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Monolayer formation and shear-resistance of human vein endothelial cells on gelatin-based hydrogels with tailorable elasticity and degradability

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Abstract

The formation of a functionally-confluent and shear-resistant endothelial cell (EC) monolayer on cardiovascular implants is a promising strategy to prevent thrombogenic processes after implantation. On the basis of existing studies with arterial endothelial cells adhering after two hours on gelatin-based hydrogels in marked higher numbers compared to tissue culture plates, we hypothesized that also venous endothelial cells (HUVEC) should be able to adhere and form an endothelial monolayer on these hydrogels after days. Furthermore, variation of the hydrogel composition, which slightly influences the materials elasticity and even more the degradation behaviour, should have no considerable effect on HUVEC. Therefore, the monolayer formation and shear resistance of HUVEC were explored on two gelatin-based hydrogels differing in their elasticity (Young's moduli between 35 and 55 kPa) in comparison to a positive control (HUVEC on glass cover slips) and a negative control (HUVEC on glass cover slips activated with interleukin-1β) after 9 days of culturing. HUVEC density after 9 days of culturing under static conditions was lower on the hydrogels compared to both controls ($p < 0.05$ each). On G10_LNCO8 slightly more EC adhered than on G10_LNCO5. Staining of the actin cytoskeleton and VE-cadherin revealed a pronounced cell-substrate interaction while the cell-cell interaction was comparable to the controls (HUVEC on glass). The secretion of vasoactive and inflammatory mediators did not differ between the hydrogels and the controls. Adherent HUVEC seeded on the hydrogels were able to resist physiological shear forces and the release of cyto- and chemokines in response to the shear forces did not

differ from controls (HUVEC on glass). Therefore, both gelatin-based hydrogels are a suitable substrate for EC and a promising candidate for cardiovascular applications.

Key words: endothelial cells, biomaterial, gelatin, hydrogel, shear stress

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1. Introduction

The initial step after exposing synthetic implant materials to blood is the nonspecific adsorption of proteins [2,22]. Especially for blood-contacting devices, such as vascular grafts or patches, adsorption of plasma proteins like fibrinogen or von Willebrand Factor occur within seconds after implantation. Following thrombocyte recruitment and activation may lead to thrombus formation, coagulation and inflammation sometimes ending up in vessel occlusion or implant failure [32]. Device failure is often caused by inadequate interaction with the surrounding biological system. Improving this interaction by mimicking the natural environment is a promising strategy to avoid the mentioned complications [30]. For this purpose, generation of a functionally-confluent endothelium on the implant's luminal surface is targeted for solving current limitations of thrombogenicity, by creating a continuously renewing antithrombogenic surface, which moreover actively regulates homeostasis of haemostasis [3,27]. However, endothelial cell adhesion and proliferation as well as stability of the cell monolayer are, especially under dynamic blood flow conditions, often limited on synthetic surfaces [15,33]. For that reason, biomimetic materials derived from macromolecules of the extracellular matrix (ECM) are used in many cases as supportive coating to enhance endothelial cell adhesion by providing a suitable microenvironment [4,18,20,30].

Gelatin derived from natural sources by partially denaturation and degradation of collagen is a tissue-compatible and degradable biomaterial, which was proved to be cyto- and immunocompatible [26,37,38]. Further, gelatin provides short cell recognition motifs for cell recruitment and adhesion. In clinical applications, gelatin or chemically modified derivatives are used for ECM-mimicking coating, as cell carrier scaffold or matrix for drug delivery [10,16,34,36]. Recently, entropy-elastic gelatin-based hydrogel networks with adjustable mechanical and degradation properties were introduced as biomaterial for medical

applications [39]. By reacting gelatin fibres with varying amounts of diisocyanates, materials elasticity, mechanical and hydrolytic stability as well as swelling could be tailored for the desired application. Hydrogel elasticity could be adjusted to the range of soft tissue, which is beneficial for the application as a coating or an artificial scaffold in the cardiovascular system. The biodegradation behaviour was directly associated with the network density, which resulted from multiple hybrid connection points formed by both, covalent crosslinks between gelatin through di-urea junctions as well as physical netpoints formed by interactions between oligo-urea side chains. This enables to tailor the period of application from several days up to month by modification of the hydrogels composition [28,31,40].

To verify our hypothesis that venous endothelial cells (HUVEC) should be able to adhere and form an endothelial monolayer on these hydrogels after several days, the endothelialization potential of gelatin-based hydrogel films was investigated. In order to characterize HUVEC interaction depending on the hydrogel network density and thereby a potential influence of the slightly different elasticity on HUVEC adherence, monolayer formation, stability and integrity as well as cell functionality and activation state were explored after nine days of static culture and after subsequent exposure to physiological shear forces, respectively.

2. Materials and methods

2.1. Synthesis and characterization of gelatin-based hydrogels

Hydrogel networks were synthesized as films in three compositions G10_LNCO3, G10_LNCO5 and G10_LNCO8 as described previously [39]. The hydrogels were prepared in petri dishes (Ø 10 cm) from an aqueous solution containing 10 wt.-% gelatin (porcine skin derived, type A, 200 bloom, GELITA USA, Sergeant Bluff, IA, USA), which was reacted, in the presence of 1 wt.-% poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Pluronic® F-108, M_n ~ 14,6 kDa, Sigma Aldrich, Steinheim, Germany), with freshly distilled L-lysine diisocyanate ethyl ester (LDI, CHEMOS, Regenstauf,

Germany) in a 3- (G10_LNCO3), 5- (G10_LNCO5) and 8-fold (G10_LNCO8) molar excess of isocyanate groups, compared to accessible primary amino groups of gelatin. After synthesis, gelatin films were freeze dried and sterilized in a DMB-SteriVIT-Automatik Typ 100 VS 12 (DMB-Apparatebau, Wörrstadt, Germany) by ethylene oxide method (gas phase: 6 vol.-% ethylene oxide, 65% relative humidity, 1.7 bar, 45 °C, gas exposure time: 3 h, aeration phase: 12 h).

Hydrogels water uptake (*H*), degree of swelling (*Q*) and Young's moduli (*E*) of G10_LNCO3, G10_LNCO5 and G10_LNCO8 were determined as described previously [23]. Water uptake and swelling in percentage was calculated by weight increase after immersing dry samples at ambient temperature in phosphate buffered saline (PBS, 138 mM NaCl, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4, Biochrom GmbH, Berlin, Germany) for 24 h to reach swelling equilibrium. Tensile tests for determining the elastic modulus *E* were performed in distilled water with a temperature-controlled tensile tester (Zwick Z2.5, Zwick GmbH, Ulm, Germany) at 37 °C. Pre-equilibrated hydrogel samples with standard dimensions (2 mm x 4 mm x 30 mm) according to ISO 527-2/1BB were analysed until break with 0.02 N pre-force and 5 $mm \cdot min^{-1}$ crosshead speed. Young's moduli of the gelatin samples were calculated from the linear region, generally around 0.5 – 5 % strain of the tensile stress–strain plot.

2.2. Sample preparation

Prior to cell culture experiments, hydrogels were swollen in 20 mL PBS until equilibrium at ambient temperature for 24 h. Subsequently, swollen hydrogel discs (Ø 25 mm) were stamped out under sterile conditions with a stainless steel punch and mounted between the two parts of poly(ether ether ketone) (PEEK) based round shaped sample holders (SmardCAD Deutschland GmbH, Neu-Ulm, Germany).The lower part of the sample holder has a centred cavity (Ø 25 mm x 1 mm depth) and the upper part has a recess (Ø 24 mm) for enabling

access to the sample after fixation by screwing together both parts of a sample holder. Glass discs (Ø 25 mm, Gerhard Menzel GmbH, Braunschweig, Germany) were used as reference material.

2.3. Cell culture

Commercially available human umbilical vein endothelial cells (HUVEC, Lonza, Cologne, Germany) were cultivated under static cell culture conditions (37 °C, 5 vol.-% CO_2) in polystyrene-based cell culture flasks (TPP, Techno Plastic Products AG, Trasadingen, Switzerland) with endothelial basal medium EBM-2 supplemented with EGM-2 Single Quots® kit and 2 vol.-% FCS (Lonza, Cologne, Germany) for no longer than three passages. For dynamic tests, confluently grown HUVEC were isolated by trypsin/EDTA treatment (0.25% v/v Trypsin and 0.53 mM EDTA in PBS, PAN-Biotech GmbH, Aidenbach, Germany), seeded on the two hydrogels ($2.6 \cdot 10^4$ cells·cm^{-2}) and cultured under static cell culture conditions in the sample holders for nine days, before starting with the shear stress experiments. As positive control HUVEC were cultivated under identical conditions on glass as standard substrate (Gerhard Menzel GmbH, Braunschweig, Germany). In addition, HUVEC were analyzed under inflammatory conditions (seeded on glass in EGM-2 supplemented with 10 ng·mL^{-1} IL-1β R&D Systems, Wiesbaden-Nordenstadt, Germany). The cell culture medium was replaced every two days and eight hours before starting shear experiments.

2.4. Cell shearing experiments

Exposure of hydrogel-adherent HUVEC to physiological shear forces was realized by cone-and-plate shear method. Briefly, a shear device (SmardCAD Deutschland GmbH, Neu-Ulm, Germany) with sterile glass-cones (25 mm diameter and 2° angle) was used. The rotation of the cones at an angular speed of 11.6 rad/s generated typical physiological shear forces of 0.3

Pa (3 dyn·cm^{-2}) [12]. HUVEC on hydrogels were treated by dynamic conditions for one hour, with HUVEC cultivated under static conditions serving as reference for non-sheared cells. Subsequently, supernatants were obtained for secretion profile analysis and the samples were further processed for cell density determination and viability assessment or fixed for immune fluorescent staining, respectively.

2.5. Cell density and viability assessment

Cell viability and density of gelatin hydrogel cultivated HUVEC (static and dynamic) were analysed by staining vital cells with fluorescein diacetate (FDA, 25 µg·mL^{-1}, Invitrogen, Carlsbad, CA, USA), whereas positive propidium iodide staining (PI, 2 µg·mL^{-1}, Sigma, Taufkirchen, Germany) indicated dead cells. The cell density was calculated by taking three pictures from different areas of each sample (n = 6) by using a confocal laser scanning microscope (cLSM) with a 10-fold primary magnification (LSM 510 META, Zeiss, Oberkochem, Germany).

2.6. Cytoskeleton and cell-cell contact

Actin cytoskeleton and VE-cadherin, as major protein of adherens junctions, were stained by specific fluorescence-labelled antibodies prior and after shearing, respectively. HUVEC were fixed with paraformaldehyde (4% v/v in 0.9% NaCl, 30 min, 4 °C) and permeabilized with Triton X-100 (0.5% v/v). F-actin was fluorescently stained with Phalloidin-Alexa555 (1:40, Molecular Probes®, Invitrogen, Germany), VE-cadherin with a monoclonal mouse anti-human VE-cadherin IgG (1:500, Abcam, Cambridge, Great Britain) and a Cy2-conjugated polyclonal goat anti-mouse IgG (1:200, Jackson ImmunoResearch, Hamburg, Germany). The genomic DNA/nuclei were tagged by using intercalating 4',6-diamidino-2-phenylindole (DAPI, 1:5000, Roth, Germany). Documentation was realized by taking images in various primary magnifications (20-, 63- and 100-fold) using the cLSM.

2.7. Secretion profile analysis

Concentrations of secreted mediators were quantified after static culturing as well as after shear stress exposure from the supernatant of HUVEC, cultivated on hydrogels (G10_LNCO3, G10_LNCO5 and G10_LNCO8) and glass. Vasoactive mediators prostacyclin (PGI2) and thromboxane A2 (TXA2) were quantified by using a competitive inhibition enzyme immunoassay for stable hydrolysis products of PGI2 (6-keto-prostaglandin F1α) and TXA2 (thromboxane B2), respectively (6-keto-Prostaglandin F1α EIA Kit from Cayman Chemical Company, Ann Arbor, MI, USA and ELISA Kit for Thromboxane A2, Cloud-Clone Corporation, Houston, TX, USA). The secretion of the potent vasodilator nitric oxide (NO) was determined by azo dye-based colorimetric detection (Total Nitric Oxide and Nitrate/Nitrite Assay, R&D Systems Inc., Minneapolis, MN, USA). Additional cytokines as well as chemokines and growth factors were quantified by magnetic bead-based Bioplex assay (Bioplex™ Cytokine Assay (Bio-Rad Laboratories Inc., Munich, Germany). As control for the HUVEC functionality, endothelium was activated by recombinant human IL-1β (10 $ng \cdot mL^{-1}$, R&D Systems Inc., MN, USA), which was added to glass-cultivated cells.

2.8. Statistics

All data are reported as arithmetic mean ± standard deviation (SD) for continuous variables and were analysed by a two-tailed Student's t-test for paired samples. A p value of less than 0.05 was considered significant.

3. Results

3.1. Hydrogel characterization

Modulating hydrogel composition resulted in the equilibrium swollen state in an altered buffer uptake (H_{G10_LNCO3} = 942±113 wt.-%, H_{G10_LNCO5} = 725±20 wt.-% and H_{G10_LNCO8} =

438±36 wt.-%) and degree of swelling (Q_{G10_LNCO3} = 1,200±190 vol.-%, Q_{G10_LNCO5} = 530±42 vol.-% and Q_{G10_LNCO8} = 360±35 vol.-%) as well as Young's moduli (E_{G10_LNCO3} = 13±3 kPa, E_{G10_LNCO5} = 35±9 kPa and E_{G10_LNCO8} = 55±11 kPa), determined above the helix dissociation temperature at 37 °C [23,39]. After swelling the hydrogels were macroscopically transparent with flat surface. On the microscopic level the hydrogel compositions showed a more rough surface and small round shaped troughs (Ø 78±28 µm; n = 60; $Ø_{max}$ = 179 µm; $Ø_{min}$ = 35 µm) were visible on the surface of G10_LNCO5. On the hydrogels G10_LNCO3 and G10_LNCO8 no troughs were visible.

3.2. HUVEC monolayer formation and shear resistance on gelatin-based hydrogels

The HUVEC monolayer formation on G10_LNCO5 and G10_LNCO8 was investigated after nine days of static culturing. For G10_LNCO3 cell detachment occurred during the incubation period resulting in only few remaining endothelial cells on the hydrogels surface after static culturing. Furthermore, in the dynamic system G10_LNCO3 hydrogels detached from the bottom of the sample holder and started to disintegrate under the rotating glass cone, making it impossible to analyse endothelialization on this hydrogel composition. The microscopic evaluation of the remaining hydrogel compositions showed a comparable cell density and morphology, which was for both mentioned aspects different compared to HUVEC on glass (Fig. 1). HUVEC on hydrogels were more spread covering a larger area. Furthermore, gaps in the monolayer were visible, especially at the troughs on the surface of G10_LNCO5.

glass (control) **glass + IL-1β** **G10_LNCO5** **G10_LNCO8**

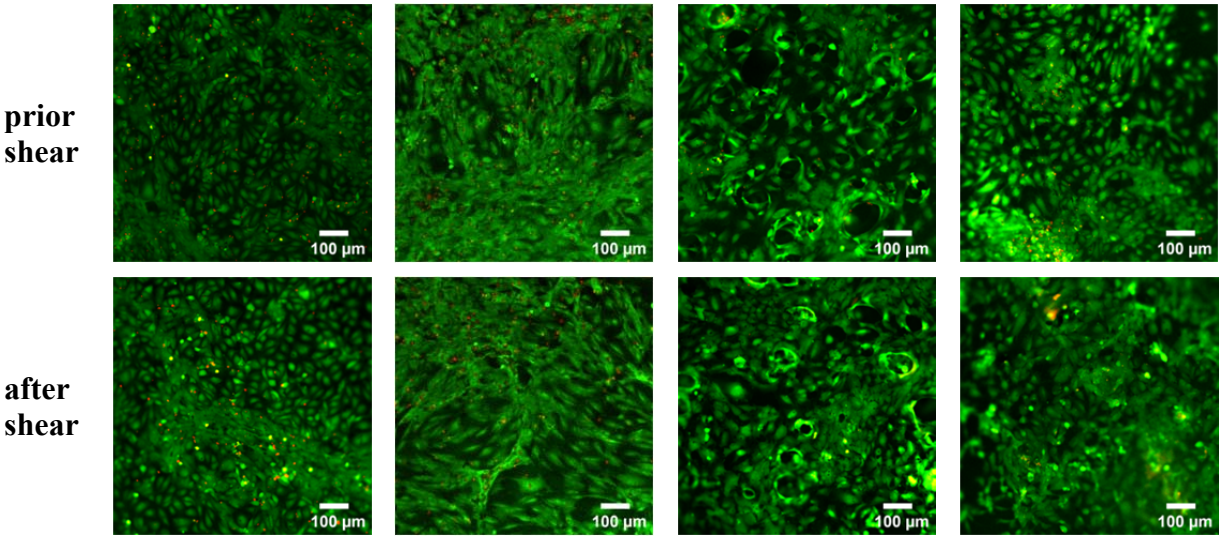

Fig. 1. Monolayer of HUVEC on gelatin hydrogels G10_LNCO5 or G10_LNCO8 compared to HUVEC on glass as substrate with and without IL-1β activation nine days after seeding prior to and after shear stress exposure. Vital cells stained with FDA (green) and dead cells with PI (red) (Scale bar: 100 µm).

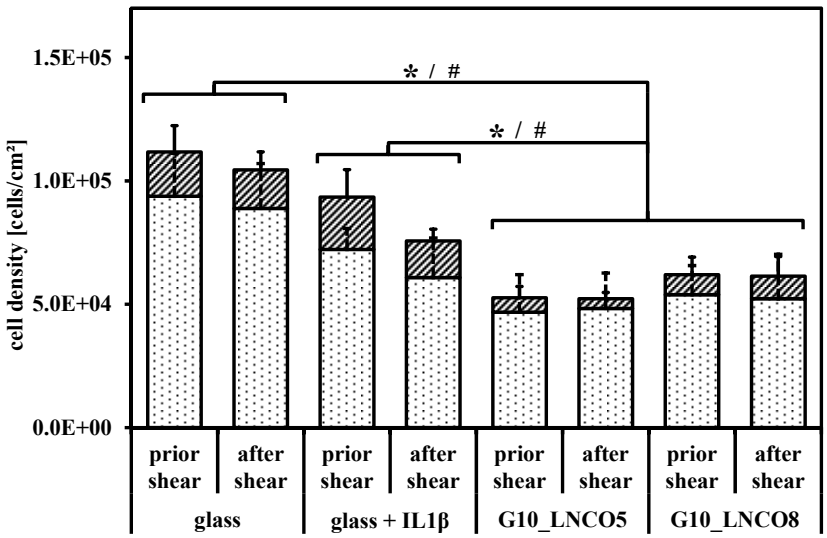

Fig. 2. Cell density of HUVEC cultivated on LDI-gelatine hydrogels G10_LNCO5 or G10_LNCO8, respectively as well as HUVEC on glass with and without IL-1β activation as control for nine days prior to and after shear stress exposure (Arithmetic mean ± standard deviation; *: significance vital cells; #: significance dead cells; *,# $p < 0.05$; $n = 6$).

This gap formation was reflected by the cell density, which was comparable between G10_LNCO5 ($N_{G10\_LNCO5\ (static)}$ = 46,784±15,241 cells·cm^{-2}) and G10_LNCO8 (N_{G10_LNCO8}

$_{(static)}$ = 48,272±11,839 cells·cm^{-2}). The HUVEC density was significantly lower on both hydrogels compared to glass ($N_{glass\ (static)}$ = 93,718±17,451 cells·cm^{-2}; p = 2.1·10^{-6} compared to G10_LNCO5 and p = 1.5·10^{-7} compared to G10_LNCO8) (Fig. 2). Vice versa. HUVEC viability was improved under static conditions on the hydrogels with 87.6% on G10_LNCO5 and 84.8% on G10_LNCO8 compared to 80.8% on glass and 70.6% on glass+IL-1β. After shear stress exposure this effect was solely visible for G10_LNCO5. Furthermore, under dynamic conditions the laminar shear flow caused a loss of adherent cells on the glass surface, but no cell detachment occurred on the hydrogels so that the cell density remained unchanged.

3.3. Cell-cell contacts on hydrogels

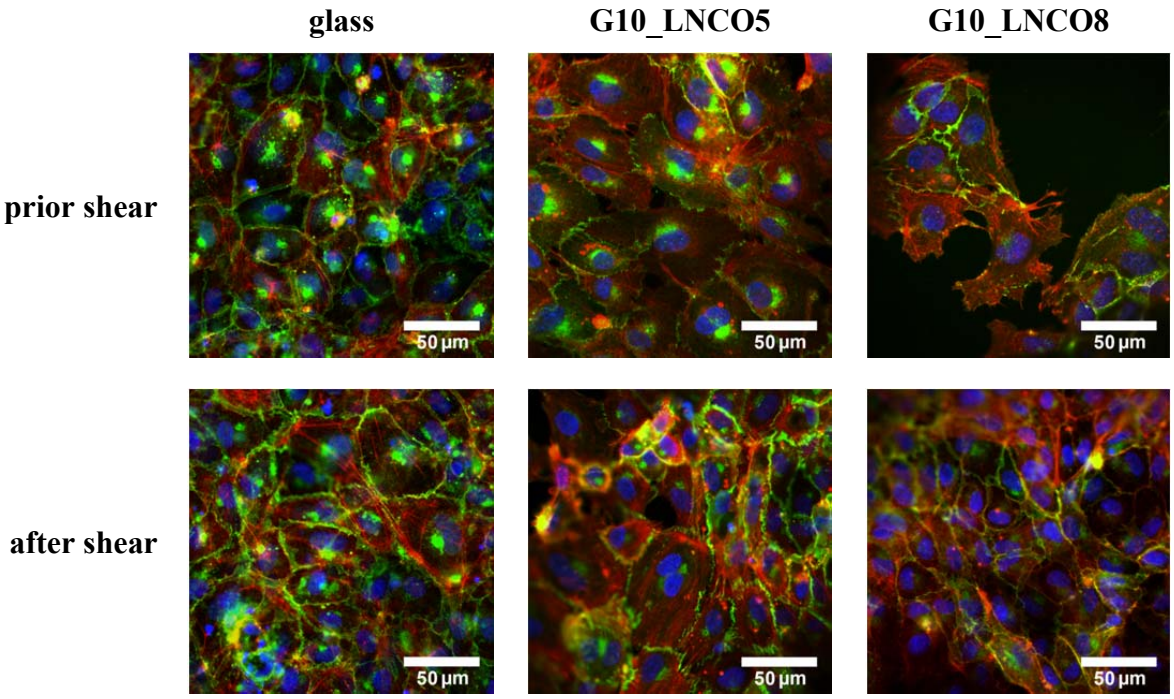

Fig. 3. HUVEC cytoskeleton and cell-cell contact marker VE-cadherin after nine days culturing on G10_LNCO5, G10_LNCO8, and glass as control, prior to and after shear stress exposure. Actin cytoskeleton (red), VE-cadherin (green), and genomic DNA (blue) were fluorescently labelled and images taken by using the cLSM with a 63-fold primary magnification.

On both, hydrogel and glass cultured HUVEC, shear stress exposure had no visible effect on the cell morphology concerning the actin cytoskeleton arrangement or the cell-cell contacts mediated by VE-cadherin (Fig. 3). HUVEC showed, regardless of the underlying substrate, close contacts on the cell rims, whereby VE-cadherin was always located as margin around the cells, connecting actin fibres of the one cell with those of the adjacent endothelial cell.

3.4. Secretion of vasoactive mediators and cytokines

Vasoactive mediators such as nitric oxide (NO), the potent vasoconstrictor thromboxane A2 (TXA2) and its physiological antagonist prostacyclin (PGI2) as well as multiple cytokines were quantified in the supernatant prior to and after shear stress exposure. This allows to analyse the activation state and the release of mediators depending on the substrate under static and dynamic conditions (Table 1). For both conditions differences in the secretion profile of PGI2 and NO were marginal between hydrogels and the control. Solely the inflammatory activation of HUVEC with IL-1β led to an increased secretion of PGI2. Application of venous shear forces always slightly increased secretion levels of PGI2 and NO on all materials. However, the secretion of PGI2 was for non-activated cells always below 100 $pg \cdot mL^{-1}$ and overall no secretion of TXA2 could be detected.

Additionally, the amounts of pro- and anti-inflammatory cytokines as well as growth factors and chemokines were measured from the supernatant of hydrogels compared to glass seeded HUVEC. Secretion of pro- (IL-1β, IL-2, IFN-γ, TNF-α) and anti-inflammatory mediators (IL-1ra, IL-10) as well as chemokines (GM-CSF, MIP-1a/b) and functional markers (FGF-b, VEGF) was below the detection limit. Furthermore, contact to the hydrogels had no influence on the secretion levels of pro-inflammatory IL-6, anti-inflammatory IL-4 as well as the chemokines G-CSF, MCP-1 and Eotaxin. Notable increases in supernatants mediator concentrations could be solely detected after shear stress exposure for pro-inflammatory

RANTES and IL-8 on G10_LNCO5 and for the functional mediator PDGF-BB on G10_LNCO8, in which only the change in secretion of IL-8 was significant. However, compared to HUVEC cultivated on hydrogel or glass, most mediator concentrations were several fold higher in the supernatants of HUVEC activated by IL-1β, both under static or dynamic conditions.

Table 1. Concentrations of multiple vasoactive mediators and cytokines determined from cell culture supernatants by ELISA or Bioplex assay prior to and after shear stress exposure of hydrogel cultured HUVEC. Cells were cultivated for 9 days on glass, on hydrogels G10_LNCO5 and G10_LNCO8, or on glass with cell culture medium supplemented with IL-1β (10 ng·mL^{-1}) (Arithmetic mean ± standard deviation; n.d. = not detectable; * $p < 0.05$ compared to static conditions; n = 6).

	Glass		G10_LNCO5		G10_LNCO8		Glass + 10 ng · ml-1 IL-1β	
	Prior shear	After shear	Prior shear	After shear	Prior shear	After shear	Prior shear	After shear
				Vasoactive mediators [pg·mL^{-1}]				
PGI2	69 ± 8	82 ± 12	87 ± 5	96 ± 9	70 ± 3	96 ± 16*	256 ± 40	343 ± 45*
NO [µM]	2 ± 0	6 ± 3*	2 ± 1	4 ± 2*	2 ± 1	3 ± 1	2 ± 0	6 ± 3*
				Pro-inflammatory mediators [pg·mL^{-1}]				
IL-6	93 ± 11	92 ± 13	84 ± 11	89 ± 13	92 ± 18	114 ± 18	39,557 ± 15,151	61,156 ± 14,917
IL-8	569 ± 124	588 ± 251	610 ± 97	1,169 ± 275*	592 ± 106	754 ± 193	100,107 ± 57,886	60,763 ± 25,940
RANTES	7 ± 1	n.d.	7 ± 1	27 ± 10	8 ± 1	n.d.	492 ± 128	379 ± 122
IP-10	85 ± 20	149 ± 166	51 ± 15	292 ± 125	58 ± 20	123 ± 71	3,661 ± 1,597	1,598 ± 546
				Anti-inflammatory mediators [pg·mL^{-1}]				
IL-4	2 ± 0	n.d.	2 ± 0	n.d.	3 ± 1	n.d.	16 ± 1	12 ± 1
				Chemokines & growth factors [pg·mL^{-1}]				
G-CSF	49 ± 11	31 ± 6	16 ± 8	20 ± 20	20 ± 20	33 ± 22	50,537 ± 23,838	95,839 ± 80,832
MCP-1	349 ± 76	231 ± 79	332 ± 32	317 ± 66	407 ± 79	383 ± 57	705 ± 74	658 ± 249
PDGF-BB	538 ± 101	57 ± 42	509 ± 97	92 ± 27	531 ± 185	207 ± 68	1,181 ± 154	131 ± 54
Eotaxin	12 ± 6	n.d.	12 ± 3	n.d.	14 ± 3	n.d.	133 ± 23	12 ± 3

4. Discussion

The use of endothelial cell recruiting ligands in the form of ECM macromolecules or cell specific ligands such as ECM-derived peptides, antibodies or aptamers is a strategy for improving cell adhesion and retention [8,18,20,21]. However, the immobilization of ECM mimicking ligands on implant biomaterial surfaces is challenging. By using collagen derived gelatin in the form of hydrogels, material immobilization of cell recruiting ligands can be omitted, because cell recognition motifs are already components of the gelatin peptide sequence structure.

Therefore, our hypothesis was that a confluent and shear-resistant HUVEC monolayer will form on gelatin-based hydrogel films. To investigate this, the *in vitro* study was performed with hydrogel compositions, differing in their degradation behaviour and elastic properties, in which Young's moduli were close to autologous vessels in the range between 35 - 55 kPa. The experiments were performed in comparison to a positive (glass) and a negative control (HUVEC under inflammatory conditions) under static and dynamic conditions. In all groups, the HUVEC were cultured for nine days under static conditions and subsequently the HUVEC monolayer was exposed to physiological shear forces. The hydrogel composition G10_LNCO3 with lower LDI content, which showed - because of less covalent and physical netpoints - altered material properties such as lower Young's modulus, higher water uptake and swelling as well as faster degradation, could not be tested [23,40]. Already during static culturing cells detached from the hydrogel surface, possibly because of the fast degradation of the underlying substrate. Further, the high flexibility of G10_LNCO3 after nine days in cell culture medium led to detachment of the material under dynamic conditions and following disintegration under the rotating glass cone of the test system.

In line with previous studies [28,31,40], about 85% of the adherent endothelial cells on the gelatin-based hydrogel networks G10_LNCO5 and G10_LNCO8 were viable and no cytotoxic influences were observed. However, in comparison to HUVEC cultured on glass, cell densities were significantly reduced on both hydrogel compositions up to nine days of

static culture. In particular, troughs on the G10_LNCO5 hydrogel surface led to gaps in the cell layer and a slightly lower cell density compared to G10_LNCO8. Considering an *in vivo* application in the cardiovascular system, the gelatin matrix would be accessible for blood components (i.e. proteins, thrombocytes) at the cell free areas. Here it might be hypothesized, that gelatin (as derivative from collagen) would be recognized as part of the subendothelial matrix, inducing intrinsic coagulation pathways [8]. While Jensen et al. [17] described a comparable thrombogenicity of gelatin and collagen, some recent studies revealed that gelatin is a low thrombogenic biomaterial, which is frequently used as a hemocompatible coating for scientific or commercial cardiovascular applications [9,42]. It can be assumed that the material processing plays a role for the accessibility of thrombogenic recognition sides on the gelatin surface.

Aside from the lower cell density, the HUVEC showed morphologically a larger spreading on both hydrogels. This counts for an adaption of the underlying gelatin matrix as a suitable ECM-surrogate by integrin mediated utilization of the available cell recognition motifs, presented in the amino acid structure of the gelatin [29]. The exposure of hydrogel cultured HUVEC to physiological shear forces revealed a substantial better adherence of the cells on both gelatin films. This can be explained by the larger cell spreading, which is directly connected with an increased number of stabilizing integrin mediated contacts (focal adhesions [6]) to the artificial ECM. Furthermore, the larger spreading may lead to a flatter cell shape with lower flow resistance. The mechanical properties of the hydrogels, mainly the elasticity, also play a substantial role. The higher flexibility of the hydrogels compared to the comparatively rigid glass surface - Young's modulus in the GPa range - enabled the distribution of acting shear forces and thereby a better compensation of the mechanical stress on the cells. However, differences in elasticity between both hydrogels do not seem marked enough to visualize different effects on the cells.

The investigation of the cell layer integrity by staining the actin cytoskeleton and VE-cadherin revealed a strong interaction between the hydrogel cultivated HUVEC. The cell layer was stabilized by multiple VE-cadherin mediated cell-cell contacts, visible as a margin around the cell cortex. VE-cadherin acts as an extracellular linker between cytoskeleton actin fibres of adjacent cells, leading to reinforcement of the cell layers integrity and mechanical strength [13]. Further, VE-cadherin is associated with other cell adhesion proteins of focal adhesions (i.e. vinculin), which are involved in cell substrate interaction, stabilization and integrin mediated mechanosensing, transmitting extracellular information into the cell [41]. In synergy with the aspects mentioned above, this marked transcellular network was stable enough on the hydrogels to completely resist physiological shear forces and retain functional integrity, despite of the imperfect HUVEC monolayer.

The secretion levels of the vasodilator NO and the antagonistic acting vasoactive mediators PGI2 and TXA2 indicated no hydrogel-induced functional changes of the HUVEC compared to the control. The detected amounts of PGI2 in the supernatant were in the physiological effective range [24,35] and no appreciable secretion could be ascertained under static and dynamic conditions for TXA2. This is possibly due to the short exposure to physiological shear forces, because induction of TXA2 secretion as well as passive release from partially damaged cells after shear stress exposure for longer periods was shown in the past [1,33]. Only slight HUVEC loss was observed on glass and nearly none on the hydrogels. The HUVEC monolayer remained unchanged regarding the cell density and morphology. HUVEC response to elevated flow conditions was more in direction of a vasodilation, visible by a slightly shear stress induced increase of NO and PGI2 secretion. This shear dependent behaviour of the endothelial cells is well studied and the amplified production of vasodilators serves under physiological conditions to counteract thrombocyte adhesion and activation [11,14].

Overall, the direct contact of HUVEC to gelatin-based hydrogels induced no changes in cell function or homeostasis for any of the samples and conditions regarding secretion of pro- and anti-inflammatory cytokines and chemokines. The sole exception was G10_LNCO5, where HUVEC secretion of pro-inflammatory mediators IL-8 and RANTES was slightly upregulated after shear stress exposure. RANTES acts together with MCP-1 as chemotactic mediator of acute and chronic inflammation, regulating the activation and recruitment of leukocytes into inflamed tissue [7]. Indeed, MCP-1 was released on all hydrogel samples, but always comparable to glass. More important is that this cytokine was not simultaneously upregulated on G10_LNCO5. IL-8 possesses several functions on endothelial cells. On the one hand, IL-8 acts, comparable to CCL5, as pro-inflammatory mediator for the chemotactic recruitment of leukocytes into inflamed sites and on the other hand as an anti-apoptotic and angiogenic factor, involved in the tissue remodeling and (neo)formation of blood vessels [5,19]. However, a substrate mediated inflammatory effect as explanation for the slightly elevated secretion levels of RANTES an IL-8 seems to be unlikely. If there would be an inflammatory effect of G10_LNCO5 on the HUVEC, secretion of other pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α should also be upregulated [25,43]. Furthermore, the quantified amounts of these pro-inflammatory mediators from the corresponding IL-1β activated controls were always substantial higher, so that the differences between G10_LNCO5 and the other samples were marginal.

5. Conclusion

Entropy-elastic gelatin-based hydrogel networks were shown to be a suitable substrate for endothelial cells. Tailoring the hydrogels degradation and mechanical properties, particularly with an elasticity in the range between 35 and 55 kPa, by alteration of the network density, showed no considerable effect on HUVEC adherence, cell density or morphology and compared to IL-1β activated HUVEC only marginal variations in the secretion profiles of

cytokines and vasoactive mediators, both under static and dynamic conditions. The cells were able to proliferate on the hydrogel substrate, but no closed cell monolayer was formed after nine days of static culture. However, HUVEC grown on the hydrogels compositions G10_LNCO5 and G10_LNCO8 showed excellent shear resistance, with no visible cell detachment after applying physiological shear forces. The shear resistance of the endothelial cell monolayer was characterized by a marked cell-cell as well as cell-substrate interaction associated with a larger cell spreading on the hydrogel surfaces. Furthermore, the functionality of the HUVEC concerning vasoactive and inflammatory response did not differ from the response to the positive controls. From these results, gelatin-based hydrogels present a promising candidate biomaterial for medical applications in the cardiovascular field. Here, the hydrogels application period could be adjusted by modifying the material composition, which associated alteration of the elasticity in the mentioned range showed no considerable effect on the endothelial cells. Utilization of the hydrogels as multifunctional scaffolds would be conceivable as patches, artificial vessels or delivery system for endothelial progenitor cells.

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