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Fabrication of biomimetic antibacterial titanium surfaces by hydrothermal oxidation

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Abstract: Within this paper we demonstrate a novel approach to create antibacterial nanostructures on the implant material titanium grade 23 by a high-pressure oxidation method. Titanium samples were oxidized and resulting nanostructures were characterized using scanning electron microscopy and contact angle measurements. Antibacterial properties were tested using Escherichia coli and Bacillus subtilis as common lab strains. In addition, cytotoxicity was determined according to ISO 10993-5 standards. Results reveal that the fabricated nanostructures have similar antibacterial properties known from different insect wings like dragonflies or cicadas and have no cytotoxic effect.

Keywords: Dental implants, titanium grade 23, antibacterial implant surface, dental abutment.

1 Introduction

The wings of different dragonflies and cicada species exhibit interesting nanostructures which show solely structural antibacterial effects [1]. Such effects can also be transferred to different materials like silicon or titanium by a wide variety of methods [1,2]. For the implant abutment material titanium grade 23 (Ti6Al4V ELI) one possible method is a special hydrothermal oxidation method well known from the fabrication of single crystals for the semiconductor industry. Within such a process, the samples are oxidized at elevated temperatures and high pressures within an aqueous solution like a weak sulfuric acid or other chemicals.
This process has so far been little used for titanium and its full potential remains unclear.

Therefore, this study was conducted to demonstrate the ability of such a process to treat the implant materials titanium grade 23 and to demonstrate the overall applicability of such a surface technology for modern implantology showing its antibacterial effects and biocompatibility.

2 Materials & Methods

2.1 Sample Preparation

Titanium grade 23 samples were prepared as described earlier [3]. In summary, samples were cut from a bar (diameter of 8 mm, length 1 m) into individual disks with a thickness of 2 mm each by wire cutting. Samples were then ground with silicon carbide grinding paper and subsequently polished to a mirror like finish. A subset of samples was put aside as reference samples (untreated) and another subset of samples was oxidized in a high-pressure autoclave as earlier described (oxidized).

2.2 Sample Characterization

2.2.1 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) analysis was performed using high voltages between 1.5 and 10 kV at working distances in the range of approx. 2 to 8 mm (Supra VP 60, Zeiss, Germany). No additional coating was applied.

2.2.2 Contact Angle Measurements

Contact angles were measured using the sessile drop method. A droplet volume of 5 µl of DI-water was used (OCA50, Data

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Physics, Germany). The droplet was carefully placed on the surfaces and the measurement routine was kept strictly to reduce errors due to evaporation of the liquid, air flow or induced by the operator.

2.3 Biological Testing

2.3.1 Bacterial Tests

Bacterial tests were performed using *Escherichia coli* (*E. coli* DSM498) and *Bacillus subtilis* (*B. subtilis*, DSM402), two typical model strains representing gram-negative and gram-positive rod-shaped, mobile bacteria strains. The antibacterial properties were determined after initial bacterial adhesion as described earlier in detail [3]. In brief, samples were incubated in a bacterial solution containing a set amount of bacteria of *E. coli* or *B subtilis* respectively for 2.5 h at 37°C. After incubation samples were washed with PBS to remove non-adherent bacteria. For microscopic evaluation bacteria were stained with DAPI for 30 min in the dark and afterwards fixated in 4% Formaldehyde. Cell count was determined by fluorescence microscopy using 6 individual images per sample adequately covering the sample surface.

2.3.2 Cytotoxicity Testing

Cytotoxicity was evaluated in accordance to ISO 10993-5 using a HL-60 cell line (300209, CLS Cell Lines Services GmbH). The cells were cultivated at 37°C with 5% CO_2. The culture medium consisted of RPMI 1640 (Thermo Fischer) with the addition of 2 mM L-glutamine, 10% fetal calf serum (TCS), and 1% of a concentrated mixture (10,000 IU/ml) of penicillin and streptomycin. Cells were cultivated in individual wells of a 24 well plate on a gelatine layer for adhesion promotion.
Titanium samples were sterilized in ethanol (25%, 50% and 75%) and rinsed with phosphate buffered saline (PBS) before they were placed on the cell lawn upside down. After 24 h, the samples were removed and the cells were stained replacing the culture medium with 500 µl PBS (containing 0.25 µMol calceine and 0.5 µMol ethidium homodimer) for 30 min in the absence of light. After staining fluorescence microscopic analysis was performed. Five images were taken per cavity in both red and green channels and cells were counted manually using the software ImageJ.

2.3.3 Statistical Analysis

For statistical evaluation, the software OriginPro 2018b (OriginLab Corporation, MA) was used. Statistical differences have been analysed using one way ANOVA and Turkey's test to show individual differences. Significance level was set to $p < 0.05$.

3 Results & Discussion

The results of surface characterization before and after oxidation and images of contact angle measurements are shown in **Fig. 1**. The topography in case of the untreated titanium samples is flat and smooth without larger distortions or higher surfaces roughness. In contrast, the oxidized samples show evenly distributed nanocrystals on top which makes the fabrication method very suitable for the fabrication of nanostructured implant surface.

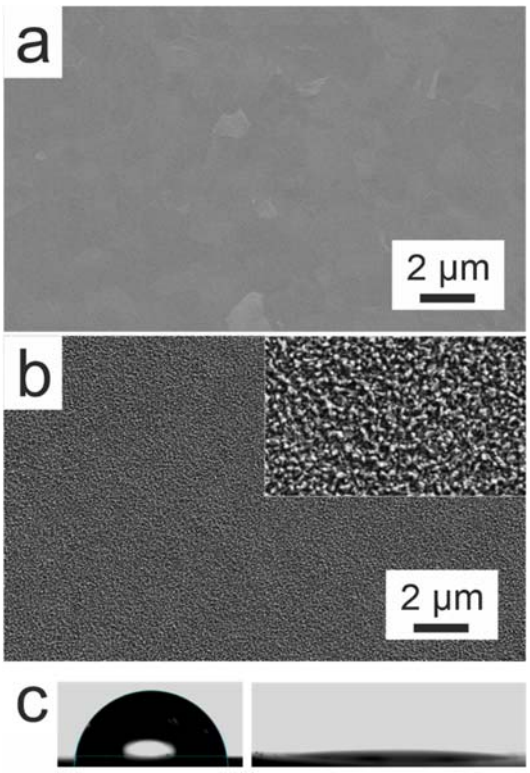

Figure 1: Results of surface characterization before (a) and after (b) oxidation and images of resulting contact angles (c) for the untreated sample (left) and the oxidized sample (right).

The contact angles for the untreated titanium samples were determined to be 74.7±0.5°. For the oxidized titanium samples the contact angles were not measurable due to a super hydrophilic behaviour (compare Fig.1 c).

Besides surface characterization the results of bacterial testing are shown in **Fig. 2**. The results are shown as normalized cell counts per area. Compared to the untreated surfaces, which represent the current state of the art in dental abutment design, a significant reduction of up to 90.4±2.3% was found for the gram-negative strain *E. coli* and a reduction of up to approx. 55±6.7% was found for the gram-positive strain *B. subtilis*.

Fig. 3 shows the results of cytotoxicity testing according to ISO 10993-5. There could be found no signs of cytotoxic effects for the oxidized titanium sample or the untreated sample.

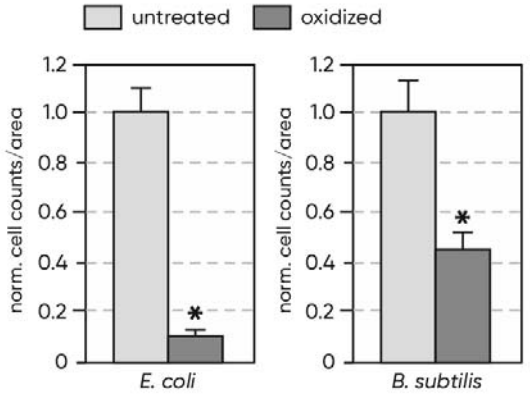

Figure 2: Results of bacterial testing of untreated and oxidized titanium samples (left: *E. coli*. right: *B. subtilis, * p < 0,05*).

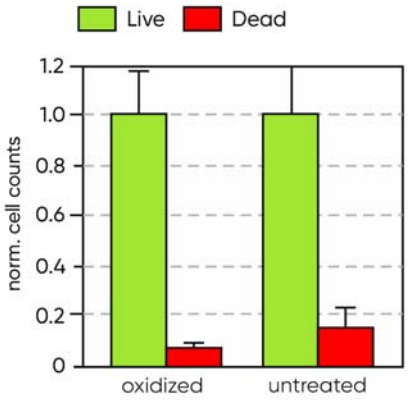

Figure 3: Results of cytotoxicity tests according to ISO 10993-5 showing normalized amounts of live/dead cells on untreated and oxidized titanium.

The presented hydrothermal oxidation method allows the fabrication of titanium nanostructures evenly on the whole sample surface (compare Fig. 2 b). These structures show an antibacterial effect for both tested strains, the gram-negative *E. coli* as well as for the gram-positive *B. subtilis*. These results are in line with recent findings that such kind of nanostructures show higher antibacterial effects for gram-negative than gram-positive strains [1-3].

Besides such in-vitro findings, the question arises, if such effects can also have a positive outcome on the treatment of patients and might lower the risk of implant infections like peri-implantitis. To answer such a question, further studies and ultimately clinical studies need to be performed. In general, nanotechnology offers interesting possibilities for future optimization of medical implants.

4 Conclusion

The demonstrated hydrothermal oxidation method is a promising way to fabricate antibacterial nanostructures on the implant material titanium grade 23. The nanostructures can be generated evenly on the whole sample surface. Especially for the gram-negative strain *E. coli* the reduction of the bacterial contamination was found to be approx. 90% while not showing cytotoxic effects. Overall these results are promising for the development of novel implant surfaces especially in oral implantology for dental abutment surfaces.

Author Statement

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