanol treated fibers (Figure 4c i). The Young's modulus, which was 21 ± 3 kPa for untreated fibers, increased to 58 ± 6 kPa for autoclaved fibers ($p <$

autoclaved fibers continued to exhibit an outstanding elongation of $447 \pm 96\%$. Overall, the fibers maintained their structural integrity and no detrimental effect on the mechanical properties was observed, suggesting that the peptide bond remains largely unaffected. This was further supported by FTIR-ATR spectra of non-autoclaved and autoclaved elastin-like fibers, that showed the same patterns in all the bands associated with the amide group of the peptide bond (Figure S7, Supporting Information).^[45]

In order to guarantee the safety and efficacy of fibers for clinical purposes, it is necessary to determine the most suitable sterilization approach at an early stage of development.^[46] Although ethanol treatment is a commonly used disinfectant approach in vitro, it should not be deemed as a sterilization method. Radiation is one of the most commonly used methods for the sterilization of protein-based scaffolds. However, radiation of water-containing systems (e.g., hydrogels) can lead to radical formation due to water radiolysis, with the concomitant detrimental effects.^[47] In the

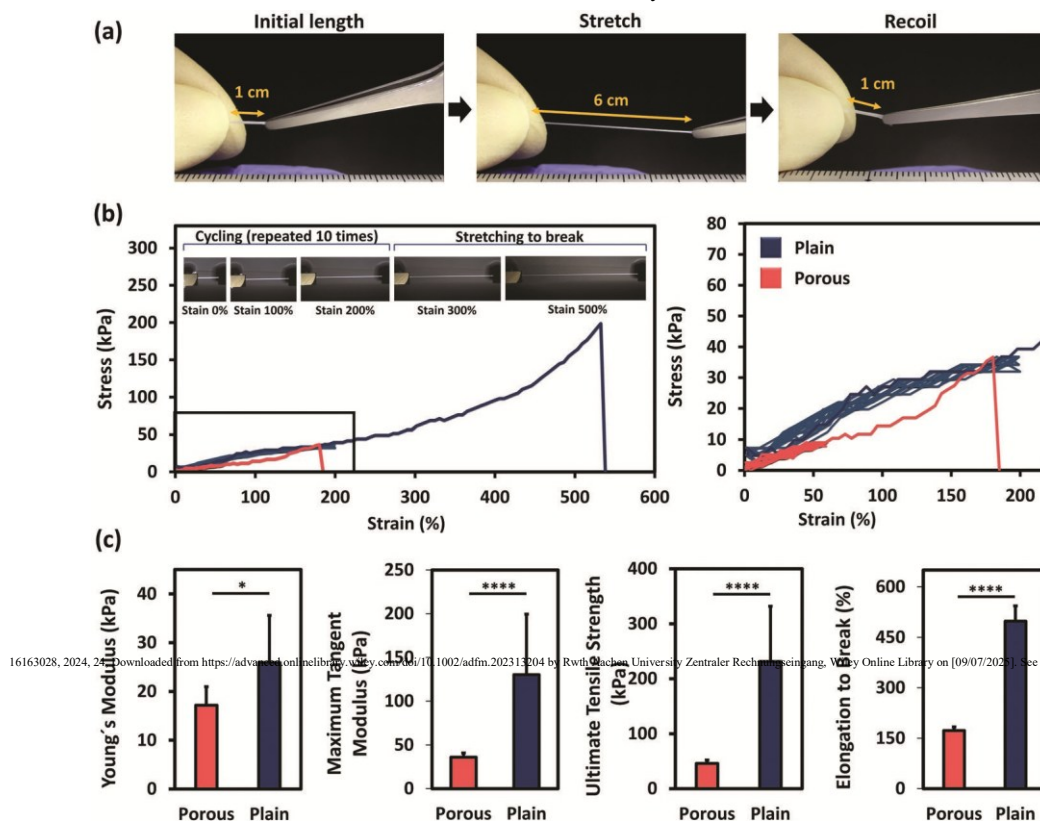


Figure 3. Mechanical characterization of elastin-like fibers prepared from ELR-DRIR and ELR-RGD. a) Captured video frames depicting the initial length (left image), the fiber stretched manually to 500% of its original length (central image), and the recoiled fiber that has returned to its original state once the stress is removed (right image). b) Representative stress–strain curves of the macroporous and plain elastin-like fibers, undergoing 10 cycles of loading and unloading followed by stretching to break. The graph on the right represents a close-up view of the stress–strain curves c) Young's moduli, maximum tangent moduli, ultimate tensile strength, and elongation at break for the macroporous and plain elastin-like fibers. Data are means $\pm$ SD ($n \geq 3$). Statistical significance was determined using a t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

0.0001). Similarly, the maximum tangent modulus increased from 98 ± 31 kPa to a remarkable 242 ± 125 kPa ($p < 0.0001$). The ultimate tensile strength also increased significantly from 193 ± 42 kPa to 431 ± 197 kPa ($p < 0.0001$). Remarkably, the elongation at break was not compromised by terminal sterilization (autoclaving) because there was no significant difference between the non-treated elastin-like fibers and of those subjected to autoclaving. The

context of clinical implementation, terminal sterilization by autoclaving^[48] is the preferred method, as recommended by the European Medicines Agency,^[49] and it is particularly advantageous due to its reliability, absence of toxicity, and lack of residual chemicals or by-products. However, the ability of biomaterials to maintain their structural, chemical and biological integrity during the sterilization process is a major challenge.^[48] Specifically,

protein-based materials (including those forming the native ECM and bioinspired counterparts) are intrinsically prone to denaturation, in which external stress (such as heat) causes them to lose their native quaternary, tertiary and/or secondary structure. Any alterations or degradation caused by autoclaving can negatively affect the overall performance and functionality of the scaffolds. This has led many authors to generalize that autoclaving and biobased materials are incompatible^[48,50,51] because thermal

treatment (121°C) would lead to detrimental effects related to their mechanical properties, bioactivity and/or structure.

The ELRs are derived from peptide sequences found in the intrinsically disordered domains of natural elastin. Therefore, their properties do not reside in specific secondary or tertiary structures prone to denaturation. Our findings show that the elastin-like fibers can indeed be autoclaved, with either no effect on their mechanical performance (macroporous fibers) or an

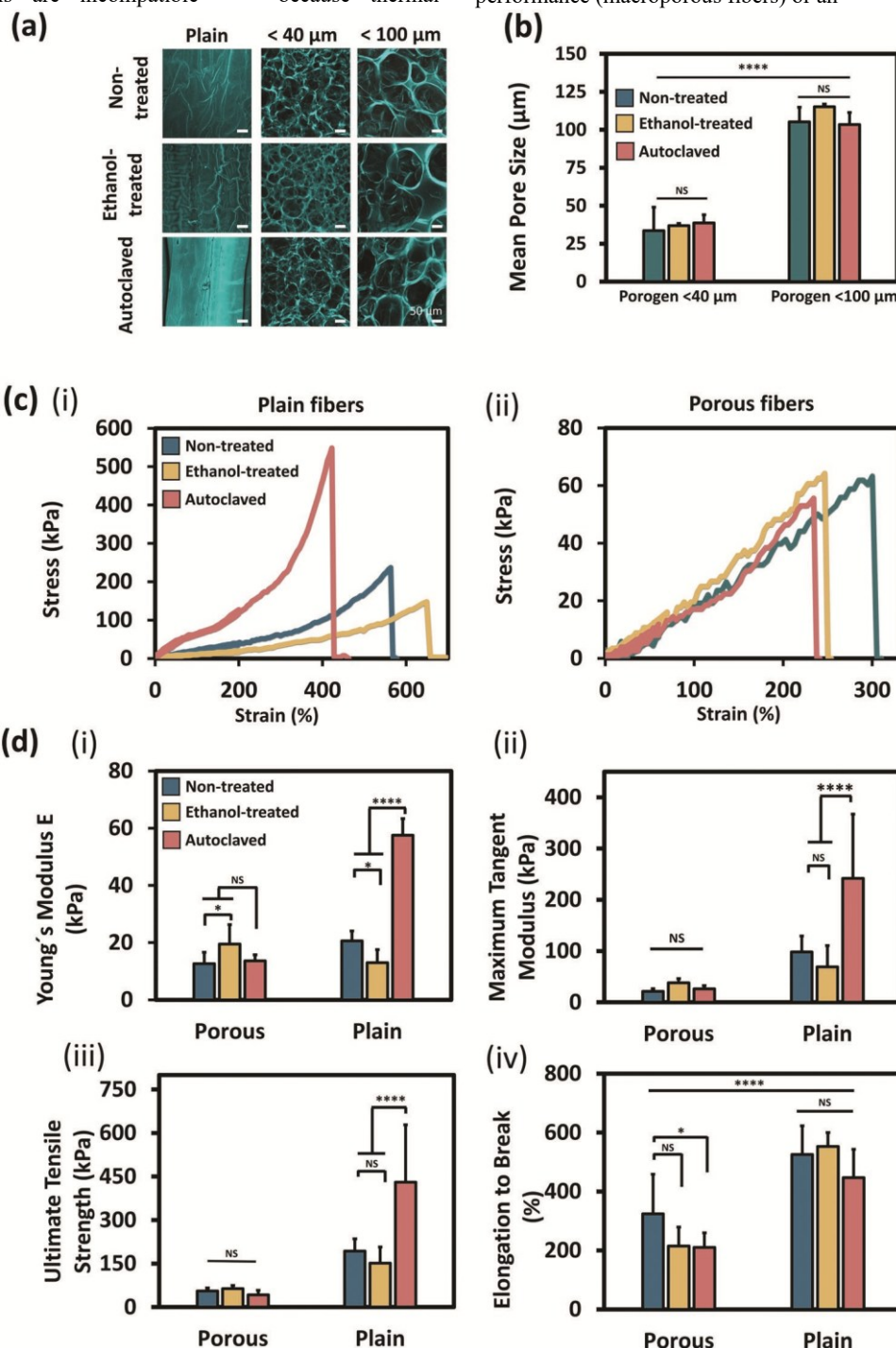


Figure 4. Impact of sterilization on the material properties of elastin-like fibers prepared from ELR-DRIR and ELR-RGD. a) Confocal images showing the microstructure of plain and macroporous fibers following different sterilization techniques. The images were taken by capturing the inherent autofluorescence of the ELRs. b) Quantification of the pore size of the fibers following different sterilization treatments. c) Representative stress–strain curves of the i) plain and ii) macroporous elastin-like fibers exposed to different sterilization techniques. The fibers were subjected to 10 cycles of loading and unloading followed by stretching to break. d) Evaluation of Young's moduli, maximum tangent moduli, ultimate tensile strength, and elongation at break for plain and macroporous elastin-like fibers treated with different sterilization methods. Data are means $\pm$ SD ($n \geq 3$). Statistical significance was determined by two-way ANOVA with Holm–Šidák comparison (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; NS = non-significant).

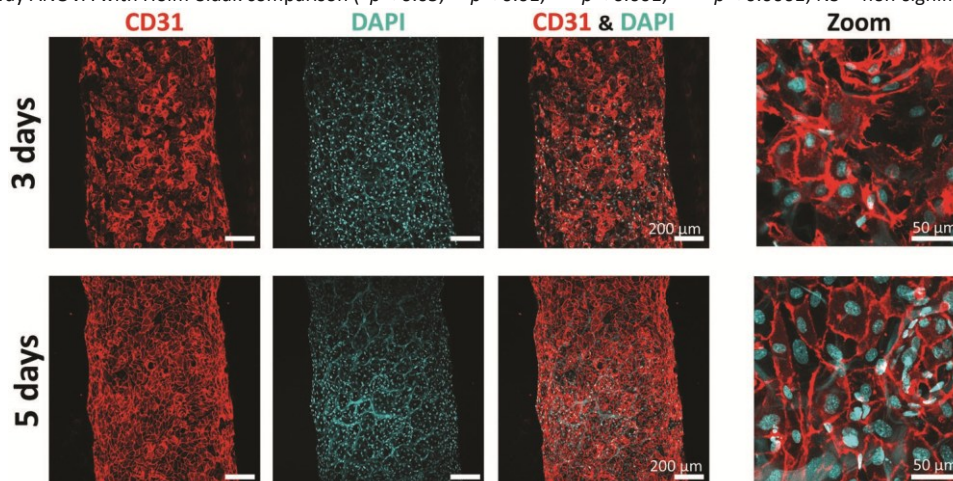


Figure 5. Confocal images of elastin-like fibers (prepared by mixing ELR-RGD and ELR-DRIR) cultured with primary human umbilical vein endothelial cells (HUVECs) for 3 and 5 days under static conditions. Endothelial cells are stained with antibodies against the CD31 marker (red) and counterstained with DAPI to reveal the nuclei (turquoise).

2.4. Biological Characterization

increase in stiffness, maximum tangent modulus, and ultimate tensile strength, while still maintaining the initial outstanding elasticity (plain fibers). This suggests that autoclaving may improve the mechanical performance of the plain fibers, making them potentially more suitable for certain applications where increased stiffness and strength are desired. Terminal sterilization by autoclaving did not compromise the ability of ELR molecules to accommodate, in a reversible manner, the typical conformational changes associated with thermo-responsive behavior (Figures S6, S8 and S9, Supporting Information). Thus, we hypothesize that the phenomena responsible for changes in the mechanical properties of the plain ELR fibers lies rather at the supramolecular level. Supramolecular interactions could be favored in the dense structure of the plain fibers, leading to further packing of the polymer chains. In the porous fiber, the open porosity would hinder tighter physical interactions due to the void spaces, explaining why the mechanical properties of the fibers before and after autoclaving are equivalent. The different impact of autoclaving plain and porous fibers shows how the suitability of a sterilization approach should be evaluated in the context of the final construct, rather than the bulk material, and at an early stage of implant development. Here, we demonstrate for the first time that engineered elastin can be autoclaved, which provides additional opportunities for the application of these protein-engineered polymers.

The fibers subjected to terminal sterilization (autoclaved) were seeded with primary human umbilical vein endothelial cells (HUVECs). A continuous and homogeneous layer with wellformed cell-to-cell junctions was observed within just 5 days, as revealed by confocal images of CD31-stained samples counterstained with DAPI (Figure 5). At this stage, the ECs exhibited an elongated and flattened morphology, with no specific orientation. Moreover, autoclaving did not compromise the ability of elastin-like fibers to support EC adhesion (Figure S10, Supporting Information) or proliferation (Figure S11, Supporting Information). The cellular performance also speaks for the absence of toxic compounds (e.g., ethanol), as also demonstrated by gas chromatography-mass spectrometry (Figure S12, Supporting Information).

The ability of elastin-like fibers to support EC attachment is highly relevant in TE, particularly for cardiovascular applications, because these cells play major roles in the regulation of vascular tone, inflammation, blood clotting, and angiogenesis.^[52] However, proper tissue morphogenesis, homeostasis, and functional regeneration require not only cellular attachment, but also coordinated cellular alignment.^[53] This is triggered by mechanical cues such as shear stress from the blood flow and cyclical stretch due to dilation across the vessel wall caused by the pulsatile nature of the blood flow.^[54]

The elastin-like fibers cultured with HUVECs were exposed to uniaxial cyclic stretching, selected to mimic the physiological values reported in human arteries.^[55,56] The stretching therefore

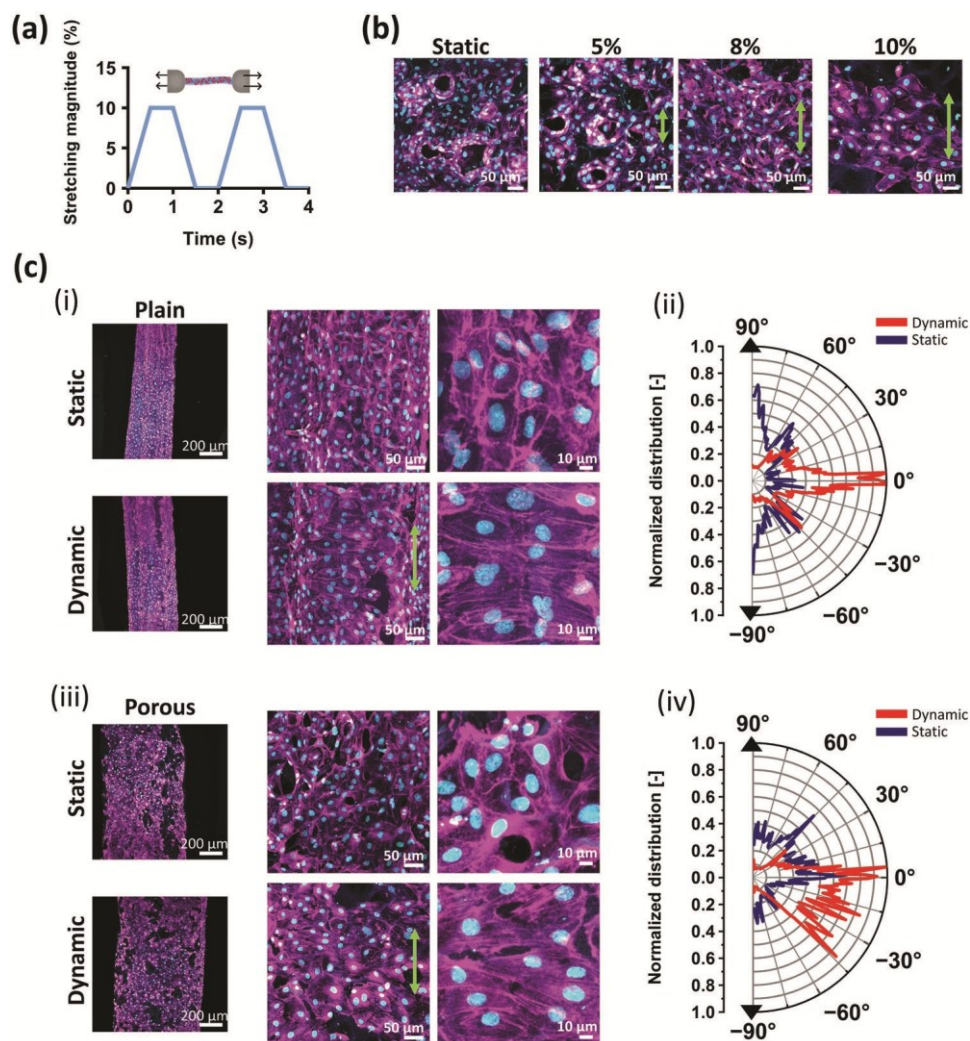


Figure 6. Cultivation of HUVECs on elastin-like fibers (ELR-RGD and ELR-DRIR) followed by mechanical stimulation. a) Diagram of the cyclic strain waveform used in the study. b) Confocal images of HUVEC-seeded elastin-like fibers subjected to various stretching magnitudes (0%, 5%, 8% and 10%). The green arrow indicates the axis of the stretching. c) Analysis of fibers. i) Confocal images of plain fibers seeded with HUVECs under static and dynamic (10% stretching) conditions. ii) Quantification of cellular alignment in the plain fibers (the direction of the stretching is represented by the black arrow). iii) Confocal images of macroporous fibers seeded with HUVECs under static and dynamic conditions (10% stretching). iv) Quantification of cellular alignment in the porous fibers. Phalloidin staining (violet) and DAPI (turquoise) were used to visualize the actin cytoskeleton and nuclei, respectively.

ranged from 5% to 10% at a frequency of 0.5 Hz for a total of 24 h (Figure 6a).^[57] We observed a clear reorientation of cells, which was largely dependent on the deformation magnitude. At stretching magnitudes of 5% and 8%, the cells continued to adapt to the topography of the fibers by assuming the shape of the pores, with a relatively chaotic organization of cytoskeletal actin filaments (Figure 6b). However, the cells began to align when the stretching magnitude reached 10%. This was confirmed by the arrangement of the actin filaments, which adopted a near parallel disposition.

The orientation of cells was quantified on the plain and porous fibers (Figure 6c). In both cases, cell elongation was followed by a collective change in cell alignment. The orientation of cells was more prominent in the plain fibers, suggesting that the macroporosity of the porous fibers hinders cell elongation and acts as a geometrical constraint on cell orientation. This highlights the significance of topographical cues in directing cell behavior.^[58–60] Remarkably, the cells exhibited a strong tendency

to align perpendicular to the direction of the stretch, which is consistent with the response of ECs to cyclic stretching as reported in the literature.^[61,62] In the vessel wall, strain is circumferential, and the tendency of ECs to reorient perpendicular to the applied cyclic strain leads to EC alignment in the axial direction of the vascular wall.^[63] Elastin-like fibers provide a soft microenvironment that closely mimics that of the ECM, making them a powerful platform for the analysis of cellular behavior in physiological environments.

2.5. Feasibility of the Bottom-Up Approach

The bottom-up assembly of biobased fibers is a promising approach for the construction of complex tissues and organs.

We tested the feasibility of using the elastin-like fibers to fabricate hierarchically organized 2D and 3D structures by knitting, winding, weaving, and braiding. Both the plain and porous elastin-like fibers displayed an exceptional ability to bend without kinking (**Figure 7a,i and iii**) and to be tied off by knotting (**Figure 7a,ii,iv**). Knot-based assembly techniques like knitting can be used to create 3D geometries, as currently implemented in the medical implant industry.^[64] Indeed, we used the weft knitting technique to produce a knitted scaffold that is highly elastic and stretchable (**Figure 7b,ii and Video S1**, Supporting Information).

We successfully collected fibers of multimeter length onto a bobbin without compromising the diameter or length of the fibers (**Figure 7b**). The elastin-like fibers were also assembled into woven (**Figure 7b,v**) and braided structures (**Figure 7**) that

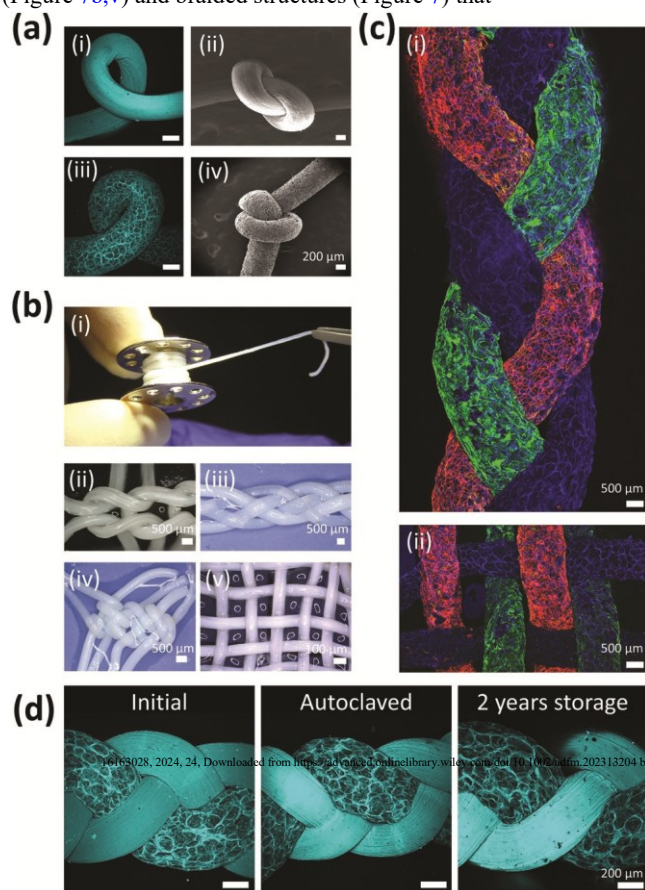


Figure 7. Feasibility of the bottom-up approach. a) Confocal and scanning electron microscopy (SEM) images showing the flexibility of the elastinlike fibers through bending and knotting, i,ii) plain fiber and iii,iv) porous fiber. b) Image of i) a multi-meter elastin-like fiber coiled on a bobbin, as well as ii) knitted, iii) braided, iv) soumak woven, and v) tabby woven elastin-like fibers. c) Elastin-like fibers cultured with human umbilical vein endothelial cells (HUVECs) and organized into textile patterns such as braiding and weaving. To facilitate the distinction of each fiber along the braided or knitted structure, each fiber was differentially stained with either an anti-CD31 antibody (red) to detect endothelial cells, or phalloidin (green) to detect the actin cytoskeleton. The blue fibers were unstained, and visualized thanks to their autofluorescence. d) Confocal images of a hybrid braided structure composed of two plain fibers and one porous fiber. The

structure was autoclaved, stored for 2 years at room temperature in a solution of 70% ethanol and washed in PBS before imaging.

were easy to manipulate. To further demonstrate the potential of this approach, we created a hybrid braided structure composed of plain and porous fibers. Confocal images of the resulting structure confirmed the successful interlocking of the porous and plain fibers (**Figure 7d**). To exploit the thermal resistance of these materials, we autoclaved and successfully stored the braided fibers for 2 years (**Figure 7d**). This confirms such textile constructs have the potential to be offered as off-the-shelf products, a preferred option in the biomedical field that promotes clinical adoption and allows the use of such products in emergency cases.^[65,66]

One significant advantage of the textile approach is its potential to automate tissue fabrication.^[5] To advance in that direction, we developed a custom-made circular loom, reducing the manual handling of the fibers and allowing us to fabricate a homogenous weaved tubular textile (**Figure 8**). To the best of our knowledge, this is the first ever tubular textile made solely from engineered elastin. Notably, fiber-like hydrogels tend to lack mechanical strength and are difficult to handle, making conventional methods for creating textile constructs challenging when using such materials.^[7] However, the elastin-like fibers were robust enough to overcome these obstacles, allowing their use for textile-like assembly.

Such tubular constructs could facilitate the realization of vascular prosthesis in the future. Vascular tissue engineering is not yet a routine clinical approach, despite the clear medical need and market demand. For example, the L'Heureux group developed vascular prosthesis derived from sheets of cell-assembled ECM that were rolled into tubes, matured in vitro, endothelialized and implanted into patients.^[67] However, the method was too complex and laborious for the economic preparation of grafts. More recently, the same group introduced textile technologies during the manufacturing scheme, which allows more versatility, and a faster and automatable production.^[68] Even so, the yarn is still based on

decellularized tissue, and requires a complex in vitro cell culture phase. The Niklason laboratory pioneered grafts based on decellularized ECM generated in vitro.^[69,70] These constructs decouple the in vitro culture and implantation phases, focusing on the use of allogenic rather than autologous cells. Accordingly, the manufacturing steps (cell isolation and expansion, tissue growth, and shipping) are carried out off-line, and the resulting grafts can be made available to patients needing rapid intervention. Phase 3 clinical trials are underway testing this concept as a means to facilitate vascular access for hemodialysis. However, these approaches still rely on tissue harvesting and/or cell isolation, with potential concomitant issues associated with unpredictable cell behavior during the production phase.

Alternative designs for tissue-engineered vascular prosthesis do not involve cells at any stage of production ("culture free") and do not rely on any donor material ("tissue free").^[71] These approaches are more material-centric,^[72] thus benefiting from potentially simpler adoption pathways. Their potential has been exemplified by the progress of some of these concepts to clinical trials.^[73] However,

these approaches has been thus far mainly focused on top-down fabrication processes such as electrospinning,^[74] and the field could benefit from strategies that better recapitulate the complex hierarchical structure of native tissues.

Despite remarkable achievements in the field, TE vascular prosthesis continue to struggle to achieve commercial success. This indicates the need for disruptive innovations that can leap over the valley of death in a true bench-to-bedside approach.^[75] Upcoming innovations should consider the pioneering work done so far in TE vascular grafts, and should implement lessons learnt into the design process. This includes a balance between graft functionality and economic feasibility, which is essential for successful translation.^[76]

The technology presented herein is built on the principles of versatility, controllability, reproducibility and storage, enabling for the first time the production of tubular elastin-like textiles. We are still far from the pioneered concepts of vascular

biomedical field and in the manufacture of everyday items. For instance, GORE-TEX is used both for the fabrication of high-value products like vascular grafts, and also as a breathable, waterproof fabric.

3. Conclusions

The platform presented herein offers precise control over the scaffold at multiple levels, ranging from the molecular level (polymers) to the micro (fibers) and macro (textile) levels. At the molecular level, we achieve this control through the custom-engineered design of the ELR chains. These ELRs serve as starting materials, which are then processed into microfibrils with a precisely controlled microstructure. These microfibrils are subsequently assembled into textiles. The toolbox that this platform embodies

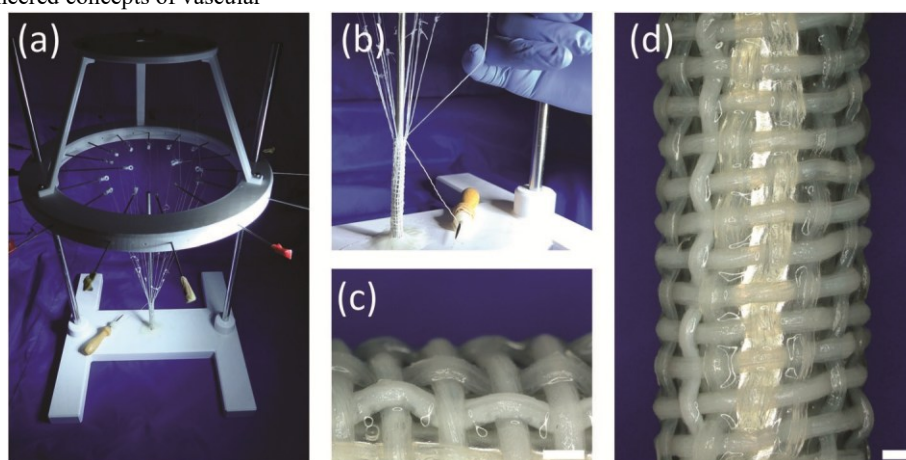


Figure 8. Textile-based assembly of elastin-like fibers (ELR-RGD and ELR-VKV) into tubular structures. a) Custom-made weaving loom, in which 15 longitudinal elastin-like fibers (15 cm) and one circumferential elastin-like fiber thread (2 m) are assembled. b) Detailed view of the fibers during the weaving process. c,d) Resulting woven tubular textile constructed from elastin-like hydrogel fibers. Scale bars = 1 mm.

prosthesis described above, but our technology provides the foundation for further exploration.

Textile-based assembly enables a further level of adaptability because the scaffold properties can be modulated not only by the inherent properties of the material but also by the hierarchical structure.^[4,34] This expands the range of properties that can be achieved, and allows local and directional fine tuning in accordance with the intended application. For instance, the warp and the weft of the construct can be prepared from ELRs with different properties, including degradation rate. By incorporating different types of fibers in both the warp and weft, precise control over the mechanical properties can be achieved.^[77] Overall, our approach integrates the customization and controllability provided by recombinant materials such as ELRs with the versatile assembly achieved using textile technologies. The new platform is not limited to high-value products in the biomedical field, but could also influence the garment industry, especially the development of long-lasting stretch fabrics. The ELR is a safe, renewable, non-animal product. This can pave the way for the manufacturing of clothes that could revolutionize the fabric industry. We have already witnessed examples in the past of material platform technologies that are applied both in the

opens up exciting new possibilities for the development of medical implants, where strict reproducibility and standardized production are essential prerequisites.

4. Experimental Section

Design and Functionalization of the ELRs: The ELRs used in this study were VKV, RGD and DRIR, each selected for their specific properties. VKV is a structural recombinamer lacking bioactive sequences, whereas RGD contains a tripeptide (Arg-Gly-Asp) that promotes cell attachment and DRIR includes the cleavage site for urokinase plasminogen activator (Asp-Arg-Ile-Arg).^[27,28] The recombinamers were chemically modified to carry azide and cyclooctyne groups by transformation of the ϵ -amine group present in the lateral chain of the lysine residue, as previously reported by us.^[78] Briefly, for the functionalization of ELRs with cyclooctyne or azide groups, (1R,8S,9S)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (SynAffix BV) or 2-azidoethyl (2,5-dioxopyrrolidin-1-yl) carbonate (GalChimia), respectively, were added to a 20 mg mL⁻¹ ELR solution in anhydrous DMF under N₂ atmosphere. The resulting mixture was stirred at room temperature for 60 h. Afterwards, diethyl ether was added at a volume ratio 3:1, resulting in a white precipitate. The supernatant was discarded, and the white precipitate was washed three times in acetone. The so-obtained product was dried under vacuum pressure and afterwards

resuspended in ultrapure water overnight (4 °C) and dialyzed against 25 L of ultrapure water at 4 °C (4 changes). The final product was lyophilized and stored at −20 °C until further use. The purity and monodispersity are systematically characterized by H-NMR and MALDI-ToF, respectively. Specifically, DRIR and VKV were modified to bear the cyclooctyne group, whereas RGD was modified to include the azide group. The detailed characteristics of the ELRs can be found in Table S1 (Supporting Information).

Fabrication of the Elastin-Like Fibers: The ELRs were dissolved in a 1:1 (v/v) mixture of phosphate-buffered saline (PBS; Thermo Fisher Scientific, USA) and ethanol (Sigma-Aldrich, USA) at room temperature to a final concentration of 100 mg mL^{−1}, as previously reported by us.^[22,30–32] Two solutions containing ELRs modified with cyclooctyne and azide groups, respectively, were loaded into separate syringes and placed into a dual injector holder (Tisseel, USA). The solutions were co-extruded through a mixing nozzle (DMG, Germany) into a silicon tube (Cole-Parmer, USA) and were left for 20 min to crosslink. The resulting fiber was ejected and washed with PBS. The diameter and length of the fiber were controlled by adjusting the size of the silicon tube. To introduce macroporosity, a SL/GF strategy was used.^[22] Specifically, NaHCO₃ particles were sieved to obtain two different size ranges (<40 μm and <100 μm) and dispersed in each ELR solution at a weight ratio of 1:10 (recombinant/particles). The solutions were co-extruded as described above, but the crosslinking reaction was extended to 30 min. The resulting elastin-like fibers were ejected and placed in 3 M citric acid for 30 min to dissolve the NaHCO₃ particles. The porous fibers were then washed three times with PBS for 16 h to eliminate any potential residues.

Sterilization of the Elastin-Like Fibers: Porous and plain ELR fibers were subjected to different sterilization schemes, and their effect on the microstructure and mechanical performance was systematically evaluated. For ethanol sterilization, the elastin-like fibers were immersed in 70% (v/v) ethanol for a minimum of 2 h followed by three washes with PBS. Alternatively, the fibers were autoclaved at 121 °C and 200 kPa for 15 min (dx-serie, Systec, Germany).

Microstructure Characterization: For SEM, the samples were first rinsed in PBS and then fixed in 3% glutaraldehyde in Sorensen's buffer at room temperature for 1 h. After fixation, the samples were dehydrated by immersing them in a graded ethanol series (40% to 100%, in 10% increments) for 15 min in each solution, followed by critical point drying in CO₂. The dried samples were then mounted on aluminum stubs and sputter coated with a 20-nm layer of gold-palladium. Images were captured using The Quattro S microscope (Thermo Fisher Scientific) with an accelerating voltage of 10 kV.

For brightfield imaging, samples were fixed in Carnoy's solution before embedding in paraffin, and were cut in 3-μm cross-sections, which were viewed using an AxioObserver Z1 epifluorescence microscope (Carl Zeiss, Germany). Images were acquired using an AxioCam MRm digital camera (Carl Zeiss) and analyzed with ImageJ software to quantify the porosity of the fibers. Three independent fibers were measured in three different regions per experimental condition.

For confocal microscopy, the samples were viewed using an inverted Zeiss LSM710 laser scanning confocal microscope fitted with an EC PlanNeofluar 10× objective. The images were acquired sequentially by unidirectional line scanning using Zen Black 2012 software (Carl Zeiss). The elastin-like fibers were imaged by exploiting their autofluorescence when excited at 405 nm, with emission in the range 410–495 nm.

Mechanical Characterization: The mechanical characterization of the fibers was carried out using a UNIVERT tensile test instrument (CellScale, Canada) equipped with a 1-N load cell. To assess the stress applied to the fibers, the diameter of each fiber was measured using a digital microscope (Keyence, Japan). These measurements were taken at a minimum of five points along the fiber and the average diameter was used to calculate the cross section of the fiber before testing. The individual fibers within each

sample type had a consistent diameter but there were minor differences between sample types (e.g., plain versus porous).

The fibers were immersed in a PBS bath heated to 37 °C and were clamped to the grips of the tensile test instrument 3–5 cm apart. To settle the % of stretching during the cycling testing, a preliminary test was done, in which the fibers were stretched until breakage, and 40% of the elongation at break for each type of fiber was selected as strain for the cyclic testing. For the systematic testing, the fibers were first subjected to 10 cycles of stretching from 0% to 200% strain, (for plain fibers) or from 0% to 60% strain (for porous fibers) using a constant strain rate of 0.15 mm s^{−1}. The fibers were then stretched to failure with the same constant strain rate. A stress-strain curve was plotted, and the initial elastic modulus (Young's modulus) and maximum tangent modulus were determined. The initial elastic modulus was calculated within the strain range of 0%–100% for the plain fibers and 0–50% for the porous fibers. The maximum tangent modulus was determined from the steepest slope, which depended on the experimental conditions. The ultimate tensile strength and the elongation to break were extracted from the stress-strain curve. The ultimate tensile strength was defined as the maximum stress reached by each sample during the test.

CD Spectroscopy: The ELRs were dissolved at a concentration of 1 mg mL^{−1} in ultrapure water. A 1:10 dilution was prepared immediately prior to each measurement, and was transferred to a quartz cuvette with a path length of 0.2 cm. The CD spectra were acquired using a Jasco J-815 150-S spectrometer, scanning over the wavelength range 190–260 nm. Points were acquired every 0.5 nm using a scan speed of 50 nm min^{−1}. Measurements were taken at temperatures of 5, 37 and 60 °C, and the reversibility of the conformational changes was also checked by recording after cooling (37 and 5 °C). Samples were equilibrated for 15 min before each measurement during heating, and for 30 min during cooling. Spectra were corrected by subtracting the corresponding blank solvent readings.

FTIR-ATR: Autoclaved and non-autoclaved elastin-like fibers (both fabricated by mixing ELR-RGD and ELR-VKV) were freeze-dried (Labconco lyophilizer) and evaluated by Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR) in an infrared spectrometer (Tensor 27, Bucker). FTIR-ATR spectra were obtained with 128 scans per spectrum at a spectral resolution of 2 cm^{−1} in the wavenumber from 4000 to 600 cm^{−1}.

Differential Scanning Calorimetry (DSC): Elastin-like scaffolds were prepared by mixing ELR-RGD and ELR-VKV at a concentration of 100 mg mL^{−1}, washed with PBS and autoclaved (when applicable) as described above. The samples were cut with a biopsy punch (Miltex) of 5 mm diameter, placed in a standard 40-μL aluminum crucible and hermetically sealed. The same volume of PBS was placed in the reference crucible. The DSC experiments were carried out in a Mettler Toledo 822e with a liquid nitrogen refrigerator by applying an isothermal stage (5 min at 0 °C) and a heating ramp (2 °C min^{−1} from 5 to 50 °C).

Gas Chromatography-Mass Spectrometry: To test whether ethanol traces could remain in the click hydrogel, the ELRs were dissolved in a 1:1 (v/v) mixture of phosphate-buffered saline (PBS; Thermo Fisher Scientific, USA) and ethanol (Sigma-Aldrich, USA) at room temperature to a final concentration of 100 mg mL^{−1}, as described above, and co-injected in a disc mold (volume 1 mL). The resulting click-elastin-like hydrogels were demolded and rinsed in PBS as follows: 1) 25 mL PBS, 30 min; 2) 25 mL PBS, 30 min; 3) 50 mL PBS, 30 min; 4) 50 mL PBS, 16 h.

Ethanol content was then determined by headspace-solid phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS) (7890A; Agilent Technologies). Briefly, the samples (1 mL) were placed in 20-mL headspace vials with silicon septa and incubated at 40 °C, 250 rpm for 15 min. Samples were analyzed with 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibers for 30 min at 40 °C. After sampling, the fiber was immediately inserted into the injection port of the GC equipment. An HP-INNOWAX column (60

m, 0.250 mm, 0.5 μm) (J & W Scientific, Folsom, CA, USA) was used. Helium flow was 1.2 mL min⁻¹ and the temperature was programmed as follows: 40 °C for 5 min, increased to 80 °C at 2.5 °C min⁻¹, and then maintained at that temperature for 5 min. A calibration curve was created with serial dilutions of ethanol in PBS from which the ethanol content in the hydrogel samples was quantified.

Cellular Studies Using the Elastin-Like Fibers: The elastin-like fibers were sterilized by autoclaving as described above and were left to cool before seeding with HUVECs isolated from human umbilical cords as previously described.^[79] Human umbilical cords were obtained after written informed consent at University Hospital Aachen, Aachen, Germany and were provided by the RWTH Aachen University Centralized Biomaterial Bank (cBMB), in compliance with its regulations, following RWTH Aachen University Medical Faculty Ethics Committee approval (cBMB project number 323). HUVECs were cultured in endothelial growth medium 2 (EGM2; PromoCell, Germany) supplemented with fetal calf serum, epidermal growth factor, basic fibroblast growth factor, insulin-like growth factor, vascular endothelial growth factor 165, ascorbic acid, heparin, and hydrocortisone. The cells were expanded under standard culture conditions in a humidified 5% CO₂ atmosphere at 37 °C. Primary cells in passages 1–3 were used for all the experiments. After the HUVECs reached 80%–90% confluency, they were trypsinized and resuspended in EGM2 medium to achieve the desired cell concentration. The elastin-like fibers were coaxially introduced into a silicon tube and clamped at one end. A cell suspension containing 1.5 mL of cells at a concentration of 0.7×10^6 cells mL⁻¹ was then introduced into the silicon tube, and the other end was also clamped. The silicon tube was placed in a sterile environment and rotated along the longitudinal axis at 1 rpm on an RS-TR 05 roller mixer (Carl Roth) for 2 h to facilitate cell attachment to the fibers. The fibers were then removed from the remaining cell suspension and placed in fresh culture medium for static cell culture.

For the dynamic culture, fibers from a 48-h static pre-culture were mounted on the MechanoCulture T6 (CellScale, Canada) in a humidified 5% CO₂ atmosphere at 37 °C and were mechanically stimulated for 24 h, undergoing uniaxial stretching (10% cyclic ramp stretch at 0.5 Hz). The control groups remained in static culture for 3 days.

Cell Staining, Visualization and Analysis of Cell Orientation: The fibers seeded with cells were washed with PBS and fixed with 4% methanol-free paraformaldehyde (Carl Roth) for 60 min at room temperature, then washed again three times with PBS. The samples were blocked for 1 h at room temperature with 0.5% normal goat serum (Agilent DaKo, USA) in 0.1% Triton X-100 (Sigma-Aldrich) and then incubated with a mouse primary monoclonal anti-CD31 antibody (Sigma-Aldrich) at a dilution of 1:10 to label the ECs. After washing as above, the samples were incubated for 1 h at room temperature with a goat anti-mouse IgG-HRP secondary antibody conjugated to Alexa Fluor 594 (Invitrogen, USA) at a dilution of 1:400. Both antibodies were diluted in 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS. To visualize actin filaments, the cells were permeabilized by incubation in 0.1% Triton-X 100 in PBS for 5 min, rinsed three times with PBS, and then stained with phalloidin-iFluor 488 conjugate (Cayman Chemicals, USA) at a dilution of 1:1000 in 1% BSA. The samples were then incubated for 90 min at room temperature, rinsed three times with PBS and incubated for 15 min with 1 μg mL⁻¹ DAPI (Carl Roth) in PBS to stain the nuclei, followed by three washes with PBS.

Samples were visualized by confocal microscopy as described above with excitation at 405 nm and emission in the range 410–495 nm for DAPI, excitation at 488 nm and emission in the range 495–630 nm for phalloidin-iFluor 488, and excitation at 561 nm and emission in the range 585–733 nm for the CD31-specific Alexa Fluor 594 staining. To determine the orientation of cells from the static and dynamic cultures, confocal images of phalloidin staining were analyzed with ImageJ^[80] using the OrientationJ Distribution function and the cubic spline algorithm with a Gaussian window of 2 pix. No cell accumulation or overlaps were added to the measurements.

Impact of Autoclaving on the Bioactivity of Elastin-Like scaffolds: Autoclaved and non-autoclaved scaffolds were prepared by mixing ELRs (ELRRGD and ELR-VKV) at a concentration of 100 mg mL⁻¹ as described above. The non-autoclaved scaffolds were produced under sterile conditions to avoid any post-treatment, and the autoclaved counterparts were sterilized as described before (121 °C, 15 min). Human umbilical vascular endothelial cells were seeded at a concentration of 0.7×10^6 cells mL⁻¹ and statically cultured for 24 h under standard culture conditions in a humidified 5% CO₂ atmosphere at 37 °C. The scaffolds were stained with an antiCD31 antibody, counterstained with DAPI, and then imaged using a confocal microscope (inverted Zeiss LSM710 laser scanning confocal microscope).

Cell Proliferation on Autoclaved Elastin-Like Scaffolds: Elastin-like scaffolds were fabricated by mixing ELR-RGD and ELR-DRIR at a concentration of 100 mg mL⁻¹, as previously described. The scaffolds were sterilized by autoclaving (121 °C, 15 min) and seeded with HUVECs at a concentration of 1.7×10^5 cells mL⁻¹, and cultured for 2 h, 24 h, 72 h and 5 days (humidified 5% CO₂ atmosphere at 37 °C). The media was changed every two days. The scaffolds were stained with an anti-CD31 antibody, counterstained with DAPI, and then imaged using a confocal microscope (inverted Zeiss LSM710 laser scanning confocal microscope). The single-cell nuclei were counted using CellProfiler Image Analyst software^[81] to quantify the number of cells at each time point. The pipeline consisted of the IdentifyPrimaryObjects module with diameter restrictions of 7 pixels to 30 pixels and adaptive thresholding parameters. The object outlines were drawn on the original image and manually inspected. Object counts were exported to a spreadsheet. The experiment was performed with $n = 3$ technical replicates for each time point.

Textile-Based Assembly: To assess the ability of the elastin-like fibers to form a higher-order 3D assembly, they were wound onto a bobbin and arranged in textile patterns using the techniques of weaving, knitting, and braiding. For the tubular weaved structure, one circumferential elastin-like-fiber (weft) was inserted between a movable and a fixed set of tensioned elastin-like fibers (warp) to create the woven textile in tubular form.

Statistical Analysis: Statistical differences were assessed using a t-test or two-way analysis of variance (ANOVA) followed by the *post-hoc* Holm-Sidak method. All experiments were conducted at least in triplicate ($n \geq 3$). The results are presented as means $\pm$ standard deviations (SD). The threshold for statistical significance was $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Any value of $p > 0.05$ was defined as nonsignificant (NS).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

bottom-up, engineered elastin, fibers, hierarchy, textile

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ADVANCED FUNCTIONAL MATERIALS

Supporting Information

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Protein-Engineered Elastin Fibers as Building Blocks for The Textile-Based Assembly of Tissue Equivalents

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Supporting Information

Protein-engineered elastin fibers as building blocks for the textile-based assembly of tissue equivalents

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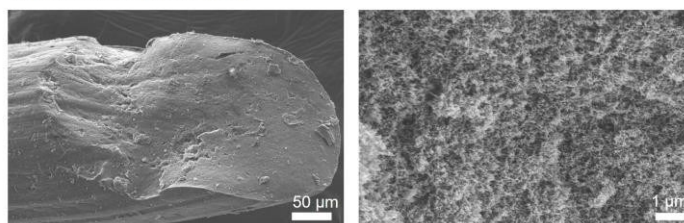
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Table S1: Amino acid sequence of the ELRs used in this study, indicating the reactive group for the catalyst-free click chemistry incorporated by the chemical modification of lysine side chains.

ELR abbreviated name	RGD Cell-binding	VKV Structural	DRIR Protease-sensitive
Sequence	MGSSHHHHHHSSGLVPRGSHMESLLP ([VPGIG] ₂ (VPGKG)(VPGIG) ₂) ₂ AVTGRGDSPASS ([VPGIG] ₂ VPGKG(VPGIG) ₂) ₆ V	MESLLP VG VPGVG [VPGKG(VPGVG) ₅] ₂₃ VPGKG VPGVG VPGVG VPGVG VPGV	MESLLP ([VPGIG] ₂ (VPGKG)(VPGIG) ₂) ₂ VYAVTGDRIIRSASPASSA ([VPGIG] ₂ VPGKG(VPGIG) ₂) ₄ V
Mw (Da)	60649.90	60450.91	42069.07
N° of amino acids	699	727	479
N° of lys	24	24	16
Type of modification	Azide	Cyclo-octyne	Cyclo-octyne
N° of lys modified	16 Lys	16 Lys	9 Lys

(a) VKV-RGD Plain (ø 1mm)



(b) DRIR-RGD Plain (ø 1mm)

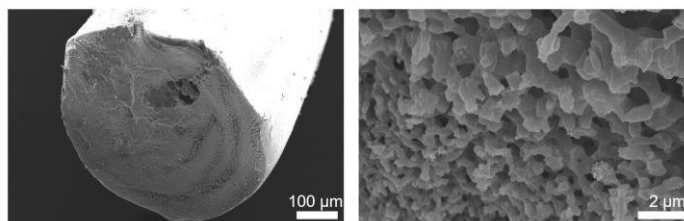


Figure S1: SEM images of the plain elastin-like fibers prepared from (a) ELR-VKV and ELR-RGD and (b) ELR-DRID and ELR-RGD. The left panels show the entire fiber cross-section and the right panels show higher magnifications revealing the detailed microstructure.

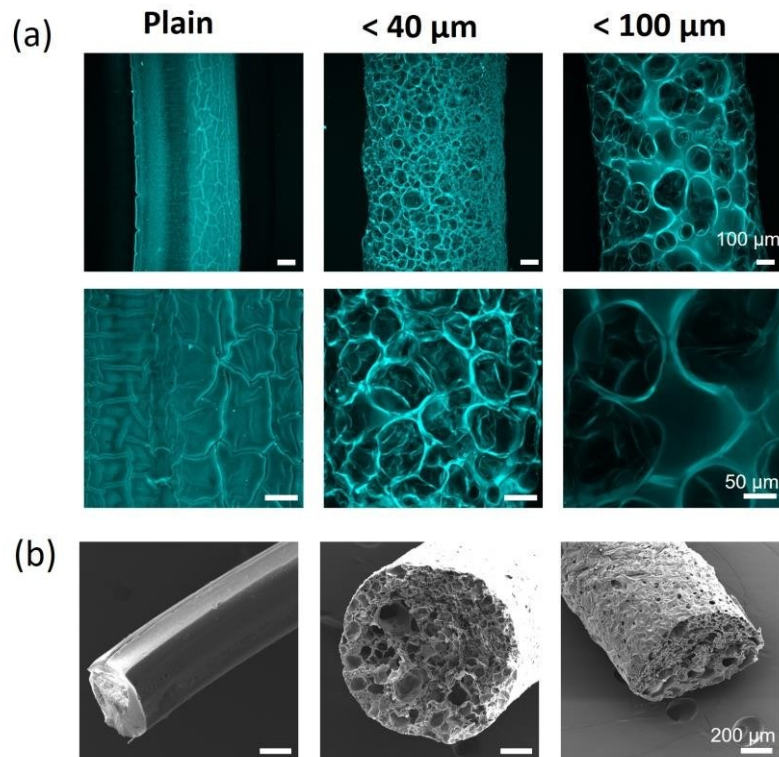


Figure S2: Microstructural characterization of elastin-like fibers prepared from ELR-DRIR and ELRRGD. (a) Confocal images. (b) Scanning electron microcopy images. The first column shows the plain fibers' microstructure and the second and third column show the fibers' microstructure obtained by implementing a SL/GF step within the fabrication process.

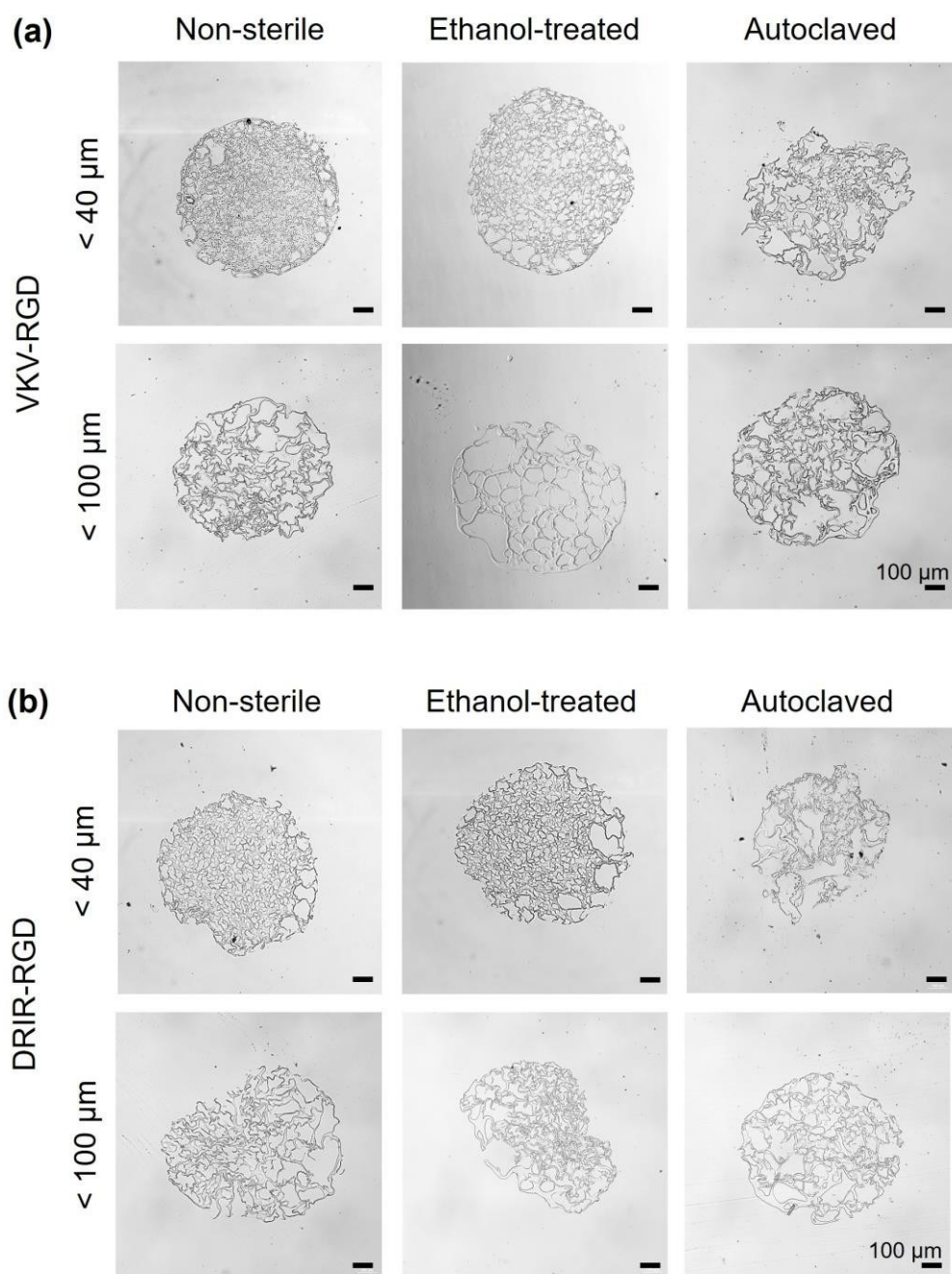


Figure S3: Brightfield images showing the microstructure of macroporous fibers following different sterilization techniques. (a) ELR-VKV and ELR-RGD. (b) ELR-DRIR and ELRRGD.

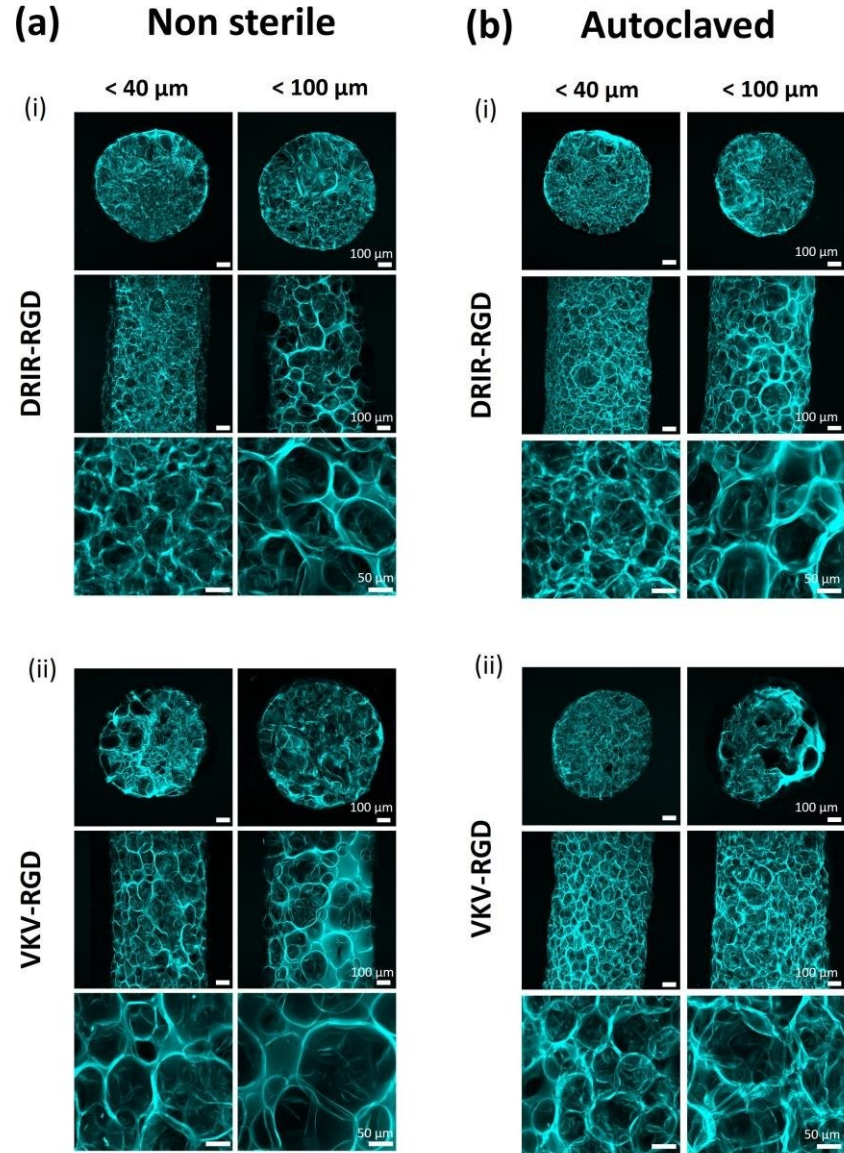


Figure S4: Confocal images showing the microstructure of macroporous fibers prepared from two different sets of ELRs (i.e., ELR-DRIR and ELR-RGD or ELR-VKV and ELR-RGD), (a) before sterilization and (b) after sterilization.

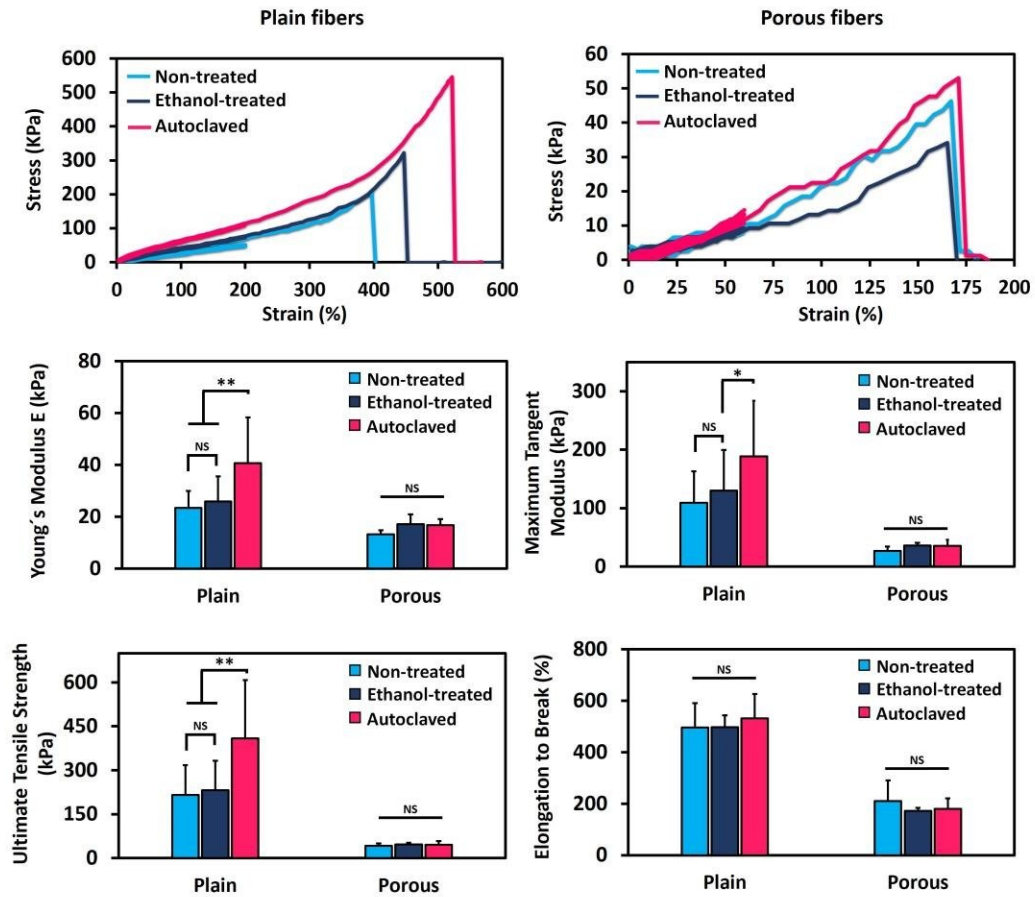
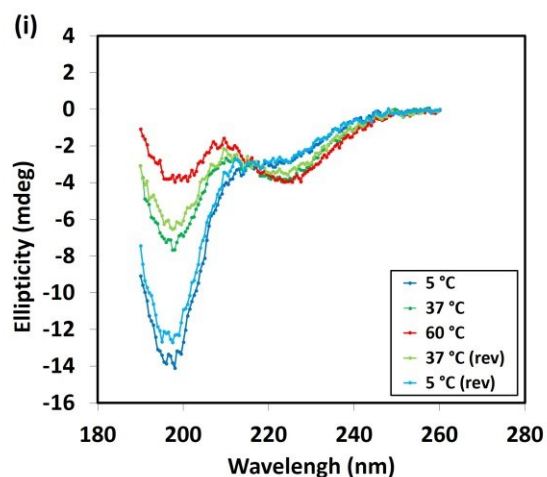


Figure S5: Impact of sterilization by ethanol treatment and autoclaving on the properties of elastin-like fibers prepared from ELR-VKV and ELR-RGD.

(a) Non-treated ELR



(b) Autoclaved ELR

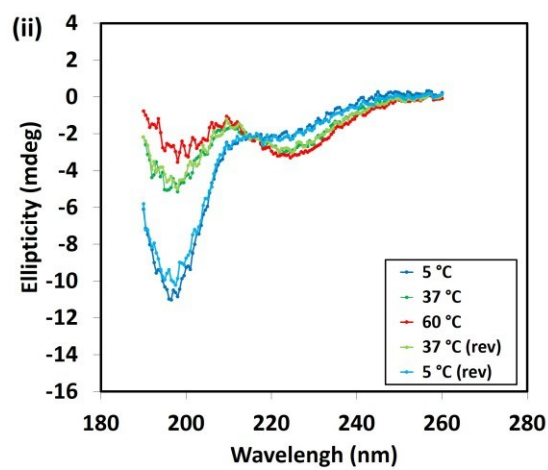
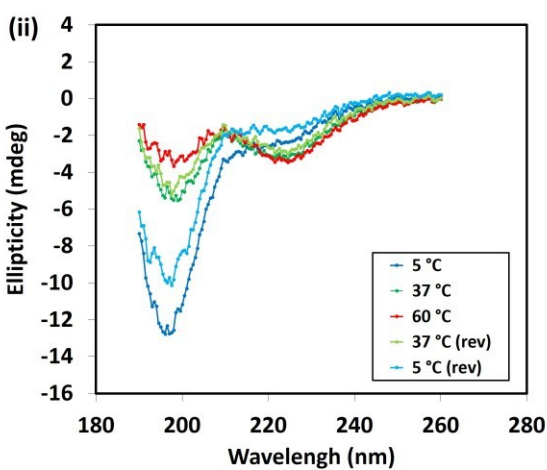
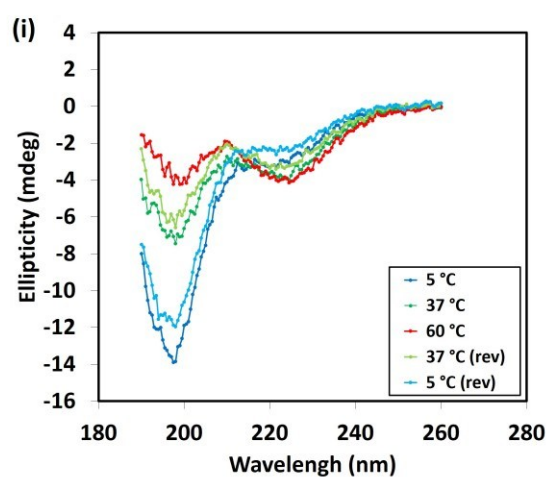


Figure S6: Circular dichroism spectra of ELRs (a) before and (b) after autoclaving. (i) ELRRGD. (ii) ELR-DRIR. The spectra were recorded at different temperatures (5, 37 and 60 °C) and the reversibility of the conformational changes was checked by recording after cooling.

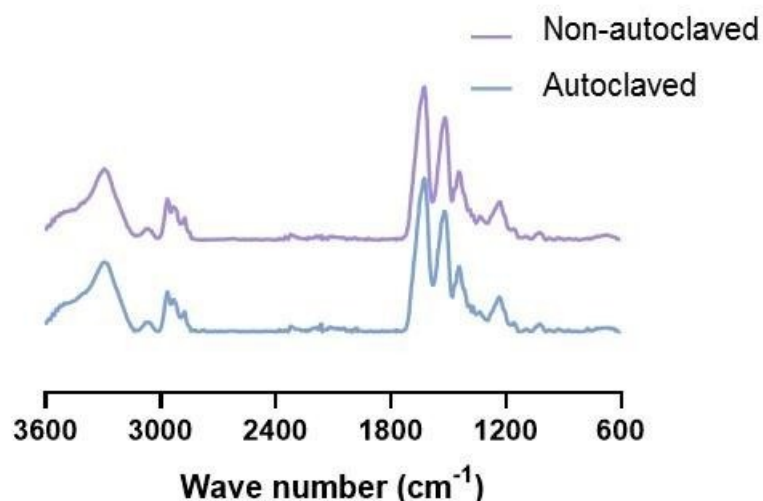


Figure S7: FTIR-ATR spectra of non-autoclaved and autoclaved elastin-like fibers. The characteristic bands associated with the amide group of the peptide bond (i.e. the Amide A peak at approx. 3300 cm^{-1} attributed to NH stretching, the Amide I peak at around 1660 cm^{-1} related to C=O stretching, and the Amide II region at 1550 cm^{-1} involving both C-N stretching and NH bending)^[1] did not experience any detectable change after autoclaving. This strongly suggests that the autoclaving process does not alter the integrity of the elastin-like molecules within the fibers.

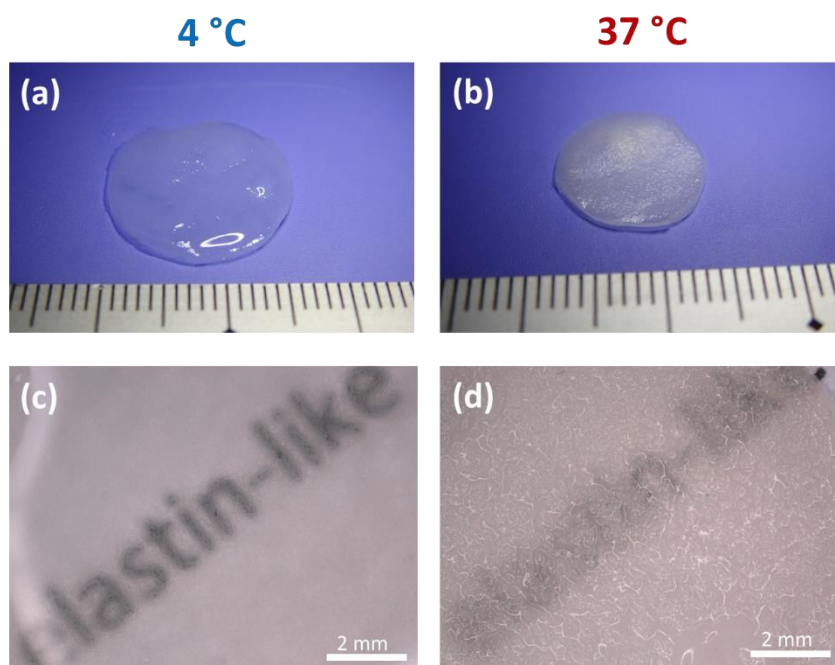


Figure S8: Autoclaved click-elastin-like scaffold (fabricated by mixing ELR-RGD and ELRVKV, and using the salt-leaching gas-foaming approach), cooled down to $4\text{ }^{\circ}\text{C}$ (a and c) and warmed up to $37\text{ }^{\circ}\text{C}$ (b and d). At $4\text{ }^{\circ}\text{C}$, the hydrogel acquires a swollen and quasi

transparent state while at 37 °C, it experiences a temperature-induced contraction and a reduction in the transparency, as typically described for these polymers [2].

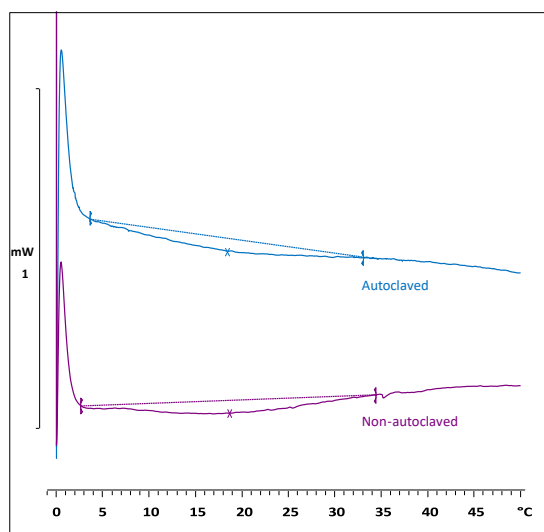


Figure S9: DSC scans for autoclaved and non-autoclaved click-elastin-like scaffolds. No detectable changes after autoclaving were detected, and both thermograms were characterized by a rather broad endothermic peak, as it is characteristic for the cross-linked state of this type of polymers [3].

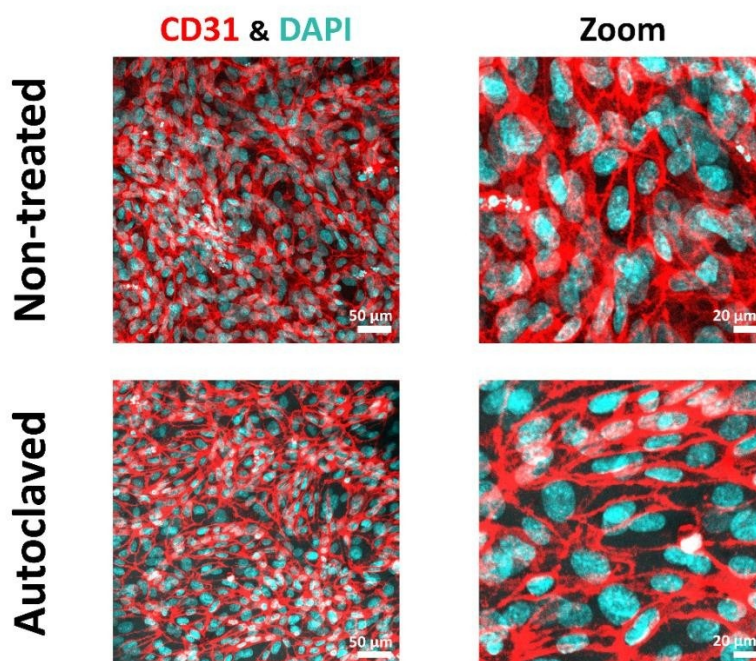


Figure S10: Confocal images of elastin-like scaffolds (ELR-RGD and ELR-VKV) autoclaved and non-autoclaved (non-treated) cultured with primary human umbilical vein endothelial cells

(HUVECs) for 1 day under static conditions. Endothelial cells are stained with antibodies against the CD31 marker (red) and counterstained with DAPI to reveal the nuclei (turquoise).

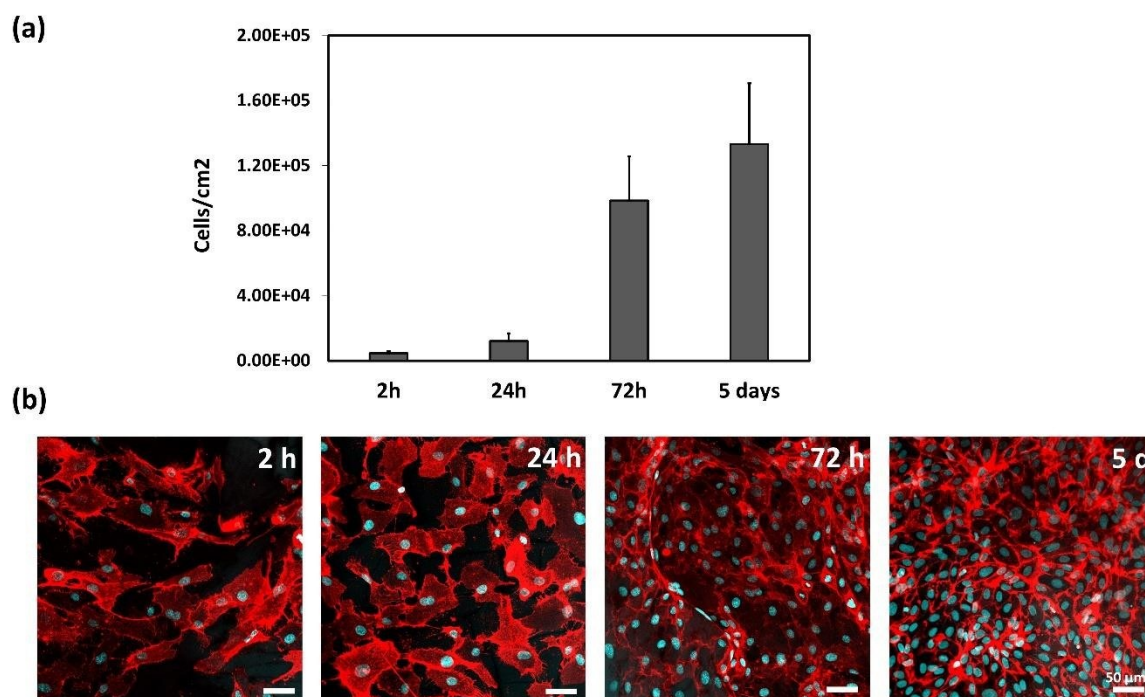


Figure S11: Cell proliferation (a) Quantification of HUVECs on autoclaved click-elastin-like scaffolds after 2h, 24 h, 72 h and 5 days of culture (b) Confocal images of autoclaved clickelastin-like scaffolds cultured with HUVECs at different time-points. Endothelial cells are stained with antibodies against the CD31 marker (red) and counterstained with DAPI to reveal the nuclei (turquoise).

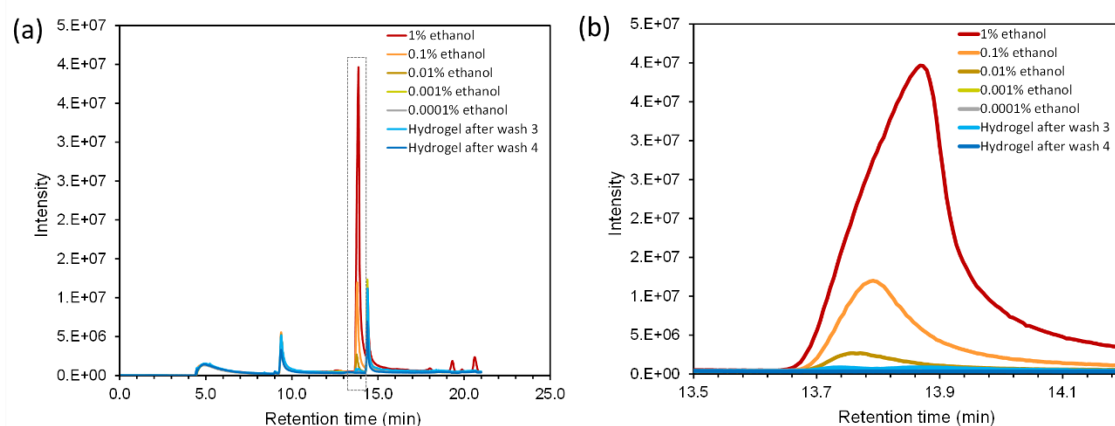


Figure S12: Assessment of ethanol removal from click-elastin-like hydrogels. (a) Mass chromatograms of serial dilutions of ethanol in PBS and of elastin-like hydrogel samples after 3 and 4 washes. (b) Close up view of the peaks used to quantify ethanol content.

Video S1: Weft-knitted scaffold prepared from elastin-like fibers, showing elastic and stretchable behavior.

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Alhamdulillah.

Affidavit according to § 5 (1) for Data Retention

Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten (I hereby declare that the original data forming the basis of this doctoral thesis are stored)

in der **Abteilung für Biohybride & Medizinische Textilien (BioTex), Institut für Angewandte Medizintechnik**, des Universitätsklinikums Aachen

hinterlegt sind.

Affidavit according to § 5 (1) and (2), and § 11 (3) 12 of the doctoral studies regulations

I, **EI Maachi Ikram**, hereby declare on oath, that I have contributed a significant part, and thus majority, of the publication:

EI Maachi I., Kyriakou S., Rütten S., Kopp A., Köpf M., Jockenhoevel S., and Fernández-Colino A.: Silk Fibroin as Adjuvant in the Fabrication of Mechanically Stable Fibrin Biocomposites; *Polymers*; 2022,14, 2251.

The contributions to the publication were as follows:

Names -⑦	El Maachi Ikram	Kyriakou Stavroula	Rütten Stephan	Kopp Alexander	Köpf Marius	Jockenhoevel Stefan	Fernández-Colino Alicia	Sum (%)
Study supervision						20	80	100
Study design/conception	20						80	100
Investigation	85	10	5					100
Collection of data	90	5	5					100
Data analysis	100							100
Statistical evaluation	100							100
Resources				10	30	50	10	100
Interpretation of data evaluation	80						20	100
Writing/draft of the manuscript	100							100
Correction of the manuscript				5	5	10	80	100

The position as the first author obviously arises from this significant contribution.

Signature of the doctoral candidate

As supervisor and /or corresponding author I confirm the statements of EI Maachi Ikram and as a representative of the non-reachable co-author Dr. Köpf Marius

Signature of the doctoral supervisor

As co-author I endorse the statement of Dr. Alicia Fernández-Colino

Kyriakou Stavroula

Rütten Stephan

Köpf Marius

Kopp Alexander

Jockenhoevel Stefan

Affidavit according to § 5 (1) and (2), and § 11 (3) 12 of the doctoral studies regulations

I, **El Maachi Ikram**, hereby declare on oath, that I have contributed a significant part, and thus majority, of the publication:

El Maachi I., Loewen A., Acosta S., Rütten S., Rodríguez-Cabello J.C., Jockenhoevel S., Fernández-Colino A.: Protein-Engineered Elastin Fibers as Building Blocks for The Textile-Based Assembly of Tissue Equivalents; *Adv. Funct. Mater.*; 2024, 34, 2313204. The contributions to the publication were as follows:

Names -⑦	El Maachi Ikram	Loewen Alexander	Acosta Sergio	Rütten Stephan	RodríguezCabello José Carlos	Jockenhoevel Stefan	Fernández-Colino Alicia	Sum (%)
Study supervision							100	100
Study design/conception	20						80	100
Investigation	95		5					100
Collection of data	85	10	5					100
Data analysis	95		5					100
Statistical evaluation	100							100
Resources					30	40	30	100
Interpretation of data evaluation	80						20	100
Writing/draft of the manuscript	100							100
Correction of the manuscript		2	2		3	3	90	100

The position as the first author obviously arises from this significant contribution.

Signature of the doctoral candidate

As supervisor and / or corresponding author I confirm the statements of El Maachi Ikram

Signature of the doctoral supervisor

As co-author I endorse the statement of Dr. Alicia Fernández-Colino

Loewen Alexander

Acosta Sergio

Rodríguez-Cabello José Carlos

Rütten Stephan

Jockenhoevel Stefan