BioCombs4Nanofibers

Project BioCombs4Nanofibers

D4.4 Example microorganisms

Reporting period	from	**01.10.2020**	to	**30.09.2022**
Report completed and released		**23.06.2021** **Anja Richter, Karin Schwibbert, Jörn Bonse**		

1. Goals and Detailed Description

Deliverable D4.4. is a public report on an example of microorganisms on laser-fabricated surface structures by **BAM**. It will be published on the **BioCombs4Nanofibers** website as well as on the project´s Zenodo open data depository page.

The current report focusses on the adhesion of microorganisms to laser-fabricated surfaces and gives revealing insight into the project results achieved within WP4 task 4.4 `Cells and microorganisms`. Former results regarding mammalian cell lines on laser-fabricated surfaces were provided by project partners **FORTH** and **INFLPR** as part of deliverable D4.3.

Micro- and nanostructures and their influence on bacterial surface adhesion

To overcome electrostatic repulsion or hydrophobicity and to initiate biofilm formation, bacterial cells can use extracellular nanofiber-like appendages: proteinaceous nanofibers such as flagella and pili, as well as components of the extracellular biofilm matrix based on polysaccharides and amyloid proteins.
Depending on the bacterial cell size and species-specific biofilm characteristics, the density of surface structures and their individual size – be it in the micrometer or nanometer range – can be used to control initial cell-surface interaction and later stages of biofilm maturation and eradication. Within the context of the **BioCombs4Nanofibers** project, laser-structuring will be used to develop bacterial-repellent surfaces with biomimetic structures.

To gain an overview on how bacteria adhere to laser-structured metal surfaces, cylinders of grade-1 titanium or grade-5 titanium alloy (diameter 24 mm, height 8 mm, top surface polished) were laser-processed at the plane top surface. Five areas (4 mm x 4 mm each) were irradiated using identical laser parameters according to Fig. 1. Afterwards, the samples have been cleaned and stored in a desiccator until further use.

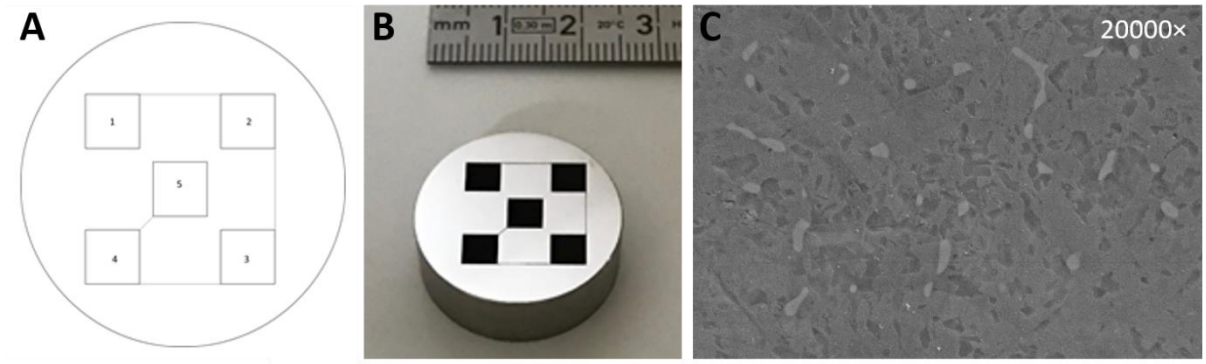

Fig. 1: (A) Scheme of fs-laser processing; (B) Photography of laser-irradiated titanium disk and (C) High-resolution top-view SEM image of non-processed polished titanium surface.

For that, the laser-processing was performed in air environment by a commercial Ti:sapphire fs-laser system (790 nm wavelength, 30 fs pulse duration) that was operated at 1 kHz pulse repetition frequency. The sample was placed in the focal region way of the loosely focused fs-laser beam and moved in a meandering way perpendicular to the incident laser radiation. Depending on the laser processing parameters, two different types of surface structures, namely hierarchical Spikes (see Fig. 2) and so-called laser-induced periodic surface structures (LIPSS, Ripples, see Fig. 3), have been obtained (Tab. 1).

Tab. 1: Laser processing parameter applied to achieve Spikes or Ripples structures on Ti or Ti alloy.

Processing parameter	**Spikes**	**Ripples (LSFL)**
Peak fluence F_0 [J/cm^2]	3.0	0.5
Effective number of pulses N_{eff_1D}	400	40
Line separation ΔS [µm]	25	50
Pulse repetition frequency [kHz]	1	1

For biofilm experiments, the samples were washed in detergent solution, disinfected for 5 min in 70% ethanol, air-dried and transferred to sterile 6-well plates. *Escherichia coli* TG1 was used as test strain. Bacterial suspension of exponentially growing cells was diluted to 10^5-10^7 cells/ml in mineral salt medium and added to the sample. After an initial sedimentation phase of 1 h the plates were further incubated with mild shaking. The sample was removed after approx. 18 h and subjected to a washing procedure to remove lightly attached cells.

Scanning electron microscopy (SEM) with an environmental scanning electron microscope (FEI XL 30, Hillsboro, OR, USA) was used to characterize bacterial adhesion on Spike-structured titanium. For SEM imaging, biofilm-samples were fixed with glutaraldehyde and subjected to a washing procedure. After dehydration using gradient ethanol solutions, samples were critical point dried with carbon dioxide and coated with a 15-30 nm conducting layer of gold. For titanium samples without preceding bacterial colonization (Figs. 1C; 2A & 2B; 3A & 3B), fixation, drying and gold sputter-coating steps were omitted. For fluorescence imaging, biofilm-samples were fixed with formaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI) and subjected to epi-fluorescence microscopy.

SEM microscopic analyses showed that deeper-lying pits and trenches of the laser-generated Spikes topography are abundantly colonized with the rod-like *E. coli*, whereas a comparatively low number of bacterial cells could be found on the topographic peaks (see Figs. 2C & 2D). Note that he maximum depth of valley of the Spikes structures accounted to 22 to 27 µm here (as measured from the level of the initial surface plane by optical microscopy). Colonization clearly took place from “bottom to top” under the conditions applied.
The dimensions of Spike-structures are many times larger compared to the size of a bacterial cell and obviously offer shelter and plenty of adhesion points to promote bacterial adhesion compared to polished surfaces.

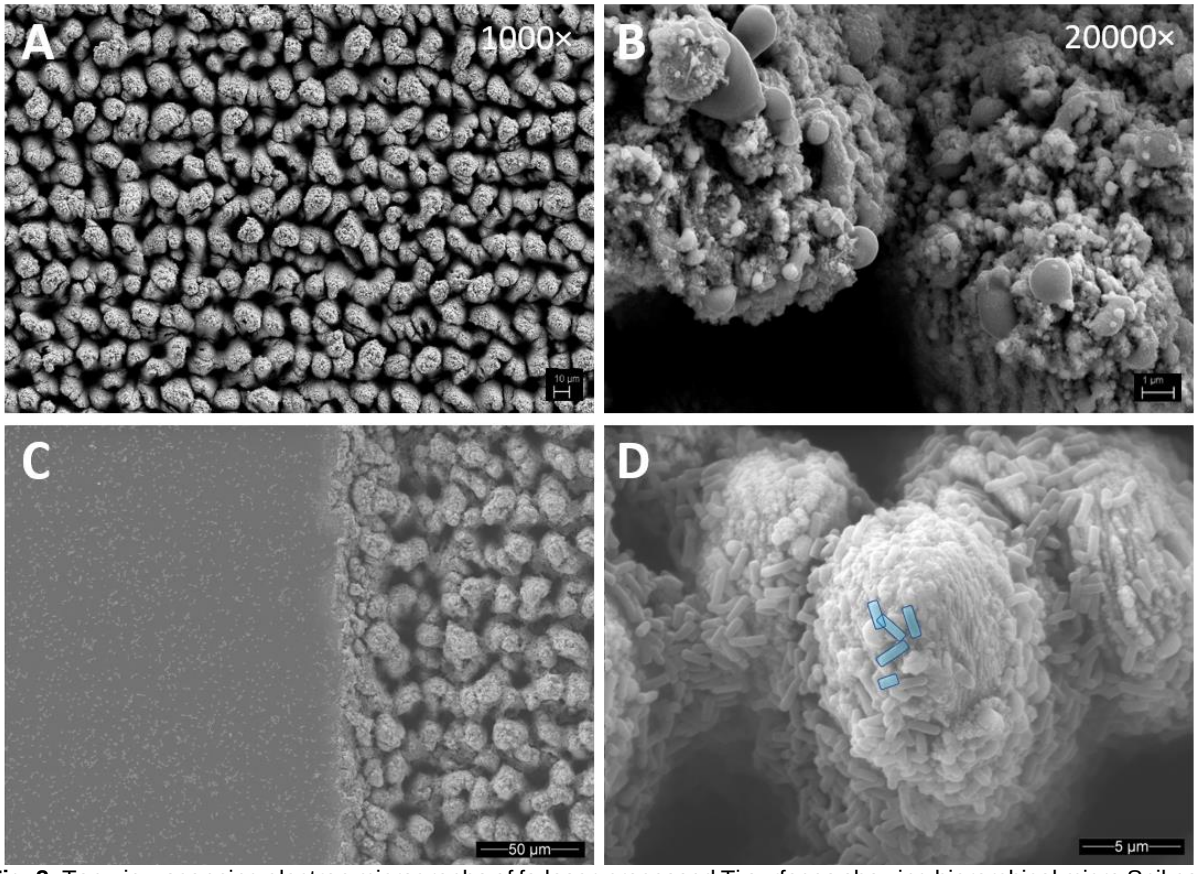

Fig. 2: Top-view scanning electron micrographs of fs-laser-processed Ti surfaces showing hierarchical micro Spikes (A & B). Representative scanning electron microscopy micrograph of fs-laser processed Ti surface after overnight incubation with *E. coli* TG1 (C & D with highlighted cells in blue). Detailed laser-processing parameters are given in the text.

First findings of epi-fluorescent biofilm images demonstrated that LSFL-Ripple-structuring of titanium surfaces altered biofilm formation of *E. coli* under static biofilm conditions (Fig. 3). On non-structured (polished) surfaces, *E. coli* TG1 biofilms consisted of densely packed cell aggregates suggesting cell proliferation and extracellular matrix production after surface attachment. However, on laser-processed surfaces with LSFL-Ripples, cell attachment was characterized by flat, mono-layered biofilms interrupted by loosely interconnected groups of cells (TG1, Fig. 3C) or smaller cell clusters for weak biofilm formers such as *E. coli* MG1655lm (Fig. 3D), suggesting changes in extracellular matrix composition and biofilm architecture compared to polished surfaces. Comparing the epi-fluorescence results in Figs. 3C and 3D indicates that the bacterial adhesion may significantly depend on the *E. coli* strain under investigation.

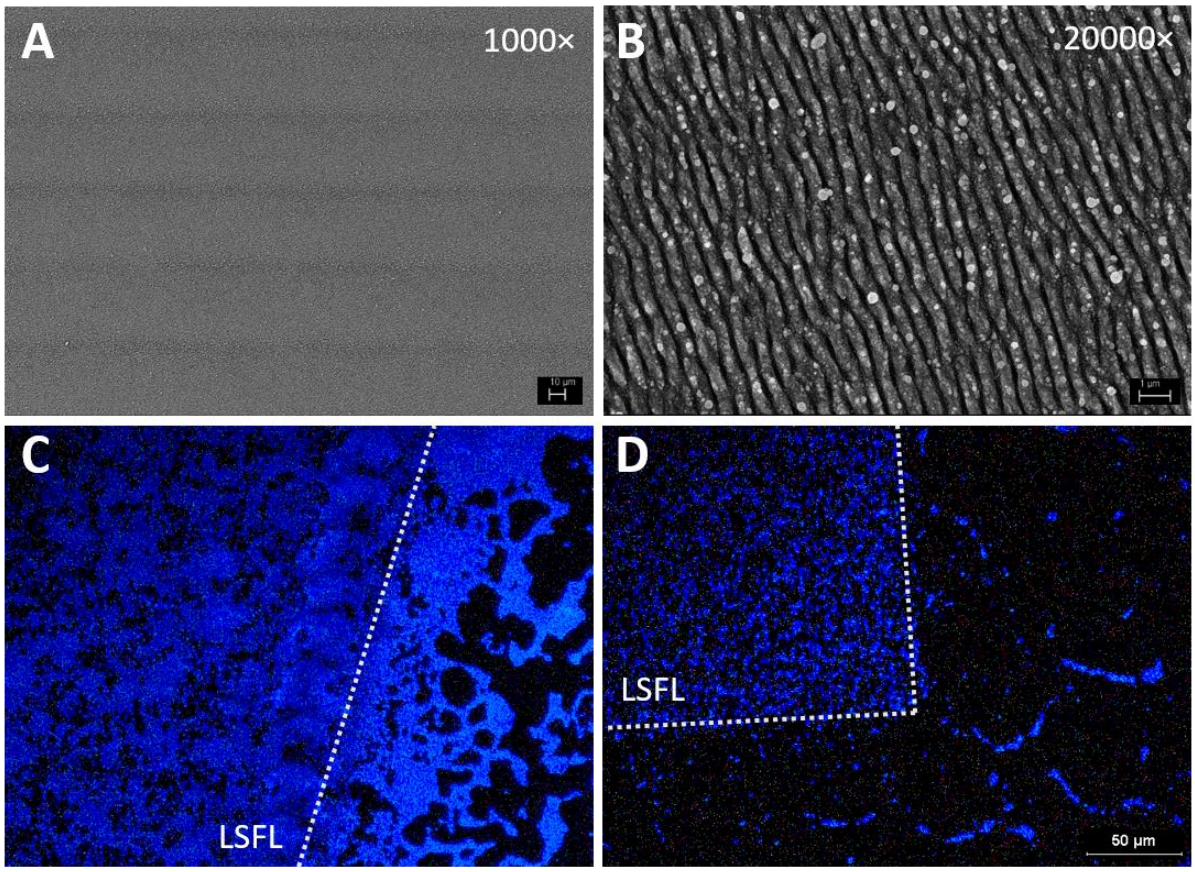

Fig. 3: Top-view scanning electron micrographs of fs-laser-processed Ti surfaces showing low spatial frequency LIPSS (LSFL) / Ripples (A & B). Representative epi-fluorescence microscopy micrograph of fs-laser processed Ti surface after overnight incubation with *E. coli* TG1 (C) and MG1655lm (D) and DAPI staining of attached cells. Detailed laser processing parameters are given in the text.

2. Evaluation of Goals and Resulting Actions

The presented results demonstrate the strong effect of laser-fabricated surface structures on bacterial adhesion and biofilm formation on titanium and titanium alloy surfaces. While on the microscopic scale extensive sample preparation and scanning electron microscopy (SEM) allows to visualize even single individual bacterial cells and their localized adhesion in the laser-processed surface structures, on the macroscopic scale, staining with DAPI and epi-fluorescence microscopy has proven to be a powerful tool for the large-area characterization of bacterial adhesions on laser-processed surfaces. First indications are found that the bacterial response does not only depend on the characteristics of the surface structures but also on the bacterial cultivation method and the type of bacteria used in the tests.

Future work

In cooperation with project partners **JKU** and **FORTH**, alternative experimental laser setups and surface materials, thus, resulting in variable surface structures, will be tested to further investigate the effect of laser-fabricated nanostructures on bacteria-surface-interaction.

In addition to rod-shaped *E. coli*, other industrially and clinically relevant model organisms with different cell sizes and shapes will be used to for biofilm-assays to further specify bacterial-repellent surfaces characteristics for a broad application range and adjusted laser-processing parameters.

This report is part of the project **BioCombs4Nanofibers** that has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 862016.