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Reducing the Endotoxin Burden of Desaminotyrosine- and Desaminotyrosyl Tyrosine-Functionalized Gelatin

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Summary: Biomaterial-induced autoregeneration requires materials with distinct tailored mechanical and thermal properties, water uptake and swelling properties as well as degradation behavior. Furthermore, before any biomaterial can be applied *in vivo*, *in vitro* studies should be performed that confirm the suitability for such applications. One facet in this process is the evaluation of endotoxin loads and immunogenic response to the material to avoid an unspecific activation of the immune system, which otherwise might cause fever and could lead to life–threatening pathologies. In this study, gelatins functionalized with desaminotyrosine (DAT) or desaminotyrosyl tyrosine (DATT) were investigated in terms of their endotoxin content and their potential to induce an inflammatory cytokine response in macrophages. Using the Limulus amebocyte lysate (LAL) test it could be shown that the endotoxin content was substantially reduced by using certified low endotoxin containing gelatin and performing the gelatin functionalization under cleanroom conditions. Furthermore, production of inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor–alpha (TNFα) of an immune relevant macrophage cell line was significantly reduced for these materials. The survival of the macrophage cell line in the presence of DAT(T)-functionalized gelatins was not influenced by both materials. Therefore, DAT- and DATT-functionalized gelatins were shown to have passed the tests concerning immunological responses important for their applicability *in vivo*.

Keywords: biocompatibility; biomaterial; gelatin; hydrogels; endotoxins

Introduction

Biomaterial-induced autoregeneration, which stimulates the endogenous patterns of tissue regeneration, is based on the temporary substitution of tissue by an implanted material, which is replaced over time by functional neo-tissue.[1] Therefore, the applied material has to act as a substitute for the extracellular matrix (ECM), and consequently has to provide mechanical and functional clues in this respect. One approach for such materials is the use of biopolymers from or derived from the ECM and to tailor their properties e.g. by the formation of polymer network systems, in which already small changes in the chemical structure can have a large effect on the macroscopic properties. Inherently, such materials provide adhesion sequences for cells important for the biological performance of the materials *in vivo*. Gelatin, which is produced by partial hydrolysis of collagen, has been shown to be a suitable starting material for the development of materials with tailorable properties as long as the random triple helix formation of gelatin chains is suppressed. This can be achieved by covalent

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crosslinking with diisocyanates,[2] but also by introducing the physically interacting aromatics desaminotyrosine (DAT) or desaminotyrosyl tyrosine (DATT) on free amino groups of the gelatin [3–4] (Figure 1).

While these materials with physical netpoints formed by π-π interactions and hydrogen bonds have mechanical properties relevant for biomedical applications, which can be tuned by processing[5] and formation of composites[6] for applications *in vivo*, it has to be assured that the materials are sterilizable, well-tolerated by cells, and contain very low endotoxin loads to avoid an unspecific activation of the immune system.

Endotoxins such as lipopolysaccharides (LPS), which are cell wall components of Gram-negative bacteria, belong to the strongest activators of the mammalian immune system. The receptors mediating such inflammatory responses in immune cells are the germline encoded Toll-like receptors (TLR), which sense a broad range of microbial products.[7] The activation of immune relevant cells via TLR4 by high concentrations of LPS can lead to severe pathologies such as septic shock syndrome.[8–9] The standard test to determine the endotoxin burden of biomaterials is the LAL test, which detects soluble LPS from Gram-negative bacteria.[10] However, other microbial products, such as bacterial DNA or fungal compounds, cannot be detected by this test but can induce an unspecific activation of the immune system similarly to LPS, which might lead to severe side effects after the implantation of contaminated biomaterials.[11] Furthermore, microbial products from Gram-positive bacteria, such as *Staphylococcus aureus* or *Listeria monocytogenes*, which are the causative reagents for pneumonia or meningitis, can also not be determined by the LAL assay.[10] Noteworthy, the cellular response strongly depends on the bacterial species from which the LPS is derived. However, the LAL test cannot distinguish between different LPS species and their subsequent cellular effects.[12] Furthermore, it needs to be considered that the activation of the same TLR can result in different cellular responses. For example, activation of macrophages via TLR4 leads to a strong pro–inflammatory response, which is characterized by the production of high levels IL-6 and TNFα.[13] In contrast, TLR4 activation of B cells induces the secretion of the anti–inflammatory cytokine IL-10 and, subsequently, such TLR4-activated B cells are able to suppress autoimmune reactions as well as immune responses to bacterial infections.[14–15] Therefore, the examination of the cellular response of distinct immune relevant cells is crucial for the evaluation of the immunogenic potential of promising biomaterial candidates. Newly synthesized biomaterials are usually directly assessed for their biocompatibility including haemo-, tissue-, and immuno-compatibility.[16] In some cases contamination with endotoxins can hardly be avoided. Inappropriate laboratory environments or contaminated starting materials are the major sources of endotoxin contaminations.[17]

Here, the DAT- and DATT-functionalized gelatins were prepared from different starting materials in a normal chemical laboratory or a cleanroom facility, respectively. These functionalized gelatins were evaluated for the resulting endotoxin con-

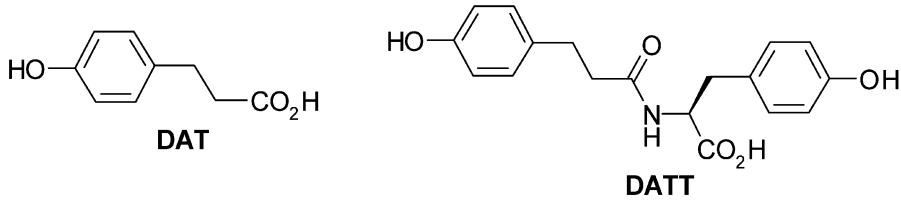

Figure 1.
Structures of desaminotyrosine (DAT) and desaminotyrosyl tyrosine (DATT).

tent determined by LAL tests as well as for their macrophage activation capacities, the influence on cell survival, and the induction of cytokine release from macrophages.

Materials and Methods

Functionalization of Gelatin

The synthesis of DATT was performed as published elsewhere.[4] Desaminotyrosine or desaminotyrosyl tyrosine (29 mmol) was activated by reaction with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) (32 mmol) and *N*-hydroxysuccinimide (NHS) (Sigma) (43 mmol) in 110 mL of dimethyl sulfoxide (DMSO) at 37 °C. After 3 h, β–mercaptoethanol (43 mmol) was added. A gelatin solution (15 g in 150 mL DMSO) was added and the mixture stirred at 37 °C for 5 h. The functionalized product was then precipitated in ethanol, filtered, washed with ethanol and acetone, and dried under vacuum. All synthesis steps were either performed under normal laboratory conditions or in the cleanroom. A 2,4,6-trinitrobenzenesulfonic acid (TNBS) colorimetric assay was performed to determine the degree of functionalization using a method described in the literature.[18] Gelatin (Type A, 200 Bloom, porcine, referred to as ''normal gelatin'') was obtained from Sigma Aldrich (Munich, Germany). The certified low endotoxin containing gelatin (Type A, 200 Bloom, porcine) was obtained from Gelita (Eberbach, Germany).

LAL Test

The material eluates were prepared according to ISO10993–12. In brief, 0.1 g of each material was incubated for 72 hours in MEM cell culture medium. The eluates were analyzed for LPS contaminations using the LAL test (Lonza, Cologne, Germany), which was performed according to manufacture instructions.

Macrophage Activation

In order to investigate the cellular response of macrophages, 5×10^5 RAW-Blue™ cells (InvivoGen, San Diego, USA) were cultured in 1 ml VLE-RPMI (Biochrom® Berlin, Germany) supplemented with 200 μg/mL Zeocin (InvivoGen, San Diego, USA) and 100 μg/mL of Primocin in a 24-well flat-bottom plate in the presence of DAT- or DATT-functionalized gelatin for 24 hours. After incubation, cell culture supernatants were harvested for cytokine secretion analysis, and cell viability was determined.

Macrophage Viability Determination

Cell viability staining was performed using fluorescein diacetate (FDA) and propidium iodide (PI) (both Sigma Aldrich) as described elsewhere.[19] In brief, after 24 h of incubation with DAT- or DATT-functionalized gelatins, 25 μg/mL FDA and 2 μg/mL PI were directly added to the cells and incubated for 3 min at room temperature. Each sample was evaluated at three different fields of view using a confocal laser scanning microscope (LSM 510 META, Zeiss) with the AxioVision (Zeiss) image analysis software.

Quantification of Cytokine Secretion

The secretion of murine IL-6 and murine TNFα (both BioRad®, München, Germany) was determined in cell culture supernatants of RAW-Blue cells incubated for 24 h with DAT- or DATT-functionalized gelatin. Both cytokines were quantified using the Bio-Plex® system (BioRad®, München, Germany). The Bio–Plex® assay was performed according to manufacture instructions.

Results

Gelatin was functionalized with DAT and DATT, respectively, in yields of 73 mol-% and 74 mol-%, respectively with respect to gelatin lysine residues[6] under cleanroom conditions to avoid endotoxin contamination during the synthesis. The degrees of functionalization by DAT and DATT for the materials synthesized in the cleanroom were slightly lower than for those synthe-

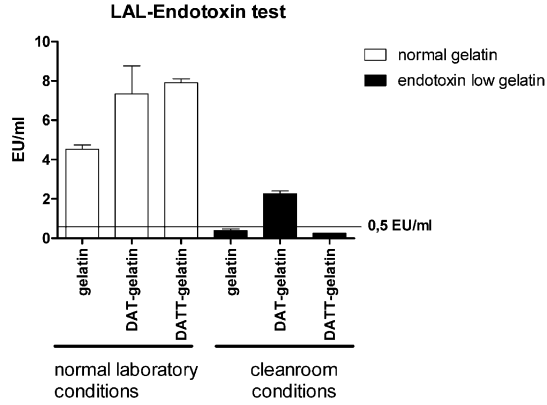

Figure 3.
Endotoxin contents of unfunctionalized and functionalized gelatin samples, determined by LAL test after incubating the samples with cell culture medium according to ISO10993–12 for 72 h. Results shown are pooled for two independent measurements.

sized in the chemical laboratory[4] (about 80 mol-%) as measured using the TNBS colorimetric assay.[18] The introduced tyrosine-derived side groups, DAT and DATT, led to the reduction of the residual helical conformation and to the formation of physical net-points by π-π interactions and hydrogen bonds.[4] The degree of swelling in water decreased with increasing the number of inserted aromatic functions (Figure 2), while Young's modulus, elongation at break, and maximum tensile strength increased.[4]

A LAL test was performed in order to compare the endotoxin burden of certified low endotoxin containing gelatin functionalized in the cleanroom with non-certified ('normal') gelatin that was functionalized under non-sterile laboratory conditions.

Figure 3 shows that using endotoxin low gelatin and performing the functionalization in the cleanroom resulted in a strongly reduced endotoxin load of the DAT(T)-functionalized gelatins compared to the reference materials that were synthesized under normal laboratory conditions.

Remarkably, the endotoxin level of DATT-functionalized gelatin could even be decreased to less than 0.5 EU/ml, which is the current US Food and Drug Administration limit for biomaterials.[17] These data indicate that the combination of a low endotoxin containing starting material and a cleanroom synthesis procedure reduced the final endotoxin level of the prepared biomaterials.

Since the LAL test only detects soluble LPS from Gram-negative bacteria, very limited information can be obtained about

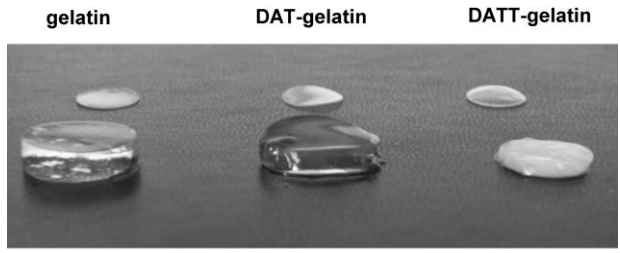

Figure 2.
Hydrogels (in front) and dry samples (back) of gelatin, DAT-gelatin, and DATT-gelatin (from left to right). The picture is representative for two independent material preparations.

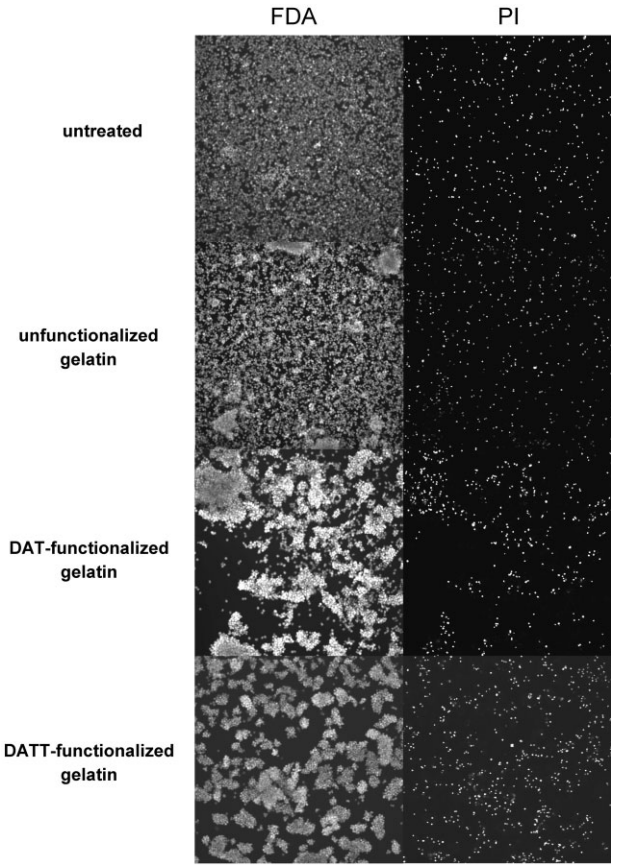

Figure 4.
RAW cell survival in the presence of low endotoxin containing unfunctionalized or functionalized gelatin. 5×10^5 RAW cells were incubated for 24 h in the presence of unfunctionalized, DAT–functionalized, or DATT-functionalized gelatin and stained with fluorescein diacetate (FDA, left), which labels live cells, and propidium iodide (PI, right), which indicates dead cells. Pictures were taken using a confocal laser scanning microscope. Representative pictures of two independent experiments are shown.

other potential microbial contaminations, such as products from Gram-positive bacteria or viral and fungal pathogens. Macrophages express a broad spectrum of TLRs and can thereby be activated by a broad range of such microbial products. Here, the murine macrophage cell line RAW-Blue™ was used to investigate the immunogenic potential of the functionalized gelatins. First, the effect that DAT- and DATT-functionalized gelatins have on the survival of murine macrophages was analyzed. Both functionalized gelatins did not induce a substantial cell death (Figure 4). Differences in cell survival between normal gelatin films prepared under normal laboratory conditions and low endotoxin containing gelatin films synthesized in the clean room could not be observed (data not shown). However, the macrophages changed their morphology and aggregation behavior on both types of gelatin. The reason for this effect could be microbial residues, which can not be detected by the LAL test but are still sufficient to induce a cellular response. Furthermore, gelatin fragments derived by the hydrolytic- or macrophage-mediated degradation could mimic ECM compounds, which might have induced this cell aggregation.

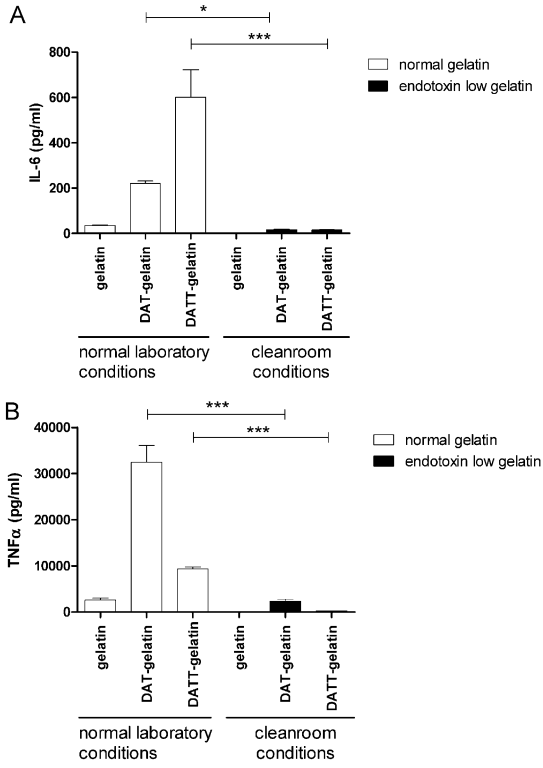

Figure 5.
Inflammatory cytokine response of RAW cells. 5×10^5 RAW cells were incubated for 24 h in the presence of unfunctionalized, DAT-functionalized, or DATT–functionalized gelatin. Supernatants were harvested after 24 h, and secreted IL-6 (A) and TNFα (B) were determined by Bio-Plex®. Data were pooled from two independent experiments. For statistical analysis the One-Way ANOVA test was performed (* for $p < 0.05$; *** for $p < 0.001$; mean ± SEM).

The activation of macrophages by endotoxins can result in the production of pro-inflammatory cytokines.[20] Here we examined whether DAT and DATT-functionalized gelatins could induce such a cytokine response in RAW-Blue cells. The production of IL-6 and TNFα was significantly reduced when graded certified gelatin with low endotoxin content was used and when all synthetic steps were performed under cleanroom conditions (Figure 5).

Discussion

The functionalization of gelatins with increasing numbers of tyrosine-derived aromatic groups resulted in a strong reduction of swelling capacity. This is a key property, which also led to increases in Young's modulus, elongation at break, and maximum tensile strength in the equilibrium swelling state.[4] These tailorable swelling and mechanical properties can be tuned for applicability in the biological environment.[6] Before newly developed materials can be used for medical applications or as medical devices, they have to be free of endotoxins, which otherwise can cause an unwanted immune reaction towards biomaterials, resulting in adverse side–effects in the patients or in rejection of the implants. Surprisingly, it was recently shown that even commercially available biomaterials or Titanium and Ti alloy prepared by implant manufactures contain

significant amounts of adherent endotoxins.[21–22] Furthermore, commercially available laboratory reagents have been found to contain endotoxins and induce cellular responses. For example, very high levels of LPS contaminations were found to be present in commercially available collagens and can lead to activation of dendritic cells, which play a central role during immune activation.[23] This finding is in agreement with our data, since we could show that commercially available gelatin, which is prepared from collagen, also contains high levels of LPS. However, when graded and certified gelatin was tested, the LPS contamination was strongly reduced.

Additionally, we analyzed the material-mediated macrophage activation. Besides their role in inflammation, macrophages also participate in the degradation of implants, in angiogenesis, and in tissue regeneration.[24] The activation of macrophages by LPS leads to a pro-inflammatory response and a subsequent T cells activation and interferon-γ (IFNγ) production.[13] With the help of IFNγ-producing T cells (T_H1) or natural killer cells, macrophages become fully activated and produce reactive oxygen species (ROS).[13] Such ROS have bactericidal capacities, but could also lead to tissue damage and may mediate the degradation of biomaterials, which subsequently impairs their functionality.[25] For this reason, the investigation of the cellular macrophages response is critical for the evaluation of the immunocompatibility of biomaterials. Our data show that when commercially available gelatin was functionalized under normal laboratory conditions, macrophages produce very high levels of IL-6 and TNFα, which would induce a strong pro-inflammatory response towards the material *in vivo*. However, when the functionalization was performed in the cleanroom and graded certified gelatin was used as starting material, the cytokine production by macrophages was significantly reduced. Both parameters, the starting material as well as the laboratory environment, have an impact on the endotoxin burden of biomaterials since we have previously observed that endotoxin contamination was still substantially high when low endotoxin containing gelatin was functionalized under normal laboratory conditions (data not shown).

The advantage of such cell-based endotoxin detection assay is that cells can get in direct contact with the material, which thereby allows the detection of material-bound endotoxins or other microbial products. Nevertheless it is still essential to perform the LAL test since the cellular effects can also be mediated by inherent material properties in addition to microbial products. In order to evaluate the immunogenic potential of any biomaterial, the investigation of the cellular response of immune relevant cells is essential. Here, the RAW macrophage cell line was used to obtain preliminary insights of the immunogenic potential of DAT- and DATT-functionalized gelatins. This RAW cell line is a murine leukaemic monocyte macrophage cell line, which is easy to handle, and since they express a broad range of pattern recognition receptors, these cells can be used to screen biomaterials for microbial contaminations. Furthermore, due to their adherent behavior, RAW cells get in direct contact with the material allowing the assessment of material-bound microbial products.[26] However, their limited clinical relevance makes the translation of data obtained from RAW cell experiments into the human system difficult. Therefore, further studies on human immune cells such as peripheral blood mononuclear cells (PBMC) or purified monocytes/macrophages, dendritic cells, T cells, and B cells will allow a conclusive evaluation of the immunogenic potential of DAT- and DATT-functionalized gelatins.

Conclusion

Here, the endotoxin contents of DAT- and DATT-functionalized gelatins were analyzed by the LAL test to detect soluble LPS from Gram-negative bacteria and by cell–

based macrophage activation assay. When normal gelatin was used and all synthetic steps were performed under normal laboratory conditions, our functionalized products showed substantial amounts of endotoxins, which were more than 15-fold above the United States Food and Drug Administration limit and led to strong macrophage activation. However, the use of low endotoxin containing gelatin and performing all synthesis steps in a cleanroom strongly decreased the endotoxin burden and almost completely abolished the macrophage activation. Therefore, DAT- and DATT-functionalized gelatins can be further studied as biomaterials and could therefore be suitable for the regeneration of functional tissues. Furthermore, our study indicates that the laboratory conditions that are used for biomaterial synthesis as well as the choice of starting material might have a strong influence on the endotoxin levels and should be considered when performing biomaterial synthesis.

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