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Engineering of a bio-functionalized hybrid off-the-shelf heart valve☆

Svenja Hinderer [a,b,c], Jan Seifert [d], Miriam Votteler [a], Nian Shen [b], Johannes Rheinlaender [d], Tilman E. Schäffer [d], Katja Schenke-Layland [a,b,e,*]

[a] University Women's Hospital, Eberhard-Karls-University Tübingen, 72076 Tübingen, Germany
[b] Dept. of Cell and Tissue Engineering, Fraunhofer Institute for Interfacial Engineering and Biotechnology (IGB), 70569 Stuttgart, Germany
[c] Institute for Interfacial Process Engineering and Plasma Technology (IGVP), University of Stuttgart, 70569 Stuttgart, Germany
[d] Institute of Applied Physics and LISA+, Eberhard-Karls-University Tübingen, 72076 Tübingen, Germany
[e] Dept. of Medicine/Cardiology, Cardiovascular Research Laboratories, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

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ABSTRACT

Currently available heart valve replacements are limited in long-term performance or fail due to leaflet thickening, lack of growth or remodeling potential. In order to address these issues, it is necessary to mimic multiple factors of the native valvular extracellular matrix (ECM) such as architecture, mechanical behavior and biochemical signals. Here, we successfully generated an electrospun PEGdma–PLA scaffold adapted to the structure and mechanical properties of native valve leaflets. Valvular interstitial cells (VICs) and valvular endothelial cells (VECs) were seeded on the scaffold and when cultured under physiological conditions in a bioreactor, the construct performed like a native leaflet. Atomic force microscopy (AFM) was employed to obtain detailed mechanical information from the leaflets, which enabled the first layer-specific measurement of the Young's modulus. Interestingly, spongiosa stiffness was much lower compared to the fibrosa and ventricularis. Moreover, investigations into human fetal heart valve development identified collagen type I and versican as important structural proteins. As a proof of principle, these proteins were introduced to the scaffold, demonstrating the ability to bio-functionalize the hybrid valve based on natures' blueprint.

1. Introduction

A major limitation of the currently clinically available heart valve replacements is their incapability to grow or remodel post-implantation [1]. The valvular extracellular matrix (ECM) is a complex fibrous network composed of structural proteins such as collagens, elastic fibers and microfibrils as well as signaling molecules like water-storing proteoglycans (PGs), glycosaminoglycans (GAGs) and growth factors [2]. Electrospinning is a suitable method to generate fibrous scaffolds that mimic the ECM structure [3–5]. A high electric field is applied to a droplet polymer fluid. As the force from the electric field overcomes the surface tension of the polymer droplet solution, a fiber is formed, which travels in spinning motions to the counter electrode, while the solvent evaporates [3]. By changing parameters, for example polymer, solvent, voltage or electrode distance, the fiber size as well as the mechanical properties of the scaffold can be adjusted [6]. Additionally, it is possible to electrospin ECM components in order to biochemically functionalize the material [5]. It has been previously shown that the presence of defined signaling molecules, three-dimensionality and appropriate mechanical properties significantly impact cell survival, adhesion, migration, proliferation and differentiation [7–10]. In natural ECM, the Young's modulus strongly varies depending on the organ. Bone tissue for example has a stiffness of 10^6–10^7 kPa, meanwhile skin is much more elastic with a modulus of 10–100 kPa [7]. The matrix density and thus the stiffness of a matrix can be used as a cue for stem cell fate decision [8]. Accordingly, mesenchymal stem cells were differentiated into neural tissue when cultured on hydrogels with a stiffness of 0.1 kPa, into myogenic tissue when exposed to 11 kPa hydrogels and osteogenic differentiation was achieved when the cells were cultured on 34 kPa matrices [8]. Maturation of neonatal rat cardiomyocytes was realized exposing the cells to 10 kPa, which was defined as the stiffness of native rat myocardium [11]. The Young's modulus is an important parameter for tissue growth and remodeling. Therefore,

* Corresponding author. Fraunhofer IGB Stuttgart, Department of Cell and Tissue Engineering, Nobelstr. 12, 70569 Stuttgart, Germany. Tel.: +49 711 970 4082; fax: +49 711 970 4158.
E-mail addresses: katja.schenke-layland@igb.fraunhofer.de, ksl@igb.fraunhofer.de (K. Schenke-Layland).

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it is important to identify the mechanical properties of the intact tissue and translate these findings to organ- and tissue-tailored biomaterials design [12].

Heart valve leaflets are permanently exposed to a high pressure of 120/80 mmHg as well as to laminar and oscillatory shear stress due to blood flow [13]. An engineered heart valve material should therefore not only mimic the mechanical properties of the native ECM, but also withstand the mechanical forces in vivo. In addition to comprehensive materials and cell–matrix interaction analyses, we employed atomic force microscopy (AFM) in order to measure the Young's modulus in each layer of native heart valve leaflets. Here, we aimed to design a hybrid bio-functionalized heart valve that mimics the native aortic valve, which can be potentially manufactured as off-the-shelf medical product.

2. Materials and methods

The studies involving human tissues were in accordance with institutional guidelines and were approved by the local Ethics Committees at the University of California Los Angeles (UCLA) and the University Hospital of the Eberhard Karls University (UKT) (UCLA IRB #05-10-093; UKT IRB #356/2008BO2 and #406/2011BO1). The research was carried out in compliance with the rules for investigation of human subjects, as defined in the declaration of Helsinki.

2.1. Electrospun scaffold fabrication

Electrospinning was performed using a customized electrospinning device as described before [5]. Equal amounts of poly(ethylene glycol) dimethacrylate (PEGdma; 687529, Sigma, Steinheim, Germany) and poly(L-lactide) (PLA; 93578, Sigma) were dissolved in 1,1,1,3,3,3 hexafluoro-2-propanol (HFP; 804515, Merck, Darmstadt, Germany). One percent 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Initiator; 410896, Sigma) was added to the solution to enable subsequent crosslinking. Electrospinning was performed in the dark using a 20 G nozzle and 18 kV. The distance of the nozzle to the target collector was 15 cm. To induce crosslinks between the dimethacrylated groups, the initiator was activated with UV light (256 nm; 3J per side) subsequently after the electrospinning process. For the pure PLA scaffolds, 0.15 mg PLA was dissolved per mL HFP. The polymeric PLA-solution was then electrospun with a distance of 20 cm using 12 kV and an 18 G nozzle. Both scaffold types were treated with 70% ethanol and washed thoroughly with sterile cell culture water prior to cell seeding. Furthermore, the scaffold performance under physiological conditions was investigated [14]. Details are displayed in the supplements.

2.2. Cell seeding and culture

Valvular endothelial cells (VECs) and valvular interstitial cells (VICs) were isolated from porcine heart valve leaflets by collagen digestion [15]. Endothelial cell growth medium (C-22010, Promocell, Heidelberg, Germany) was used for VECs and smooth muscle cell growth medium (C-22062, Promocell) was used for the culture of VICs. The medium was exchanged every second day. For cell–matrix interaction studies, 2.5×10^5 cells were seeded per PLA or PEGdma–PLA scaffold and cultured for 4 days at 37 °C and 5% CO_2. Cell viability was determined using an MTT assay.

2.3. Scanning electron microscopy (SEM)

Native tissues as well as unseeded and cell-seeded electrospun scaffolds were analyzed using a scanning electron microscope (1530 VP, Zeiss, Jena, Germany). All samples were rinsed with DPBS and incubated in a 2% glutaraldehyde solution for 45 min. To remove remaining water, the samples were passed through ascending alcohol concentrations (25%, 50%, 70%, 2 × 96% ethanol, isopropanol). Subsequently, the samples were dried at room temperature, mounted onto stubs and sputtered with platinum for 60 s.

2.4. Electron spectroscopy for chemical analysis (ESCA)

ESCA was performed to determine the atomic composition of the scaffold surfaces. Electrospun scaffolds were placed into the analysis chamber of the Axis Ultra device (Kratos Analytical, New York, USA) and a pressure of 10^{-9} mbar was applied. All samples were activated via X-rays and the kinetic energy of emitted auger and photoelectrons was measured. To validate the presence of both polymers (PEGdma and PLA) on the fiber surface, the obtained results were compared with known values from the literature for the single components.

2.5. Atomic force microscopy

Fresh dissected valve leaflets ($n = 4$) and electrospun scaffolds ($n = 3$) were immersed in water-soluble tissue freezing medium (14020108926, Leica, Nussloch, Germany) and immediately frozen at −80 °C. Cryosections with a thickness of 10–20 μm were prepared using a cryotome (Microm HM 560, Thermo Scientific, Waltham, MA, USA). The slides were thawed and brought to room temperature prior to AFM measurements. A commercial AFM setup (MFP3D Bio, Asylum Research, Santa Barbara, CA) was used to perform force mapping [16] on the sample sections. The measurements were performed in phosphate buffered saline (PBS) using a single sphere-tip cantilever (FM-M-SPL, Nanoworld, Neuchâtel, Switzerland) [17] with a radius of 980 nm and a spring constant of 4.0 N/m, determined by the thermal noise of the cantilever [18]. Force–distance-curves were recorded within the scan area on 30 × 30 points (on the ventricularis, spongiosa and fibrosa layer of each leaflet) or on 40 × 40 points (on the scaffold sections). The scan area was chosen between 40 × 40 μm^2 and 90 × 90 μm^2, depending on the size of each layer. The force curve rate was 2.5 Hz, resulting in a force curve velocity of 20 μm/s. Each force–distance-curve was analyzed by fitting the spherical Hertz model [19], giving images of the local Young's modulus $<E>$ as a dimension of local stiffness ("stiffness images").

For each stiffness image the layer-specific mean Young's modulus was calculated as the geometric mean, because the Young's modulus of tissues is log-normally distributed [20–22]. Statistical significance was determined by using Student's t-test on the logarithmic values. In addition, the mean moduli were averaged separately for each layer to give a total modulus $<E>_{total}$ for each layer.

2.6. Uniaxial tensile testing

Native leaflets and electrospun scaffolds were cut into 10 mm × 40 mm rectangular pieces and clamped into the uniaxial tensile testing device (Zwick Roell, Ulm, Germany). The exact sample dimensions were determined before each measurement and recorded with the software for further calculations of Young's modulus, tensile strength and elongation. An initial load of 0.1 MPa was used and the scaffolds were then stretched with a velocity of 5 mm/min. Native leaflets were directly measured after dissection. For better comparison, the scaffolds were also measured in wet state. All measurements were performed at room temperature.

2.7. Contact angle measurement

Hydrophilicity of the electrospun substrates was analyzed with an OCA 40 (DataPhysics Instruments GmbH, Filderstadt, Germany). A water drop with the volume of 2 μl was placed onto the sample and the contact angle was measured using a video setup and the SCA20 software (DataPhysics Instruments).

2.8. Swelling ratio

To determine the water-holding capacity of the native valve tissues and electrospun scaffolds, the samples were weighed in their dry (W_{dry}) and wet state (W_{wet}) after 4 h swelling in water. The swelling ratio was calculated with the following formula:

$$\text{Swelling ratio}[\%] = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100$$

2.9. Immunofluorescence staining

Four days after VIC and VEC seeding, the scaffolds were rinsed with PBS and processed for antibody staining. Unspecific binding sites were blocked using goat serum. Paraffin-embedded human tissues were treated as previously described [23]. As primary antibodies served: anti-vWF (1:200; A0082, Dako, Hamburg, Germany), anti-αSMA (1:500; A2547, Sigma), anti-FAK (1:100; ab4803, Abcam, Cambridge, UK), anti-vinculin (1:500; MAB3574, Millipore, Darmstadt, Germany), anti-collagen type I (1:100; HPA008405, Sigma) and anti-versican (1:375; HPA004726, Sigma). After overnight incubation at 4 °C, the antibody solution was removed and the scaffolds were carefully rinsed with PBS. A fluorescence-conjugated secondary antibody (1:250; Alexa Fluor® 488, Molecular Probes, Darmstadt, Germany) served to visualize the protein. Alexa Fluor 546-conjugated phalloidin (1:50; A12381, Alexa Fluor® 546, Molecular Probes, Darmstadt, Germany) was added to the secondary antibody solution and the mixture was incubated for 25 min in the dark. Further washing steps followed prior to DAPI staining. Images were taken with a Laser Scanning Microscope (LSM710 inverted confocal microscope, Carl Zeiss, Jena, Germany).

2.10. Generation of a bio-functionalized leaflet

Electrospinning of PEGdma-PLA was performed as described earlier. A copper negative mold of a valve served as the collector (Suppl. Fig. 1). After electrospinning, the valve was removed from the mold and a collagen type I gel-coating was performed to mimic the fibrosa. For gel formation, a ratio of 2:1 collagen type I (10 mg/mL in acetic acid) and neutralization buffer was mixed and subsequently added onto the scaffold [24]. The construct was placed in an oven and dried at 60 °C for approximately 1 h. Furthermore, 50 μL human versican (H00001462-P01, Novus Biologicals, Littleton, USA) was mixed in PBS, containing 15% PEGdma and 0.01% photoinitiator. The tips of the leaflets were dipped in a versican–PEGdma solution and additionally crosslinked using 365 nm UV light in order to mimic the thick versican-containing tips of native heart valve leaflets. After fixation using 4% paraformaldehyde and paraffin embedding, 10 μm sections were generated and Alcian blue (visualization of PGs and GAGs) as well as Saffron (visualization of collagen-containing structures) staining were performed to visualize the engineered constructs and to compare it to native tissues.