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# Immunocompatibility and non-thrombogenicity of gelatin-based hydrogels

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**Key words:** gelatin-based hydrogels, immune cells, platelets, *in vitro* testing, mouse model

## Abstract

Immunocompatibility and non-thrombogenicity are important requirements for biomedical applications such as vascular grafts. Here, gelatin-based hydrogels formed by reaction of porcine gelatin with increasing amounts of lysine diisocyanate ethyl ester were investigated *in vitro* in this regard. In addition, potential adverse effects of the hydrogels were determined using the "Hen's egg test on chorioallantoic membrane" (HET-CAM) test and a mouse model.
The study revealed that the hydrogels were immunocompatible, since complement activation was absent and a substantial induction of reactive oxygen species generating monocytes and neutrophils could not be observed in whole human blood. The density as well as the activation state of adherent thrombocytes was comparable to medical grade polydimethylsiloxane, which was used as reference material. The HET-CAM test confirmed the compatibility of the hydrogels with vessel functionality since no bleedings, thrombotic events, or vessel destructions were observed. Only for the samples synthesized with the highest LDI amount the number of growing blood vessels in the CAM was comparable to controls and significantly higher than for the softer materials. Implantation into mice showed the absence of adverse or toxic effects in spleen, liver, or kidney, and only a mild lymphocytic activation in the form of a follicular

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hyperplasia in draining lymph nodes (slightly increased after the implantation of the material prepared with the lowest LDI content). These results imply that candidate materials prepared with mid to high amounts of LDI are suitable for the coating of the blood contacting surface of cardiovascular implants.

## Introduction

Regeneration supported by materials aims to restore the function of damaged or degenerated tissue. Materials envisioned for cardiovascular implants including vascular grafts, stents, or heart valves have to be sterilizable, an indispensable condition for implant materials, non-toxic, and have to be synthesized and processed so that they are ready for use implants and are not contaminated with immunogenic impurities.[1] The implant material should also be non-thrombogenic per se so that during the process of endothelialisation, which can take some months, no thrombotic events occur.[2] The materials should allow the adjustment of the mechanical properties to the elasticity of the vascular wall. Ideally, the material would degrade over time leaving an anatomically and functionally completely restored vessel behind without any adverse effects on organs involved in the detoxifying process.

At present, no material fulfills all these requirements for properties and functions. However, hydrogels seem to meet most of the requirements outlined above.[3] Notably, a mechanical mismatch between implant and surrounding tissue can be avoided, which is reported as one possible cause of device failure.[4] It is of utmost importance that implants do not exhibit a higher elastic modulus than the surrounding tissue, because relative movements of the tissue result in stresses at the interface between implant and tissue inducing inflammatory signals, possibly facilitating the formation of a fibrotic capsule.[5] One possible approach for such materials is based on components of the extracellular matrix (ECM). Collagens, as main proteins of the connective tissues, are widely applied in tissue regeneration.[6,7] They are described to be histocompatible, easily available, and highly versatile,[8] while induction of adverse immunogenic host responses,[9] thrombotic responses,[10] and difficulties in tailoring their mechanical properties have also been reported.[11]

In order to yield materials possessing the positive properties of collagens, while avoiding their drawbacks, gelatin-based hydrogels were introduced.[12] These can be tailored in their physicochemical properties and degradation behavior by network formation.[13] Hydrogels synthesized by reacting gelatin with lysine diisocyanate ethyl ester (LDI) in water applying a variable excess of isocyanate groups per mol of amino groups present in gelatin (3, 5 or 8 mol isocyanate groups per mol primary amino group)[14] in particular have been shown to be sterilizable and non-cytotoxic according to ISO 10993[15] with an endotoxin load below the limit[16] set by the U.S. Food and Drug Administration.[17] Varying the amount of LDI in the process allows to tailor the macroscopic as well as the local Young´s modulus of the materials to 15 – 55 kPa (tensile tests)[14] and 1.1-10.3 kPa (AFM indentation)[18] which is below the Young´s modulus of the vascular wall, reported to be 34-100 kPa,[19,20] but similar to the properties of material supporting the growth of endothelial cells.[21,22] Recent *in vitro* studies revealed that arterial endothelial cells as well as mesenchymal stem cells were able to migrate

into the gelatin-based hydrogels remaining in a vital state.[15,23] In addition, two animal studies showed an effect of the LDI amount on degradation, inflammatory response, and capsule formation.[23] In particular, an excellent integration of the hydrogels into the surrounding tissue without any substantial inflammatory reactions was observed .[23]

In the framework of the current study, three gelatin-based hydrogels with increasing molar ratios of LDI to gelatin in the starting material mixture were evaluated (in the following referred to as "hydrogels") in view of a potential application as cardiovascular implants. For this purpose, in two first *in vitro* studies the innate immune response of human immune cells as well as the thrombogenicity of the hydrogels were investigated. Further on, the influence of the hydrogels on the growth of vessels of the developing embryo and the vasoregulation was explored using the hen´s egg chorionallantoic membrane test (HET-CAM test). Finally, a safety study was performed using immuno-competent hairless mice with subsequent histopathological evaluation of various inner organs to exclude potential toxic effects.

## Material and Methods

### Materials

Hydrochloric acid, diethylether, sodium bicarbonate, 2,4,6-trinitrobenzenesulfonic acid (TNBS), poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (Pluronic® F-108, average $M_n$ ~ 14,600 $g \cdot mol^{-1}$) were purchased from Sigma-Aldrich, Steinheim, Germany. Lysine diisocyanate ethyl ester (LDI) was acquired from CHEMOS, Regenstauf, Germany, and gelatin (from porcine skin, 200 bloom, type A, low endotoxin content) from GELITA USA, Sergeant Bluff, IA, USA. VLE-RPMI was from Biochrom, Berlin, Germany, lipopolysaccharide (LPS) from the *E. coli* strain O111:B4 from Axxora GmbH, Lörrach, Germany, the Phagoburst® assay kit from ORPEGEN Pharma, Heidelberg, Germany, pooled normal human plasma from Precision BioLogic Inc. Dartmouth, Nova Scotia, Canada, zymosan and paraformaldehyde (4 wt-% solution) from Sigma-Aldrich, St Lois, MO, USA, the C5a ELISA kit from R&D, Minneapolis, MI, USA, DPBS buffer from Life Technologies GmbH, Frankfurt/Main, Germany, polydimethylsiloxane (PDMS, silicone) from Bess Medizintechnik GmbH, Berlin, Germany, rat tail type I collagen and fibronectin from neuVitro, El Monte, USA, FITC-conjugated CD42a antibody from Beckman Coulter, Marseille, France, AlexaFluor 555-conjugated phalloidin from Life Technologies GmbH, Darmstadt, Germany, VALO SPF eggs of the white Leghorn species from Lohmann Tierzucht GmBH, Cuxhaven, Germany, frozen section medium Neg-50™ and Cytoseal™ XYL from Richard-Allan Scientific™, Thermo Scientific, Waltham, USA, and hemalaun solution as well as eosin G solution from Roth, Karlsruhe, Germany.

### Material synthesis and degradation

The G10_LNCO3, G10_LNCO5, and G10_LNCO8 hydrogels were synthesized by reacting a 10 wt.-% aqueous gelatin solution with varying amounts of freshly distilled lysine diisocyanate ethyl ester, using a 3-, 5-, or 8-fold excess of isocyanate (LNCO*x*)groups compared to amino groups of gelatin, which were quantified with a TNBS assay.[24] The reaction was performed

in the presence of 1 wt.-% poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol). All samples were sterilized by ethylene oxide sterilization (gas phase: 6 vol.-% ethylene oxide, 45 °C, 65% relative humidity, 1.7 bar, gas exposure time: 3 h, aeration phase: 12 h) in a DMB-SteriVIT-Automatik Typ 100 VS 12 (DMB-Apparatebau, Wörrstadt, Germany).

Hydrolytic degradation experiments were performed on hydrogel discs (6 mm diameter), which were incubated at 37 °C in PBS for 21 days. Buffer was exchanged every third day. After 3 h and 1, 3, 6, 9, 14, and 21 days, three replica for each time point were dried, weighed, and the remaining relative mass $\mu_{rel}$ was calculated using equation (1), where $m_d$ and $m_0$ are the mass at time point d and the mass before degradation, respectively.

$$\mu_{rel} = \frac{m_d}{m_0} x100 \qquad \text{(Equation 1)}$$

## Analysis of the innate immune response

### Determination of cytokine secretion and oxidative burst activity by immune cells

Cytokine and ROS expression analyses were conducted as previously described.[25] In brief, the cytokine secretion was quantified in whole human blood, which was diluted 1:10 in VLE-RPMI and incubated for four hours with the three different hydrogels. To simulate inflammatory conditions, lipopolysaccharide (LPS) from the *E. coli* strain O111:B4 was used at 1 $\mu g \cdot mL^{-1}$. The Bioplex® 200 (Biorad, München, Germany) multiplex system was used to analyze the secretion of IL-1β, IL-6, IL-10, and TNF-α according to manufacturer's instructions. To determine the ROS generation, whole human blood was incubated for 10 min on the three hydrogels, using the Phagoburst® assay kit. The oxidative burst activity, which is indicative for ROS generation, was monitored by flow cytometry utilizing the MACSQuant® analyzer (Miltenyi Biotec B.V. & Co. KG, Bergisch-Gladbach, Germany). The obtained data were analyzed with the FlowJo software (Tree Star, Ashland, USA). Monocytes, neutrophils and lymphocytes were discriminated according to their forward and sideward scatter parameters as previously described.[26]

### Detection of C5a release

To detect the release of C5a as indicator for complement activation, 300 µL of pooled normal human plasma was added to the hydrogels or TCP (Tissue Culture Test Plates, TPP, Trasadingen, Switzerland) as negative control and incubated for 30 minutes at 37 °C. As positive control, 100 $\mu g \cdot mL^{-1}$ zymosan was added to the plasma. An enzyme-linked immunosorbent assay (ELISA) kit) was used according to manufacturer's instructions to determine the amount of released C5a fragments in plasma, which was diluted 1:100 in DPBS.

### Thrombogenicity

Thrombogenicity of the hydrogels was evaluated in a standardized static platelet adherence test.[27] Medical grade smooth polydimethylsiloxane (PDMS) as well as glass coverslips coated with rat tail type I collagen and fibronectin were used as negative or positive control substrates, respectively.[27,28] The experiments were performed according to the guidelines of

the International Society on Thrombosis and Haemostasis; the protocol was approved by the institutional committee of the Charité University Medicine Berlin. Blood samples were drawn from the cubital vein into S-Monovettes® (Sarstedt, Germany; sodium citrate, final concentration 0.106 $mol \cdot L^{-1}$; ethylenediamine-tetraacetic acid (EDTA)). Based on the haemogram, platelet aggregation index, platelet function (PFA-100) and amount of C-reactive protein, donors exhibiting early inflammatory processes and abnormalities in platelet count or function were excluded.[27,29]

Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by centrifugation of sodium citrated whole blood (PRP: 140 g for 20 minutes, PPP: 1,500 g for 20 minutes). The platelet count was adjusted to $5x10^4$ thrombocytes$\cdot \mu L^{-1}$, by diluting PRP with PPP. The polymers were placed into 24 well suspension cell culture plates (CELLSTAR, Greiner Bio-One, Frickenhausen, Germany). Test polymers were covered with 400 µL of adjusted PRP for one hour (37 °C humidified atmosphere, 5% $CO_2$) and washed with PBS afterwards. Material surface adherent thrombocytes were fixed with paraformaldehyde and labelled with FITC-conjugated CD42a antibody as well as AlexaFluor 555-conjugated phalloidin. Thrombocytes were visualized by confocal laser scanning microscopy (LSM 510 Meta, Carl Zeiss, Jena, Germany, 100 fold primary magnification). The degree of platelet activation was quantified as described earlier using ImageJ (National Institutes of Health, USA).[30]

**HET-CAM test**

*The Hen's egg test on chorioallantoic membrane* (HET-CAM test) was performed as described previously.[31] VALO SPF eggs of the white Leghorn species were used. After a four day storage at 11 °C and 24 h at 18 °C, the eggs were incubated for seven days at 37 °C with 65% relative humidity and automatic egg rotation. On day eight, the top part of the shell was separated from the CAM, which allowed for a small (2.0 $cm^2$) window to be cut out without damaging the embryonic structures. Specimens of the three different hydrogels with a surface area of 125 $mm^2$ were placed on the CAM and fixed with a Thermanox™ ring ($n$ = 10, per group), or a Thermanox™ ring alone was placed (control group). After 2 days, blood vessel lysis, haemorrhage, coagulation, as well as the number of blood vessels in the CAM were counted and microscopically evaluated (stereomicroscope MZ16A, Leica Camera AG, Germany). Representative examples are shown in Figure 1.

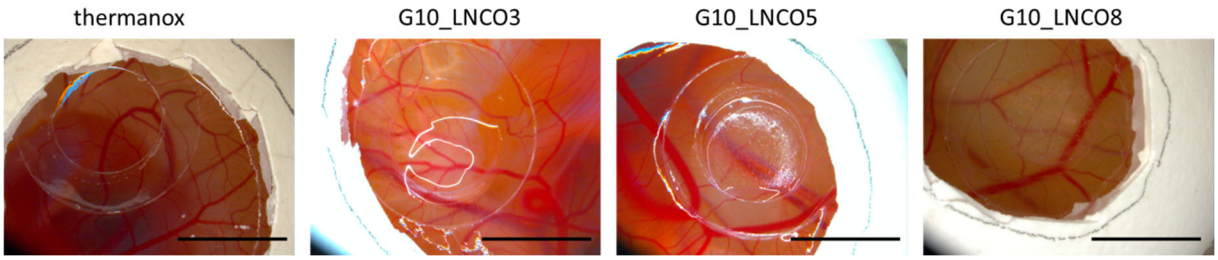

Figure 1: Representative images of the CAM with the hydrogels, G10_LNCO3, G10_LNCO5 and G10_LNCO8 within the Thermanox™ ring after 2 days of incubation. Blood vessel lysis, haemorrhage or coagulation was not observed. Scale bar: 8 mm

To analyze blood vessel diameters, the CAM was explanted and treated with 4 vol-% paraformaldehyde solution (4 °C) with subsequent embedding in frozen section medium Neg-50™. Frozen samples (-20 °C) were cut in 10 µm cryo slices (Kryostat HM560M, Microtom, Walldorf, Germany), then incubated with hemalaun solution and eosin G solution and subsequently mounted in Cytoseal™ XYL. The evaluation of the Hematoxylin-Eosin stained samples was performed using the microscope Axio Imager Z2m (Zeiss, Jena, Germany). The diameters of the uppermost blood vessels were measured at five different locations of the CAM (n = 10) (Figure 2).

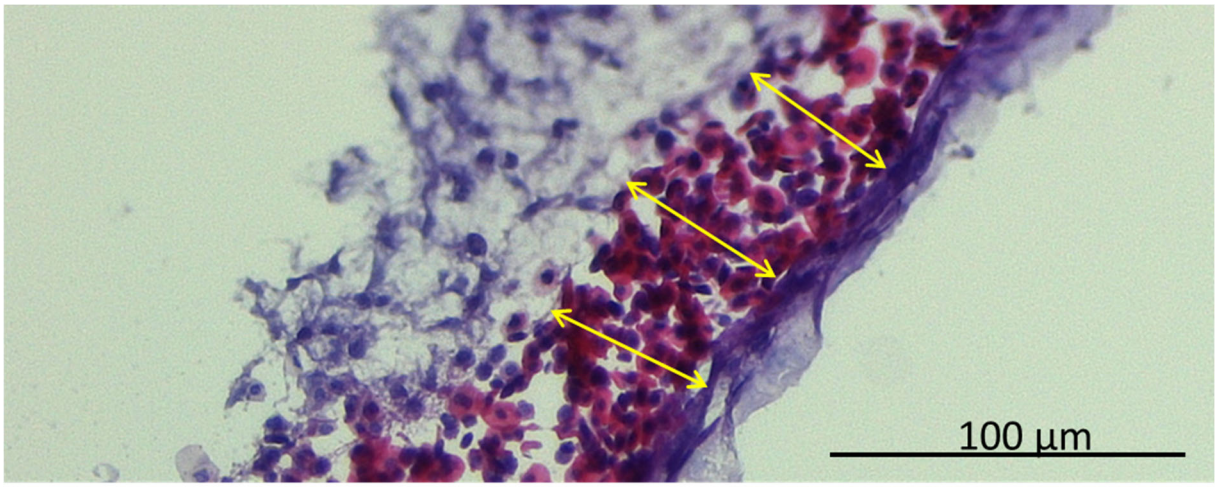

**Figure 2:** Representative image of a histological CAM section after HE staining. The image shows the measurement, which was performed at five different sites to determine the average blood vessel diameter (yellow arrows).

***In vivo* experiments**

Animal experiments were performed as described previously [23,32]. Briefly, hydrogels were implanted subcutaneously in the lower dorsal area of immunocompetent hairless SKH1 mice (1 hydrogel/animal, 10 animals/hydrogel type or sham-operated). At day 35 after implantation, mice were sacrificed by heart puncture and blood was collected. Figure 3 shows the implantation procedure with the hydrogel and an MRI image with the implanted hydrogel.

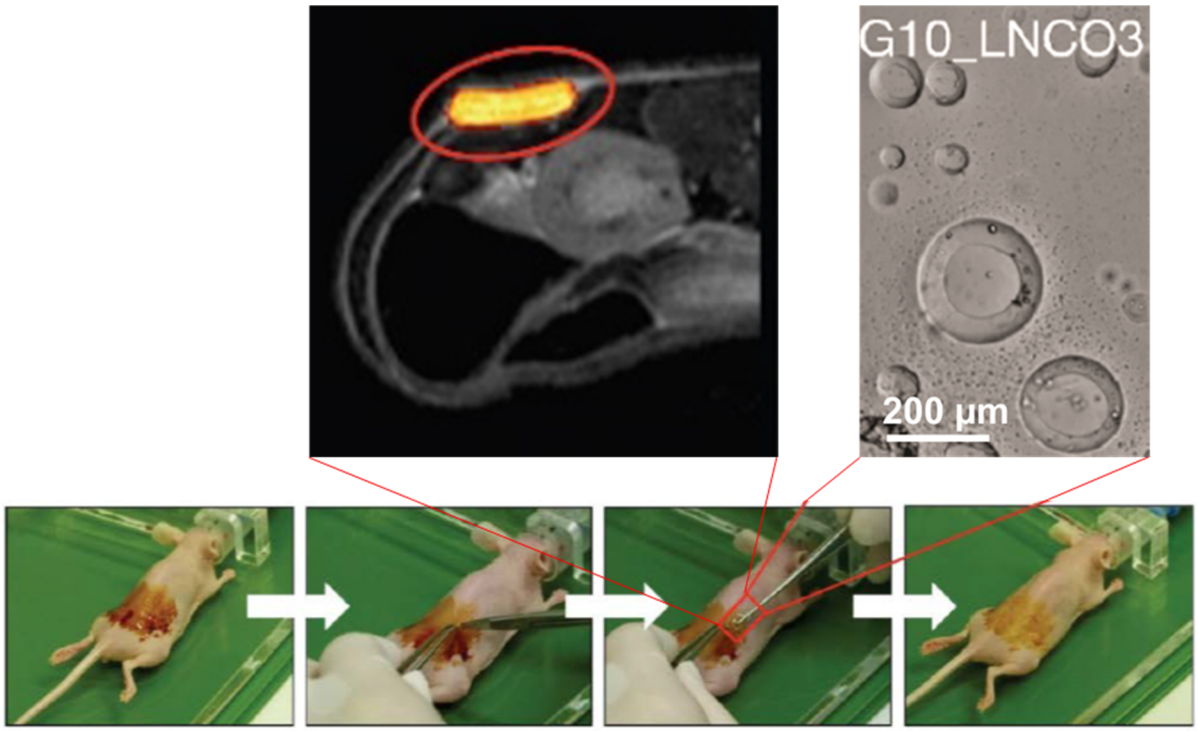


Figure 3: Confocal microscopy image of one of the three hydrogels. The implantation workflow is shown in the lower panel. First: mice skin is disinfected, second: incision and skin pocket is formed, third: pre-swollen hydrogel is implanted, fourth: incision is closed by the use of a spray-plaster. Above, a representative axial MRI images on day 1 after implantation and a confocal microscopy image of the surface structure of G10_LNCO3 are shown. Images reprinted from Ref. [32].

To investigate whether hydrogel degradation caused systemic effects, inguinal lymph nodes, spleen, liver, and kidneys were fixed in formalin and embedded in paraffin. Representative, 1 to 2 µm thin sections of the fixed organs were stained with Hematoxylin and Eosin (HE) and completely evaluated by a board-certified pathologist.

**Statistics**

For all samples arithmetic mean ± standard deviation are given. Data were statistically analyzed using GraphPad Prism (La Jolla, CA 92037, USA). One-way ANOVA for repeated measurements with post hoc analyses (adherent platelets, complement factor, ROS generation, and cytokine analysis) was performed. p-values less than 0.05 were considered significant.

## Results

### *In vitro* analysis of gelatin-based hydrogels

### *In vitro* degradation

To determine the influence of different LDI amounts used in the synthesis of the hydrogels on the degradation behavior, the mass loss of the three hydrogels was compared (Figure 4). The degradation data of G10_LNCO3 and G10_LNCO8 were already published by Ullm *et al.*.[23] The G10_LNCO5 samples showed a mean mass loss of around 35 wt.-% after 21 days, which was slower than the degradation of G10_LNCO3 (55 wt.-%) and higher than of G10_LNCO8 (8 wt.-%).

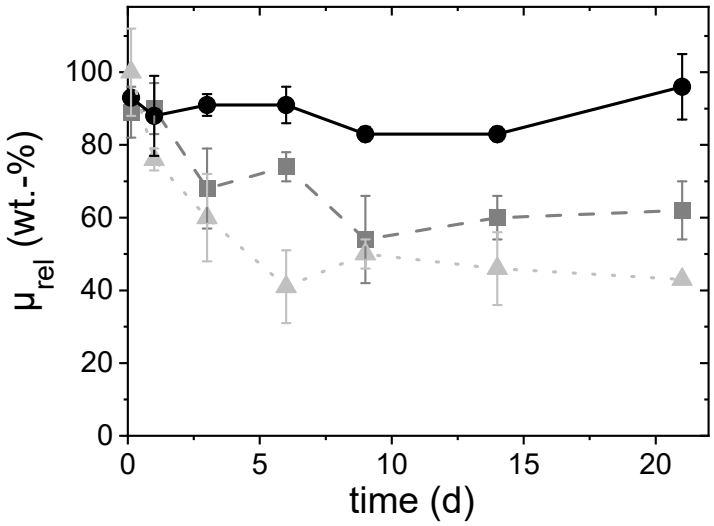


**Figure 4:** Relative mass $\mu_{rel}$ left of G10_LNCO5 samples in comparison to G10_LNCO3 and G10_LNCO8, which were published by us [23], after incubation in PBS buffer at 37°C for different time points. Samples were prepared from a 10 wt.-% aqueous gelatin solution reacted with a 3- (G10_LNCO3: -▲-), 5- (G10_LNCO5: -■-), or 8-fold (G10_LNCO8: -●-) excess of isocyanate groups per mol of amino groups present in gelatin (arithmetic mean ± standard deviation of n=3 samples. For two data points, no error bar is visible in the graph, which is due to the size of the data point and the low error (±2 in both cases)). Curves shown for G10_LNCO3 and G10_LNCO8 are reprinted from [23], Copyright (2014), with permission from Elsevier.

**Immunocompatibility**

The number of reactive oxygen species (ROS)-producing monocytes and neutrophils was determined in whole human blood. In response to heat inactivated *E. coli,* an elevated number of monocytes and neutrophils produced ROS. In contrast, untreated blood as well as the blood in contact with the hydrogels did not show increased levels of ROS-producing monocytes (Fig. 5A). Although the numbers of ROS-producing neutrophils were significantly elevated for G10_LNCO3 and G10_LNCO5, they were only slightly enhanced. As expected, ROS generation in lymphocytes could not be detected (data not shown).

The inflammatory capacity of the hydrogels was evaluated in whole human blood using the release of cytokines as indicator for potential inflammatory response (Fig. 5B). The addition of 1 $\mu g \cdot mL^{-1}$ LPS induced a strong release of IL-1β, IL-6, and TNF-α, which was reduced when only 0.1 $\mu g \cdot mL^{-1}$ of LPS was used. The LPS amount of 0.1 $\mu g \cdot mL^{-1}$ is the equivalent to 0.5 $EU \cdot mL^{-1}$, the endotoxin limit set by the U.S. Food and Drug Administration for eluates of

medical devices. For all hydrogels, the amounts of IL-1β released were above the amount released after stimulation with 0.1 $\mu g \cdot mL^{-1}$ LPS. While IL-6 secretion was induced by all samples, it was still below the amount induced after the treatment with 0.1 $\mu g \cdot mL^{-1}$ LPS. The TNF-α release revealed a similar trend as the IL-6 release; only G10_LNCO3 induced a slightly higher TNF-α secretion than when 0.1 $\mu g \cdot mL^{-1}$ LPS were added. Interestingly, the LPS induced release of IL-6 and TNF-α was reduced when blood was incubated with the gelatin films.

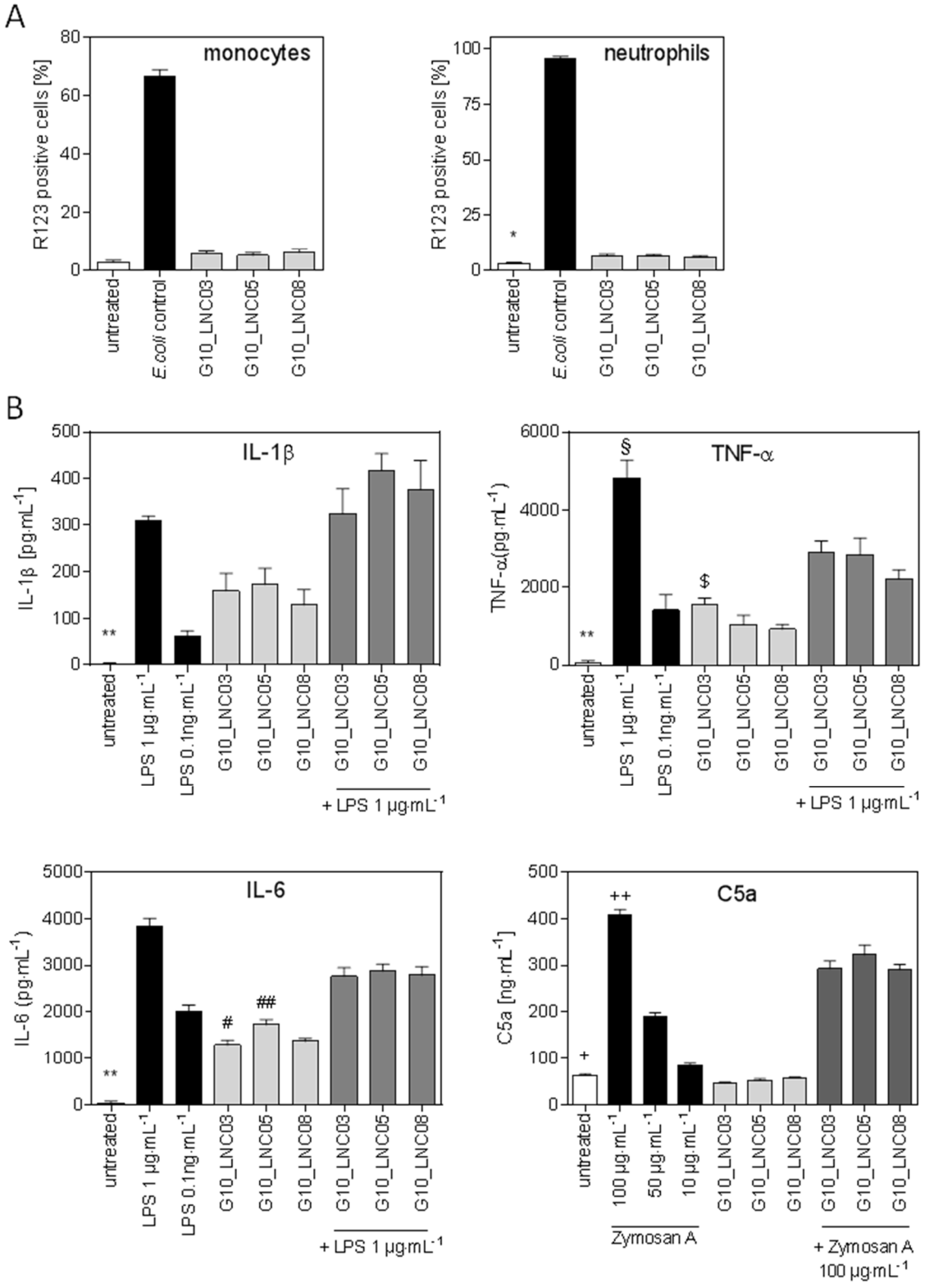


**Figure 5: Immunocompatibility of the gelatin-based hydrogel films**. (A) ROS production in whole human blood monocytes and neutrophils after incubation with the different gelatin-based hydrogels. (B) Cytokine and C5a release. IL-1β, IL-6, and TNF-α release were determined in whole human blood after contact with the gelatin-based hydrogels. C5a was quantified from human serum detected by ELISA after exposure to the different hydrogels. All experiments

were performed three times each with two independent material samples. (*: significant versus G10_LNCO3, G10_LNCO5); **: significant versus G10_LNCO3, G10_LNCO5, G10_LNCO8; #: significant versus G10_LNCO5; ##: significant versus G10_LNCO8; $: significant versus G10_LNCO8; §: LPS 1µg·mL$^{-1}$ significant versus G10_LNCO3, G10_LNCO5, G10_LNCO8 activated with LPS 1µg·mL$^{-1}$; +: significant versus G10_LNCO3; ++: Zymosan A 100 µg·ml$^{-1}$ significant versus G10_LNCO3, G10_LNCO5, G10_LNCO8 + Zymosan 100 µg·ml$^{-1}$)

In order to analyze the capacity of the hydrogels to activate the complement cascade, the release of C5a was analyzed in human plasma. The hydrogel films did not induce C5a, as the detected amounts were similar to untreated plasma (Fig. 4B). However, a slight reduction of C5a was observed when G10_LNCO3 and G10_LNCO5 were incubated with human serum. Additionally, the influence of the different hydrogels on activated complement was investigated by co-incubation of Zymosan and the different hydrogels with human serum. Compared to the Zymosan-treated serum, the C5a levels were slightly but significantly reduced for all hydrogel compositions.

**Thrombogenicity analysis**

The thrombogenicity of the hydrogels was evaluated versus a negative control (medical-grade PDMS) and two positive controls (collagen- or fibronectin-coated glass). The collagen-coating was used, because collagen leads to a strong activation of the adherent thrombocytes, while fibronectin induces a severe increase of the number of adherent thrombocytes (Figure 6). For the evaluation of the thrombogenicity of the hydrogels, the number per area and the degree of thrombocyte activation (as area of single adherent thrombocytes) were investigated (Table 1). Experiments were performed in duplicate. Six donors were included.

The number of adherent thrombocytes differed only slightly but not significantly for the three hydrogels and was comparable to PDMS. It was significantly reduced on G10_LNCO3, G10_LNCO5 and G10_LNCO8 compared to glass coated with fibronectin and also compared to collagen.

Table 1: Density of adherent thrombocytes [1/µm$^2$] and mean surface covered substrate area per single thrombocyte [µm$^2$] on PDMS, collagen-coated and fibronectin-coated glass, G10_LNCO3, G10_LNCO5 and G10_LNCO8. Arithmetic mean ± standard deviation of 6 donors is shown. (*: Significant versus: G10_LNCO3, G10_LNCO5, G10_LNCO8, glass coated with fibronectin, glass coated with collagen; #: Significant versus: glass coated with fibronectin, glass coated with collagen; $: *: Significant versus: PDMS, G10_LNCO3, G10_LNCO5, G10_LNCO8, glass coated with collagen; §: Significant versus: PDMS, G10_LNCO3, G10_LNCO5, G10_LNCO8, glass coated with fibronectin)

| Substrate | Thrombocyte density<br>Thrombocytes·µm$^{-2}$ | Area of single thrombocytes<br>µm$^2$ | p |
|---|---|---|---|
| PDMS | 469±198 | 17.7±7.7 | * |
| G10_LNCO3 | 721±649 | 20.3±7.4 | # |
| G10_LNCO5 | 586±262 | 22.8±8.6 | # |
| G10_LNCO8 | 1,096±467 | 27.1±13.3 | # |
| glass coated with fibronectin | 7,508±2,501 | 17.9±7.4 | $ |
| glass coated with collagen | 3,819±2,756 | 37.6±13.9 | § |

The analysis of the activation state of adherent thrombocytes revealed similar results (Fig. 6). The degree of thrombocyte activation was similar for the three hydrogels and comparable to PDMS as negative control. On G10_LNCO3, G10_LNCO5, and G10_LNCO8 a significantly reduced degree of activation of thrombocytes was observed compared to glass coated with collagen.

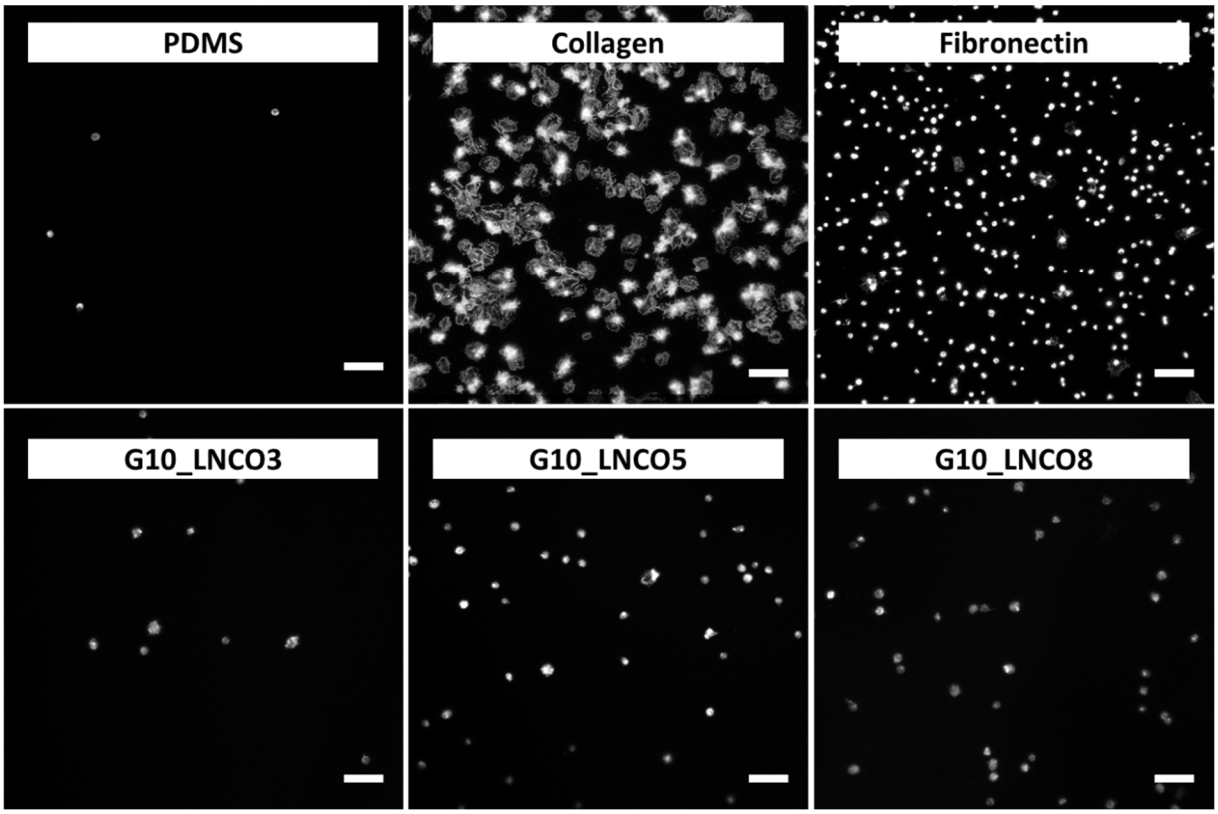

Figure 6: Representative confocal laser scanning microscopy images of adherent thrombocytes on PDMS, hydrogels, and glass coated with collagen or fibronectin (63 fold primary magnifications). Scale bar represents 20 µm.

## Systemic compatibility of gelatin-based hydrogels

### Influence of gelatin-based hydrogels on vascular growth and blood vessel diameter

To investigate whether the hydrogels have an influence on the formation of newly growing blood vessels, a HET-CAM test was conducted. The number of blood vessels was significantly reduced for G10_LNCO3 and G10_LNCO5 compared to the control (Thermanox™ ring without hydrogel, Fig. 7A). In contrast, for G10_LNCO8 the number of blood vessels was comparable to the control and significantly higher compared to G10_LNCO3 and G10_LNCO5 ($p<0.001$ each).

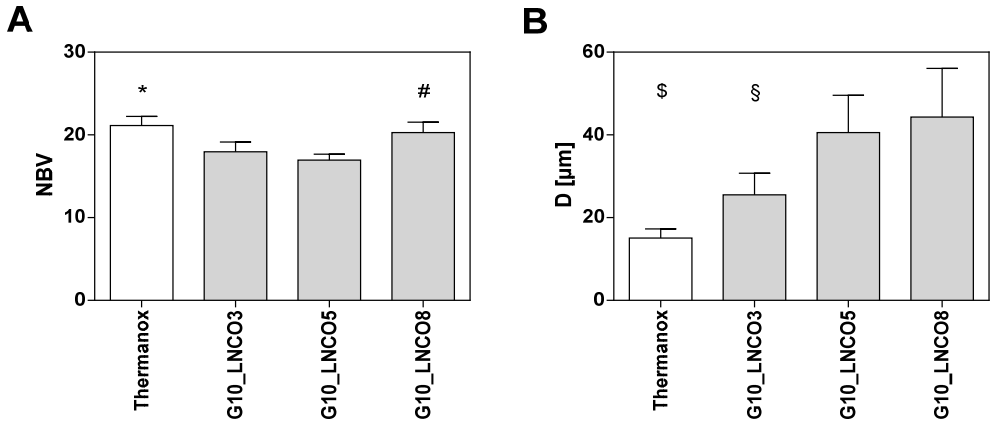


**Figure 7:** A) Numbers of blood vessels in the CAM (NBV) after 48 h incubation with only a Thermanox™ ring (control), G10_LNCO3, G10_LNCO5 and G10_LNCO8. Number of blood vessels was determined inside the Thermanox™ ring, which was used to fix the hydrogels on the CAM. Results are presented as mean ± SD of n=10 eggs. (B) Blood vessel diameters (B) after an incubation period of 48 h with a Thermanox™ ring, G10_LNCO3, G10_LNCO5 and G10_LNO8 on the CAM. Histological sections of the CAM of n=10 eggs were analyzed at 5 locations after HE-staining. (*: significant versus G10_LNCO3 and G10LNCO5; #: significant versus G10_LNCO3 and G10_LNCO5, $: significant versus G10_LNCO3, G10_LNCO5, G10_LNCO8, §: significant versus G10_LNCO8)

The exposition of the CAM to the hydrogels did not result in blood vessel lysis, haemorrhage, or thrombotic processes. However, a significant increase in vessel diameters in the CAM in comparison to the control was observed. This increase correlated with increasing amount of LDI used in the synthesis of the hydrogels (Figure 7B).

**Mouse study**

Implants bear the risk to systemically interfere with the organism leading to tissue damage, inflammation, granulomatous lesions, or necrosis.[33] Therefore, the effect of the subcutaneous implantation of the hydrogels in the lower dorsal area of ten mice (three slices per organ) was examined in regional lymph nodes, spleen, liver, and kidney 35 days after implantation. The organs revealed a physiological appearance. However, in draining lymph nodes a follicular hyperplasia – as a sign of a local inflammation - had developed. This response was only slightly increased after the implantation of G10_LNCO3 (Fig. 8).

No signs of organ necrosis were observed in any of the investigated organs after implantation. In the same group one mouse showed a small focal necrosis in a liver section.

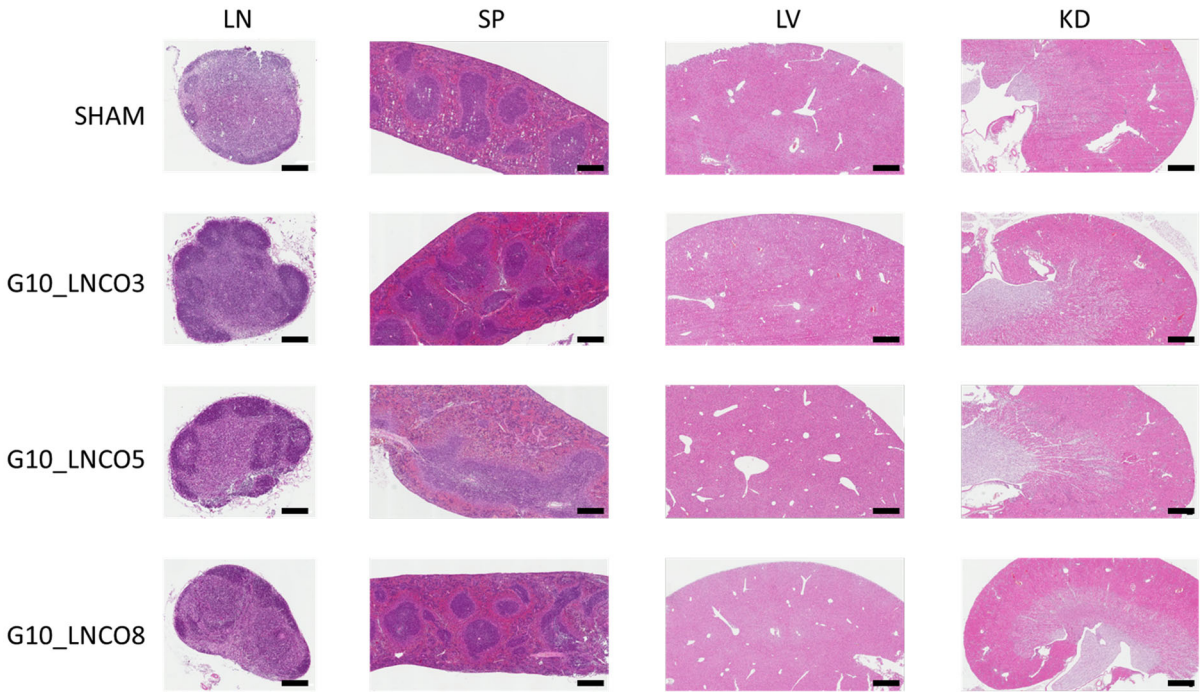


**Figure 8:** HE staining of representative sections of lymph node (LN), spleen (SP), liver (LV) and kidney (KD) of mice 35 days after implantation. The bar corresponds to 200 µm.

**Discussion**

Aim of the project was to evaluate whether gelatin-based hydrogels synthesized with variable amounts of LDI are capable to perform with an appropriate host response when used in cardiovascular implants. Former studies revealed that these hydrogels were sterilizable, non-cytotoxic according to ISO 10993, and exhibited an endotoxin load below FDA limits, fulfilling the imperative requests for implant materials. The present results revealed that the three hydrogel compositions (1) did not activate the complement system, (2) exhibited a haemocompatibility comparable to the negative control, (3) did not harm the growing vasculature in the chorioallantoic membrane, and (4) degraded in mice without toxic responses in spleen, liver, or kidney, and only a mild lymphocytic activation in draining lymph nodes was observed.

The hydrolytic degradation profile of G10_LNCO5, in comparison to the G10_LNCO3 and G10_LNCO8 hydrogels studied earlier,[23] showed that in the studied material system a decreasing rate of hydrolytic degradation correlates with an increasing amount of LDI used in the synthesis. This can be rationalized by the fact that the increasing amount of LDI is related to an increasing number of physical and covalent netpoints leading to a slower rate of diffusion in denser networks as well as a higher number of bonds required to be cleaved to yield soluble fragments.[18] As the hydrogels are degraded not only by hydrolysis but also by proteolytic enzymes *in vitro*,[16] a release of proteases by cells in culture or *in vivo* is likely to increase the rate of degradation. Furthermore, mechanical stress, which might occur in vivo, can lead to degradation by chain scission or reduction of crosslinks [34].

It is a major prerequisite for applications in humans that the implant material does not bear intrinsic immunogenic properties. The data presented indicate that the three hydrogels did not activate the complement system. Activation of the complement cascade can occur on surfaces with nucleophilic end groups such as hydroxyl or amino groups, whereas negatively charged groups such as carboxyl and sulfate, sialic acid, and bound heparin appear to be non-activating.[35,36] Although amino acids with hydroxyl and amino end groups such as threonine, tyrosine, serine and (hydroxyl)lysine are present in type A gelatin, their density seemed not to be sufficient to activate the complement system. About 35 lysines and (hydroxyl)lysine, which have free amino groups, are among 1,000 amino acids in type A gelatin. Several of these amino groups have reacted with the LDI to form, in addition to grafted oligoureas, covalent crosslinks, which reduce the number of free amino groups in the final hydrogel.[37] The slightly reduced C5a release could be explained by the use of PBS pre-swollen hydrogel films, which could cause an osmotic exchange of PBS and serum and subsequently the dilution of C5a in the serum. Another reason for the reduced C5a levels could be the adsorption of the C5a on the hydrogel surface or the diffusion of the protein into the network leading to lower C5a concentrations in the analyzed serum.

The hydrogels did not induce ROS production in monocytes and only low amounts of neutrophils were seen to be ROS positive compared to untreated blood and more than 10 times lower than after stimulation with E. coli, indicating that this elevation might have no substantial biological impact. In fact, low concentrations of ROS induce proliferation, migration, and apoptosis of endothelial cells, which could be beneficial for tissue remodeling and regeneration, while elevated ROS levels could induce or support inflammatory reactions and accelerate implant degradation and device failure *in vivo*.[38] A release of pro-inflammatory cytokines induced by materials is undesired since such cytokines could facilitate the foreign body reaction, platelet activation, pro-inflammatory endothelial cell responses, and implant failure.[39,40] However, a controlled release of inflammatory cytokines such as IL-6 is also described to support regenerative processes.[41] The hydrogels induced low levels of IL-1β, IL-6, and TNF-α, similar to the low dose LPS stimulation. Since the LPS concentration was adapted to the endotoxin limit set by the U.S. Food and Drug Administration for eluates of medical devices, it can be hypothesized that the gelatin-based hydrogels might not induce severe immunological adverse effects when applied *in vivo*.

The non- or low-thrombogenicity of implant materials envisioned for a cardiovascular application is of crucial importance in order to avoid thrombus formation.[42,43] Thrombi may lead to the failure of devices or in case of embolization to life-threatening events such as myocardial infarction, stroke or even the death of patients .[44] The study revealed all three hydrogels as low thrombogenic. The density as well as the activation status of adherent thrombocytes was comparable to a medical grade PDMS used as negative control in haemocompatibility studies (Table 1).[45-47] These results comply with recent publications showing an improved haemocompatibility by modifying polymers using gelatin.[48,49] Recently, it was reported that only very low amounts of fibrinogen adsorbed to thin films synthesized from gelatin and LDI.[50] As fibrinogen is a protein, which mediates the adherence of thrombocytes to surfaces particularly at low shear rates,[51] the low adsorption rate of

fibrinogen to the hydrogels might be one reason for the good haemocompatibility of the investigated hydrogels.

*In vitro* studies can only partially predict the potential of implant materials, as metabolic processes occurring *in vivo* cannot be fully mimicked.[52] The interaction between a biomaterial and the surrounding cells/tissue is of eminent importance for its successful application. The oxygen and nutrient transport to the surrounding tissue must be maintained and at the same time degradation and metabolic waste products must be removed to avoid microcirculatory disturbances.[53] Pion et al. clearly showed that the perfusion in the CAM vessels increases during the vessel growth [54]. A technique of directly visualizing material-induced changes in the formation of the vasculature, like the induction of bleedings, thrombotic processes or vessel wall destruction *in vivo* or angiogenesis/vasculogenesis, is the investigation of the biomaterial positioned directly on the chorioallantoic membrane (CAM) of a developing chicken embryo.[55] The prerequisite is of course the transparency of the hydrogel, as in the present case. Therefore, the HET-CAM test was performed to elucidate the angiogenic potential of the hydrogels *in vivo*. In addition, the impact on CAM vessel diameters was analyzed histologically, revealing that the number of blood vessels below the hydrogels differed between the samples. The number as well as the vasodilation of blood vessels was significantly higher for G10_LNCO8 than for the hydrogels synthesized with lower amounts of LDI, G10_LNCO3 and G10_LNCO5. In line with the increased release of VEGF from HUVEC,[18] a cytokine which was shown to increase the blood vessel diameter[56] and to affect angiogenesis ,[57] the highest pronounced vasodilation was found in CAM vessels exposed to G10_LNCO8. This agrees well with the observation from Ullm et al. who reported that only in capsules around G10_LNCO8 blood vessels were found - not in capsules around G10_LNCO3.[23]

A recent study also showed an increased diameter of CAM vessels after contact with gelatin functionalized with desaminotyrosine.[31] A direct effect of the degrading gelatin can be ruled out as plain gelatin did not influence the vessel diameters.[31] There are two possible mechanisms, which can contribute to the vasodilation. Firstly, a slight dilation of the vessels can be induced by degradation products of the hydrogels such as lysine or urea-linked lysine oligomers. In cats[58] as well as in humans,[59] the potential of lysine to dilate blood vessels was demonstrated. The vasodilatory effect was mediated by responses to ATP-sensitive potassium (KATP) channels.[58,59] Secondly, recent *in vitro* studies revealed that after contact of HUVEC to G10_LNCO3, $TXA_2$ was released[18] (TXA2 is mainly released from activated platelets but also from endothelial cells ),[60] which induces a constriction of blood vessels. This effect could possibly explain why the vasodilation was lowest for the hydrogel, which degrades faster than the others (G10_LNCO3). Vasodilation around an implant improves the blood supply of the surrounding tissue with oxygen and nutrients in addition to newly formed blood vessels and might support the healing process after implantation.

According to the European regulation 1272/2008 (CLP), gelatin is classified as not relevantly harmful for humans and it is known that gelatin is a non-immunogenic material, provided it is endotoxin-free.[61] Low immunogenicity was discussed to be associated with the absence of aromatic rings.[62] In addition, there are scarce reports about late adverse reactions like granuloma formation with collagen as filler material.[63] Beyond, there is a general discussion

that all kinds of chemical stabilization techniques leave potentially toxic residues in collagen-based biomaterials.[64] Therefore, a histopathological examination of inguinal draining lymph nodes, liver, spleen, and kidneys was performed. In a former study, it was shown that after the implantation of gelatin-based hydrogels in mice no local signs of toxic responses around the implanted materials occurred up to 35 days.[23] This study revealed that 35 days after implantation no adverse inflammatory reactions in the spleen or necrotic processes in organs mediating detoxification processes, such as liver and kidney, had developed. However, in draining lymph nodes a mild lymphocytic activation in form of a follicular hyperplasia was observed with a slightly increased response after the implantation of G10_LNCO3. At day 35 after implantation, the degradation of the hydrogels is still ongoing[23,32] and as G10_LNCO3 is degraded faster than the other studied compositions, much more degradation products have to be disposed and eliminated. These results confirm the principle biocompatibility of the gelatin-based hydrogels, stabilized by reaction with LDI.

In conclusion the study revealed a low immunogenic and thrombogenic response after contact of the hydrogels with human blood. The subsequent HET-CAM test confirmed these *in vitro* results and showed no harmful effects. Instead, a vasodilation of the vasculature surrounding the hydrogel occurred, which could facilitate the supply of the surrounding tissue with phagocytic cells, oxygen and nutrients as well as disposing degradation and metabolic waste products with an advantage for the hydrogels synthesized with higher amounts of LDI. The consistent results of the *in vitro* studies, the low inflammatory response (*in vitro* as well as *in vivo*) and the absence of toxic effects in spleen, liver, or kidney with a low follicular infiltration of the draining lymph nodes demonstrated that the hydrogels prepared with the higher amounts of LDI appeared to be candidate coating material for cardiovascular implants. These results might supplement our understanding of the excellent compatibility without any signs of inflammation of the hydrogel into the tissue, which could be demonstrated in former animal studies.[23,32]

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