Biomedical Materials

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Native aortic valve derived extracellular matrix hydrogel for three dimensional cultural analyses with improved biomimetic properties

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Abstract

Introduction: Calcific aortic valve disease (CAVD) is the most common acquired heart valve disease with complex underlying pathomechanisms that are yet not fully understood. Three-dimensional (3D) cell culture models as opposed to conventional two-dimensional (2D) techniques may reveal new aspects of CAVD and serve as a transitional platform between conventional 2D cell culture and in vivo experiments. Methods: Here we report on fabrication and characterization of a novel 3D hydrogel derived from cell-free native aortic valves. A detailed analysis containing protein composition, rheological behavior, cytotoxic and proliferative effects as well as results of 3D cell culture experiments are presented. Moreover, this aortic valve derived hydrogel (AVdH) is compared to commercially available biological extracellular matrix (ECM) components to evaluate and classify AVdH with respect to other currently used ECM solutions, i.e. Collagen type I and Matrigel®. Results: On the biochemical level, a complex composition of native proteins was detected. Using different techniques, including mass spectrometry with Gene Ontology network and enrichment analysis, different fundamental biological functions of AVdH were identified, including peptidase-, peptidase inhibitor-, growth- and binding activity. No cytotoxic effects were detected and AVdH showed positive effects on cell growth and proliferation in vitro when compared to Collagen type I and Matrigel®. Conclusion: These results suggest AVdH as an organotypic ECM supporting sophisticated 3D cell culture model studies, while mimicking the native environment of the aortic valve to a greater level for enhanced in vitro analyses.

1. Introduction

1.1. Clinical background

Calcific aortic valve disease (CAVD) is the most frequent acquired heart valve disorder in the Western world with a prevalence up to 2.8% in the population aged above 75 years [1]. Up to date the complex pathomechanisms underlying initiation and progression of CAVD are yet not fully understood. Inflammatory processes [2, 3], accumulation of extracellular matrix (ECM) composites [4], changing biomechanical properties [5, 6] as well as a change in tissue specific differentiation status (i.e. chondro-osteogenic transformation) affecting valvular endothelial cells as well as valvular interstitial cells (VIC) [7, 8] and are suggested to play an important role. A growing body of evidence indicates that 3D culture conditions have an impact on fundamental cell
biological events governing tissue remodeling [9]. However, much of our knowledge in the field of heart valve biology has been derived from studies employing conventional two-dimensional (2D) tissue culture conditions, whereas only very few studies have been conducted using three-dimensional (3D) models. Likewise, more sophisticated in vitro models are needed as a crucial [10] experimental step between conventional in vitro models and in vivo studies. Here different ECM preparations have been applied in the past to yield 3D cultures, e.g. Matrigel®, collagen type I, elastin and hyaluronan as the main ECM components. Recently, native tissue derived ECM has been evaluated as a scaffold for preparation of 3D culture of various organ and tissue systems [10–13]. Considering the complex interaction of cellular and extracellular components within native tissue [14], native cell-free ECM is expected to combine the benefit of tissue specific architecture with an organ specific composition of biologically active components, i.e. matrix-bound growth factors, cytokines and ligands of cellular receptors. Recently native ECM derived organ specific hydrogels have been introduced in life sciences for in vitro and in vivo studies as well as for clinical application focusing on diverse types of tissues and organs, e.g. myocardium [11], urinary bladder [10], lung, and the liver [15].

In front of this background and considering the above-mentioned limitations of previous work on VIC biology in context of CAVD, the aim of this study was to establish a novel 3D cell culture model utilizing an ECM that is derived from native aortic valve tissue.

Starting from native aortic valves a cell-free ECM was achieved and further processed to a novel biomimetic and tissue specific hydrogel, termed aortic valve derived hydrogel (AVdH). To reach a comparison to already available and established matrix material we have compared AVdH with collagen type I (concentration range 1–4 mg ml⁻¹) as well as Matrigel® (concentration range 1–8 mg ml⁻¹). AVdH was subsequently seeded with primary aortic VIC. By multilevel characterization of AVdH and the resulting 3D VIC cultures we demonstrate the feasibility of this approach for more sophisticated in vitro studies in 3D. This approach provides novel perspectives in scientific field of CAVD as it is both, tissue specific and flexible in handling of re-seeded cultures.

2. Materials and methods

2.1. Processing of native aortic valve cusps for donor cell removal

Ovine hearts were obtained from a local abattoir (Laarme, Wuppertal, Germany) immediately after slaughtering. The aortic cusps were excised and treated with a detergent solution containing 0.5% sodium dodecyl sulfate (SDS) (Carl Roth, Germany), 0.5% sodium-deoxycholate (DCA) (Merck, Germany) and 0.05% sodium azide (NaN₃) (Carl Roth, Germany), under agitation for a total of 48 h (figure 1(A)). Detergent solution was changed twice a day. Afterwards, aortic valves were rinsed with 0.05% NaN₃ for 24 h and three times with phosphate buffered saline (PBS) (Invitrogen, ThermoScientific, USA) containing 1% penicillin/streptomycin (Life Technologies, USA) to remove residual detergent solution. Subsequently, cell-free aortic cusps were collected and treated with 100 μg ml⁻¹ DNase from bovine pancreas (˃400 units mg⁻¹, Sigma Aldrich, Germany) to remove remaining double stranded DNA (dsDNA). Leaflets of 25–30 hearts were processed simultaneously to fabricate one batch of AVdH. Before and after DNase digestion, a sample of the resulting matrix was cryo-embedded and analyzed after hematoxylin (Thermo Scientific, USA) -eosin (Sigma) staining, as well as 4,6-diamidino-2-phenylindole (DAPI) staining (VectorShield, Vector Laboratories, USA) for gross estimation of decellularization efficacy. Visualization was performed with a Leica DM2000 microscope, equipped with a digital camera DFC 425C (Leica, Germany) using the manufacturer’s software (Leica Application Software version 3.8).

2.2. Processing of cell-free aortic valve matrix to yield a hydrogel

Cell-free aortic valve matrix was minced (fragments of ca. 1 mm²) and lyophilized using a freeze-drying system.
(alpha 1-4 LSC, Christ) followed by mechanical homogenization using a commercial tissue homogenizer system (Precellys®, 24, VWR, USA). Therefore, 10 mg of lyophilized aortic valve matrix and 6 ceramic beads (2.8 mm diameter, VWR, USA) were agitated in two ml tubes at 6800 rpm for 35 s twice to create a homogeneous powder (figure 1(B)), which was stored at −20°C until further use. To generate a hydrogel, pepsin from gastric mucosa (Sigma Aldrich, Germany) dissolved in 0.01 M HCl (Carl Roth, Germany) was added to the homogeneous powder in a pepsin matrix ratio of 1:10. The matrix was digested for 48 h at room temperature under constant stirring. Pepsin was inactivated by raising pH to 7–7.5 through the addition of 0.1 M NaOH (Carl Roth, Germany) and the hydrogel preparation was diluted with PBS to a final concentration of 2, 4, 6 or 8 mg ml⁻¹, respectively. Final preparations of AVdH were kept on ice until characterization or gelation (figure 1(C)).

2.3. Biochemical composition of AVdH
Resuspended AVdH was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to access protein fragment content. Pure rat-tail collagen type I (Corning, USA) and Matrigel® (Corning) were run on adjacent wells to compare ECM protein content. Solutions were run on a 7.0% polyacrylamide gel in Tris/glycine/SDS buffer (Thermo Scientific, USA). Gel electrophoresis was performed in an XCell Surelock MiniCell (Invitrogen, USA) and stained with flamoing fluorescent gel stain according to manufacturer’s instructions (BioRad, USA).

Collagen content quantification was performed using a commercial hydroxyproline assay according to manufacturer’s instructions (BioVision, USA). Solutions of AVdH, collagen type I and Matrigel® were supplemented with 12 N HCl (ratio 2:5) and hydrolyzed at 95°C for 4 h. Absorbance at 560 nm was measured using a microplate reader Infinite M1000PRO (Tecan, Switzerland).

Soluble glycosaminoglycan (sGAG) content of the ECM was quantified using a commercial assay (Bicolor, UK). Preparations of AVdH were supplemented with an extraction reagent containing 5 mg papain (Sigma Aldrich, Germany), 400 mg sodium acetate (Sigma Aldrich, Germany), 200 mg EDTA (Sigma Aldrich, Germany) and 40 mg cysteine HCl (Sigma Aldrich, Germany) in 0.2 M sodium phosphate buffer (Sigma Aldrich, Germany) and incubated at 65°C for 3 h. After centrifugation at 10 000 g for 10 min, supernatants were used for colorimetric quantification measuring the absorbance at 656 nm according to manufacturer’s instructions using a microplate reader Infinite M1000. Pure collagen type I and Matrigel® were used as reference.

For characterization of the gelation kinetics a turbidimetric evaluation was applied by time-dependent spectrophotometrical analysis as previously described [10]. Solutions of AVdH, collagen type I and Matrigel® were transferred to a cold 96-well plate (100 μl/well) in triplets. The change in turbidity of each well was measured at 405 nm every 2 min for 90 min at 37°C using a microplate reader Infinite M1000PRO (Tecan, Switzerland).

For mass spectrometric (MS) analysis protein containing gel lanes were cut out of gelled AVdH, reduced and alkylated and digested with trypsin as described elsewhere [16]. Peptides were extracted with 0.1% trifluoroacetic acid (Merck Milipore, Germany) and subjected to liquid chromatography. For peptide separation over a 130 min LC-gradient, an Ultimate 3000 Rapid Separation liquid chromatography system ( Dionex/Thermo Scientific equipped with an Acclim PepMap 100 C18 column (75 μm inner diameter, 25 cm length, 2 nm particle size from Thermo Scientific, USA)) was used. MS analysis was carried out on an Orbitrap Elite mass spectrometer (Thermo Scientific, USA) operating in positive mode and equipped with a nano electrospray ionization source. Capillary temperature was set to 275°C and source voltage to 1.4 kV. Survey scans were carried out in the Orbitrap mass analyzer over a mass range from 350 to 1700 m/z at a resolution of 60 000 (at 400 m/z). The target value for the automatic gain control was 10000 and the maximum fill time 200 ms. The 20 most intense peptide ions (minimal signal intensity 300, excluding singly charged ions and ions with a charge state of four and up) were isolated, transferred to the linear ion trap (LTQ) part of the instrument and fragmented using collision induced dissociation. Peptide fragments were analyzed using a maximal fill time of 200 ms and automatic gain control target value of 100 000. The available mass range was 200–2000 m/z at a resolution of 5400 (at 400 m/z). Already fragmented ions were excluded for fragmentation for 45 s.

2.4. Scanning electron microscopy (SEM) imaging
AVdH was brought to the concentration of 2, 4 and 6 mg ml⁻¹ and gelation was induced. The samples were fixed using 2.5% glutaraldehyde (GA) (Serva, Germany) and 4% paraformaldehyde (PFA) (Merck, Germany) in 0.1 M cacodylate buffer (Serva, Germany) (pH 7.4) and kept at 4°C for a couple of days. Then, samples were rinsed and 1% osmium tetroxide (Plano, Germany) was used for contrasting in 0.1 M cacodylate buffer for 2 h with a following rinsing using bidistilled water. Dehydration was achieved using acetone (50%, 70%, 90% and 100%) and subsequent critical point drying (Acetone and CO₂) using a Leica EM CPD030. Afterwards, samples were coated with a 20 nm gold layer (Cressington Sputter Coater 108auto; Cressington Scientific Instruments, UK). SEM images were obtained using a JEOL JSF-35CF Scanning Microscope at 25 kV (JEOL, Japan). The fiber width was determined by measuring the diameter of 50
representative fibers in one image from 3 different AVdH batches using ImageJ software.

2.5. Rheological measurements
A Kinexus Ultra+ rheometer (Malvern Instruments, UK) was used to determine the mechanical properties of AVdH (4 and 6 mg ml⁻¹, n = 7 from three different batches for each concentration), collagen type I (4 mg ml⁻¹, n = 3) and Matrigel® (4 and 6 mg ml⁻¹, n = 3 for each concentration). A cone-plate geometry (model CP4/40 SR2938) 40 mm in diameter was used. Each sample was dispensed on the precooled rheometer plate (5 °C) while in liquid state. The cone was lowered and the excess solution was discarded. The temperature of the rheometer was then increased to 37 °C while in liquid state. The cone was lowered and the excess solution was discarded. Strain sweeps with amplitudes ranging between 0.01% and 20% at 1 Hz were performed in order to define the linear viscoelastic region and to determine the storage modulus (\(G'\)), the loss modulus (\(G''\)) and the loss factor tan(\(δ\)) (\(G''/G'\)) within this region.

2.6. In vitro experiments with VICs
Primary VIC were isolated from ovine aortic valve cusps. Aortic cups were excised from fresh ovine hearts that were obtained from a local slaughterhouse (Laame, Wuppertal, Germany). Cusp tissue was minced to small fragments of ca. 1 mm² diameter and transferred to tissue culture flasks containing Dulbecco’s Modified Eagle’s Medium (DMEM), (Sigma Aldrich, Germany) supplemented with penicillin/streptomycin, L-glutamine, non-essential amino acids (Life Technologies, USA) as well as 10% fetal calf serum (FCS), (Sigma Aldrich, Germany). Tissue fragments were cultivated at 37 °C and 5% CO₂ to allow VIC to migrate from the tissue fragments and to proliferate. VIC in passage 3–6 were used for the following assays.

2.7. Impact of ECM on cell viability
Solutions of AVdH, collagen type I or Matrigel® containing VIC were transferred to pre-cooled 96-well plates (100 μL/well, 10,000 VIC/well) to form a hydrogel at 37 °C for one h and allowed to attach overnight in DMEM with 10% FCS, followed by an additional 8 h period of cultivation in DMEM containing 0.5% FCS. Medium supernatants were collected and free lactate dehydrogenase (LDH) concentration was determined using a commercial assay according to the manufacturer’s recommendations (LDH cytotoxicity assay kit, Thermo Fisher, USA) as an indicator for cytotoxicity. Signals were visualized and evaluated using a Tecan Reader (Infinite M1000PRO, Tecan, Switzerland). The mean value of triplicates was used for further analysis.

To assess viability of VIC cultured in 3D hydrogels, a live-dead assay was performed. Therefore, 3 x 10⁶ cells were resuspended in 750 μl of AVdH, collagen type I or Matrigel® and poured into transwells to form a 3D culture. The transwell chamber was fitted with a circular rim of Velcro® placed at the bottom of the transwell prior to pouring the culture in order to inhibit shrinkage of the gel during further cultivation, as described previously [17]. After an initial gelation time of 1 h, medium was added to the culture. After 2 d of cultivation cell viability was evaluated using a commercial assay (Live Dead Assay, Life Technologies, USA) according to manufacturer’s instructions. In brief, 3D cultures were incubated for 30 min within the staining solution and visualized using confocal microscopy (LSM 710, Zeiss, Germany). For quantification, 6 series of Z-stacks were collected and the fluorescence intensity of the linear region was analyzed using ImageJ software. At the end of the experiments, scaffolds were rinsed, submerged in PBS and cryo-embedded in freezing medium for histological stainings.

2.8. Cell proliferation
Samples that have been generated and used along the in vitro experiments (see 2.7) have also been used to determine cell proliferation rate applying the Bromodeoxyuridin (BrU) cell proliferation kit according to the manufacturer’s recommendations (Cell Signaling Technology, USA). Signals were visualized and evaluated using a Tecan Reader (Infinite M1000PRO, Tecan, Switzerland). The mean value of triplicates was used for further analysis.

2.9. Characterization of VIC
VIC were cultivated as previously described. Based on Gwanmesia et al [14] circular cover glasses (Menzel, Germany) with a diameter of 5 mm were coated either with AVdH, collagen type I or Matrigel®. Therefore, solutions of AVdH, collagen type I or Matrigel® were prepared by adjusting pH and in the appropriate concentration. The glass slides were covered with the respective ECM solution, transferred in a 6-well plate and incubated for 2 h at 37 °C to receive a coated surface. Afterwards the 6-wells were washed two times with warm PBS (Invitrogen, USA) and 150 000 VIC/well were seeded onto the glass slides. The cells were cultivated for 48 h as previously described, washed with warm PBS and fixed with cold methanol (VWR, USA) for 5 min and cold acetone (Carl Roth, Germany) for 20 s. For immunofluorescent staining, the cells were incubated with 0.1% T-X-100 for 5 min followed by 3 x 5 min washing steps with PBS. Next, primary antibodies against vimentin (GP53, Progen, Germany) and alpha smooth muscle actin (α-SMA) (Ab5694, Abcam, UK) were incubated for 60 min followed by 3 x 5 min washing steps with PBS. Fluorescent secondary antibodies (Dianova, Germany) were incubated for 30 min. The glass slides were
then washed again 2× with PBS and rinsed in aqua dest. for 1 min, followed by a fixation step in 100% ethanol. Afterwards, nuclear staining with DAPI was added and glass slides were mounted on microscope slides and pictures were taken with a Leica DM2000 fluorescence microscope (Leica, Germany).

### 2.10. Histological staining

Samples of VIC were suspended in AVdH to generate 3D cultures and cultivated over 7 d with medium changed every second day (see 2.7). Cryo-sections (10 μm) were stained with hematoxylin-eosin and Movat pentachrome staining for evaluation of gross ECM structure and cell distribution. Visualization was performed with a Leica DM2000 microscope using the manufacturer’s software (Leica Application Software version 3.8).

### 2.11. Statistics

All data are presented as the mean ± standard deviation. For direct group comparisons Kruskal–Wallis tests with Dunn’s Multiple Comparison tests were performed as indicated except for data derived from rheological measurements, where Tukey’s test was applied. A p-value of $p < 0.05$ was considered significant. Data were analyzed with GraphPad Prism (version 6.0 h, GraphPad Software, USA) and SPSS software (version 20, IBM, USA).
3. Results

3.1. Evaluation of donor cell removal
For the generation of a homogenous hydrogel of cell-free aortic valve cups removal of endothelial cells, VIC as well as cell-free dsDNA was required. Sodium azide, SDS and DCA effectively removed cellular components as shown in figure 2. Nevertheless occasionally nuclear staining was observed (figures 2(A) and (B)), which was markedly reduced by using an additional DNase digestion step (figures 2(C) and (D)) compared to control (figures 2(E) and (F)) and decellularisation without DNase digestion. Ultrastructural analysis of AVdH by SEM did not show any signs of microbiological contamination.

3.2. Hydrogel composition
Comparing the protein band pattern and intensity of four different batches of AVdH, which means all samples originate from different decellularisation and production processes, suggests a high level of reproducibility in terms of size distribution of major protein bands upon SDS PAGE separation (figure 3(A)). Protein content of AVdH was compared to that of rat trail collagen I and Matrigel®, respectively. Gel electrophoresis followed by protein staining revealed that all major bands observed in collagen type I preparations were also present in AVdH, suggesting collagen type I as the major constituent of AVdH. However, AVdH showed additional protein bands that were distinct from the profile of purified collagen type I (figure 3(B)). Some of these bands distinguishing AVdH from collagen type I were also detected in Matrigel® (figure 3(B) arrows). In another quantitative analysis using hydroxyproline assay, the observed hydroxyproline concentrations in four different batches of AVdH were 8.2 ± 0.4 ng ml⁻¹ and therefore lower than in commercially available pure collagen type I (15.9 ± 2.2 ng ml⁻¹). Matrigel® preparations contained only a small amount of hydroxyproline (0.4 ± 0.06 ng ml⁻¹) (figure 3(C)).

Furthermore, sGAG content of AVdH was 0.89 ± 0.18 μg ml⁻¹ (figure 3(D)). Matrigel® contained roughly four times more sGAG (2.89 ± 0.1 μg ml⁻¹) than AVdH, while almost no sGAG were present in commercially available collagen type I preparations.

For in-depth analysis of preserved proteins in AVdH, MS analysis was performed. The extraction method was based on previously published work by Didangelos et al [18]. By this approach we identified 257 proteins in total (216 non-redundant proteins), which could be allocated to membrane (26.5%), extracellular space (15.1%), cytoplasm (14.2%), nucleus (10.1%) and other organelles (34.1%) (multiple annotations per protein possible). All of these proteins are also known to be found in the ECM (85 proteins), the extracellular space (102 proteins) or within extracellular exosomes (145) according to Gene Ontology (GO) Slim terms (24 August 2017; version). Enrichment analysis of the identified proteins using the FunRich Software also depicted a clear enrichment of extracellular located proteins (figure 3(E)). A signal peptide function is predicted for 128 of these proteins according to UniProt. The main biological processes of these proteins were involved in the regulation of biological processes (50.58%), cell organization and biogenesis (31.13%), response to stimulus (30.74%), metabolic processes (30.74%), and transport (15.18%) (figure 3(F)). Furthermore, the classification of these proteins revealed 17 types of collagen and 21 proteins with peptidase or peptidase inhibitor activity as well as 16 proteins with growth factor or growth factor binding activity [19, 20]. Regarding collagen type I, two different chains have been detected (COL1A1, COL1A2) based on MS spectra, 40 COL1A1 spectra were detected which classifies this protein as high abundant. COL1A2 with an amount of 9 spectra could be classified as moderate abundant. Network and enrichment analysis using StringDB. The STRING database in 2017 [21] revealed that for example response to wounding, the regulation of proteolysis and endopeptidase activity and blood coagulation are overrepresented and show a high degree of connection among each other (supplemental figure S1 is available online at stacks.iop.org/BMM/14/035014/mmedia). Furthermore, regulation and response to transforming growth factor beta (TGF-beta), glycosaminoglycan catalytic processes and collagen associated processes as well as blood vessel development and cell-substrate junction assembly are enriched among the AVdH proteome (supplemental figure S1).

3.3. Ultrastructural characteristics
SEM imaging showed a fibrillar appearance of the AVdH at concentrations of 2, 4 and 6 mg ml⁻¹. This fibrillar structure was reproducible in three different batches. The fibers showed an approximate diameter of 40–140 nm (figures 4(A) and (B)). Figures 4(C) and (D) show the growth pattern of the cells and the distribution of the GAG’s and collagen of the AvdH after a cultivation time of 7 d stained with Movat pentachrome staining (figure 4(C)) and hematoxylin-eosin satining. For further description see results section 3.6 (Feasibility of VIC culture in 3D culture using AVdH).

3.4. Gelation kinetics
To further characterize AVdH in direct comparison to collagen type I or Matrigel® a turbidity assay was applied (figures 5(A) and (B)). Hereupon turbidity of AVdH and collagen type I showed a sigmoidal shape (figure 5(B)). The observed absorbance increased in particular during the first 30 min of the gelation process, while the turbidity of Matrigel® remained at a low level. Collagen type I (4 mg ml⁻¹) showed the highest turbidity followed by AVdH. The maximum
Figure 3. Characterization of aortic valve derived hydrogel (AVdH). (A) Fluorescence protein staining of 4 different batches AVdH in a concentration of 8.3 mg ml$^{-1}$. The sharp, unaltered band pattern and intensity indicates a homogenous isolation of aortic valve matrix proteins. (B) Fluorescence protein staining of SDS-PAGE gel showing AVdH (4 mg ml$^{-1}$ (line 1), 6 mg ml$^{-1}$ (line 2)), collagen type I (2 mg ml$^{-1}$ (line 3), 4 mg ml$^{-1}$ (line 4)), and Matrigel® (4 mg ml$^{-1}$ (line 5), 2 mg ml$^{-1}$ (line 6)). SDS-PAGE shows the typical band pattern for collagen type I, with a doublet at apparent molecular masses of 115, 130 kDa, and another doublet at 215 and 235 kDa. Additional bands were detected that are not contained in purified Collagen type I specimens but are also detected in Matrigel® (ca. 150–160 kDa, see arrows). (C) and (D) ECM analysis of AVdH, collagen type I and Matrigel®, adjusted concentration of 4 mg ml$^{-1}$ by using a hydroxyproline and sGAG assay. Hydroxyproline content (C) and sGAG content (D) clearly demonstrate the distinct nature of AVdH as compared to collagen type I or Matrigel®. (E) Pie chart showing 7 most abundant cellular compartments within the AVdH proteome using GO slim annotation (multiple annotation per protein possible). (F) Bar chart showing the most prominent biological processes in the proteome of AVdH according to their GO Slim annotation. Dominant processes are the regulation of biological processes, response to stimulus, metabolic processes, cell organization as well as biogenesis and transport. Data represent mean ± SD *: p < 0.05. Col I: collagen type I.
turbidity values rised for the four different AVdH batches with increasing concentrations and lay between the maximal values of collagen type I and Matrigel® (figure 5(A)).

The time required to reach half of the final turbidity ($t_{1/2}$) as well as the gelation rate ($S$) was smaller in the AVdH compared to collagen type I (figure 5(C)), indicating an enhanced gelation kinetic. In comparison to AVdH the gelation rate was significant higher for Matrigel® ($p < 0.05$). Interestingly AVdH concentration had no impact on the observed gelation kinetics. Both hydrogel concentrations showed a similar $t_{1/2}$, speed of gelation ($S$) and lag phase ($t_{lag}$).

3.5. Rheological characterization

In order to determine the mechanical properties of the hydrogels, rheological measurements were performed and $G'$, $G_{elas}$ ($G'$) and $G_{visc}$ ($G''$) were recorded (figure 5(A)). AVdH displayed complex moduli in the range of 50–200 Pa, with a clear dependence on the concentration. Matrigel® at equivalent concentrations had significantly lower moduli as compared to collagen type I ($p < 0.001$) and AVdH ($p < 0.001$), respectively. All of the tested gel preparations had a prevalently elastic behavior, demonstrated by the fact that the respective complex moduli are similar to the storage moduli. For AVdH this behavior was more marked for the 6 mg ml$^{-1}$ samples, which displayed a $\tan(\delta)$ value of 0.20 ± 0.04 Pa, whereas the 4 mg ml$^{-1}$ showed $\tan(\delta)$ values of 0.30 ± 0.04 Pa.

3.6. Assessing cytocompatibility and growth of AVdH in 2D

As a first step towards assessing biocompatibility, we examined the ability of VIC to grow in 2D on standard plastic cell culture dishes/glass slides coated with either hydrogel, AVdH, collagen type I, Matrigel® or with no hydrogel (control condition in this setting). VIC grown on standard glass slides formed typical monolayers with an aligned growth pattern (figure 7(A)). The growth pattern on the coated surfaces depended on the used concentration. Here we tested concentrations 2, 4 and 6 mg ml$^{-1}$ in AVdH and Matrigel® and 2 and 4 mg ml$^{-1}$ for collagen type I. A fluorescent staining of $\alpha$-SMA and vimentin have been used to characterize VIC on different coatings. In this experiment vimentin served as a maker which is basically expressed in VIC, and $\alpha$-SMA as a marker of VIC activation. Interestingly VIC grown on Collagen type I and AVdH showed a similar growth pattern and formed a homogeneously distributed layer. The cells were not aligned like in the control group, rather the VIC spread in all directions. Additionally the pattern and signal intensity for $\alpha$-SMA differed between the used concentration with a maximum achieved with...
2 mg ml\(^{-1}\) of AVdH and 4 mg ml\(^{-1}\) of collagen type I. Staining results for VIC on 4 or 6 mg ml\(^{-1}\) AVdH were similar (figure 7(A)). Matrigel\(^{\text{\textregistered}}\) coating led to colonies of stellar configuration in a concentration of 4 and 6 mg ml\(^{-1}\) and monolayers in a concentration of 2 mg ml\(^{-1}\). The \(\alpha\)-SMA expression of Matrigel\(^{\text{\textregistered}}\) was similar in all concentrations and less strong as compared to collagen type I and AVdH as well as to controls (figure 7(A)). Cell proliferation rate measured by BrdU assay for Matrigel\(^{\text{\textregistered}}\) (2 and 4 mg ml\(^{-1}\)) was unchanged as compared to cells cultured on plastic but significantly enhanced when VIC were cultured on collagen type I or AVdH (2 and 4 mg ml\(^{-1}\)). The maximal proliferation rate was observed in VIC cultured on AVdH (4 and 6 mg ml\(^{-1}\)). As compared to Matrigel\(^{\text{\textregistered}}\) AVdH led to significantly increased VIC proliferation rate at the concentration of 4 mg ml\(^{-1}\), while this difference was lost at 6 mg ml\(^{-1}\) (figure 7(B)).

### 3.7. Feasibility of VIC culture in 3D culture using AVdH

Free LDH in the medium was determined as a potential indicator of cytotoxicity. None of the evaluated hydrogel coatings led to an increased LDH activity in the supernatants of the cultured VIC (figure 7(C)).

Next, VIC were cultured in 3D within AVdH for a period of 2 and 7 d, respectively. To determine the effects of AVdH on VIC viability in 3D cultures live-dead staining was performed after 2 d of cultivation and cultures were subjected to analysis by laser scanning microscopy (figure 8). To visualize cell distribution in different sections of the hydrogel, Z-stacks were subdivided in three sections—top, center and bottom as further described (figures 8(B)–(D)). Analysis of fluorescence intensity within each of these three sections revealed that vital cells survived in all three sections but with a greater concentration within the top and center section. A small number of dead cells was detected in all three sections, with slight but statistically significant increase in the center section (figure 8(E)).

After 7 d of cultivation within AVdH a non-homogeneous growth pattern with prominent amount of VIC clusters was observed in the upper part, which narrowed towards the bottom part (also see figures 4(C) and (D)). In some gels VIC formed a cell layer at the top. Movat pentachrome staining showed both, green colored GAG and yellow colored collagen fibers. Interestingly, in the histological stainings, GAG were the most abundant ECM proteins. The content of stained collagen fibers partly varied between the individual batches of AVdH \((n = 3)\).

### 4. Discussion

The use of tissue-specific ECM derived from native tissues has become increasingly popular in the field of regenerative medicine due to its complex protein
composition and the preserved microstructure of such scaffolds. Moreover, such technique may also facilitate disease modeling strategies. In this study we transformed the cell-free matrix derived from ovine aortic valve cusps in a viscous hydrogel to result in a highly cytocompatible and versatile biomaterial with high fidelity on the biochemical level to the native tissue. This ECM obtained from valvular cusp tissue, here termed AVdH, is further evaluated in vitro for its properties to enhance 3D cell culture applications, as a transitional model between 2D cell culture and in vivo studies. The concept of manufacturing tissue derived hydrogels has been previously demonstrated for other types of organs or tissues, such as myocardium \[11\] and lung \[22\]. However, such approach has yet not been established for hydrogels derived from aortic valve matrix. The valuable benefits of organ specific hydrogels speak in favor of using such ECM solutions for a number of in vitro purposes, including bioprinting techniques, and also for an in vivo implementation.

The complex structures and functions of extracellular matrices of various native organs are not completely understood. In previous work it has been demonstrated that each tissue consists of its own protein composition and network created by tissue resident cells \[23, 24\]. The ECM serves as a communication platform. Besides the possibility of fibers to bind to cellular surface receptors, ECM-associated growth factors and other bioactive molecules collectively contribute to a considerable level of biological functionality of ECM. Previous studies suggest a remarkable impact of ECM components on differentiation pathways, e.g. in the setting of human embryonic stem cell culture, and the influence of its regenerative potential, e.g. in post myocardial infarction zones \[25\] emphasizing the enormous effect of ECM and underlining its importance for 3D cell culture models. These findings warrant further emphasis on natural (i.e. organotypic) ECM as supporting scaffold for in vitro studies.

In the field of heart valve biology such scaffold is ideally generated by native aortic valve cusps subjected to a common decellularization procedure. However, adequate in vitro repopulation of intact decellularized heart valves has remained considerably cumbersome \[17\]. Similarly, results from translational in vivo studies and anecdotic clinical reports have not been able to demonstrate full repopulation of decellularized heart valves \[26, 27\]. In contrast, by using hydrogels for in vitro generation of 3D cultures of VIC the aforementioned limitations maybe overcome. AVdH further adds the biological value of native ECM to the experimental advantages of a hydrogel scaffold for in vitro studies on VIC biology. Based on the results presented here a number of bioactive ECM components has been detected by MS, including proteins with peptidase or peptidase inhibitory activity, growth factors and proteins with growth factor binding activity demonstrating successful preservation of structural proteins as well as bioactive ones. Obtaining a complex protein content with the composition coming close to

**Figure 6.** Mechanical properties of 4 different hydrogel batches as determined by the rheological measurements. Data are reported as mean ± SD. AVdH specimens display moduli from 45 to 150 Pa depending on the concentration, similar to collagen type I and Matrigel® at the tested concentrations. : p ≤ 0.05; ***: p ≤ 0.001, Col I: collagen type I.
that of the native aortic valve depends on the applied processing techniques [28]. The herein presented results demonstrate that a variety of proteins typical for native aortic valve cusps may be preserved. Since collagens are the most abundant ECM proteins in native aortic valves, high level of consistency has been observed between the protein band patterns of collagen type I and AVdH. The high collagen content in AVdH was further confirmed by a hydroxyproline assay and MS. Other bands which were also present in Matrigel® indicate that further ECM components have been preserved, e.g. laminin and entactin, which have been identified by MS to be preserved in AVdH. The obtained GAG content was lower than described in previous reports on hydrogels derived from other organs [10, 29, 30]. This might depend on the GAG content of the valves in general. Alternatively, a technical limitation of the applied assays to detect and quantify the GAGs may also be the reason for the here obtained data [30]. Additionally it may also be related to a different degree of loss of GAGs during the respective procedures used for cell removal [31, 32].

Figure 7. Biocompatibility of AVdH®. (A) Phase contrast images of VICs (first line, 100×) seeded on glass covers, non-coated (control) and coated with collagen type I, AVdH or Matrigel® (showed concentration 4 mg ml\(^{-1}\)) immunostained for α-SMA (red), vimentin (green) and nuclei (blue) (400×). Essentially, VICs confirm a similar morphology when seeded on collagen type I and AVdH as compared to Matrigel® after 48 h of cultivation. VICs express α-SMA in all conditions with a maximum signal when grown on collagen type I and AVdH. Cell proliferation (B) and evaluation for cytotoxic effects (C) as determined by BrdU (B) and LDH assay (C) revealed a high level of biocompatibility for AVdH comparable to collagen type I and Matrigel®. (B) Measured antibody absorbance at different AVdH concentrations. (n = 3; *, p < 0.05; **: p < 0.01, data are represented as mean ± SEM.) (C) LDH activity measured in cell culture supernatants after 24 h. Col I: Collagen type I. Scale bars = 200 μm.
Nevertheless, MS analysis indentified a large number of proteins present in AVdH, underlining the value of AVdH with regards to the complexity of the native aortic valve ECM. The detected proteins are known to play a role in several fundamental pathways, e.g. regulation of biological processes, responses to stimuli, cell organisation as well as transport and biogenesis. Moreover, some of these proteins have the ability to bind growth factors with the result that AVdH may not only act as a protein reservoir but it may also exert mediatory effects. Future studies are needed to show whether AVdH may provide further options as a drug delivery depot. It may be expected that AVdH harbors signal transporting proteins that more closely resemble native aortic valve tissue conditions. In contrast, deficient cell behavior and differentiation and artificial signaling have been reported for culture conditions using Matrigel\(^{[33, 34]}\). Interestingly in this study the growth of VIC on Matrigel\(^{[33, 34]}\) showed a clearly different growth pattern as well as \(\alpha\)-SMA expression compared to cells grown on collagen type I and AVdH, which could support this findnings. Future studies will show how AVdH influences VIC signaling pathways \textit{in vitro}.

Along the herein performed functional assays, the growth of cells cultivated on standard plastic culture dishes was significant lower than cell growth on ECM coated surfaces. Furthermore, live-dead assay and histological stainings underlined favorable growth performance of VIC within the AVdH for the tested period of 7 d.

Beside the aforementioned positive aspects of AVdH matrix there also exist some challenges and limitations which should be exposed. One aspect may be the batch to batch variability, which will remain as an inherent limitation to all biologically derived products. A large amount of native aortic valves need to be prepared, in our hands one batch AVdH of 20–25 ml contains approximately aortic valve cusps from 25 to 30 different lambs. Using tissue from different donors helps to reduce the variations of the valve protein content of each individual donor, but most likely will not entirely eliminate this limitation. Another aspect is the high technical effort needed for production of AVdH. Other organotypic hydrogels, e.g. derived from myocardium or lung tissue, may have a favorable outcome in terms of quantitative harvest because a higher amount of initial donor tissue is available from the same donor animal.

Further, the valve is a three-layered structure. In this study we created a powder which contains ECM components from the whole valve leaflet. In their natural form, VIC are not exposed to a mixture of all these components simultaneously. Ideally, a three-layered architecture would be preserved, however up to now, this is out of experimental reach. Our working group could show in a previous publication\(^{[35]}\) that there are some unsolved challenges in reseeding a three-layered valve with a homogenous distributed and reproducible, stable number of cells (VIC). Therefore, we have chosen a different approach due to these previously experienced difficulties. Even if VIC are exposed to a mixture of all components the specific layers contain, the ingredients of the hydrogel are still naturally derived in the sense of that they come from the original source and are not artificially synthetized by other sources, e.g. tumor cells in case of Matrigel\(^{[33, 34]}\), which contains a variation of additional components, the cells might never be exposed under physiological conditions.

Our results are of particular interest as they vividly demonstrate the important effect of matrix components on cell behavior, calling for a higher level of

![Figure 8. Three-dimensional imaging of VIC seeded in AVdH after two days of cultivation. (A) 3D image of Z-stacks recorded by a confocal microscope (100×, 0.5 zoom) after Live–dead staining. (B) The 30 images of one Z-stack were divided into 3 equal parts, representing the different regions of the AVdH (B)–(D). Green represents living cells, red represents dead cells. (E) Fluorescence intensity was analyzed for each of the three spatial zones using Image J. *: \(p < 0.05, n = 4\).](image-url)
awareness when performing in vitro experiments as a model for in vivo disease progress or treatment testing. For further studies it might be interesting, what kind of additional effects the complex mixture of matrix components have on VIC behavior. Furthermore previous studies indicate a relation between elastic modulus of the matrix and transformation of VIC in an actice myofibroblast cell type [36]. In this study we mainly investigated the AvDH in a protein concentration range of 1–8 mg ml⁻¹ with a focus on in-depth analysis of the most promising concentration of 6 mg ml⁻¹. We decided to show mainly the concentrations of 4 mg ml⁻¹ in order to have a comparison of all three gels at the same concentration as well as 6 mg ml⁻¹ as the concentration we decided to do the in-depth analysis on. For mechanistic studies on biomechanics, AvDH of higher concentration or hybrid scaffolds may be used, employing the herein described AvdH in combination with mechanically tunable matrix materials e.g. gelatin Methacryloyl hydrogels (GelMa) to achieve a matrix with a reproducible stiffness and the possibility to adjust the rigidity of the material [37]. Finally, rat collagen type I has been used here as a control hydrogel matrix, while VIC were isolated from ovine heart valves. Currently available ovine collagen preparations may lead to other results when used for VIC culture, although the lack of biological complexity as opposed to ovine AvDH may still be expected to remain.

In this project we only focused on aortic valves, however we would like to point out that the herein presented principles can be adapted to other heart valves, e.g. to the mitral valve. Collectively this model may be a good compromise in the wide experimental gap between 2D cell culture and in vivo models.

5. Conclusion

An organotypic hydrogel may be generated from native aortic valve tissue for further use as a novel scaffold for 3D culture models. This approach provides high biochemical similarity and enhanced supportive effect cell growth. Considering current limitations of conventional 2D models and 3D models using artificial scaffolds, native aortic valve derived hydrogel represents a promising alternative for in vitro studies on heart valve biology.

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Disclosures

All authors declare that there are no conflicts of interest.

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