Letter

Katharina Doll[a,*], Elena Fadeeva[a], Nico S. Stumpp, Sebastian Grade, Boris N. Chichkov and Meike Stiesch

Reduced bacterial adhesion on titanium surfaces micro-structured by ultra-short pulsed laser ablation

DOI 10.1515/bnm-2015-0024
Received December 21, 2015; accepted March 15, 2016; previously published online April 12, 2016

Abstract: Implant-associated infections still pose serious problems in modern medicine. The development of fabrication processes to generate functional surfaces, which inhibit bacterial attachment, is of major importance. Sharklet™-like as well as grooves and grid micro-structures having similar dimensions were fabricated on the common implant material titanium by ultra-short pulsed laser ablation. Investigations on the biofilm formation of *Staphylococcus aureus* for up to 24 h revealed similarly reduced bacterial surface coverage on all micro-structures investigated compared to smooth titanium surfaces. This study is a prove-of-principle and could serve as basis for further investigations towards a structure-based biofilm-inhibiting implant.

Keywords: implant-associated infection; micro-structured surface; reduced bacterial adhesion; Sharklet™ surface; ultra-short pulsed laser ablation.

Despite enormous advance in modern implant medicine, the prevalence of patients suffering from implant-associated infections is still in the order of 20% [1]. These infections are caused by bacterial biofilms, a sessile multi-microbial community surrounded by self-produced extracellular polymers. Intrinsic resistance to antibiotic agents and host immune defense mechanisms make the treatment of biofilms difficult [2]. Implant research aims at avoiding biofilm formation from the early beginning by developing novel implant materials, which inhibit bacterial adhesion and growth. A multitude of chemical implant functionalizations, ranging from metallic nanoparticles to antimicrobial polymers, were already reported to inhibit microbial colonization *in vitro* [3–6]. However, chemical modifications may be an obstacle for medical device approval. This problem could be avoided by creating bacteria-repellent properties solely from surface structuring. A micro-structure design with potential anti-adhesive effects is the Sharklet™ surface. It is inspired by the skin of fast-moving sharks and consists of diamond-shaped, geometrically ordered micro-structures [7]. It was shown that Sharklet™ surfaces produced from poly(dimethyl siloxane) elastomers (PDMS) reduce bacterial adhesion of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Mycobacterium abscessus* [8–12]. Moreover, the structure also decreases attachment of algal spores, blood platelets, endothelial and epithelial cells [11, 13, 14].

The aim of the present study was to generate a Sharklet™-like micro-structure on the common implant material titanium and to assess bacterial biofilm formation on this surface. The exact mechanism of the Sharklet™ effect has not yet been completely understood. It is hypothesized that particular arrangement of topographical features with appropriate dimensions create a stress gradient within the cell membrane during initial contact resulting in lower attachment rates [15]. Therefore, in addition to Sharklet™, groove and grid structures, which have similar dimensions, were examined. All structures were generated by ultra-short pulsed laser ablation. SEM images of micro-structured titanium are shown in

[a]**Katharina Doll and Elena Fadeeva:** These authors contributed equally to this work
***Corresponding author: Katharina Doll,** Stadtfelddamm 34, 30625 Hannover, Germany, Phone: +49 (0)511-532 1421, E-mail: Doll.Katharina@mh-hannover.de; and Department of Prosthetic Dentistry and Biomedical Materials Science, Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hannover, Germany
Elena Fadeeva and Boris N. Chichkov: Department of Nanotechnology, Laser Zentrum Hannover e.V., Hannover, Germany
Nico S. Stumpp, Sebastian Grade and Meike Stiesch: Department of Prosthetic Dentistry and Biomedical Materials Science, Hannover Medical School, Hannover, Germany

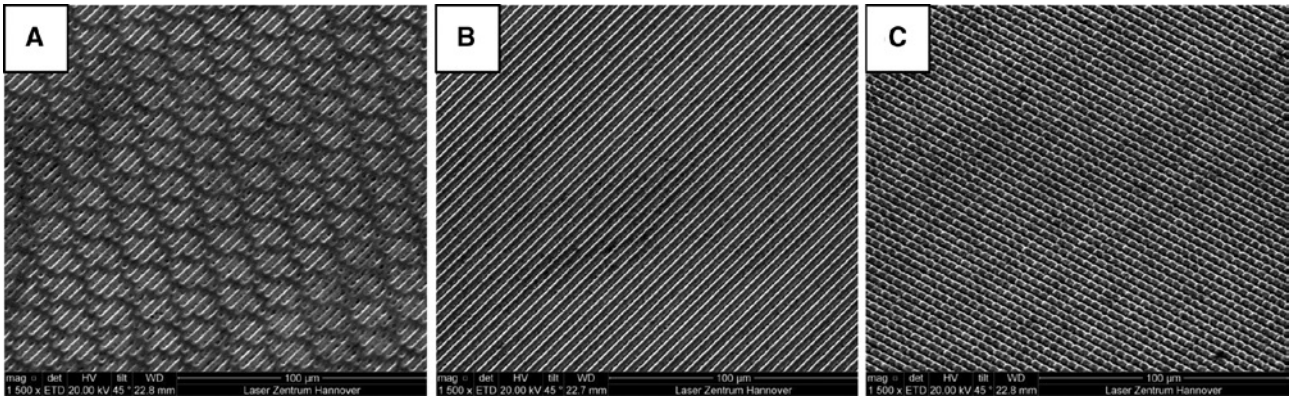

Figure 1: SEM images of analyzed micro-structures.
All micro-structures were generated on disk shaped titanium specimen (grade 4, Ø 12 mm) as 1×1 mm squares by ultra-short pulsed laser ablation. For surface machining of titanium samples, a commercially available amplified Ti-Sapphire femtosecond laser system (Femtopower Compact Pro, Femtolasers Produktions GmbH, Vienna, Austria) was used. This system delivers sub-30-fs pulses at 800 nm wavelength with energy of up to 1 mJ, and a repetition rate of 1 kHz. Three different structural designs were implemented: (A) the Sharklet™ like design consisted of 2 µm wide grooves of varying lengths (from 4 µm to 16 µm). These grooves were arranged into a periodic, diamond-like pattern at a fixed spacing of 2 µm between adjacent features. The grooves depth was ≥2 µm; (B) linear grooves of 2 µm width, >=2 µm depth and a fixed spacing of 2 µm between adjacent grooves; and (C) grid structures generated by orthogonal overlapping of two groove structure with dimensions the same as B. A mask projection technique was used for structuring. In doing so, a 100 µm wide square mask (for Sharklet™ like) and 100 µm wide line mask (for groove and grid structure) was imaged onto the sample surface with a 50-times magnification using a 50× microscope lens (Leica HCX PL APO L 50×/0.55 UVI, Leica Microsystems, Mannheim, Germany) integrated in an autofocus system (INH200, Vistec, Milpitas, CA, USA). This technique enables high quality, reproducible structuring and a finer depth control of the surface features.

Figure 1. The samples were colonized with *S. aureus*, a major implant-associated pathogen. Biofilm was grown up to 24 h and bacterial surface coverage was quantified at the structured/smooth titanium interface.

As shown in Figure 2A, with beginning of biofilm growth, a reduced surface coverage on Sharklet™ like micro-structures could be observed. This trend was maintained up to 24 h of cultivation (Figure 2A, B). Statistical analysis revealed a total p-value of 0.074 for differences between smooth and Sharklet™ like structured titanium. After 24 h, the differences reached statistical significance (Table 1). The detailed p-values are listed in Table 1. The reduced biofilm surface coverage is in line with findings from Chung et al. [8]. They detected a similar effect for *S. aureus* on Sharklet™-structured PDMS surfaces. The rate of reduction was higher than 40%, as also found by Reddy et al. [12] and May et al. [10]. The reduction rate determined in the present study was about 15%. The most plausible explanation is that this is related to the different materials used. Whereas *S. aureus* biofilm formation on PDMS typically takes 14 days [8], biofilm formation on titanium needs only several hours. This may influence the effectiveness of the Sharklet™-like micro-structured titanium.

Interestingly, the groove and the grid micro-structures showed the same effect as found for the Sharklet™-like structure (Figure 2C, E). Bacterial surface coverage was decreased on structured surfaces compared to smooth ones from the beginning of the biofilm growth up to 24 h (Figure 2D, F). Statistical analysis revealed a total p-value of 0.013 (for groove structures) and 0.017 (for grid structures) and therefore statistically significant differences for structured/smooth titanium. The detailed p-values are listed in Table 1. As for the Sharklet™-like structure, the rate of reduction was approximately 15% for both structures. Equivalence testing also supported that biofilm surface coverage on all micro-structures was largely comparable (Table 2).

The patented Sharklet™ structure was originally developed for inhibition of settlement of swimming algal spores [13]. Afterwards, their effectiveness was also proven for bacterial attachment [8–12]. The mechanism proposed to explain inhibition of algal spore attachment – the increasing of intracellular stress gradients due to cell contact with two geometrically different features [15] – cannot be used for explanation of reduced bacterial attachment. The dimension of a single bacterial cell (e.g. *S. aureus* has a diameter of 1 µm) is smaller than the 2 µm spacing between neighboring feature of the Sharklet™ structure. A single cell cannot contact two neighboring features and experience an impact of stress gradient. Other mechanisms should be responsible for the anti-adhesive effect of these micro-structured surfaces. According to the results of this study, micro-structured surfaces become unfavorable for initial cell attachment even if the size is larger than the bacterial cell. Possibly, further

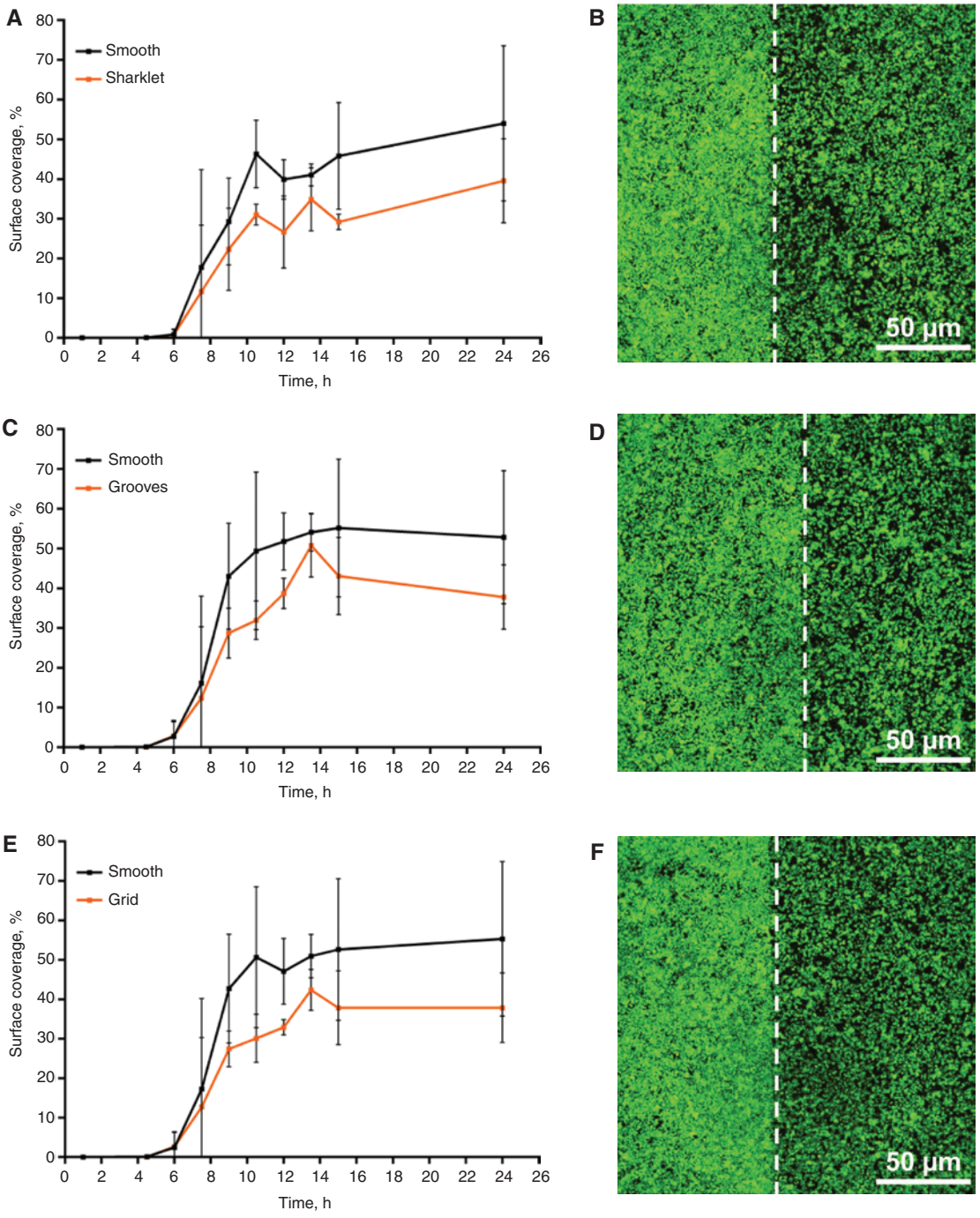

Figure 2: Bacterial colonization on micro-structured titanium

Prior to each experiment, *Staphylococcus aureus* (DSM 2569, German collection of microorganisms and tissue culture cells, Braunschweig, Germany) was pre-cultivated in tryptic soy broth supplemented with 10% yeast extract (TSB) for 96 h to reach late stationary phase. Pre-cultures were diluted in 50% TSB supplemented with 25 mM glucose and adjusted to an optical density at 600 nm of 0.001 (approx. 1×10^6 cfu/mL). Cells were allowed to adhere to titanium specimens for 1 h at 37 °C under gentle rotation at 50 rpm. Then, medium was exchanged and biofilm was cultivated up to 24 h at 37 °C without agitation. After 1, 4.5, 6, 7.5, 9, 10.5, 12, 13.5, 15 and 24 h, test specimens were removed, fluorescently stained using LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Life Technologies, Darmstadt, Germany) and fixed using 2.5% glutardialdehyde in PBS. Bacterial colonization was analyzed by CLSM (Leica TCS SP2, Leica Microsystems, Mannheim, Germany). For each structure, 4 pictures were taken at the structure/smooth interface. Percent surface coverage on structured and smooth titanium was calculated using ImageJ 1.48v (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij/). Surface coverage over time and standard deviation are depicted in A, C and E for Sharklet™-like, groove and grid micro-structures, respectively. B, D and F show representative microscopic images of bacterial colonization after 24 h at Sharklet™-like, groove and grid interphase, respectively. Smooth titanium is shown on the left side of the dotted line and micro-structured titanium on the right side.

Table 1: Statistical analysis of differences in bacterial surface coverage on structured and smooth titanium.

Time, h	Sharklet vs. smooth		Groove vs. smooth		Grid vs. smooth	
	p-Value	Significance	p-Value	Significance	p-Value	Significance
1	>0.999	ns	>0.999	ns	>0.999	ns
4.5	>0.999	ns	>0.999	ns	>0.999	ns
6	>0.999	ns	>0.999	ns	>0.999	ns
7.5	>0.999	ns	>0.999	ns	>0.999	ns
9	>0.999	ns	0.016	[a]	0.029	[a]
10.5	0.031	[a]	0.003	[a]	0.002	[a]
12	0.083	ns	0.033	[a]	0.052	ns
13.5	>0.999	ns	>0.999	ns	0.685	ns
15	0.016	[a]	0.057	ns	0.038	[a]
24	0.047	[a]	0.011	[a]	0.010	[a]
Total	0.074	ns	0.013	[a]	0.017	[a]

Experiments were performed three times. Data were analyzed using GraphPad Prism 6.01 Software (GraphPad Software, Inc., La Jolla, CA, USA). p-Values were calculated using two-way ANOVA followed by Bonferroni multiple comparison correction. Familywise significance threshold was set to α=0.05. The total p-value is a two-way ANOVA calculated summary of all time points; ns, not significant; [a]significant.

Table 2: Equivalence testing of bacterial surface coverage on differently structured titanium.

Time, h	Sharklet vs. groove		Sharklet vs. grid		Groove vs. grid	
	p-Value	Equivalence	p-Value	Equivalence	p-Value	Equivalence
1	>0.999	e	>0.999	e	>0.999	e
4.5	0.745	e	0.736	e	0.908	e
6	0.414	e	0.463	e	0.950	e
7.5	0.961	e	0.943	e	0.982	e
9	0.411	e	0.479	e	0.784	e
10.5	0.787	e	0.818	e	0.701	e
12	0.101	e	0.305	e	0.079	e
13.5	0.070	ne	0.244	e	0.199	e
15	0.072	ne	0.189	e	0.540	e
24	0.829	e	0.840	e	0.993	e

Equivalence testing was done with GraphPad Prism 6.01 Software (GraphPad Software, Inc., La Jolla, CA, USA) using multiple t-test with Sidak-Bonferroni correction of multiple comparisons and a confidence interval of 90%; e, equivalent; ne, not equivalent.

reduction of structural dimensions can increase the anti-adhesive effect, because bacterial cells will experience intracellular stress gradients proposed by Schumacher et al. [15]. This question will be clarified in further studies. Apart from the stress gradient, it can be assumed that physical interactions between attached bacterial cells and surrounding surface features negatively influence biofilm development [8, 9]. Even though the effect mechanism for reduced *S. aureus* surface coverage is not known yet, the current study together with previously published investigations demonstrate an attachment inhibitory effect of micro-structures per se. The essential question whether micro-structured surfaces mainly reduce cell attachment or if they also alter bacterial biofilm development should be addressed in future studies.

In conclusion, the present study demonstrates that (1) solely due to micro-structuring of titanium samples reduced surface coverage of *S. aureus* could be achieved; and (2) different pattern designs with similar structure feature dimensions show similar effectiveness. Therefore, it can be presumed that the effect of Sharklet™ is more a result of structure feature dimensions than structure design.

Concerning clinical applications, the effectiveness of the micro-structures should be further improved (e.g. by optimization of structure dimensions) and evaluated for their interaction with host tissue. The latter may be important for the structure design choice. This study serves as prove-of-principle, showing that micro-structures carry potential for anti-bacterial functionalization of titanium

surfaces, and is therefore a promising basis for further investigations.

Acknowledgments: This work has been carried out as an integral part of the BIOFABRICATION FOR NIFE initiative, which is financially supported by the ministry of Lower Saxony and the Volkswagen Foundation.

References

1. Mombelli A, Müller N, Cionca N. The epidemiology of peri-implantitis. Clin Oral Implants Res. 2012;23:67–76.
2. Flemming HC, Wingender J. Biofilme – die bevorzugte Lebensform der Bakterien. Biologie in Unserer Zeit. 2001;31:169–80.
3. Grade S, Eberhard J, Jakobi J, Winkel A, Stiesch M, Barcikowski S. Alloying colloidal silver nanoparticles with gold disproportionally controls antibacterial and toxic effects. Gold Bulletin. 2014;47:83–93.
4. Harris LG, Richards RG. Staphylococci and implant surfaces: a review. Injury. 2006;37:S3–14.
5. Pfaffenroth C, Winkel A, Dempwolf W, Gamble LJ, Castner DG, Stiesch M, et al. Self-assembled antimicrobial and biocompatible copolymer films on titanium. Macromol Biosci. 2011;11:1515–25.
6. Winkel A, Dempwolf W, Gellermann E, Sluszniak M, Grade S, Heuer W, et al. Introducing a semi-coated model to investigate antibacterial effects of biocompatible polymers on titanium surfaces. Int J Mol Sci. 2015;16:4327–42.
7. Sharklet Technologies. Understanding sharklet™ surface protection products. Aurora: Sharklet Technologies Inc.; 2011.
8. Chung KK, Schumacher JF, Sampson EM, Burne RA, Antonelli PJ, Brennan AB. Impact of engineered surface microtopography on biofilm formation of staphylococcus aureus. Biointerphases. 2007;2:89–94.
9. Kim E, Kinney WH, Ovrutsky AR, Vo D, Bai X, Honda JR, et al. A surface with a biomimetic micropattern reduces colonization of mycobacterium abscessus. FEMS Microbiol Lett. 2014;360;17–22.
10. May RM, Hoffman MG, Sogo MJ, Parker AE, O'Toole GA, Brennan AB, et al. Micro-patterned surfaces reduce bacterial colonization and biofilm formation in vitro: potential for enhancing endotracheal tube designs. Clin Transl Med. 2014;3:8. eCollection 2014.
11. May RM, Magin CM, Mann EE, Drinker MC, Fraser JC, Siedlecki CA, et al. An engineered micropattern to reduce bacterial colonization, platelet adhesion and fibrin sheath formation for improved biocompatibility of central venous catheters. Clin Transl Med. 2015;4:9. eCollection 2015.
12. Reddy ST, Chung KK, McDaniel CJ, Darouiche RO, Landman J, Brennan AB. Micropatterned surfaces for reducing the risk of catheter-associated urinary tract infection: an in vitro study on the effect of sharklet micropatterned surfaces to inhibit bacterial colonization and migration of uropathogenic escherichia coli. J Endourol. 2011;25:1547–52.
13. Carman ML, Estes TG, Feinberg AW, Schumacher JF, Wilkerson W, Wilson LH, et al. Engineered antifouling microtopographies – correlating wettability with cell attachment. Biofouling. 2006;22:11–21.
14. Magin CM, May RM, Drinker MC, Cuevas KH, Brennan AB, Reddy ST. Micropatterned protective membranes inhibit lens epithelial cell migration in posterior capsule opacification model. Transl Vis Sci Technol. 2015;4:9.
15. Schumacher JF, Long CJ, Callow ME, Finlay JA, Callow JA, Brennan AB. Engineered nanoforce gradients for inhibition of settlement (attachment) of swimming algal spores. Langmuir. 2008;24:4931–7.