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*Title*

**Biocompatibility and Inflammatory Response *in vitro* and *in vivo* to Gelatin-based Biomaterials with Tailorable Elastic Properties**

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## Abstract

Hydrogels prepared from gelatin and lysine diisocyanate ethyl ester provide tailorable elastic properties and degradation behavior. Their interaction with human aortic endothelial cells (HAEC) as well as human macrophages (M $\phi$ ) and granulocytes (G $\phi$ ) were explored. The experiments revealed a good biocompatibility, appropriate cell adhesion, and cell infiltration. Direct contact to hydrogels, but not contact to hydrolytic or enzymatic hydrogel degradation products, resulted in enhanced cyclooxygenase-2 (COX-2) expression in all cell types, indicating a weak inflammatory activation *in vitro*. Only M $\phi$  altered their cytokine secretion profile after direct hydrogel contact, indicating a comparably pronounced inflammatory activation. On the other hand, in HAEC the expression of tight junction proteins, as well as cytokine and matrix metalloproteinase secretion were not influenced by the hydrogels, suggesting a maintained endothelial cell function. This was in line with the finding that in HAEC increased thrombomodulin synthesis but no thrombomodulin membrane shedding occurred. First *in vivo* data obtained after subcutaneous implantation of the materials in immunocompetent mice revealed good integration of implants in the surrounding tissue, no progredient fibrous capsule formation, and no inflammatory tissue reaction *in vivo*. Overall, the study demonstrates the potential of gelatin-based hydrogels for temporal replacement and functional regeneration of damaged soft tissue.

## Keywords

*Cyclooxygenases* ● *Cytokines* ● *Endothelial cells* ● *Hydrogels* ● *Macrophages* ● *Matrix metalloproteinases* ● *Receptor for Advanced Glycation Endproducts* ● *Thrombomodulin*

## Introduction

Biopolymer-based materials resembling components of the extracellular matrix (ECM), like collagen or gelatin, are promising candidates for soft tissue regeneration since they provide recognition motifs for cell adhesion and differentiation and are degradable under physiological conditions [1]. Current clinical applications of collagen or chemically modified collagen rest on its advantages of being tissue compatible and available from natural resources [2], but also display drawbacks as immunogenic responses [3], difficulty to tailor mechanical properties [4], and limitations in retaining dimensional stability. In contrast, gelatin, a product of thermal and chemical partial degradation of collagen, displays better solubility in water, a low level of immunogenicity [5], and is supposed to be angiogenic [6]. Formation of gelatin-based networks is one strategy to tailor, e.g., the elasticity and swellability of gelatin-based materials also during the degradation phase. For this purpose, covalent crosslinking can be employed, e.g., by photopolymerization of glycidyl methacrylate-functionalized gelatin. Functionalization of gelatin with the amino acid derived groups desaminotyrosine or desaminotyrosyl tyrosine [7] has been employed to enable a directed physical crosslinking. Entropy-elastic hydrogels synthesized by reacting gelatin with lysine diisocyanate in water were shown to have tailorable elasticities closely to tissues and suitable biodegradation behavior [8]. These favorable functions derive from a combination of direct covalent crosslinks via di-urea junction units with physical crosslinks formed by oligo-urea side chains. The hydrogels have been positively evaluated as non-toxic for L929-fibroblasts and supportive for mesenchymal stem cell culture [9]. A major aspect of the applicability *in vivo* of biopolymer-based materials is the acute and chronic inflammatory host response following their implantation, initiating the regeneration of the injured tissue and/or the formation of fibrous capsules (foreign body reaction, FBR) [10]. These processes can partially be simulated *in vitro*. Therefore, essential cell types of inflammation (granulocytes and macrophages) and angiogenesis (endothelial cells), also acting as key players in regenerative as well as foreign body reaction processes, were cultivated with gelatin-based materials of different crosslinking degree and subsequently investigated regarding cell adhesion, viability, and infiltration. Additionally, cells were cultivated with material eluates obtained after incubation of the hydrogels in buffer or trypsin solution to study the influence of degradation products. In order to characterize cell functionality in more detail the following parameters were analyzed: *i*) expression of the inducible pro-inflammatory enzyme cyclooxygenase-2 (COX-2) and its constitutively expressed isoform COX-1, *ii*) expression of the

inflammation mediating receptor for advanced glycation end products (RAGE), *iii*) secretion of cytokines and potentially hydrogel degrading matrix metalloproteinases (MMPs), *iv*) endothelial cell monolayer integrity characterized by the expression of tight junction proteins occludin and claudin-5, and *v*) endothelial expression of anticoagulant thrombomodulin. This *in vitro* approach will reveal the possible influence of material composition and properties like elasticity, as well as degradation behavior and products on the biocompatibility and inflammatory response of cells involved in inflammation, tissue regeneration and FBR. Moreover, a pilot animal experiment employing subcutaneous implantation of the materials in immunocompetent hairless mice was performed to compare the *in vitro* results with an *in vivo* situation and gain further insights into the reactions of the organism regarding an acute or chronic inflammation and material integration, respectively.

## Materials and Methods

### Hydrogel Synthesis

Dried, sterile films of gelatin-based networks G10\_LNCO3 and G10\_LNCO8 were synthesized as described before [8, 9]. Briefly, an aqueous 10 wt.-% gelatin solution (from porcine skin, 200 bloom, type A, low endotoxin content, GELITA USA, Sergeant Bluff, IA, USA) was reacted with ethyl lysine diisocyanate (LDI; 3- and 8-fold NCO/NH<sub>2</sub> (gelatin) molar ratio - G10\_LNCO3 and G10\_LNCO8; freshly distilled) (CHEMOS, Regenstauf, Germany) in the presence of 1 wt.-% poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (Pluronic® F-108, average M<sub>n</sub> ~ 14,600 g × mol<sup>-1</sup>, Sigma-Aldrich, Steinheim, Germany). 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). Soluble LPS contamination was determined by the LAL test (Lonza, Cologne, Germany) according to manufacturer's instructions after incubation of material discs (3 cm<sup>2</sup>) in 1 ml of MEM cell culture medium for 72 h. All materials contained < 0.2 EU × ml<sup>-1</sup> soluble LPS (FDA limit = 0.5 EU × ml<sup>-1</sup>). Materials were used for further experiments as material eluate or as swollen hydrogel. Hydrolytic material eluate is the supernatant after 48 or 72 h incubation time of a biomaterial film (diameter 10 cm) in 35 ml of suitable serum-free medium containing 1 U/ml penicillin/streptomycin (P/S, Biochrom, Berlin, Germany) at 37 °C, whereas enzymatic material eluate was prepared by addition of trypsin as a model protease in a concentration of 5 × 10<sup>-6</sup> wt.-% trypsin (250 USP U/mg) and 2 × 10<sup>-6</sup> wt.-% EDTA (Biochrom) to the elution medium. Material eluates were subsequently supplemented with 2 vol.-% fetal calf serum (FCS, Biochrom). For substrate contact experiments a material film was swollen in 20 ml of phosphate buffered saline (PBS, pH 7.4, 274 mM NaCl, 5.4 mM KCl, 3 mM KH<sub>2</sub>PO<sub>4</sub>, 14.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub> × 6 H<sub>2</sub>O, 1 mM CaCl<sub>2</sub> × 2 H<sub>2</sub>O, chemicals from Sigma-Aldrich) overnight at 37 °C with following removal of the supernatant and punching of 6-well or 24-well sized hydrogel pieces.

### Hydrogel Water Uptake (*H*), Degree of Swelling (*Q*,) and Tensile Tests

Water uptake and swelling of the materials were determined at room temperature. Samples were immersed in PBS (140 mM NaCl, 12 mM Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) at 37 °C for 24 h to reach swelling equilibrium. Water uptake and swelling were then calculated from mass difference in dry and swollen state (see Supporting Information). Mechanical tests were performed at 37 °C on a tensile

tester (Zwick Z2.5, Zwick, Ulm, Germany) equipped with a water tank with temperature control. Film samples of standard dimensions (ISO 527-2/1BB), equilibrated in water at 37 °C, were tested with 0.02 N pre-force and 5 mm × min<sup>-1</sup> crosshead speed, until break. E-moduli of the different gelatin films were calculated from the linear region, generally between 0.5-5% elongation ( $\epsilon$ ) in the stress–strain plot.

### **Hydrogel *in vitro* Degradation Studies**

In order to investigate the degradation behavior of the hydrogels, crosslinked gelatin film samples (6 mm diameter discs) were incubated at 37 °C in PBS for 21 days. Buffer was exchanged every third day. After 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.

$$\mu_{rel} = \frac{m_d}{m_0} \cdot 100\% \quad (\text{Eq. 1})$$

### **Cells**

Primary Human Aortic Endothelial Cells (termed as HAEC) were purchased from PelloBiotech (Planegg, Germany) and cultured in Endothelial Cell Growth Medium enhanced containing 0.5 ng/ml soluble vascular endothelial growth factor (VEGF) (PelloBiotech). THP-1, a human acute monocytic leukemia cell line, and HL-60 cells, a human acute myeloid leukemia cell line, were obtained from the European Collection of Cell Cultures (ECACC, Porton Down, UK), and maintained in RPMI 1640 medium (Biochrom) supplemented with 10 vol-% FCS and 1 U/ml P/S. HL-60 and THP-1 cells were differentiated with 64 nM 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich) for 72 hours into macrophage-like cells (HL-60 M $\phi$  and THP-1 M $\phi$ ) [11]. HL-60 cells were differentiated with 1.3 vol-% dimethyl sulfoxide (DMSO; Fisher BioReagents, Schwerte, Germany) for 6 days into granulocyte-like cells (HL-60 G $\phi$ ) [12]. These established human leukemia derived cell models are easily available and provide a homogeneous genetic background [13, 14]. In order to determine the effect of hydrolytic material eluates on HL-60 and THP-1 cells,  $1 \times 10^5$  cells/well were seeded in a 24-well plate in 2 ml hydrolytic eluate (elution for 72 h) of G10\_LNCO3, G10\_LNCO8, medium or medium supplemented with 64 nM TPA as positive control for differentiation. Cells were imaged with microscope Axioimager A1 (Carl Zeiss, Jena, Germany) over a period of 7 days.

### **Mitochondrial Activity**

The mitochondrial activity of HAEC, HL-60 M $\phi$ , THP-1 M $\phi$  and HL-60 G $\phi$  after 24 h or 48 h cultivation with hydrolytic material eluates or cell culture medium was determined via MTT assay (see Supporting Information).

### **Cell Adhesion Experiments**

Initial adhesion of calcein-stained HAEC, HL-60 M $\phi$ , THP-1 M $\phi$  and HL-60 G $\phi$  to the hydrogels was evaluated in substrate contact experiments (see Supporting Information). As controls, uncoated tissue culture plastic (TCP) and gelatin-coated TCP were used.

### **Cell Infiltration**

In order to determine whether cells were able to infiltrate the hydrogels,  $5 \times 10^5$  cells were seeded on 24-well sized hydrogel pieces of G10\_LNCO3 and G10\_LNCO8. After 2 and 7 days cells were fluorescence stained via a 30 min incubation time in a  $4 \mu\text{M}$  calcein solution. Hydrogel pieces were placed reverse on microscope slides. Slides were studied using confocal laser scanning microscopy (FV10\_ASW, Olympus, Hamburg, Germany) via Z-stack images and section shots.

### **Protein Expression**

In order to investigate protein expression as specific response after substrate contact, cells were cultivated directly on hydrogels G10\_LNCO3 and G10\_LNCO8 in 6-well plates ( $1 \times 10^6$  cells/well in 5 ml medium) for 48 h. As positive control for COX-2 induction in endothelial cells,  $0.1 \mu\text{M}$  TPA was added to cultivation medium [15]. In order to determine the effect of hydrolytic and enzymatic material degradation products, cells were seeded in 6-well plates like for mitochondrial activity studies ( $5 \times 10^5$  cells/well HAEC,  $3 \times 10^6$  cells/well for HL-60 M $\phi$  and THP-1 M $\phi$ , and  $1 \times 10^7$  cells/well for HL-60 G $\phi$ ) and incubated with eluates (5 ml/well) for 48 h. Cells were detached using a 2 mM EDTA in PBS solution for 30 min, centrifuged at  $300 \times g$  for 5 min and cell pellet was collected in 100  $\mu\text{l}$  RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP40, 0.5 wt.-% SDS, 7  $\mu\text{g/ml}$  leupeptin, 1 mM PMSF, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM DTT, 7 mM NaF). Cell lysis, SDS-PAGE and Western blotting were performed as described previously [16]. Blots were incubated with primary and secondary antibodies as shown in Table S1. Western Blots were evaluated using densitometric analysis (see Supporting Information)

### **Secretion of Cytokines and MMPs**

The secretion of cytokines (Tables 1 and 2) and MMPs (Tables S3 and S4) in the supernatant of HAEC and THP-1 M $\phi$  was quantified via Bioplex Multiplex System (Bio-Plex200®, Bio-Rad, München, Germany) using Bio-Plex Pro Human Cytokine 27-plex Assay from Bio-Rad and MMPs Luminex Performance Assay MMP Base Kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions [17].

### **In vivo Experiments**

Animal experiments were performed in accordance to the guidelines of the German Regulations for Animal Welfare. The protocol was approved by the local Ethical Committee for Animal Experiments (reference number 24-9168.11-4/2013-1). In this pilot animal experiment the hydrogels were subcutaneously implanted in immunocompetent hairless SKH1 mice and followed up to 35 days. Female mice (SKH1-Elite; age 8-15 weeks, weight 22-30 g; Charles River, Sulzfeld, Germany) were housed in controlled-airflow cabinets with 12 h light cycle, at a temperature of  $27 \pm 1$  °C and humidity of  $55 \pm 5\%$ . Animals were anesthetized under an air flow of 0.8-1 liter/min with 40% oxygen and 10-12% desfluran. Dried, sterile material pieces (3 x 10 mm) were swollen in PBS for 1 h before they were implanted subcutaneously in the lower dorsal area. Animals received the analgesic Rimadyl (0.05 mg/10 g) and wound closure was achieved by spray-on plaster. At selected time points, hydrogels with surrounding tissues were explanted, fixed in a 4 wt.-% paraformaldehyde solution for 24 h and in a 30 wt.-% sucrose solution for 4 days at room temperature. Subsequently, the tissue was stored at -65 °C. For cutting, samples were embedded in 7.5% gelatin solution with 20% sucrose and frozen sections (10  $\mu$ m) were prepared with a cryostat (Leica CM1850, Leica Biosystems, Nussloch, Germany) at -30 °C. Slides were subjected to Masson Goldner stain for detection of hydrogel degradation and capsule formation, and were analyzed using Axio Imager Z2m microscope (Carl Zeiss) and the appropriate AxioVision software package (Carl Zeiss). For immunohistochemical staining, sections were pretreated in 10 mM citrate buffer (pH 6.0) and heated for 20 min by microwave. After quenching of endogenous peroxidase for 10 min in a 3 vol.-% hydrogen peroxide solution, unspecific binding sites were blocked with a solution of 10 vol.-% FCS in Tris-buffered saline (pH 7.4) containing 0.05 vol.-% Tween 20 (TBS-T). Tissue sections were incubated with primary antibody or isotype control for 2 h at room temperature and overnight at 4 °C, and with secondary antibody for 1 h at room temperature (see Table S2). The secondary antibody was visualized by incubation for 30 min with ExtrAvidin peroxidase (Sigma Aldrich, dilution 1:50 in TBS-T), and for

10 min with AEC substrate kit (BD Biosciences, Heidelberg, Germany). Finally, sections were counterstained with Mayer's hematoxylin and embedded in aqueous solution. Slides were analyzed with AxioImager.A1 microscope and the appropriate AxioVision software package (Carl Zeiss).

## Results

### Hydrogel Synthesis and Characterization

Hydrogel films were prepared from 10 wt.-% gelatin solutions by reacting with 3- (G10\_LNCO3) or 8-fold (G10\_LNCO8) excess of isocyanate groups compared to the amount of amino groups of gelatin, resulting in entropy elastic hydrogels with tailored degradation time, Young's moduli ( $E = 13 \pm 3$  and  $55 \pm 11$  kPa), elongation at break, and tensile strength ( $7 \pm 3$  kPa at  $43 \pm 25\%$  and  $19 \pm 11$  kPa at  $30 \pm 11\%$ ) [18], water uptake ( $1350 \pm 150$  and  $390 \pm 25$  wt.-%), and swelling ( $1200 \pm 190$  and  $360 \pm 35$  vol.-%). As mechanical data and swelling were determined at  $37^\circ\text{C}$ , i.e. above the helix dissociation temperature, an influence of potential triple helices acting as physical netpoints can be neglected. G10\_LNCO8 samples showed around 5 to 15% mass loss over a period of 21 days, while G10\_LNCO3 materials showed already around 40% mass loss after 3 days and up to 60% mass loss after 21 days (Figure 1). Material eluates for investigating the influence of degradation products were prepared by incubation of hydrogels in cell culture medium (hydrolytic material eluates) or cell culture medium and trypsin (enzymatic material eluates).

### Cell Differentiation and Mitochondrial Activity

THP-1 and HL-60 cells incubated with 64 nM TPA adhered to the TCP, spread and formed elongated cell protuberances after 48 h (Figure S1). In contrast, cells cultivated with cell culture medium and hydrolytic material eluates stayed round, small and in suspension. The mitochondrial activity of HAEC cultivated with hydrolytic material eluates decreased significantly after 48 h compared to cultivation with cell culture medium. In contrast, the mitochondrial activity of HL-60 M $\phi$  and THP-1 M $\phi$  increased markedly in presence of hydrolytic material eluates. HL-60 G $\phi$  revealed contradictory reactions to hydrolytic material eluates: briefly lowered mitochondrial activity after incubation with the eluate of G10\_LNCO3 and elevated mitochondrial activity in response to the eluate of G10\_LNCO8 (Figure 2).

### Cell Adhesion and Infiltration

Relatively high cell numbers were used to enable the detection of an initial cell adhesion after a short period of time. HAEC adhered better to the hydrogels than to TCP, whereas adhesion to gelatin-coated TCP was even higher than adhesion to the hydrogels (Figure 3). Adhesion of HL-60 M $\phi$ , THP-1 M $\phi$  and HL-60 G $\phi$  to the hydrogels was significantly lowered compared to uncoated and gelatin-coated TCP. All cells showed higher adhesion to the higher crosslinked G10\_LNCO8 than to G10\_LNCO3.

HAEC, HL-60 M $\phi$ , THP-1 M $\phi$  and HL-60 G $\phi$  were able to infiltrate the hydrogels within 7 days of cultivation (Figure 4). Directly after seeding, cells accumulated in small pores and cavities on the macroscopically smooth-appearing hydrogel films, where they proliferated (HAEC) and finally infiltrated the hydrogels. First hydrogel infiltrating cells were detected after 2 days, whereas no differences between the two hydrogels and the cell types could be detected. After 7 days, all cells infiltrated G10\_LNCO3 to a higher extent than G10\_LNCO8.

### **Expression of Inflammatory Proteins**

Expression of COX-2, COX-1, and RAGE was determined via Western blotting (Figure S2) and densitometric analysis (Figure 5). In HAEC, HL-60 M $\phi$  and THP-1 M $\phi$  contact to hydrogels G10\_LNCO3 and G10\_LNCO8 induced an enhanced expression of COX-2. HAEC showed increased reaction to G10\_LNCO8, even more than to induction with TPA as positive control, whereas in all other cell types expression of COX-2 did not differ between G10\_LNCO3 and G10\_LNCO8. In HAEC, cultivation with hydrolytic as well as enzymatic material eluates also enhanced COX-2 expression, although to a lesser extent than substrate contact. In HL-60 M $\phi$ , only eluates of G10\_LNCO8 led to increased COX-2 expression, whereas the other cells did not react to material eluates. In some cases, the expression of COX-1 was slightly enhanced, but no recurrent pattern could be observed between cell and material types. The expression of RAGE also varied, but seemed to be independent from the material type, mostly enhanced in HAEC, and mostly diminished in HL-60 M $\phi$  and THP-1 M $\phi$ . In HL-60 G $\phi$ , RAGE expression was slightly increased after substrate contact, but with a high variability.

### **Endothelial Cell Function**

The expression of the tight junction proteins occludin and claudin-5 was not influenced by contact to eluates or hydrogels of G10\_LNCO3 and G10\_LNCO8, in contrast to TPA-treated positive control, where claudin-5 was downregulated (Figure S3). The expression of membrane thrombomodulin remained constant after contact to material eluates, whereas contact of the cells to hydrogels induced an enhanced expression (Figure 6). However, soluble thrombomodulin could not be detected in cell culture supernatants. After incubation of HAEC with TPA, soluble thrombomodulin was detected in cell culture supernatants.

### **Secretion of Cytokines and MMPs**

Several pro- and anti-inflammatory cytokines, chemokines and functional markers were measured in supernatants of HAEC and THP-1 M $\phi$  after contact to the hydrogels (Tables 1 and 2). In case of

HAEC, neither secretion of pro- (IL-6, IL-8, IFN- $\gamma$ , RANTES, TNF- $\alpha$ ) nor anti-inflammatory mediators (IL-1Ra, IL-4), nor secretion of chemokines (G-CSF, MCP-1) and functional markers bFGF and PDGF-BB were influenced by materials, whereas incubation with TPA as positive control enhanced secretion notably or even significantly. Pro-inflammatory IL-1 $\beta$  and IL-2, as well as anti-inflammatory IL-10 and functional marker VEGF were below the detection limits in supernatants of HAEC. More complex effects were observed for THP-1 M $\phi$ : Secretion of pro-inflammatory mediators was mainly enhanced by contact to G10\_LNCO3 (IL-6, IFN- $\gamma$ , RANTES, TNF- $\alpha$ ), except for IL-1 $\beta$  (unchanged), as well as IL-8 and IL-12(p70) (diminished), whereas contact to G10\_LNCO8 did not alter secretion of pro-inflammatory mediators in most cases (IL-1 $\beta$ , IL-6, IL-8, IFN- $\gamma$ , TNF- $\alpha$ ), except for RANTES (enhanced) and IL-12(p70) (diminished). The secretion of anti-inflammatory mediators (IL-1Ra and IL-10) and chemokines (MCP-1 and MIP-1 $\alpha$ ) followed the same principle, increased after contact to G10\_LNCO3, but unchanged or decreased after contact to G10\_LNCO8. Secretion of chemokines G-CSF and MIP-1 $\beta$  was not altered after hydrogel contact, but secretion of functional markers PDGF-BB and VEGF was influenced by hydrogel contact (enhanced and diminished, respectively).

Different MMPs, whose secretion is partially known from literature, were measured in supernatants of HAEC and THP-1 M $\phi$  after contact to the hydrogels (Tables S3 and S4). In HAEC, substrate contact did not enhance MMP secretion, whereas cultivation with 0.1  $\mu$ M TPA induced or enhanced the secretion significantly (MMP-3 and MMP-9, as well as MMP-10 and EMMPRIN, respectively). In all samples, the release of MMP-1 exceeded the detection limit of 91 ng/ml. In THP-1 M $\phi$ , substrate contact did not alter secretion of all measured MMPs.

### **In vivo Experiments**

Both hydrogels degraded with time but were still visible after 35 days of implantation. Masson Goldner stain revealed that collagenous connective tissue formed a fibrous capsule around the implants, whereas the time course of the formation of the capsules differed between the hydrogels (Figure 7 and Table S5). For both hydrogels the capsule at the cutaneous side was thicker than at the musculo-fascial side (G10\_LNCO3: Mann-Whitney U-test,  $p < 0.001$ ,  $n = 45$  slices out of 3 animals; G10\_LNCO8: Mann-Whitney U-test,  $p = 0.015$ ,  $n = 45$  slices out of 3 animals). The total capsule thicknesses (musculo-fascial plus cutaneous side, Figure 8) around the hydrogel G10\_LNCO3 increased from day 7 to day 14, followed by a decrease from day 14 to day 35. In contrast, the capsule around G10\_LNCO8 increased continuously from  $163.8 \pm 62.4 \mu\text{m}$  after 7 days to  $345.2 \pm 179.1 \mu\text{m}$

after 35 days (Holm-Sidak test,  $p = 0.017$ ). At day 35 post implantation the capsule around hydrogel G10\_LNCO8 was substantially thicker compared to G10\_LNCO3.

In addition, both hydrogels integrated very well into the surrounding tissue showing no separation between hydrogel and the capsule from day 14 on for G10\_LNCO3, and from day 35 on for G10\_LNCO8 (Figure 7). Using Masson Goldner staining in or around G10\_LNCO3 no foreign body giant cells could be detected. In the capsule of G10\_LNCO3 no blood vessels were found (5 sections from 3 mice at 3 time points with overall 45 images analyzed), while in the capsule of G10\_LNCO8 small microvessels (with diameters clearly less than 100  $\mu\text{m}$ ) were formed. Figure 9 shows three vessels in the capsule around G10\_LNCO8 parallel to the implant at day 35 after implantation. Moreover, some monocytes/macrophages and foreign body giant cells were observed in the transition region between capsule and implant 7 and 14 days after implantation, respectively (Figures S4 and 10). Immunohistochemical analysis showed that COX-2 expression in the surrounding tissue was not altered by the implanted materials. This could be confirmed by expression analysis in tissue lysates via Western blotting (Figure 11 and Figure S5).

## Discussion

The tissue response towards two gelatin-based hydrogels differing in elastic properties and degradation behavior was studied *in vitro* and *in vivo*. For this purpose, an *in vitro* study using human aortic endothelial cells and immune cells which are key players in tissue regeneration, inflammation, and FBR was performed, followed by a pilot *in vivo* experiment in immunocompetent hairless mice. Besides direct contact of hydrogels with cells also the influence of degradation products was investigated. Therefore, degradation of the materials was explored *in vitro* by preparation of hydrolytic and enzymatic hydrogel eluates. This study revealed an influence of material composition and properties on the biocompatibility and inflammatory response *in vitro* and, moreover, provided further insights into material integration and inflammation *in vivo*.

### Hydrogel Synthesis and Properties

Synthesis of sterile hydrogels was easily feasible under cleanroom conditions. With increasing diisocyanate concentration during synthesis the hydrogels get stiffer, show lower water uptake and swelling, and degrade slower *in vitro*. Higher LDI concentrations led to hydrogels which contain more grafted oligo-urea chains as well as oligo-LDI crosslinks. Both are expected to influence swelling behavior and cell contacts [8]. The oligo-urea side chains are probably engaging in hydrogen bonds [19]. These hydrogen bonds are primarily directed inwards the hydrogel thus forming physical crosslinks contributing to lower swellability, or outwards the hydrogel thus exhibiting target sites for protein and cell attachment. The hydrogels showed macroscopically smooth-appearing surfaces, whereas light-microscopy revealed that the material surfaces display some inhomogeneities like micropores and cavities. Incubation of the materials in cell culture medium led to partial and random hydrolysis of peptide bonds and therefore release of fragments. The trypsin treated hydrogels additionally are likely to be selectively cleaved after arginine residues in the chain, as the amino groups of lysines were reacted in the hydrogel formation step, preventing trypsin cleavage at these sites. The enzymatic digestion led to enhanced degradation of the hydrogels, and therefore higher concentrations of fragments in the enzymatic material eluates than in the hydrolytic material eluates.

### Endothelial cell function

In line with previous studies showing no cytotoxic effects of both hydrogels for L929 fibroblasts and human mesenchymal stem cells [9], also HAEC remained viable after contact to eluates containing

degradation products of the materials. The reactions of HAEC to eluates of G10\_LNCO3 and G10\_LNCO8 did not differ, most probably because both contain the same structural elements (gelatin backbone, LDI crosslinks and oligo-urea grafts), and thus should form similar degradation products which only differ in their concentrations. HAEC showed increased adhesion to hydrogels compared to TCP, which was intended when creating hydrogels that mimic the extracellular matrix. Here, higher crosslinking of the hydrogel led to higher cell adhesion. This may be explained by the different surface properties of the two hydrogels like the wettability [20] or surface roughness, which change during degradation processes [21]. Additionally, available data imply that also the higher substrate stiffness of G10\_LNCO8 supports cell adhesion [22]. HAEC infiltrated the hydrogels within a few days, while the less crosslinked G10\_LNCO3 was infiltrated to a higher extent. These findings agree very well with the determined degradation behavior of the hydrogels *in vitro*, showing G10\_LNCO3 to be faster degraded, thus allowing more extensive cell infiltration. Starting from small pores and cavities on hydrogel surfaces HAEC migrated and proliferated in a three-dimensional manner, thereby performing steps that are essential for angiogenesis during tissue regeneration. It is noteworthy that HAEC still adhered on the degrading hydrogel surface suggesting that the inherent adhesion sequences in gelatin are accessible during the whole degradation process.

As a key player in inflammation, COX-2 is a leading enzyme in the COX-pathway converting arachidonic acid in several inflammatory (prostaglandin E<sub>2</sub>, prostacyclin) or pro-resolution metabolites (prostaglandin D<sub>2</sub>). COX-2 is inducible by inflammatory stimuli and regulates the resolution of inflammations [23]. HAEC significantly enhanced their synthesis of COX-2 after contact to the hydrogels (3-fold for G10\_LNCO3 and 7-fold for G10\_LNCO8), whereas contact to hydrolytic and enzymatic material eluates induced only a slight increase in COX-2 expression. This, and the significant difference of COX-2 induction between the hydrogels, indicates an important influence of the hydrogel surface or elasticity on COX-2 expression. An influence of the substrate topography on the inflammatory response of HAEC has recently been reported by McKee et al. [21]. In line with the enhanced adhesion of HAEC to G10\_LNCO8 compared to G10\_LNCO3, it can be hypothesized that surface topography, wettability, and stiffness changing upon degradation may influence integrin-mediated cell adhesion and COX-2 induction [20, 24-26]. In contrast, HAEC only slightly regulated their expression of RAGE after hydrogel contact, suggesting that RAGE is not involved in the inflammatory response of HAEC to the studied gelatin-based hydrogels.

It is well known that during inflammation elevated cytokine secretion by activated endothelial cells can be detected. The contact of HAEC to the hydrogels did not induce changes in their cytokine secretion pattern, indicating that the cells maintained homeostasis and did not react in an inflammatory manner. In contrast, incubation with TPA resulted in a significantly enhanced cytokine secretion even after 48 h [27].

MMPs, a family of zinc-dependent endopeptidases [28], are capable of degrading all kinds of extracellular matrix proteins. They are involved in the breakdown of collagen and, putatively, of the gelatin-based hydrogels, and have a decisive function in the remodelling of the extracellular matrix during cellular ingrowth and angiogenesis. The release of MMPs by HAEC remained unchanged after contact to the hydrogels compared to the control, indicating that cells were not stimulated to degrade the materials, while TPA supplementation led to a significant increase of different MMPs. Nevertheless, HAEC infiltrated the hydrogels, indicating that either MMPs are cell surface associated and not secreted [29] or basal levels of secreted MMPs were sufficient for partial degradation of the gelatin-based hydrogels, or other types of proteases contribute to the degradation. One should have in mind that at least for G10\_LNCO3 hydrolytic degradation contributes to mass loss in the timeline of the cell culture experiment.

Changes in the endothelial barrier function and permeability, maintained by tight junction proteins, are involved in many inflammatory diseases [30]. HAEC did not alter the expression of the tight junction proteins occludin and claudin-5 after contact to materials, indicating a maintained permeability. In contrast, TPA reduced the expression of claudin-5, suggesting a reduced cell-cell binding. The expression of occludin was not influenced, supporting the hypothesis that cells are able to modulate tight junctions independent of occludin expression [31]. However, expression of tight junction proteins is downregulated by pro-inflammatory cytokines like IL-1 $\beta$ , IFN- $\gamma$  and TNF- $\alpha$  [30], thereby reconstituting the link to elevated cytokine secretion by HAEC after treatment with TPA.

The transmembrane glycoprotein thrombomodulin as potent anticoagulant plays an important role in attenuation of the inflammatory response in various clinical settings, e.g., endotoxin- and radiation-induced tissue damage, cytokine production, and atherogenesis [32, 33]. Thrombomodulin is widely expressed in a variety of cell types, e.g., monocytes and macrophages, and is predominantly located on the luminal surface of normal vascular endothelial cells. In the present study the upregulatory action on thrombomodulin synthesis in HAEC indicated anti-thrombotic/anti-inflammatory stimulation of endothelial cells by direct contact to the hydrogels. This is consistent with the observation that no

soluble thrombomodulin could be detected after exposure of HAEC to hydrogels. In this regard, experimental and clinical data demonstrate that inflammatory activation is associated with rapid and sustained loss of endothelial thrombomodulin by ectodomain shedding catalyzed by metalloproteinases and serine proteases [34], which we could simulate by incubation of HAEC with TPA.

### **Reaction of macrophages and granulocytes**

Hydrolytic eluates of materials G10\_LNCO3 and G10\_LNCO8 did not induce adherence and differentiation of THP-1 and HL-60 cells, which corresponds to the confirmed endotoxin-free production of the gelatin based hydrogels [9]. Nevertheless, an increased mitochondrial activity of HL-60 M $\phi$  and THP-1 M $\phi$  was detected 48 h after incubation with hydrolytic material eluates of G10\_LNCO3 and G10\_LNCO8, indicating an activation of the cells [35], which could be caused either by gelatin fragments or also by lysine [36] or lysine-based oligo-ureas in the material eluates. In contrary, HL-60 G $\phi$  exhibited contradictory effects with high variability, which may be due to a defective and incomplete differentiation resulting in a more heterogeneous cell population [13].

In contrast to endothelial cells, both macrophages and granulocytes showed reduced adhesion to hydrogels compared to TCP. This may be due to the rather low E-moduli of the materials (10-60 kPa), as earlier studies showed increasing macrophage attachment to materials with increasing Young's modulus, preferentially > 100 kPa [37]. Additionally, highly hydrophilic materials (like gelatin-based hydrogels) tend to show low macrophage adhesion [38]. *In vivo*, this might prevent their fusion to foreign body giant cells and thereby reduce the formation of fibrous scar tissue and the development of a chronic inflammation [39]. On the other hand, adherent macrophages as well as granulocytes were able to infiltrate the hydrogels within a few days, dependent on the crosslinking degree of the material. This again agrees very well with the determined degradation behavior of the investigated materials and correlates also to the ability of HAEC to infiltrate the hydrogels. The adherence and infiltration of endothelial cells is important for angiogenesis and tissue regeneration, while macrophage infiltration might support material degradation, likewise a prerequisite for full tissue regeneration.

After contact to the hydrogels, both THP-1 M $\phi$  and HL-60 M $\phi$  enhanced their expression of COX-2, indicating a regulating mechanism similar to HAEC [40]. A basal expression of COX-2 is induced by the differentiation of THP-1 and HL-60 with TPA, which activates protein kinase C and, thereby, forces the induction of COX-2 [15]. As in HAEC, RAGE expression was not influenced by material contact. In

contrast, HL-60 G $\phi$  showed enhanced expression of RAGE after material contact, whereas COX-2 expression was not influenced. Here, the high standard errors again underline the heterogeneity of the cell population.

In order to further investigate the response of macrophages to hydrogel contact, the cytokine and MMP release by THP-1 M $\phi$  was quantified. The macrophages showed mainly increased secretion in reaction to G10\_LNCO3 and unchanged or diminished secretion after contact to G10\_LNCO8. This implies, that the material's surface properties differently influenced the cytokine secretion of macrophages [41]. In contrast, THP-1 M $\phi$  did not enhance their secretion of MMPs, confirming that cells were not stimulated to degrade the materials. Macrophages secreted lower levels of MMP-1 compared to HAEC, whereas most other MMPs were secreted at higher levels.

### **Animal experiments**

*In vitro* no signs of an acute inflammatory response to the hydrogels were found. In the organism the initial injury from the implantation procedure causes a perturbation of homeostasis such as induction of inflammatory reactions. It is widely accepted that the host response to biomaterials is divided into several overlapping phases: blood-material interactions, acute inflammation, chronic inflammation, FBR, and fibrous encapsulation [10]. Therefore, subcutaneous implantation of the hydrogels in immunocompetent hairless mice was performed to further investigate the biomaterial-tissue interaction, particularly, chronic inflammation, FBR, and fibrous encapsulation *in vivo*. In this pilot experiment the almost complete absence of both macrophages and foreign body giant cells as well as changes in COX-2 expression revealed that the hydrogels did not initiate a substantial local inflammation, wherefore, tissue regeneration may not be slowed down or impaired [42]. More detailed, in or around G10\_LNCO3 no foreign body giant cells could be detected until 35 days after implantation, while a few monocytes/macrophages and foreign body giant cells appeared in the transition region between capsule and G10\_LNCO8. On the other hand, around both hydrogels COX-2 expression did not increase during the follow-up period of 35 days. These results are in good agreement with the prior performed *in vitro* studies using hydrogel eluates, in which also no signs of an acute inflammatory response to the hydrogels were found. The extent of encapsulation, initiated by FBR, varies depending on several factors, e.g., physical and chemical properties as well as shape of the implanted material as well as the location of the implant and the time of retention within the implantation situs [10]. In the present study, collagenous connective tissue formed a fibrous capsule

around both implants, whereas the time course of the formation of the capsules and the tissue integration differed between the hydrogels. Here, the less crosslinked hydrogel showed a more promising behavior, which possibly can be explained by differences in hydrogel degradation. This seems to be supported by red-coloured areas observed after Masson Goldner staining up to 35 days after implantation predominantly in G10\_LNCO3 (Figure 7). These red-coloured areas indicate the appearance of negatively charged components and thus in this study are a surrogate marker of differences in degradation between the two hydrogels. In this regard, the integration of polymers into surrounding tissues is influenced by the concentration and structure of the degradation products arising at the polymer-tissue interface as well as their metabolic clearance [10]. Beyond the different degradation behavior differences in the formation of new blood vessels were observed. While in the capsule of G10\_LNCO8 small microvessels were formed already 14 days after implantation no blood vessels were found in the capsule of G10\_LNCO3. It can be assumed that the monocytes/macrophages observed around and in the more crosslinked hydrogel emigrated from surrounding vasculature, particularly, from these newly formed vessels in the capsule.

## Conclusions

Due to their different degree of chemical and physical crosslinking, the investigated hydrogels differed in elasticity, swellability, ratio of gelatin and lysine oligo-urea chains, as well as rate of degradation. Especially direct cell-hydrogel contact influenced cell reactions. Exemplarily, adhesion of endothelial cells was higher on G10\_LNCO3 while macrophages preferred G10\_LNCO8. The different degree of crosslinking led to faster cell invasion into G10\_LNCO3 than into G10\_LNCO8. Likely, the interdependent properties of matrix elasticity and swellability might play a major role. The influence of hydrogen bond forming oligo-urea chains, of which more are present in G10\_LNCO8 than in G10\_LNCO3, also could be important and might be investigated in the future by studying model compounds. Degradation is supposed to contribute to changes in surface topography and, therefore, indirectly influences cell behavior. After implantation of the hydrogels a capsule formation around both hydrogels occurred, markedly differing over time. Noteworthy is the decrease of capsule size around G10\_LNCO3 showing a very good integration in the surrounding tissue. Overall, the study revealed that the two gelatin-based hydrogels with crosslinking-dependent tailorable properties and behavior did not induce substantial inflammatory reactions neither *in vitro* nor *in vivo*. This indicates these hydrogels to be promising polymer candidates for soft tissue replacement and regeneration.

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## Tables

**Table 1: Concentrations [pg/ml] of multiple cytokines** measured by magnetic bead-based bioplex assay in cell culture supernatants of HAEC. Cells were cultivated for 48 h on tissue culture plastic (TCP), on hydrogels G10\_LNCO3 and G10\_LNCO8, or on TCP with addition of 0.1  $\mu$ M TPA to the cell culture medium.  $n \geq 3$ , mean  $\pm$  SEM, \*  $p < 0.05$  vs. medium, ANOVA, Bonferroni *post hoc* test.

Cytokines	HAEC			
	Concentration [pg/ml]			
	TCP	G10_LNCO3	G10_LNCO8	TCP + 0.1 $\mu$ M TPA
<b>pro-inflammatory mediators</b>				
IL-1 $\beta$	n.d.	n.d.	n.d.	n.d.
IL-2	n.d.	n.d.	n.d.	n.d.
IL-6	452 $\pm$ 306	499 $\pm$ 222	497 $\pm$ 247	2455 $\pm$ 1211
IL-8	4316 $\pm$ 1775	6144 $\pm$ 2829	6187 $\pm$ 2505	14430 $\pm$ 3251 *
IFN- $\gamma$	33.2 $\pm$ 13.0	35.6 $\pm$ 26.9	26.6 $\pm$ 5.7	85.5 $\pm$ 10.3 *
RANTES	n.d.	n.d.	n.d.	55.5 $\pm$ 10.7
TNF- $\alpha$	n.d.	n.d.	n.d.	19.4 $\pm$ 2.4
<b>anti-inflammatory mediators</b>				
IL-1Ra	24.8 $\pm$ 9.7	27.3 $\pm$ 13.4	27.7 $\pm$ 8.8	58.1 $\pm$ 7.8 *
IL-4	1.7 $\pm$ 0.5	1.8 $\pm$ 1.0	1.5 $\pm$ 0.3	2.9 $\pm$ 0.2 *
IL-10	n.d.	n.d.	n.d.	n.d.
<b>chemokines</b>				
G-CSF	53 $\pm$ 23	31.9 $\pm$ 11.8	38.3 $\pm$ 16.4	162 $\pm$ 16 *
MCP-1	651 $\pm$ 113	541 $\pm$ 96	596 $\pm$ 113	1052 $\pm$ 333
<b>functional marker</b>				
bFGF	n.d.	n.d.	n.d.	143 $\pm$ 62
PDGF-BB	277 $\pm$ 66	354 $\pm$ 125	363 $\pm$ 97	3278 $\pm$ 1906
VEGF	n.d.	n.d.	n.d.	n.d.

**Table 2: Concentrations [pg/ml] of multiple cytokines** measured by magnetic bead-based bioplex assay in cell culture supernatants of THP-1 M $\phi$ . Cells were cultivated for 48 h on tissue culture plastic (TCP) or on hydrogels G10\_LNCO3 and G10\_LNCO8. n  $\geq$  3, mean  $\pm$  SEM, \* p < 0.05 vs. medium, # p < 0.05 vs. G10\_LNCO3, ANOVA, Bonferroni *post hoc* test.

Cytokines	THP-1 M $\phi$		
	Concentration [pg/ml]		
	TCP	G10_LNCO3	G10_LNCO8
<b>pro-inflammatory mediators</b>			
IL-1 $\beta$	1786 $\pm$ 975	2532 $\pm$ 904	3037 $\pm$ 2007
IL-6	184 $\pm$ 57	1062 $\pm$ 344 *	138 $\pm$ 38 #
IL-8	19965 $\pm$ 5625	10087 $\pm$ 1863 *	18912 $\pm$ 6354
IL-12(p70)	157 $\pm$ 9	132 $\pm$ 12 *	138 $\pm$ 7 *
IFN- $\gamma$	153 $\pm$ 25	213 $\pm$ 28 *	151 $\pm$ 37
RANTES	2783 $\pm$ 861	5370 $\pm$ 2045	8717 $\pm$ 5687
TNF- $\alpha$	676 $\pm$ 283	4776 $\pm$ 1956 *	387 $\pm$ 137 #
<b>anti-inflammatory mediators</b>			
IL-1Ra	3418 $\pm$ 617	4620 $\pm$ 389 *	3366 $\pm$ 206 #
IL-10	31.1 $\pm$ 13.4	44.4 $\pm$ 22.5	27 $\pm$ 9
<b>chemokines</b>			
G-CSF	376 $\pm$ 45	465 $\pm$ 50	414 $\pm$ 86
MCP-1	422 $\pm$ 39	666 $\pm$ 166 *	262 $\pm$ 50 *, #
MIP-1 $\alpha$	7790 $\pm$ 6610	25909 $\pm$ 41090	8835 $\pm$ 11284
MIP-1 $\beta$	3253 $\pm$ 832	2928 $\pm$ 1181	3925 $\pm$ 1224
<b>functional markers</b>			
PDGF-BB	18.6 $\pm$ 5.5	27.5 $\pm$ 7.4	25.2 $\pm$ 11.4
VEGF	5514 $\pm$ 1557	2957 $\pm$ 307 *	3811 $\pm$ 1238

## Figure Legends

**Figure 1: Biomaterial characteristics.** Representative G10\_LNCO8 hydrogel film in **(A)** swollen and **(B)** dry state. **(C)** Mass loss during degradation represented as relative remaining mass  $\mu_{rel}$  of G10\_LNCO3 and G10\_LNCO8 samples. 21 day degradation in PBS (pH 7.4) at 37 °C. n = 6, mean  $\pm$  SD.

**Figure 2: Mitochondrial activity of HAEC, HL-60 G $\phi$ , THP-1 M $\phi$  and HL-60 M $\phi$**  cultivated with medium (set to 100%) or hydrolytic material eluates (\_h) of G10\_LNCO3 or G10\_LNCO8 determined using MTT test. n  $\geq$  12 out of at least 4 independent experiments, mean  $\pm$  SEM, \* p < 0.05 vs. medium 24 h or 48 h, ANOVA, Bonferroni *post hoc* test.

**Figure 3: Adhesion of HAEC, HL-60 G $\phi$ , THP-1 M $\phi$  and HL-60 M $\phi$**  to tissue culture plastic (TCP, set to 100%), gelatin-coated TCP or hydrogels G10\_LNCO3 or G10\_LNCO8 determined after 2 h (THP-1 M $\phi$ , HL-60 G $\phi$ ) or 4 h (HAEC, HL-60 M $\phi$ ). n  $\geq$  12 out of at least 4 independent experiments, mean  $\pm$  SEM, \* p < 0.05 vs. medium 24 h or 48 h, ANOVA, Bonferroni *post hoc* test.

**Figure 4: Infiltration of HAEC, HL-60 G $\phi$ , THP-1 M $\phi$  and HL-60 M $\phi$  into hydrogels G10\_LNCO3 or G10\_LNCO8** after 2 days or 7 days. Cells were grown on the hydrogels, fluorescence stained with calcein and imaged using confocal laser scanning microscopy via Z-stack images. Hydrogels were examined upside down on microscope slides, wherefore the hydrogel surface is situated at the bottom of the figures. Scale bars represent 100  $\mu$ m.

**Figure 5: Relative expression of the pro-inflammatory proteins COX-2 and RAGE and the constitutively expressed isoform COX-1**, related towards  $\beta$ -actin expression as loading control and cultivation with cell culture medium (set to 100%), in HAEC, HL-60 G $\phi$ , THP-1 M $\phi$  and HL-60 M $\phi$ . Cells were cultivated for 48 h with hydrolytic (\_h) and enzymatic (\_e) material eluates of G10\_LNCO3 and G10\_LNCO8, or on hydrogels G10\_LNCO3 and G10\_LNCO8, or with medium and in case of HAEC medium with 0.1  $\mu$ M TPA. Cultivation with medium was set to 100%. n  $\geq$  3, mean  $\pm$  SEM, \* p < 0.05 vs. medium, # p < 0.05 vs. G10\_LNCO3, ANOVA, Bonferroni *post hoc* test.

**Figure 6: Representative Western Blot analysis of HAEC lysates and cell culture supernatants** after cultivation for 48 h with hydrolytic (\_h) and enzymatic (\_e) material eluates of G10\_LNCO3 and G10\_LNCO8, or on hydrogels G10\_LNCO3 and G10\_LNCO8, or with medium and medium with 0.1  $\mu$ M TPA. Expression of extracellular and membrane thrombomodulin, as well as the housekeeping protein  $\beta$ -actin is shown.

**Figure 7: Degradation and capsule formation** in SKH1 mice around hydrogels G10\_LNCO3 and G10\_LNCO8 up to 35 days after implantation, representative examples. Scale bars represent 500  $\mu$ m. After Masson Goldner staining red-coloured areas were observed up to 35 days after implantation predominantly in G10\_LNCO3, and only very limited in G10\_LNCO8.

**Figure 8: Capsule thickness** (summarized over cutaneous and musculo-fascial side) around implanted G10\_LNCO3 and G10\_LNCO8 in SKH1 mice from day 7 to day 35 after implantation. n = 45 out of 3 independent experiments, mean  $\pm$  SD, \* and § p < 0.05, # p < 0.05 vs. G10\_LNCO3, two-way ANOVA, Holm-Sidak *post hoc* test.

**Figure 9: Three vessels in the capsule** (see arrows) around G10\_LNCO8 35 days after implantation in SKH1 mice (detail from Figure 7, G10\_LNCO8 at day 35).

**Figure 10: G10\_LNCO8 14 days after implantation** in SKH1 mice. **(A)** Section of the complete implant. Scale bar represents 500  $\mu$ m. **(B)** Detail with some foreign body giant cells in the transition region between capsule and implant **(C)** Further magnification with foreign body giant cells.

**Figure 11: COX-2 expression *in vivo*.** **(A)** Relative expression of COX-2 related towards GAPDH expression as loading control in tissue lysates of SKH1 mice implanted with either G10\_LNCO3 or G10\_LNCO8. Specimens were taken at indicated time points after implantation. Normal skin (-) and induced inflammation (+) were used as control. Control inflammation was induced by 3 injections of 100  $\mu$ l of a 100  $\mu$ M TPA solution. Normal skin was set to 100%, n = 3, mean  $\pm$  SEM, \* p < 0.05 vs. medium, ANOVA, Bonferroni *post hoc* test. **(B)** Representative immunohistochemical staining of COX-2 in tissue sections of SKH1 mice implanted with either G10\_LNCO3 or G10\_LNCO8. Specimens were taken 21 days after implantation, fixed, cryosectioned and stained for COX-2 or isotype control. Scale bars represent 200  $\mu$ m.