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Evaluation of *Streptococcus oralis* adhesion and biofilm formation on laser-processed titanium

Abstract: To prevent implant-associated infections, surface modifications need to be developed that prevent bacterial colonisation and biofilm formation. In the present study, titanium surfaces were processed by nanosecond-pulsed laser ablation to generate a variety of different structures (anatase, rutile, Osteon, as well as Osteon additionally coated with silver and clove nanoparticles). Analysis of adhesion and biofilm formation of the oral pioneer bacterium S*treptococcus oralis* could demonstrate antibacterial properties of anatase surfaces. For clinical translation, the effect should be enhanced by further adaption and combined with the osseointegrative Osteon structure.

Keywords: antibacterial surface, titanium, laser processing, anatase, *Streptococcus oralis*, biofilm.

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1 Introduction

Dental implants are well established to replace missing teeth, thereby restoring functional occlusion and aesthetic appearance. However, they suffer from a comparable high rate of implant-associated infections [1]. Due to their exposed transmucosal position in the oral cavity, they are prone to bacterial colonisation followed by adverse immune reaction, which can lead to soft tissue loss (peri-implant mucositis) or even hard tissue loss (peri-implantitis). Bacterial colonisation in the oral cavity starts with the adhesion of commensal pioneer bacteria, like *Streptococcus oralis*, which induce the formation of a biofilm [2]. Through growth and incorporation of further – also pathogenic – bacterial species a three-dimensional agglomerate surrounded by a self-produced matrix made from polymeric substances arises. These biofilms are inherently resistant against immune defence and antibiotic therapy [3].

To avoid biofilm development, current research focusses on the development of innovative titanium implant surface modifications that prevent bacterial attachment – either by antiadhesive or antibacterial properties. At the same time, bone tissue integrative characteristics should be preserved or even enhanced. As metallic dental implants are comparable hard and exhibit complex geometries, surface modification requires a versatile technology. Pulsed laser ablation has already been used for multiple different metal surface processing approaches [4-7]. Here, the surface is irradiated with short laser pulses, which leads to sublimation of the topmost material layer resulting in desired surface modifications.

The aim of the present study was to analyse the effect of different laser-processed titanium surfaces on the adhesion and biofilm formation of *Streptococcus oralis*. An ytterbium nanosecond-pulsed fibre laser was used to generate different surface crystal structures (anatase and rutile) as well as bone-biomimetic Osteon structures with additional adsorbed nanoparticles. Bacterial colonisation of these surfaces was evaluated using fluorescent staining and confocal laser-scanning microscopy.

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2 Material & methods

2.1 Laser-assisted surface modification

The commercially available laser technology complex MiniMarker2™ (Laser Center, Saint-Petersburg, Russia), based on a pulsed ytterbium fiber laser (wavelength $\lambda = 1.064$ µm, pulse duration for this experiment $\tau = 100$ ns, spot diameter in focus 50 µm), was used for surface processing. One flat side of Ti-6Al-4V discs (Ø 6 mm, height 2 mm) were processed by laser radiation. The first group of samples "Anatase" was made using average power of P = 18.8 W, scanning speed of $V_s = 150$ mm/s, repetition rate of $f = 900$ kHz and a minimum scanning step size along the y-axis of $M_Y = 50$ µm. The second group of samples "Rutile" was made using P = 18.8 W, $V_s = 350$ mm/s, $f = 900$ kHz and $M_Y = 50$ µm. The chemical composition and oxide film thickness of "Anatase" and "Rutile" are presented in our previous work [8]. The third group of samples "Osteon" was made using P = 4.5-6.5 W, $V_s = 190$ mm/s, $f = 50$-60 kHz and $M_Y = 8$ µm. A detailed description on Osteon relief's formation will be given in our upcoming work [9]. Samples of "Osteon" group were further modified: "Osteon AgNP" and "Osteon CloveNP" were coated with silver and clove nanoparticles, respectively. Both nanoparticles were made using laser ablation in liquid technique, as described in [10], and complex MiniMarker2®. Distilled water was used as a liquid environment and dried cloves and silver plates were used as nanoparticle source. Colloidal solutions of clove and silver nanoparticles were applied in the form of drops on the surface of "Osteons" under ambient conditions and dried in air. "Osteon Anatase" samples were generated by a second laser radiation following the laser mode with P = 6.5 W, $V_s = 200$ mm/s, $f = 60$ kHz and $M_Y = 50$ µm. Polished Ti-6Al-4V discs served as "Control" group. Ten samples per type (N = 4 per experiment) were generated. Prior to experiments, Anatase, Rutile and Osteon Anatase samples were sonicated in distilled water for 90 min at 37 °C. All surfaces were sterilized by UV irradiation for 20 min.

2.2 Bacterial adhesion and biofilm formation

Streptococcus oralis (ATCC 9811, American Type Culture Collection, Manassas, USA) was pre-cultivated in Todd-Hewitt-Broth (Oxoid Limited, Hampshire, UK) supplemented with 10 % yeast extract (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 20 hours under agitation. Bacteria were harvested and adjusted to an optical density at 600 nm of 0.05 in phosphate-buffered saline (PBS, Sigma-Aldrich®, St. Louis, USA). To analyse initial adhesion, structured specimens were incubated in bacterial suspension for 5 hours at 37 °C and 5 % CO_2 under continuous rotation with 300 rpm. For subsequent biofilm formation, bacterial suspension was replaced by tryptone soy broth (Oxoid Limited) supplemented with 10 % yeast extract and 50 mM glucose (Carl Roth) and specimens were incubated for a total of 24 hours at 37 °C and 5 % CO_2 without agitation.

2.3 Fluorescence staining and confocal laser-scanning microscopy

Structured specimens with adhering bacteria or developed biofilms were rinsed once with PBS. For fluorescent staining, the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Life Technologies, Darmstadt, Germany) was used with the fluorescent dyes Syto®9 and propidium iodide diluted 1:2000 in PBS, each. Bacteria were fixed in 2.5 % glutardialdehyde and kept in PBS for microscopy. Microscopy was done using a confocal laser-scanning microscope (TCS SP8, Leica Microsystems, Mannheim, Germany) with a 488 nm excitation laser line and emission detection at 500-550 nm for Syto®9 and a 552 nm excitation laser line and emission detection at 650-750 nm for propidium iodide. Per specimen, five images at different positions were taken with z-step-size of 3 µm and 630x and 400x magnification for initial adhesion and biofilm formation, respectively. For initial adhesion, the ImageJ software (version 1.48v and WCIF ImageJ, Wayne Rasband, National Institute of Health, USA) was used to calculate area fraction colonized by bacteria and live/dead distribution. For biofilm formation, the Imaris software package (version 8.1.2, Bitplane AG, Zurich, Switzerland) was used to quantify biofilm volume and live/dead distribution. GraphPad Prism software (version 8.4.2, GraphPad Prism Software Inc., La Jolla, USA) was used for statistical analysis and data visualisation. Data were standardised to Control samples and were analysed for normal distribution by D'Agostino & Pearson Omnibus normality test. To identify significant differences, adhesion results were tested using mixed-models analysis (REML) with Dunnett's multiple comparison correction, biofilm results were evaluated using Friedman test with Dunn's multiple comparison correction; and live/dead distributions were tested with Two-way ANOVA with Dunnett's multiple comparison correction. Significance level was set to $\alpha = 0.05$ and is indicated by *.

3 Results

The different laser-processed surfaces showed uniform structures as presented in Fig. 1. Whereas Anatase and Rutile are of nanometre scale, Osteon samples exhibit surface geometries of micrometre scale. For the following bacteria experiments, Anatase and Rutile were directly compared to Control, while Osteon modifications were compared to the unmodified Osteon surface.

Quantification of adhering bacteria showed a clear increase on all Osteon samples compared to Control (Fig. 2A). However, neither differences between Anatase/Rutile compared to Control nor between modified compared to unmodified Osteon could be detected. Analysis of bacterial membrane integrity showed 80-90 % living cells on all structures. Only the comparison between Osteon Anatase and unmodified Osteon yielded a slight but significant decrease in living cells.

For biofilm formation, a significant decrease in biofilm volume (approx. 50 %) compared to Control could be detected on Anatase but not on Rutile samples (Fig. 2B, C). For Osteon samples, the biofilm volume again increased to Control. When comparing the modifications to unmodified Osteon, mean variation of values was reduced, especially on Osteon AgNP and Osteon Anatase, but did not reach statistical significance. Regarding membrane integrity, all biofilms showed approx. 70 % living and 30 % dead cells.

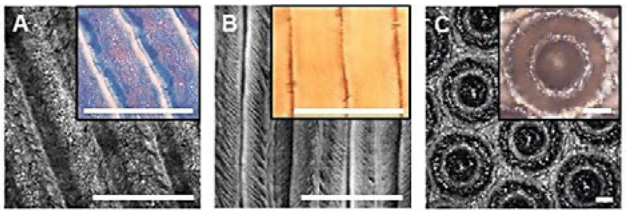

Figure 1: Representative images of laser-processed titanium surfaces (grey images generated by CLSM reflection mode, colorful images generated by light microscopy): A) Anatase, B) Rutile, C) Osteon. Scale bar = 100 µm.

4 Discussion

Aim of the present study was to analyse the effect of different laser-processed titanium surfaces on adhesion and biofilm formation of the oral pioneer bacterium *Streptococcus oralis* to identify possible candidates for infection-resistant implant modifications.

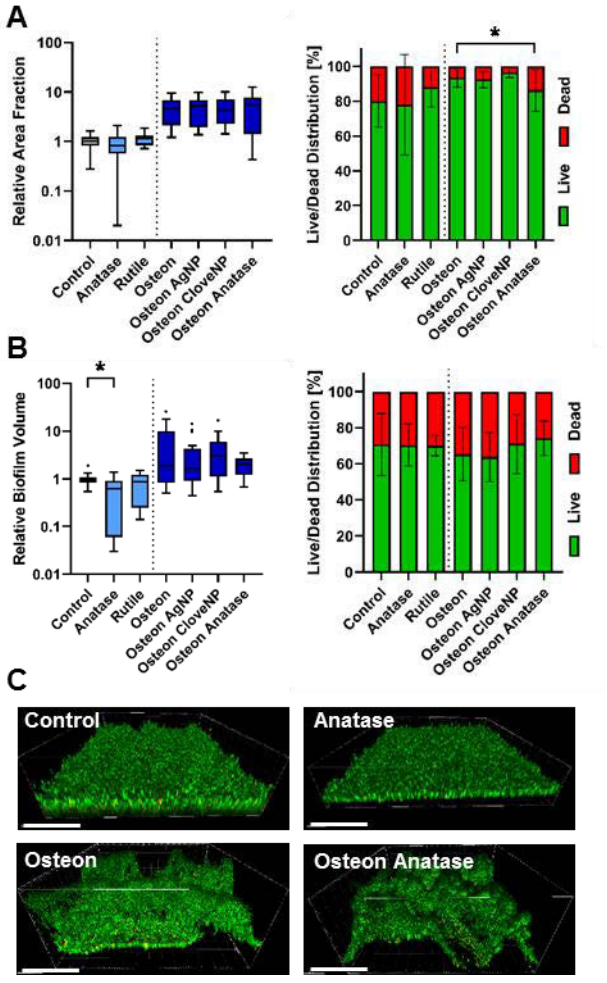

Figure 2: Bacterial adhesion and biofilm formation on laser-processed titanium surfaces: Tukey box plots of A) relative area fraction covered by adhering bacteria or B) biofilm volume and respective mean ± standard deviation of live/dead distribution. * indicate statistically significant differences. C) Representative 3D-reconstructed CLSM images of biofilms. Living bacteria are shown in green; dead bacteria in red/orange. Scale bar = 100 µm.

Titanium dioxide is a semiconductor and its crystal morphotypes anatase and rutile exhibit high photoactivity upon irradiation with UV light. This leads to formation of hydroxyl radicals, which stepwise disrupt bacterial membranes and finally completely mineralize dead cells [11]. Previous studies could show approx. 50 % reduction in oral bacterial colonisation on anatase coated substrates [12-14]. In the present study, the antibacterial effect of directly laser-generated anatase surfaces was demonstrated, with a similar extend as coated surfaces. As in previous studies, anatase showed stronger antibacterial properties than rutile, which is mainly attributed to higher adsorption capacity and greater hole trapping [11; 14]. Future studies with increased group sizes should address how the antibacterial effect of anatase

could be further increased to already reduce adhering bacteria, e.g., by combining anatase and rutile crystals and elongated UV exposure times [11; 14].

Osteon structures are biomimetic surfaces of compact bone with the main objective to enhance mesenchymal stem cells attachment, proliferation and its osteogenic differentiation for successful long-term tissue integration [15]. Due to their increased surface area available, they inherently increase bacterial adhesion likewise – as could be observed in this study. Thus, equipping these structures with antibacterial properties is of particular importance. Silver (i.e., its ions) and clove (i.e., the essential oil eugenol) are well established antibacterial agents in medicine [16; 17]. However, the local concentration used in this study was most probably not sufficient to achieve antibacterial effects. Osteon Anatase slightly increased the proportion of dead cells but did not reduce bacterial colonisation. Nevertheless, if the photoactive effect could be enhanced on the flat surfaces as described above, an antibacterial effect on the more complex Osteon structure could be achieved. As such surface would exhibit osseointegrative and antibacterial properties by physical surface modification only – which would be of great advantage for medical device approval – further studies should be conducted with this focus.

5 Conclusion

In the present study, an antibacterial effect of laser-generated titanium anatase could be demonstrated on biofilm formation of oral pioneer bacterium *Streptococcus oralis*. For application on dental implants, further efforts should be taken to enhance this effect. Ideally, it should be combined with osseointegrative Osteon structure to achieve an infection resistant implant solely by physical surface modification.

Author Statement

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