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Influence of physically crosslinked gelatins on the vasculature in the avian chorioallantoic membrane

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Abstract

Gelatins functionalized with desaminotyrosine (DAT) or desaminotyrosyl tyrosine (DATT) form physically crosslinked hydrogels, due to the interactions between the introduced aromatic moieties and gelatin triple helices, whose extent depends on the thermal treatment of the material. The G-modulus of these hydrogels can be tailored to the range of the natural extracellular matrix by adjusting the degree of crosslinking. While these gelatin-based materials have been shown to be not angiogenic, the aim of the study was to evaluate whether these biomaterials influence the regulation of blood vessels when positioned on the chorionallantoic membrane (CAM) of fertilized eggs.

The results clearly indicate that the DAT-functionalized gelatin led to an increase of the diameter of the blood vessels in the CAM, which at the same time is probably associated with an increased blood flow in these CAM vessels. The vessel diameters of the four groups (DAT-functionalized gelatin, DATT-functionalized gelatin, plain gelatin, control group without gelatin, each n = 10) differed significantly ($p<0.0001$). Vessels in the CAM exposed to the DAT-functionalized gelatin showed with 36.4 µm ± 3.4 µm the largest mean diameters compared to the mean diameters of the samples exposed to DATT gelatin (16.0 µm ± 0.8 µm; $p<0.05$) and the plain gelatin (21.2 µm ± 1.0 µm; $p<0.05$), which both did not differ significantly from the vessels of the control group.

The biocompatibility of the materials *in vitro* motivates the exploration of their application as matrix in local drug-release systems with short half-life times (one hour up to several days).

Key words: HETCAM assay, gelatin, tyrosin

1. Introduction

Regenerative medicine aims to restore damaged or missing tissue especially by stimulation and control of endogenous regeneration. The regeneration of tissues often requires degradable materials, which temporarily substitute a tissue or organ function and induce regeneration. While the new functional tissue is formed, the implant degrades. Angiogenesis is an important process for wound healing and the formation of neotissue [13,27]. Materials based on components or derivatives of the extracellular matrix (ECM) are attractive candidates for biomaterials as they can provide epitopes for cell-attachment and are principally able to induce different biological processes [31]. An appealing compound for this purpose is gelatin, a mixture of partially degraded and denatured collagen. Collagen is represented by 29 distinct types, which differ in structure, affinity to the other elements of ECM as well as the amount and distribution in tissues. It is responsible for the maintenance of integrity, tensile strength and elasticity of connective tissue and plays a crucial role in functioning of the blood vessel walls [6,9,15]. However, tailoring the mechanical properties of collagen-based materials is challenging. Collagen was also reported to cause potentially immunological responses [4]. On the contrary, gelatin is non-toxic, histo- and immunocompatible, and reduces inflammatory responses to body foreign materials. However, gelatin readily dissolves under physiological conditions. Covalent or physical crosslinking of gelatin can be applied for increasing the residence time of the biomaterial at the side of implantation [31]. In this work, physically crosslinked gelatin based on gelatin functionalized with the amino acid derived groups desaminotyrosine (DAT) or desaminotyrosyl tyrosine (DATT) was investigated [17]. The G-modulus and degree of swelling of the hydrogels formed by functionalized gelatins can be tailored by adjusting the density of physical crosslinks. These are formed by the aromatic moieties as well as by triple helices of gelatin chains. The crosslink density can be influenced by the degree of functionalization and the thermal treatment of the material before wetting [35]. It could be shown that drying conditions of DAT- and DATT-functionalized gelatins affect the thermal and mechanical properties, as well as the helical content of gelatin samples. Drying at a temperature above the helix-to-coil transition temperature of gelatin ($T > T_c$, called υ_{short}, 40 °C and 40.83 g·m^{-3} absolute humidity) generally resulted in gelatins with relatively lower triple helical content ($X_{c,t}$ = 1-2%) than drying at lower temperatures ($T < T_c$, called υ_{long}, 10 °C, 2.81 g·m^{-3} absolute humidity) ($X_{c,t}$ = 8-10%). Drying at υ_{short} conditions enabled higher chain mobility in the gelatin samples during a short drying time, which inhibited helix formation and resulted in more amorphous films. While different helical

contents affected the thermal transition temperatures only slightly, the mechanical properties were strongly affected for equilibrium swollen hydrogels (E = 4-13 kPa for υ_{long}; E = 120-700 kPa for υ_{short}) [35]. Due to the higher gel strength and additionally higher elasticity at equilibrium swelling, the υ_{short} treated materials should be further explored herein as potential materials in regenerative therapies. The functionalized gelatins can be produced free of endotoxins and are non-cytotoxic [22], which raised interest in more specific studies of biofunctionality.

The interaction between a biomaterial and the surrounding tissue is of crucial importance for its successful application. A suitable biomaterial should not hinder, but ideally support the transport of oxygen and nutrients to the surrounding tissue. Furthermore, degraded and metabolic products need to be removed to avoid microcirculatory disturbances [5,21,22]. Inadequate interactions between the microvasculature and implanted materials may limit the suitability for a variety of biomedical applications including *in vivo* tissue engineering [3,12,26,28]. Therefore, the influence of a biomaterial on angiogenesis and blood vessel morphology is of high interest. The HETCAM-test (Hen's egg test on chorioallantoic membrane) initially described by Luepke in 1985 [14] can be used as a test system for biocompatible materials [29]. It is accepted as *in vivo* system [32], which is not an animal test when eggs to a maximum fertilized time of 10 days are used. When polymer samples are placed on the chorioallantoic membrane (CAM), the blood vessel diameter in the CAM as associated with the regulation of the blood flow can be determined. High blood flow guarantees the transportation of oxygen and nutrients to the surrounding tissue to support endogenous repair in regenerative medicine. To visualize material-induced changes in the diameter of blood vessels *in vivo*, the CAM can be explanted after two days of exposure and evaluated by cryosections for vessel diameters [8,29,32]. Recently, it was shown that DAT- and DATT-functionalized gelatins did not alter the number of formed blood vessels compared to non-functionalized gelatin or untreated controls, and additionally did not induce thrombus formation or haemorrhage [11]. This is in accordance with studies on other polymers based on DATT [30]. In addition to the vessel number, the vessel diameter is an important parameter that affects blood flow.

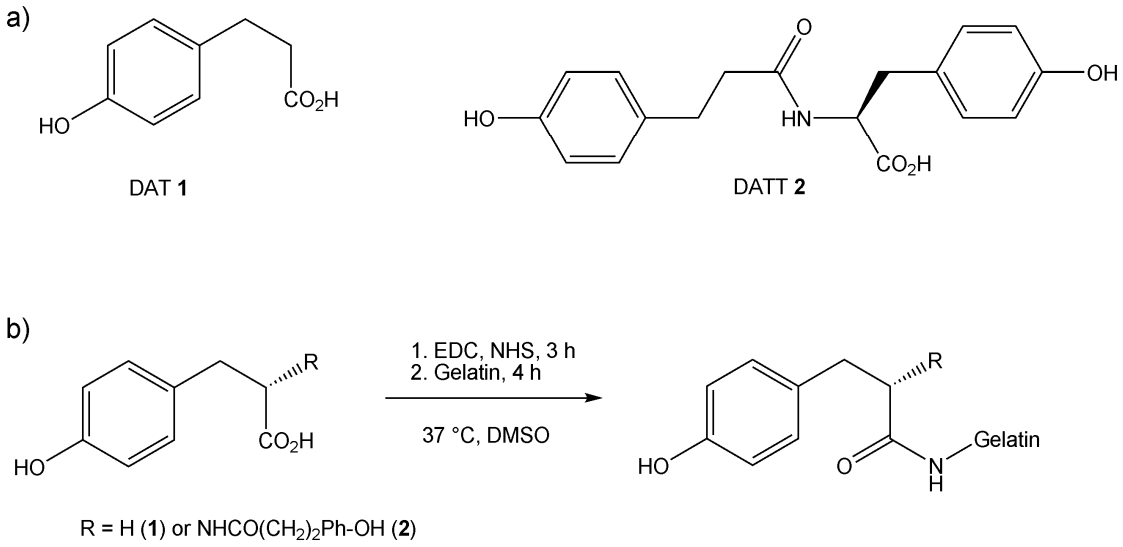

Fig. 1: a) Chemical structures of desaminotyrosine (DAT) **1** and desaminotyrosyl tyrosine (DATT) **2**.
b) Functionalization of gelatin with DAT or DATT by EDC-NHS coupling.

Therefore, in this work, the influence of DAT- or DATT-functionalized gelatin (compared to plain gelatin and control eggs without gelatin) on the blood vessel diameter in the CAM was explored.

2. Material and Methods

2.1. Gelatin-based materials

Desaminotyrosine (DAT, **1**) or desaminotyrosyl tyrosine (DATT, **2**) (Fig. 1a) were used to functionalize gelatin. The synthesis of DATT was performed as published in [17].

For the functionalization of gelatin (Fig. 1b), DAT or DATT (29 mmol) was activated by reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 32 mmol; Sigma, Taufkirchen, Germany) and N-hydroxysuccinimide (NHS; 43 mmol; Sigma, Taufkirchen, Germany) in 110 mL of DMSO (Merck, Darmstadt, Germany) in a round bottom flask at 37 °C.

After 3 h, β-mercaptoethanol (43 mmol) (Sigma, Munich, Germany) was added. After 30 minutes, a gelatin (Type A from porcine skin, Sigma, Munich, Germany) solution (15 g in 150 mL DMSO) was added and the mixture stirred at 37 °C for 4 h. The functionalized product was then precipitated in ethanol, filtered, washed with ethanol and acetone, and dried under vacuum. The degree of functionalization was determined by TNBS. A functionalization up to 90 mol.-% was achieved. Films were prepared by casting a 5 wt.-% aq. solution into

polystyrene Petri dishes and subsequent drying at 40 °C and 40.83 g·cm^{-3} absolute humidity (υ_{short}) until constant weight was achieved.

2.2. Sterilization

Samples as synthesized (details see [17,35]) were sterilized by Medicoplast (Illingen, Germany) according to DIN EN ISO 13485:2003 standards using ethylene oxide (EO). The sterilization step consisted of 3 h of contact time with 600 mg/L EO at 44 °C, which was followed by a desorption step (35 – 45 °C for 3 d).

2.3. HETCAM-test

The effect of different gelatin-based materials (DATT-functionalized gelatin, DAT-functionalized gelatin, and plain gelatin) on the blood vessel diameter of the chorioallantoic membrane (CAM) of fertilized chicken eggs was studied by histological evaluation. The research was conducted and the manuscript was written in accordance with the ethical guidelines of the journal [1].

For the HETCAM-test fertilized VALO SPF eggs of the white Leghorn species (Lohmann Tierzucht GmBH, Cuxhaven, Germany) were used. The eggs were breeded for eight days under standard conditions (breeding temperature: 37 ± 0.5°C, relative humidity: 65 ± 7.5%) in an incubator (BSS 300 MP GTFS, Grumbach GmbH, Asslar, Germany). The egg rotation occurred automatically (5x per day). Temperature, humidity and the rotation were regulated by an actual-value-control. Due to the continuous rotation of the eggs during the breeding time, a consistent development of the embryos was accomplished. At day seven of the egg breeding, the hen's egg was raised (the angular part of the egg was directed down). By this act the air bubble migrated to the stump end of the egg. After egg removal out of the incubator, the breeding was tested with a sheer lamp. Eggs containing no embryo were abolished. By using the lamp, the position of the air bubble was marked. So, the upper part of the eggshell could be removed, the egg membrane became accessible and was moistened with 0.9% NaCl for one minute and then removed with a forceps [11]. The dry polymer films (DATT-gelatin, DAT-gelatin, plain gelatin) were pretreated by swelling in PBS (Biochrom AG) for 45 minutes and punched out with a Biopsy Punch (Ø 8 mm, Stiefel) to obtain circular samples for biological evaluation. The samples were applied on the chorionallantoic membrane and fixed in a thermanox™ ring (external diameter = 13 mm, internal diameter: 8 mm) (n = 10). As control, eggs incubated with a thermanox™ ring without gelatin were used (n = 10). To

analyse the material effect on angiogenesis in the CAM histologically, the thermanox™ ring was detached after the microscopically assessment of the CAM [8], the CAM was moisted with 4 vol% paraformaldehyde solution (precooled on ice to 0 – 4 °C) and incubated at room temperature for four hours. Subsequently, the CAM (20×20 mm^2) was removed, embedded in "Frozen Section Medium" (Neg 50; Richard-Allan Scientific) and frozen for 10 min at -20 °C. Then 5 – 10 µm cryo slices were fabricated (Kryostat HM560M, Microm) and fixed on glass slides (Superfrost Plus; Thermo Scientific, Waltham, Massachusetts, USA). Afterwards, a staining with hematoxylin- eosin was performed. For this, the cyro slices were incubated with hemalum solution (Roth, Karlsruhe, Germany) for three minutes, rinsed with Aqua dest, water for 15 min. and additional Aqua dest. Subsequently, the slides were stained with eosin G solution 0.5% (Roth, Karlsruhe, Germany) for additional three minutes, followed by rinsing with Aqua dest. The evaluations of the HE-stainings were performed using the light microscope Imager Z2m (Zeiss, Germany). The diameters of the CAM vessels were measured at the top side of the CAM (average of three measurements per vessel are given, see yellow arrows in Fig. 3). The histological preparation was performed at the 10th day of fertilization.

2.4. SDS-Page

The molecular weight distribution of gelatin fragments after hydrolysis in PBS (pH 7.4) at 37 °C for different time points (0 h, 12 h, 24 h, 48 h, 72 h) was determined by SDS-PAGE on 20% Ready Gels (Bio-Rad Laboratories) in a Mini-Protean system (Bio-Rad). A pre-stained protein mixture standard (M_w= 10-250 kDa) was used for the determination of molecular weight. Samples of gelatin and DAT(T)-gelatin were first swollen in PBS at room temperature for 3 h. The time after swelling is defined as time point 0 h, where only the supernatant was analyzed. After swelling, the samples were incubated at 37 °C for different times (12 h, 24 h, 48 h, 72 h). All samples of 2 $mg \cdot mL^{-1}$ or 3 $mg \cdot mL^{-1}$ concentration were diluted 1:1 with a 2x sample buffer, then heated to 95 °C for 5 min, and cooled down. 15 µL of each sample were loaded onto the respective lane of the gel and a current of 200 V was applied for approximately 30 min. Protein bands were visualized after a 60 min staining (0.1 wt.-% Coomassie Blue, 10 vol-% acetic acid, and 40 vol.-% methanol in water) and 120 min treatment in a destaining solution (10 vol.-% glacial acetic acid, 20 vol.-% methanol in water). The molecular weight of the gelatin fragments was approximated by measuring the relative mobility of the standard protein molecular weight markers. The pictures of the SDS-gels were taken, using a LI-COR imager (Odyssey Classic 2.0, LI-COR Biosciences GmbH, Bad Homburg).

2.5. Statistics

Mean values and standard errors of the mean (SEM) are given for continuous data. A one-factorial variance analysis was used for group comparisons including Tukey test for two-group comparisons. *P* values less than 0.05 were considered significant.

3. Results and Discussion

Sterility of samples is an essential precondition for sample analysis by HETCAM, which is why samples were sterilized with ethylene oxide. Another impact factor may be endotoxins, which are not removed by ethylene oxid sterilization. However, previously, a very low endotoxin content of the DATT-gelatin was shown, which was clearly below the FDA level of 0.5 EU/ml. The plain and the DAT-functionalized gelatin samples were slightly above the FDA level [22]. Additionally, the previously analyzed integrity of the cell membranes (LDH release) and the mitochondrial activity as measured on L929 cells after 48 h of incubation both yielded the low cytotoxicity level 1 for plain gelatin, which was comparable to DAT functionalized gelatin. On the contrary, the increases of both values were stronger in case of DATT-gelatin (LDH release: level 4; mitochondrial activity: level 2) [22].

After two days of incubation, the CAM was explanted and cryo-sectioned. The thickness of the explanted CAM was between 100 and 150 µm, which corresponds well with earlier reported studies [18]. The average diameter of vessels in the control eggs without gelatin on the 10^{th} day after fertilization was 23.0±1.4 µm. Ribatti and coworkers reported similar diameters of CAM vessels; at day 8 (beginning of their experiment) they described diameters between 10-15 µm, and diameters between 10 µm and 85 µm between day 10 and 12 [20].

Figure 2 shows typical microscopic images of the blood vessels in the CAM stained with hematoxylin-eosin after two days of exposition with the three different gelatins.

Figure 3 shows matured vessels with an intact and confluent endothelial inner lining (black arrows) as could be expected since the development of the CAM vessels is already completed at day six to seven [7]. Clearly recognizable are the nuclei in the erythrocytes as was expected for chicken. The erythrocytes show a normal size and shape.

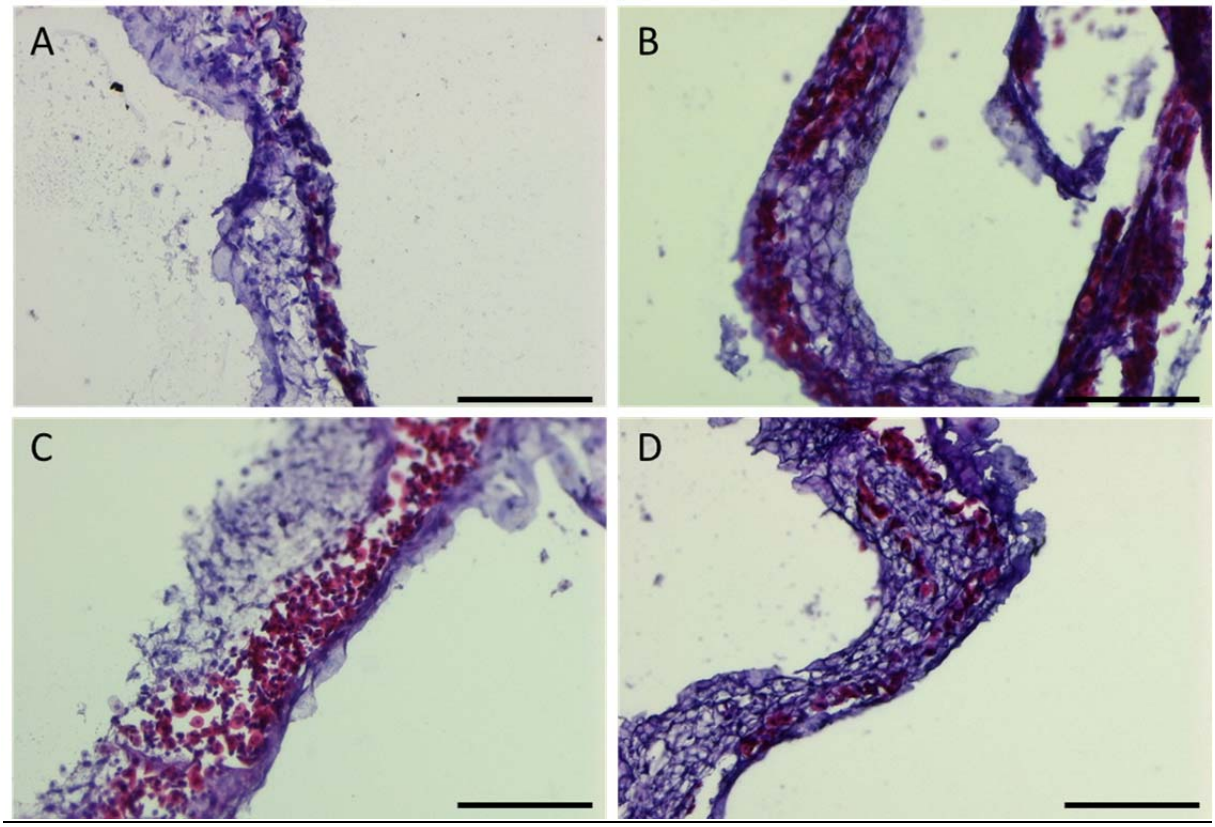

Figure 2: Typical microscopic images of sections of the explanted chorioallantoic membrane two days after application with the thermanox™ ring without gelatin (**A**; 23 µm vessel diameter), plain gelatin (**B**; 21 µm vessel diameter), the DAT-functionalized gelatin (**C**; 76 µm vessel diameter), and of DATT-functionalized gelatin (**D**; 16 µm vessel diameter). HE-staining; primary magnification 1:20, Bar: 100 µm

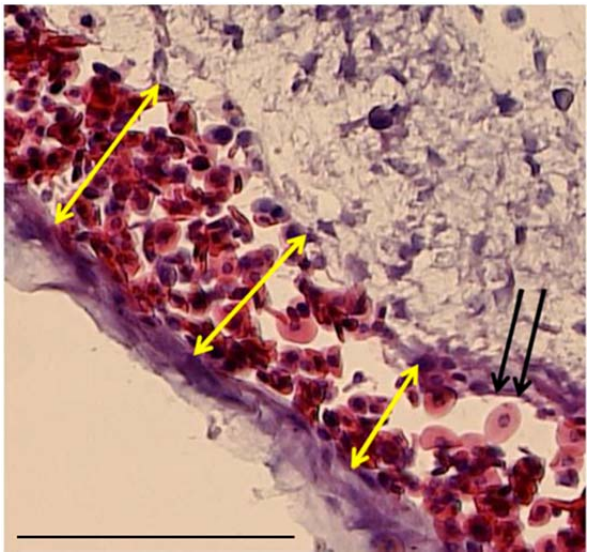

Figure 3: Typical example for the quantification of blood vessel diameters in the chorioallantoic membrane (yellow arrows) (control egg without gelatin). The endothelial cell layer was confluent and intact (black arrows). Bar: 75 µm

The vessel diameters of the four groups differed significantly ($p<0.0001$) after two days of exposition to the materials (10 days after fertilization) (see Figure 4). Vessels in the CAM

exposed to the DAT-functionalized gelatin showed with 36.4 ± 3.4 µm the largest diameter being greater than the mean diameters of the samples exposed to DATT gelatin (16.0 ± 0.78 µm; $p<0.05$) and the plain gelatin (21.2 ± 1.0 µm; $p<0.05$).

The increase of vessel diameter is within the range of changes observed in vasodilation upon physiological demand or because of drug action [10]. As only vessels were evaluated in this study, which were formed at the beginning of the experiment, it is likely that the observed increase in vessel diameter is a physiological, putatively reversible response to the material rather than due to an increased growth of the vessel.

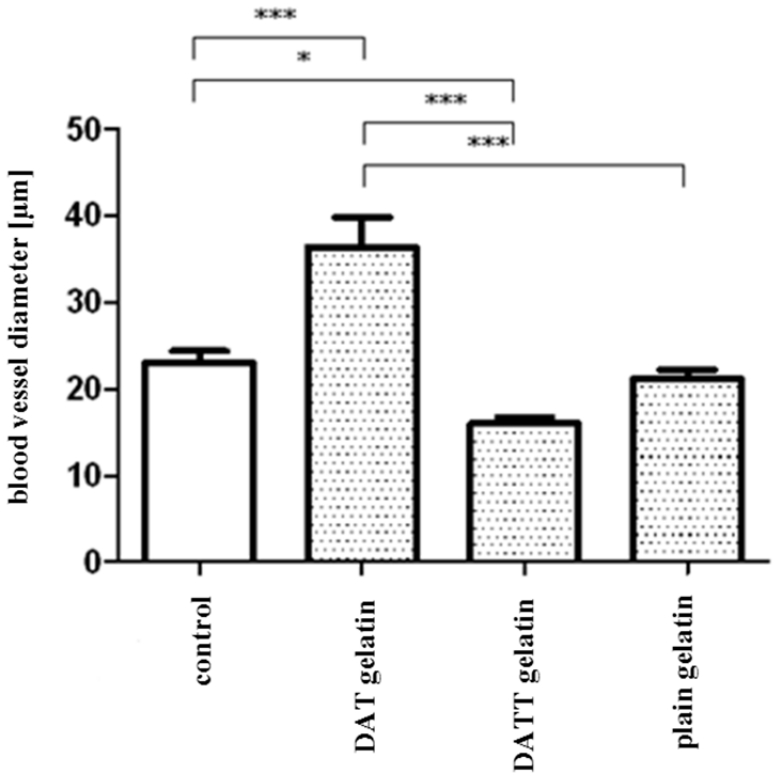

Figure 4: Mean vessel diameters in the chorioallantoic membrane after two days of exposition with different gelatins in comparison to controls
(mean values ± SEM)

Furthermore, due to the increase of the vessel diameter, an enhanced blood flow and, in addition, an improved supply of the embryo with oxygen and nutrients can be assumed.

No remains of degraded gelatin could be detected on any of the CAMs on the second day of exposition. It should be noted that the three types of gelatin discs dissolved within different time points after positioning on the CAM: non-functionalized gelatin and the DATT-functionalized gelatin were no longer visible after 90 minutes, while this was the case for DAT-functionalized gelatin already after 30 minutes [11]. The removal of the gelatin films can be explained by dissolution and/or hydrolytic or enzymatic degradation of the samples

with subsequent diffusion into the CAM, potentially followed by subsequent metabolization of the materials. These soluble macromolecules and their fragments and metabolites could potentially influence the blood vessel diameter. For the materials studied here, fragments of the gelatin itself as well as the introduced aromatic compounds might be involved in these processes.

So far only limited knowledge exists about the influence of circulating gelatin and gelatin fragments on the dilatory capacity of blood vessels. First studies showed, that chicken collagen hydrolysate exerted an antihypertensive effect in spontaneous hypertensive rats [24]. Recently, this effect was confirmed in a clinical study [23]. Additionally, collagen-derived inhibitory peptides for angiotensin I converting enzyme (ACE) have been isolated as the active entity [25]. Also, an increase of the NO production in human and murine endothelial cells upon exposure to collagen was reported recently [33]. Under the assumption, that similar degradation products might be released upon hydrolysis of the peptide backbone from the studied gelatin materials compared to collagen, both mechanisms (i.e. inhibition of ACE or induction of NO production) could theoretically induce the vasodilation of the CAM vessels.

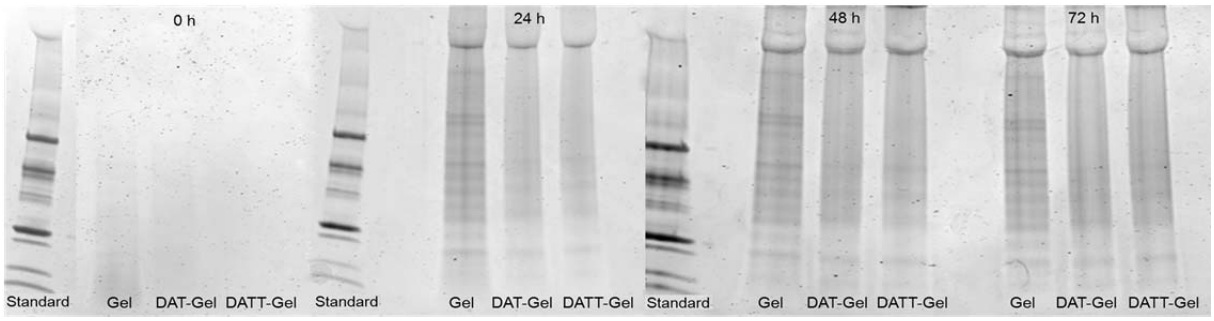

Figure 5: SDS PAGE band pattern of non-functionalized gelatin (Gel) and functionalized gelatins (DAT-Gel, DATT-Gel) from a degradation study in PBS at 37 °C. Data shown for 0 h, 24 h, 48 h, 72 h.

However, as the vasodilation is only observed for DAT-functionalized gelatins, it may be hypothesized that different degradation products were formed from the different gelatin materials. Therefore, initial degradation studies of gelatin and the functionalized gelatins in PBS at 37 °C were performed for different degradation time points (0 h, 12 h, 24 h, 48 h, 72 h). The hydrolysis products were investigated by SDS PAGE. In these experiments (see Figure 5), no clear difference in the resulting fragments from the different materials as indicated by the position of the bands in the gel could be identified. At the time point for 0 h, no presence of degradation products could be observed, possibly because potential low amounts of degradation products could not be detected by SDS PAGE or were not extracted from the samples at room temperature. The results for a kinetic study with analysis at 12 h

(data not shown), 24 h, 48 h, and 72 h illustrated that no difference in the position of bands corresponding to degradation products could be detected for each individual sample between these time points. Nevertheless, a difference between non-functionalized and functionalized gelatin was observed with apparently higher concentrations and more bands for non-functionalized gelatin. In contrast, no difference in the degradation products of DAT-gelatin compared to DATT-gelatin was detected. However, it is possible that selective enzymatic cleavage in the CAM will produce different and possibly bioactive fragments of the materials.

Since degradation kinetics may not be substantially different between DAT- and DATT-functionalized gelatin, the results may suggest that there is an influence of the functionalizing group or its degradation products on the vessel diameter. Typically, phenols easily oxidize under physiological conditions giving catechols and potentially also orthoquinones. Phenols and catechols, e.g. taken up with food, in general were shown to influence vasoconstriction or –dilatation. However, at least desaminotyrosine and the oxidation product 3-(3',4'-dihydroxyphenyl) propionic acid were shown not to induce NO release [2] as a substance leading to an increase of the vessel diameter [16]. An increase of the vessel caused by an inflammation reaction only in case of the DAT-functionalized gelatin is rather unlikely, especially since the immune system of the chicken embryos is not fully developed at the time of the examination [19,34]. An exact elucidation of the molecular basis for the vasodilation observed for DAT-gelatin in this study would require a detailed analysis and identification of all possible degradation fragments, their synthesis or isolation as pure compounds, and testing of each individual substance. This could be approached in the future.

4. Conclusion

This study revealed that hydrogels based on gelatin functionalized with desaminotyrosine (DAT) or desaminotyrosyl tyrosine (DATT) and/or its degradation products have the potential to influence blood vessel diameters. A circulation-enhancing effect of such a biomaterial could potentially lead to an improvement of oxygen and nutrient supply of the adjacent tissue and additionally support the removal of metabolic end products. At the same time, more cells potentially involved in the regeneration of the tissue - during degradation of the implant material - may be transported by the blood flow into the surrounding tissue, which might accelerate regenerative processes.

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