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METHODS ARTICLE

Multiple-Step Injection Molding for Fibrin-Based Tissue-Engineered Heart Valves

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Heart valves are elaborate and highly heterogeneous structures of the circulatory system. Despite the well accepted relationship between the structural and mechanical anisotropy and the optimal function of the valves, most approaches to create tissue-engineered heart valves (TEHVs) do not try to mimic this complexity and rely on one homogenous combination of cells and materials for the whole construct. The aim of this study was to establish an easy and versatile method to introduce spatial diversity into a heart valve fibrin scaffold. We developed a multiple-step injection molding process that enables the fabrication of TEHVs with heterogeneous composition (cell/scaffold material) of wall and leaflets without the need of gluing or suturing components together, with the leaflets firmly connected to the wall. The integrity of the valves and their functionality was proved by either opening/closing cycles in a bioreactor (proof of principle without cells) or with continuous stimulation over 2 weeks. We demonstrated the potential of the method by the two-step molding of the wall and the leaflets containing different cell lines. Immunohistology after stimulation confirmed tissue formation and demonstrated the localization of the different cell types. Furthermore, we showed the proof of principle fabrication of valves using different materials for wall (fibrin) and leaflets (hybrid gel of fibrin/elastin-like recombinamer) and with layered leaflets. The method is easy to implement, does not require special facilities, and can be reproduced in any tissue-engineering lab. While it has been demonstrated here with fibrin, it can easily be extended to other hydrogels.

Introduction

Semilunar heart valves are highly complex and specialized components of the circulatory system that ensure the unidirectional blood circulation. Valvular dysfunction has drastic consequences and, if untreated, it can lead to increased disability and mortality.[1,2]

Current replacement therapies imply long-term anticoagulation therapy (mechanical prostheses) or structural degeneration (biological prostheses) and lack the potential for growth and remodeling, so that reoperations are needed for pediatric patients to match the size of the valve to the changing size of the patient's heart.[3] Reinterventions are also needed in elderly patients who outlive a degenerated bioprosthesis because of the increased life expectancy.[4,5]

In the field of tissue engineering (TE), various approaches to create living semilunar heart valves with growing and remodeling capabilities have been developed based on different scaffold materials as well as on cell sources.[6–8] This is a very challenging task, as native heart valves are structures that are optimized for their function: The tissue of the vascular wall and that of the leaflets differ in cellular and extracellular matrix (ECM) composition and, as a consequence, in the biomechanical properties.[9] The vascular wall in the aortic and pulmonary roots contains mostly smooth muscle cells (SMCs) characterized by their contractility and the expression of α smooth muscle actin (α-SMA).[10,11] The predominant cell type of semilunar leaflets is the valvular interstitial cell (VIC), which, in health, appears in its α-SMA-negative, fibroblast-like quiescent phenotype, with only an α-SMA-positive subpopulation in the subendothelial layer. The leaflets of semilunar heart valves are composed of layers with specific functions and are mechanically anisotropic.

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Despite the well-known complexity, heterogeneity, and anisotropy of the native semilunar heart valves[7,8] and the well-investigated relationship between structure and function,[9] most polymer-based TE concepts employ one cell type that is capable of ECM synthesis for the whole construct (vascular part and leaflets) and one scaffold material or one combination of materials without any regional optimization.

To date, only a few attempts have been reported in the direction of fabrication of a tissue-engineered heart valves (TEHVs) with biomimetic design. Tedder *et al.* realized layered leaflets that should then be sutured to the native wall[12] by combining layers of decellularized tissue of different origin with the goal of mimicking the physiological structure of native valves. The leaflets resulted in being stiffer than the native ones, probably due to glue used to hold the different components together. Hockaday *et al.* showed the applicability of three-dimensional (3D) printing as a method to create a scaffold with two different PEG-DA compositions for the wall and the leaflets,[13] which was then cultivated for 21 days in static conditions. Neither dynamic functionality of the valve nor tissue formation was reported. The method relies on the presence of a photoinitiator and requires access to a (modified) 3D printer.

Our group and others[14–16] use fibrin as the key scaffold component for TEHVs because of the numerous advantages it offers, including its autologous origin,[17] the rapid polymerization, the tunable degradation via protease inhibitors,[14] and the manufacturability into complex 3D geometries.[18]

Here, we exploit the versatility of fibrin as a scaffold material and of the fibrin-based molding technique first reported by Jockenhoevel *et al.*[18] by developing a multiple-step molding process that opens up possibilities toward the fabrication of biomimetic heart valves with controlled spatial heterogeneity in terms of material composition and cell phenotype. The process relies on the capability of fibrin to crosslink with other synthetic and natural polymers and on the possibility to confine the different materials and combinations of materials and cells to specific substructures (leaflets, vascular wall, layered leaflets) by the molding technique without the need of gluing or stitching.

Specifically, we demonstrate the potential of the process to obtain valves with (1a) different cell lines in the leaflets and in the wall; (1b) different gel compositions in the leaflets and the wall (demonstrated by the use of an elastin-like recombinamer (ELR)-fibrin hybrid gel); and (2) structurally layered leaflets. The method delivers heart valves as whole constructs, with leaflets connected to the wall, that can withstand the opening and closing cycle in bioreactors and continuous dynamic cultivation.

Materials and Methods

Fibrin synthesis

Human fibrinogen (Calbiochem, Darmstadt, Germany) was dissolved in purified water (Milli-Q™; Millipore, Schwalbach, Germany) and dialyzed with a cut-off membrane (Novodirect, Kehl, Germany) of 6000–8000 MW overnight against Tris-buffered saline (TBS). The fibrinogen concentration after sterile filtration was estimated by measuring absorbance at 280 nm with a spectrophotometer (Spectronic Genesys™6; Thermo Fisher Scientific GmbH, Dreieich, Germany). The final concentration of the fibrinogen solution was adjusted to 10 mg/mL with sterile TBS. The fibrin gel for the TEHV wall consisted of 2.5 mL fibrinogen solution (10 mg/mL), 1 mL TBS, and 0.75 mL 50 mM $CaCl_2$ (Sigma-Aldrich, Seelze, Germany) in TBS. Fibrin polymerization was initialized by adding 0.75 mL of thrombin solution. For the valvular leaflets, the amounts were as follows: 1 mL fibrinogen solution, 0.4 mL TBS, 0.3 mL of 50 mM $CaCl_2$ in TBS, and 0.3 mL thrombin solution (40 U/mL; Sigma-Aldrich).

Hybrid ELR-fibrin gel synthesis

For the implementation of a different scaffold material, we used HRGD6, which is an ELR that bears a bioactive sequence (RGD) to improve cell adhesion and proliferation. It has been previously described by Costa *et al.*[19] HRGD6 was constructed using standard genetic engineering techniques. Its purification was performed with several cycles of temperature-dependent reversible precipitations as described by Girotti *et al.*[20] The obtained ELRs were dialyzed against milli-Q water and then lyophilized. Purity and molecular weight of HRGD6 were verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy in a Voyager STR (Applied Biosystems). Amino-acid composition analysis was also performed. Additional characterization of the ELR was accomplished by infrared spectroscopy, differential scanning calorimetry, and nuclear magnetic resonance techniques.

The solution for the hybrid gel contained the ELR and fibrinogen (Calbiochem) at a 1:1 weight ratio with a final concentration of 20 mg/mL in TBS. The polymerization of the hybrid gel was initialized by adding thrombin and $CaCl_2$ with the following amounts: 1 mL fibrinogen-ELR solution (20 mg/mL), 0.4 mL TBS, 0.3 mL of 50 mM $CaCl_2$ in TBS, and 0.3 mL thrombin solution.

Molding procedures

To realize spatially selective cell embedding and/or material confinement in semilunar heart valves, a new multiple-step injection molding process was developed. The mould consists of a vascular, a ventricular stamp, and two outer shells (Fig. 1A) machined from polyoxymethylene (POM; ThyssenKrupp Schulte GmbH, Düsseldorf, Germany) and a silicone ring realized by molding of a two-component silicone rubber (Elastosil M4641; R&G Faserverbundwerkstoffe, Waldenbuch, Germany). The concept is demonstrated to create the following:

(1) *Valve with different wall and leaflet composition:* The molding procedure is depicted schematically in Figure 1B (steps 1–7) and illustrated by Figure 1C–F as carried out in the laboratory. In both cases, the fibrin used in the two steps is differently colored for visualization purposes. For the first molding step, the mold was assembled as shown in Figure 1C. The fibrin gel components with the addition of blue colorant were delivered in the volume defined by the silicone ring. The vascular stamp was placed and after 5 min of polymerization time, the silicone ring was removed by cutting it open longitudinally, leaving the newly formed leaflets (Fig. 1D). Subsequently, the outer shells were assembled and the fibrin gel components with the addition of green colorant were inserted in the annular space between the outer

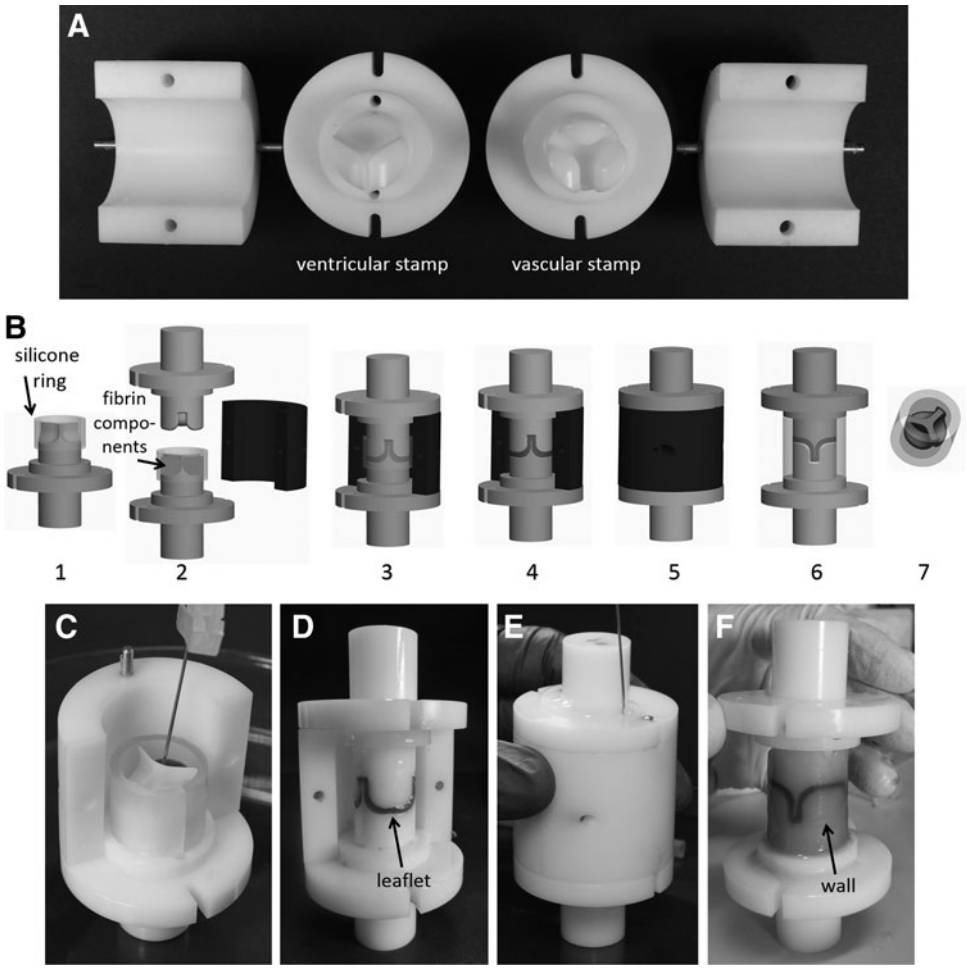

FIG. 1. **(A)** Mold parts: Ventricular and vascular stamp and shells. **(B)** Schematic representation of the molding procedure: (1) The ventricular stamp and a silicone ring are assembled to define the volume needed to mold the leaflets; (2) assembling the vascular stamp to obtain the leaflets whose thickness is defined by placing one of the shells; (3) polymerization of the fibrin gel; (4) after polymerization, the silicone ring is cut open and carefully removed; and (5) the second shell is also assembled to define the volume corresponding to the wall. The whole assembly is turned upside down to allow for the injection of the fibrin components that are injected through a hole in the ventricular stamp (visible in **A)**; after polymerization of the wall, (6) the shells are disassembled first and (7) subsequently, the valve is carefully released by removing the ventricular and vascular stamps. **(C–F)** Images from the corresponding molding: **(C, D)** Molding of the leaflets; **(E)** injection of the fibrin components for the realization of the wall; **(F)** Complete valvular construct after removal of the shells.

shells and the stamps of the mold through a hole in the ventricular stamp (Fig. 1E) and they were allowed to polymerize for 45 min before demolding. For the release of the complete valvular construct, first the shells were disassembled (Fig. 1F) and then the stamps were carefully removed.

(a) *Valve with different cell types in wall and leaflet:* Two different cell lines were used to mold a TEHV as described earlier with the addition of cells (10×10^6 cell/mL) to the fibrin components. Cells used in the leaflets were stained for 60 min with a 2.5 μg/mL HOECHST 33258 (Sigma-Aldrich) solution in a humidified incubator. The other cell line was stained with a Calcein AM (Molecular Probes, Darmstadt, Germany) solution for 45 min in a humidified incubator. Sections of the TEHV were viewed with a fluorescence microscope equipped for epi-illumination (AxioObserver Z1; Carl Zeiss GmbH, Jena, Germany). Images were acquired using a digital camera (AxioCam MRm; Carl Zeiss GmbH).

(b) *Valve with different scaffold materials in wall and leaflet:* A TEHV was molded as described earlier using a fibrinogen solution for the wall and a hybrid ELR-fibrinogen solution for the leaflet. To localize the hybrid gel, blue colorant was added to the solution. After demolding, the valve's commissures were cut open and it was placed in a bioreactor system to demonstrate the functionality.

(2) *Valve with layered leaflets:* The molding technique for creating multi-layered leaflets is schematically depicted in Figure 2 for the case leaflets consisting of two layers. The first layer of the leaflet was molded as described in (1) (Fig. 1B, steps 1–4). On polymerization, the silicone ring and then the vascular stamp were removed (Fig. 2, step 1) and the silicone ring was installed again in a slightly upward shifted

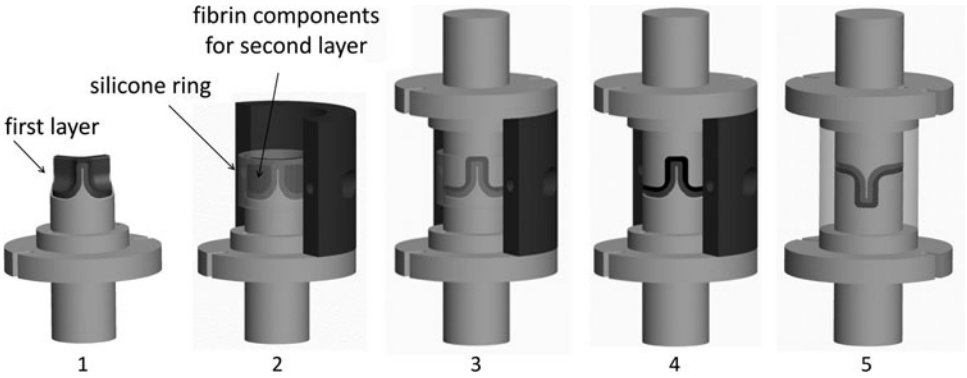

FIG. 2. Schematic representation of the molding technique for two-layered leaflets. (1) The first layer is molded as depicted in Figure 1B (steps 1–4). The vascular stamp and the shell are released. (2) A silicone ring is placed around the stamp to define the volume of the components needed for the second layer. By placing the shell and (3) a new vascular stamp with modified geometry and height, the shape and thickness of the second leaflet layer are defined. (4) After polymerization of the leaflets, the silicone ring is cut open and carefully removed to allow for (5) molding of the wall as described in Figure 1B (steps 5–7).

position according to the new layer thickness. The molding process was repeated by injecting the fibrin components with green colorant added (Fig. 2, step 2). Another vascular stamp with slightly different geometry and increased distance from the ventricular stamp was positioned (Fig. 2, step 3). When the second layer had been polymerized for 5 min, the silicone ring was carefully removed, leaving a two-layered leaflet (Fig. 2, step 4). At this point, a third injection step was performed with fibrin to form the wall of the valve as described in (1) (Fig. 1B, steps 5–7); afterward, the valve molded in three steps could be released from the mold as described earlier (Fig. 2, step 5). The concept was also shown with layers containing cells stained with calcein for one layer and Hoechst for the second layer.

Cell isolation and culture

For isolation of arterial cells, ovine carotid arteries (OCA) and ovine umbilical arteries (OUA) were harvested under sterile surgical conditions. All animals received humane care in compliance with the European Convention on Animal Care. The isolation protocol was the same previously used for cell isolation from the OCA.[21] After removing the adventitia and after removal of endothelial cells by 1 mg/mL collagenase (Sigma-Aldrich) pretreatment, the arteries were minced into 1 to 2 mm rings. The tissue pieces were bathed in primary cell culture media (Dulbecco's modified Eagle medium [DMEM; Invitrogen, Karlsruhe, Germany] with 10% fetal calf serum [FCS; PAA, Cölbe, Germany] and 1% antibiotic/antimycotic solution [Invitrogen]) for primary explant culture, and they were maintained in a humidified incubator at 37°C and 5% CO_2. On confluency, the cells were serially passaged (1:2 to 1:3) using 0.25% trypsin/0.02% EDTA solution (PAA) until a sufficient cell number for generation of a valve construct was obtained. Cells in passage six and seven were used.

Immunocytochemistry

Cells were seeded in 96-well plates (Greiner, Frickenhausen, Germany) and cultured until at least 80% confluence was reached. After fixing with ice-cold methanol (VWR, Darmstadt, Germany) and blocking with 5% normal goat serum (NGS; Dako, Glostrup, Denmark) in 0.1% Triton-phosphate-buffered saline (Sigma-Aldrich), the cells were stained with anti–α-SMA monoclonal antibody (dilution 1:1000; Sigma-Aldrich) and von Willebrand factor (dilution 1:200; Dako) for 1 h at 37°C. Subsequently, they were incubated for 1 h at 37°C with rhodamine-conjugated goat-anti-mouse and goat-anti-rabbit secondary antibodies (Alexa Fluor 594, 1:400; Invitrogen). Nuclei were counterstained with DAPI nucleic acid stain (Molecular Probes). As negative controls, samples were incubated with the secondary antibody only.

The OCA cells exhibited abundant α-SMA expression (Fig. 3A), while the OUA cells were mostly quiescent fibroblasts containing no α-SMA (Fig. 3B). Endothelial phenotype was excluded by the absence of von Willebrand factor.

Cultivation of TEHV with different cell types in the leaflets and the wall

TEHVs ($n=3$) were molded as described in (1a) using OUA cells for the leaflets and OCA cells for the wall (10×10^6 cells/mL). To be able to position the valve into a bioreactor for dynamic cultivation, an outer silicone ring was used to support the valve temporarily. A polyethylenterephthalate (PET) mesh sutured to the silicone ring (Prolene 4-0; Ethicon GmbH, Norderstedt, Germany) was permeated by the fibrinogen solution during polymerization, thus ensuring the attachment of the conduit to the silicone ring.

The valves were cultivated for 2 weeks statically and then for 2 weeks dynamically in a bioreactor system as previously described[22] with an increasing signal amplitude at 37°C, 5% CO_2, and 21% O_2. The initial pulse frequency of 30 bpm was increased to 40 bpm after 7 days of cultivation. The pressure difference across the valve was 15 mm H_2O. All bioreactor components were sterilized using low-temperature hydrogen peroxide gas plasma (STERRADs 100S Sterilization System; ASP, Norderstedt, Germany) 1 week before use. The valves were cultured in low-glucose DMEM with 10% FCS, antibiotic/antimycotic solution, 1.0 mM L-

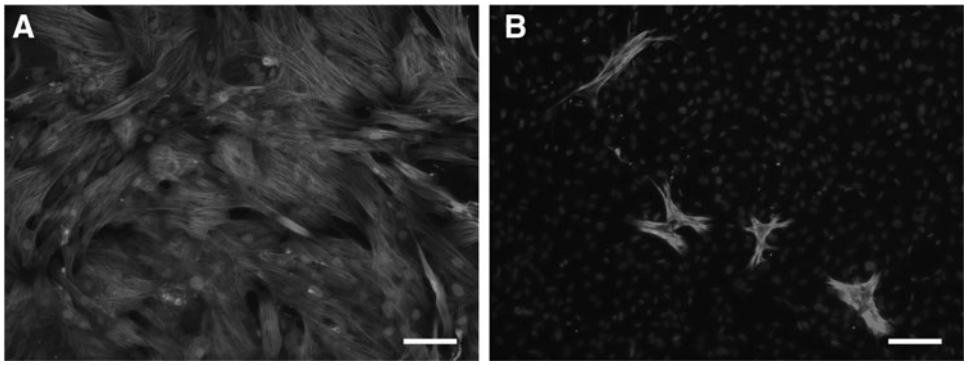

FIG. 3. **(A)** Ovine carotid artery (OCA) and **(B)** ovine umbilical artery (OUA)-derived cells stained with α-SMA-antibody. OCA cells are characterized by abundant α-SMA, while only a few OUA cells demonstrate α-SMA expression. Scale bar 100 μm. α-SMA, α smooth muscle actin.

ascorbic acid 2-phosphate (Sigma-Aldrich), and 1.6 μL/mL tranexamic acid (1000 mg/mL; Pfizer, Berlin, Germany). Culture medium within the bioreactor was replaced every 7 days. Culture conditions were monitored by measuring lactate, glucose, pO_2, pCO_2, and pH levels on a blood-gas analyzer (Blood gas analyzer ABL 800 Flex; Radiometer, Copenhagen, Denmark).

Immunohistochemistry

Nonspecific sites on Carnoy's-fixed, paraffin-embedded sections from native ovine aortic valves and tissue-engineered valves were blocked, and cells were permeabilized with 5% NGS in 0.1% Triton-phosphate-buffered saline. Sections were incubated for 1 h at 37°C with the following primary antibodies: 1:1000 mouse monoclonal anti-α-SMA (Sigma-Aldrich) and 1:200 rabbit monoclonal anti-type I collagen (Acris Antibodies GmbH, Hiddenhausen, Germany). The sections were then incubated for 1 h at 37°C with either fluorescein- or rhodamine-conjugated secondary antibodies (Alexa Fluor 488 and Alexa Fluor 594, 1:400; Invitrogen). Cells were counterstained with DAPI nucleic acid stain. Native ovine aortic valve tissue samples served as positive controls. As negative controls, samples were incubated with the secondary antibody only. Samples were viewed using a microscope equipped for epi-illumination (AxioObserver Z1; Carl Zeiss GmbH). Images were acquired using a digital camera (AxioCam MRm; Carl Zeiss GmbH).

Results

Proof of principle

Figure 4A and B illustrate the feasibility of the described molding concept. It depicts a semilunar heart valve molded in two steps with fibrin as a scaffold. The colorants show the clear division between the leaflets molded in the first step (blue) and the wall (green). The leaflets were firmly attached to the wall. A further proof of principle was given by the TEHV molded with live stained cells. Cells stained with HOECHST 33258 (blue) were equally distributed throughout the leaflet, while cells stained with calcein AM (green) were localized within the wall (Fig. 4G).

In a further step, a different scaffold material was implemented for the multi-step-injection method. We applied an ELR-hybrid gel for the TEHV leaflets as depicted in Figure 4C and D. They were well attached to the fibrin wall, and the valve could be actuated in a bioreactor system.

Apart from a heterogeneous composition of wall and leaflet, we extended the multi-step-injection method to producing two-layered leaflets. The proof of principle is shown in Figure 4E, in which the two layers of fibrin colored in blue and green can be distinguished. A third molding step was performed to complete the valve by fabricating the wall (fibrin without colorant). As can be seen in Figure 4F, this resulted in a whole valve with leaflets attached to the wall. A clear division between the layers is verified by florescence imaging of the leaflet in which life-stained cells, with either calcein (green) or Hoechst (blue), are embedded in the layers (Fig. 4H).

Conditioned TEHV with spatially selective cell embedding

The native aortic valve showed the typical distribution of α-SMA-positive SMCs in the wall, while the leaflets contained nonpositive quiescent VICs (Fig. 5A and B). Minor α-SMA expression was exhibited by cells in the subendothelial layer on the ventricular side of the leaflet. In the TEHV, α-SMA could also be found throughout the complete conduit wall (Fig. 5D). In the leaflet, the density of cells was lower, and they were mostly located on the vascular side and α-SMA-negative (Fig. 5E).

Collagen I was located in the wall of the TEHV with higher deposition toward the vessel's lumen (Fig. 5J) comparable with the deposition of collagen in the native aortic wall (Fig. 5G). In the leaflets, a homogenous distribution of collagen could be found (Fig. 5K).

Discussion

Heterogeneity is one of the key features of heart valves and a crucial characteristic for their functionality. It comprehends both the complex structure of the ECM and the distribution of different cell types. Mimicking these characteristics is an essential step toward an efficient and long-lasting TEHV.[23] Most attempts to tissue engineer heart valves, however, concentrate on using one scaffold material without local diversity in its deposition as well as one cell source for ECM production. With the ultimate goal of producing a biomimetic TEHV, we developed a multi-step-injection molding technique that enables the spatially selective embedding of different cell lines in a fibrin scaffold and the use of different hydrogel compositions for different parts of the valve. The process is easy to implement, does not require expensive equipment, and can be performed in

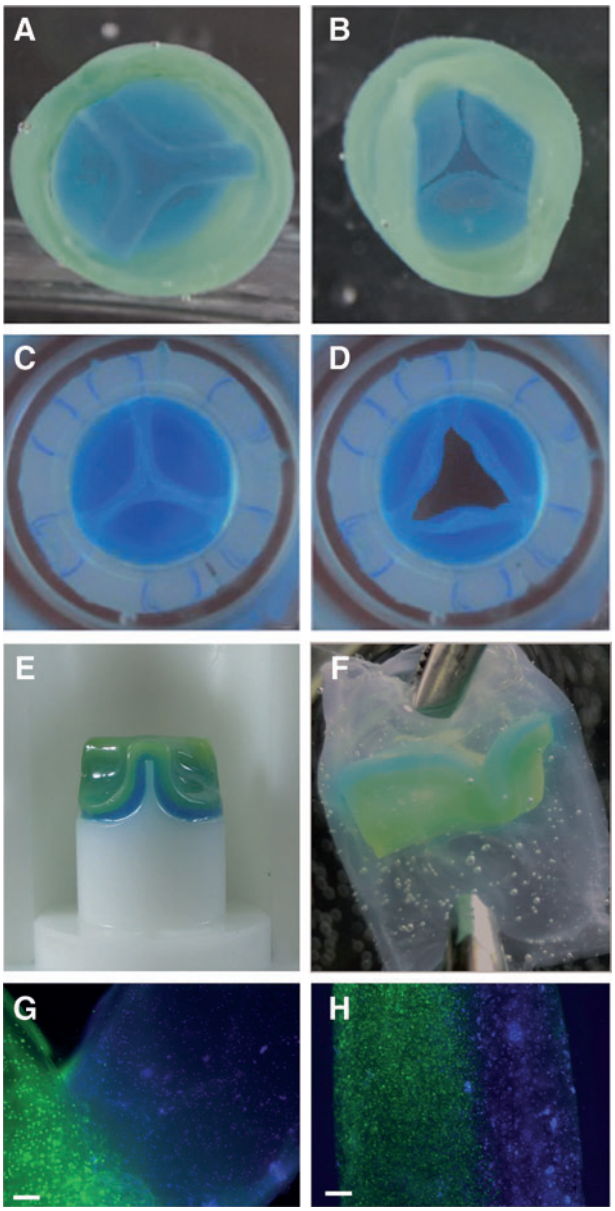

FIG. 4. Multi-step injection molding: proof of principle. Fibrin valve scaffold where leaflets and wall were molded in consecutive steps. Colors have been added to the fibrin-gel components for visualization purposes. View from **(A)** vascular and **(B)** ventricular side. **(C, D)** TEHV with leaflets made of an ELR-fibrin hybrid gel (*blue*) and wall composed of fibrin. **(E)** Leaflets consisting of two separate layers of fibrin (first, the *blue colored layer* was molded and subsequently, the *green one*). **(F)** Three-step molding of TEHV with two-layered leaflets (*green* and *blue colored layers*) attached to the wall (*colorless*). **(G)** Commissure area of a TEHV molded with two different cell populations in the leaflets and in the wall. The nuclei of the cells embedded in the leaflet were stained with Hoechst 33258 (*blue*), while the cytoplasm of the cells in the valvular wall were stained with calcein AM (*green*). **(H)** Layered leaflet with two different cell populations to distinguish the layers that show a clear division and are attached to each other. One layer contains Hoechst stained (*blue*), and the other contains calcein-stained (*green*) cells. Scale bar 200 μm. ELR, elastin-like recombinamer; TEHV, tissue-engineered heart valve. Color images available online at www.liebertpub.com/tec

any TE lab. There is no need for gluing or suturing parts together, processes that could result in too stiff leaflets[12] or the presence of calcification points.[24]

The method relies on the capability of creating different components of the heart valve in consecutive steps. This was achieved by confining specific volumes in the molding process by means of silicone rings.

In a first step, we applied the method to fabricate a TEHV with a heterogeneous composition of the leaflets and the conduit wall. We demonstrated that it can be used to implement different cell types (OUA and OCA derived) as well as different scaffold materials (ELR-fibrin hybrid gel). The introduction of different scaffold materials in selective parts of a TEHV opens the possibility to define the mechanobiology and, ultimately, the biomechanical characteristics of the valve. A variety of materials and mixtures of materials that can crosslink with fibrin can be employed, such as collagen,[25] glycosaminoglycans,[26] and fibronectin[27,28] among others. Moreover, chemically modified fibrin compositions developed to enhance specific cell interactions[29–31] can be applied. Here, we demonstrated the potential of the method with a fibrin-ELR hybrid gel as a possible solution for the long existing problem of poor elastogenesis in tissue-engineered constructs.[32] The elastic network plays a crucial role in the biomechanics of semilunar heart valves, and the lack of it jeopardizes the long-term durability of TEHVs.[33] Although elastin production *in vitro* has been reported,[34,35] it remains unclear whether it was organized in a functional network contributing to the tissue's elasticity. ELRs are genetically engineered polymers consisting of the repetition of pentapeptide sequences present in the natural elastin that offer an interesting possibility of enhancing the elastic properties of the scaffold while being biocompatible and nonimmunogenic.[19] Furthermore, they can be functionalized with bioactive groups for cell attachment and proliferation.[36] As proof of principle, we showed the molding of a heart valve scaffold with leaflets made of a hybrid gel obtained by the crosslinking of ELR and fibrin while the wall was in plain fibrin. The optimization of the hybrid gels and their use for TEHVs are the focus of ongoing research.

The multiple-step injection technique was extended to fabricate layered leaflets. Also here, toward a biomimetic design, different materials can be used to recreate the composition according to the specific function of each layer as in the native valve.[9] Another peculiarity of the aortic leaflet is the high anisotropy due to the oriented distribution of the collagen bundles in the fibrosa, which results in a higher elastic modulus in the circumferential direction than in the radial one. Electrospinning of oriented fibers has been extensively applied to recreate the anisotropy of different tissues, showing the tendency toward biomimetic scaffolds in the field of TE. Specifically for heart valves, Courtney *et al.* demonstrated that electrospinning of oriented poly (ester urethane) urea fibers resulted in an anisotropic layer with different young moduli in the fiber orientation direction and in the perpendicular one, closely matching the circumferential and radial moduli of the pulmonary valve leaflet.[37] Although such a construct has not been applied to produce a TEHV, it could be easily implemented in our molding method by placing the e-spun substrates over the mold and proceeding with the careful injection of the fibrin components. We have

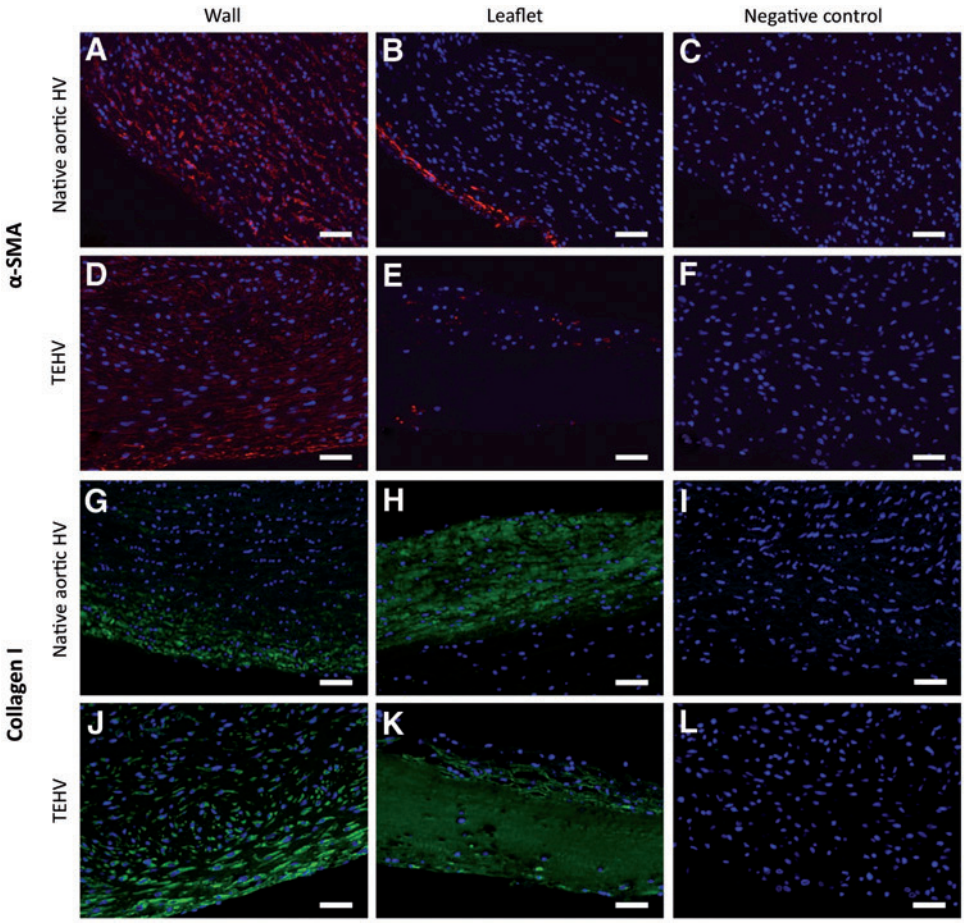

FIG. 5. Fluorescence immunohistochemical micrographs of native aortic valve and TEHV produced with different cell types for wall and leaflet stained for **(A–F)** α-SMA and **(G–L)** collagen type I. DAPI (*blue*) was used as a cell nuclear counterstain in all samples studied. Negative controls demonstrated undetectable levels of staining in native valve and TEHV tissue. Scale bar 50 μm. Color images available online at www.liebertpub.com/tec

demonstrated that the presence of textile reinforcements is compatible with the molding process and results in enhanced mechanical properties of the construct.[22,38] Nowadays, with the multiple-step injection molding, tissue-specific textile reinforcement can be even selectively combined with different materials or different cell lines.

We demonstrated the spatially selective embedding in the leaflets and in the wall of different cell lines, specifically of vessel-derived α-SMA-negative and -positive cells. Although we were inspired by the α-SMA expression of the cells in the native aortic valve where the wall is rich in α-SMA-positive cells while in the leaflets predominantly α-SMA-negative cells are found (Fig. 5A, B), we chose OCA and OUA cells exclusively for visualization purposes. α-SMA staining enabled us to distinguish them after *in vitro* cultivation and to confirm their location in the valve. Indeed, we could identify α-SMA-positive cells in the wall and mostly α-SMA-negative cells in the leaflet, thus indicating the feasibility of spatially selective cell embedding within the molding system. Viability of the cells was underlined by ECM production, here shown by staining against collagen type I (Fig. 5J, K). In the leaflet of the TEHV, cells were preferentially located on the vascular side. While using the optimal cell sources was not the goal of this article, it certainly remains an issue of great concern and the subject of extensive research.[39–41]

Conditioning of TEHVs in bioreactor systems is necessary to achieve an adequate ECM to withstand the mechanical loads in the corporeal circulation. This is applied especially for hydrogel scaffolds due to their initial fragility. Hockaday *et al.* fabricated a heterogeneous hydrogel-based valve with different compositions of wall and leaflets by 3D printing.[13] They demonstrated good shape fidelity of the valve as well as cytocompatibility of the applied materials. However, the quality of the leaflets' attachment to the wall was not described and since the valves were cultured in flasks under static conditions, no information on their functionality was given. In our study, the *in vitro* conditioning proved that the scaffold was robust enough to undergo dynamic mechanical stimulation according to protocols routinely used in our lab.

We believe the multiple-step molding technique is an interesting and versatile tool toward the fabrication of biomimetic TEHVs featuring heterogeneous material and cell composition. The method is easy to implement, does not require special facilities, and can be reproduced in any tissue-engineering lab. While demonstrated here with fibrin, it can easily be extended to other hydrogels.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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