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Immobilizing hydrolytic active Papain on biodegradable PLLA for biofilm inhibition in cardiovascular applications

Abstract:

The use of biomaterials in medicine is becoming increasingly important. One of the main concerns is the foreign body associated infection caused by direct microbial contamination or clinical infections. The bacterial biofilm formation on biomaterials depends on their surface properties. Therefore, several anti-adhesive surface modifications were developed. Nevertheless, the demand for antimicrobial agents that prevent bacterial colonisation is still largely unmet. The immobilization of active antimicrobial agents, such as antibacterial peptides or enzymes, offers a potential approach to achieve long-lasting effectiveness.

In this investigation, the hydrolytic enzyme papain with its published antibacterial activity was covalently immobilized on the well-established biodegradable biomaterial poly-L-lactic acid (PLLA). For the characterization of the enzymes on the PLLA surfaces, the protein content and enzyme activity were determined. A biofilm assay was performed to test the effect of the papain-modified PLLA samples on the biofilm-forming bacterial strain *Clostridioides difficile*, one of the most frequently occurring human nosocomial pathogens. The investigated hydrolytic enzyme papain could be immobilized by coupling via the crosslinker EDC to the PLLA surface. Detection was performed by determination of the amount of protein and the reduced biofilm growth after 24 h and 72 h compared to the reference.

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1 Introduction

The use of biomaterials in medicine, especially in the field of cardiology, is becoming increasingly important [1]. However, there are also limitations associated with their use. A main concern are foreign body associated infections. In US hospitals alone, the number of nosocomial infections was about 1.7 million in 2002, with 100,000 cases ending in death [2]. These infections can be caused by direct microbial contamination or clinical infections [3].

Although the number of contaminating bacteria is reduced by using aseptic surgical techniques, over 90% of implants still contain microorganisms [4]. The bacterial biofilm formation on biomaterials is highly dependent on their physical and chemical properties [5]. To inhibit biofilm formation, various antibacterial coatings have been developed to generate anti-adhesive surfaces by optimizing these properties. Hydrophobic surfaces and biomaterials coated with albumin or heparin have been described as causing less bacterial adhesion [6, 7]. Another strategy is the immobilization of active biocides as a drug delivery system (DDS), which leads to the elimination of the bacteria. Although some of the strategies are appropriate for specific applications, there is still a high demand for antimicrobial agents that prevent bacterial colonisation [8]. Therefore, further development of functionalised implant surfaces with a biofilm inhibiting effect is essential [5]. The immobilization of antimicrobial agents, such as antibacterial peptides or enzymes, offers a successful approach to achieve long-lasting effectiveness [9].

In the context of this work, the hydrolytic enzyme papain, with published antibacterial activity [10], was covalently immobilized on the well-established biomaterial poly-L-lactic

acid (PLLA) [11]. For the characterization of the enzymes on the PLLA surfaces, techniques for the determination of protein content and enzyme activity, normally developed for applications in solution, have been adapted. Furthermore, a biofilm assay was performed to test the effect of the papain modified (PLLA) samples on the biofilm-forming bacterial strain *Clostridioides difficile*. This strain is ubiquitously distributed in nature and is one of the most frequently occurring human nosocomial pathogens [12].

2 Materials and Methods

Poly-L-lactide (PLLA, L210) was purchased from Evonik (Schwerte, Germany) and films were prepared according to with a thickness of about 100 µm [13]. The chemicals 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), PBS buffer, Tween20, ethanol, *tris*(hydroxymethyl)-aminomethane (TRIS), bicinchoninic acid (BCA) kit, L-Cystein-HCL, yeast extract, crystal violet and sodium lauryl sulfate (SDS) were purchased from Sigma Aldrich (Munich, Germany). BHI (Brain Heart Infusion) Broth was purchased from HIMEDIA Laboratories (Einhausen, Germany).

2.1 Papain immobilization

After washing with 70% ethanol and ultrapure water the PLLA surfaces were activated using a radio frequency (RF) plasma generator (frequency 13.56 MHz, Diener electronic GmbH & Co. KG, Ebhausen, Germany) with oxygen plasma for 30 sec at 10 W and 0.3 mbar according to [13] generating functional carboxyl groups. Samples were shaken for 60 min at 300 rpm at room temperature in a 0.2 M EDC/NHS PBS solution (pH 7.4), washed three times with PBS and finally incubated for 130 min in PBS with papain (5 µg/mL) at 300 rpm and room temperature. Samples were finally washed with TRIS and PBS with 0.05% w/w Tween20. Samples for biological studies were disinfected after plasma activation with 70% ethanol for 5 min and sterile filtered solutions under a sterile bench were used.

2.2 Determination of papain amount

The determination of the amount of protein was performed with a BCA kit according to the manufacturer's instructions. The protocol was adapted for the PLLA films as enzyme carriers. The PLLA samples (Ø=15 mm) were cut and transferred to a well of a 96-microtiter plate. After addition of 200 µL of the specified reagent solution, the samples were incubated for 40 min at 60°C. 100 µL of the supernatant solution were then removed and transferred to a new well. The absorbance of the samples was measured with the microplate reader FLUOstar Omega (BMGLabtech, Germany) at a wavelength of 540 nm. Bovine serum albumin (BSA) was used as standard. Three independent experiments (n=3) were performed, where three samples were measured in each case. As reference samples were treated the same way like for the papain immobilisation, but no enzyme was added in PBS after EDC/NHS incubation.

2.3 Determination of Papain activity

The determination of the enzyme activity of papain was performed with a Pierce Colormetric Protease Assay Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The protocol was adapted to the PLLA discs (Ø=15 mm). One disc was cut and transferred to a well of a 96-microtiter plate. A succinylated casein solution of 100 µL (kit) was added and incubated for 20 min at room temperature. Then, 50 µL trinitrobenzene sulfonic acid solution (kit) were added and the samples were incubated for another 20 min at room temperature. 100 µL of the supernatant solution were transferred to a new well of a 96-microtiter plate and the absorbance at 450 nm was measured. Three independent experiments (n=3) were performed, where three samples were measured in each case. Same samples like for the determination of papain amount were used as reference.

2.4 Biofilm inhibition test

The *Clostridioides difficile* VPI 10463 bacteria culture was cultivated in Brain Heart Infusion supplemented with 5 g/l yeast extract and 0,1% L-cysteine-HCL (BHIS). All experimental steps concerning bacterial growth and biofilm formation took place under anaerobic conditions (nitrogen atmosphere with max. 5% hydrogen) at 37°C. For biofilm cultivation, an overnight grown culture was adjusted to an optical density at 600 nm of 0.01. The microbiological investigation of the biofilm inhibiting effect of the papain modified PLLA films was based on the crystal violet staining. Enzyme coupling was performed under aseptic conditions as described.

First a PLLA disc was incubated with 1 mL of the *C. difficile* bacteria suspension in a 24-microtiter plate for either 24 or 72 h. For the samples with 72 h incubation the medium was changed after 24 h and 48 h. After incubation, the supernatant was removed, and the resulting biofilm was

washed with 1 mL PBS. The biofilms were stained with 400 µL of a 0.1% crystal violet solution for 15 min at room temperature. The excess crystal violet solution was discarded, and the biofilm was washed twice with PBS. Solubilisation of the biofilm was performed in 1 mL of a 1% SDS solution for 20 min at room temperature while shaking. 20 µL of the solution were transferred to a 96-microtiter plate, diluted 1:5 with the used SDS solution and the absorbance at 540 nm was measured. Three independent experiments (n=3) were performed, where four samples were measured in each case. Same samples like for the determination of papain amount were used as reference.

2.5 Statistical analysis

Statistical analysis for significance ($p < 0.05$) were performed with GraphPad Prism® Version 6 using the unpaired two-sided t-test.

3 Results and Discussion

The results for the amount of immobilized papain on one PLLA sample (Ø=15 mm) was significant higher with 33.6 ± 17.3 µg papain per sample in comparison to the reference (plasma activated and with EDC/NHS treated without papain coupling) with 26.8 ± 9.9 µg per sample (see Table 1). The high results for the reference can be caused by interactions occurring on the PLLA surface. Li *et al* [14] also showed increased protein amount results on even unmodified PLLA microcarriers in a BCA test.

Table 1: Results of the determination of papain mass (n=3) and mass of active papain (n=3) on modified PLLA discs (Ø=15 mm) with and without (reference) papain. * are significant from reference.

Characterization	Papain-EDC/NHS-PLLA	EDC/NHS-PLLA
Mass papain	33.6 ± 17.3 µg/sample*	26.8 ± 9.9 µg/sample
Mass active papain	9.2 ± 0.4 µg/sample	9.0 ± 0.7 µg/sample

The analysis of active papain reveals an amount of 9.2 ± 0.4 µg per sample which is not significant different from the reference (plasma activated and with EDC/NHS treated without papain coupling) with 9.0 ± 0.7 µg per sample (see Table 1). The small difference in amount of active papain seems to be due to the type of immobilization, which took place in two steps and thus prevented cross-linking of the papain, which could have resulted in a higher immobilised amount.

The reduction of the biofilm compared to the reference was significantly at 24 h (24.8%) (see Figure 1). After 72 h is a slightly reduction still to observe (10.0%). A possible reason for the decreasing effect over the time may be the low stability of the enzyme or the low immobilized amount of papain (see Table 1).

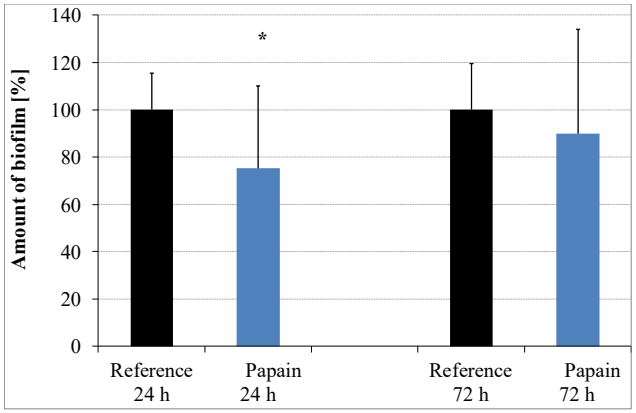

Figure 1: Results for the *in vitro* determination of biofilm amount from *Clostridioides difficile* VPI 10463 after crystal violet staining and measurement at 540 nm. Reference (modified PLLA without immobilized papain) were normalized to 100% and compared with the immobilized papain samples after 24 h and 72 h (n=3). * are significant from reference.

4 Conclusion

It was demonstrated that the investigated hydrolytic enzyme papain could be immobilized by coupling via the crosslinker EDC to the PLLA surface and has a significant biofilm reducing effect for *C. difficile*. The biofilm reducing effect decreases over time but is still observable after 72 h. Furthermore, proof of papain coupling was performed by the determination of the amount of protein and the reduced biofilm after 24 h and 72 h Therefore we also demonstrate the successful adaption of the BCA-Assay for immobilized papain on PLLA films and a suitable bioassay for analysing the biofilm inhibition.

To reduce infections of cardiovascular implants by enzyme surface modifications EDC as crosslinker is suitable. However, this requires determination of the different bacterial species causing the infections as well as suitable enzyme mixtures in combination with active agents from a drug delivery system for an effective antibacterial coating.

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