Colloids and Surfaces B: Biointerfaces 145 (2016) 617–625

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

# Nanorough titanium surfaces reduce adhesion of *Escherichia coli* and *Staphylococcus aureus* via nano adhesion points

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ARTICLE INFO

*Article history:*
Received 9 February 2016
Received in revised form 26 April 2016
Accepted 17 May 2016
Available online 20 May 2016

*Keywords:*
Nanoroughness
Microbial adhesion
Titanium
SEM
Focused ion beam

ABSTRACT

Microbial adhesion to natural and synthetic materials surfaces is a key issue e.g. in food industry, sewage treatment and most importantly in the biomedical field. The current development and progress in nanoscale structuring of materials surfaces to control microbial adhesion requires an advanced understanding of the microbe-material-interaction. This study aimed to investigate the nanostructure of the microbe-material-interface and link it to microbial adhesion kinetics as function of titanium surface nanoroughness to gain new insight into controlling microbial adhesion via materials' surface nanoroughness. Adhesion of *Escherichia coli* and *Staphylococcus aureus* was statistically significantly reduced ($p \leq 0.05$) by 55.6 % and 40.5 %, respectively, on physical vapor deposited titanium thin films with a nanoroughness of 6 nm and the lowest surface peak density compared to 2 nm with the highest surface peak density. Cross-sectioning of the microbial cells with a focused ion beam (FIB) and SEM imaging provided for the first time direct insight into the titanium-microbe-interface. High resolution SEM micrographs gave evidence that the surface peaks are the loci of initial contact between the microbial cells and the material's surface. In a qualitative model we propose that the initial microbial adhesion on nanorough surfaces is controlled by the titanium surface peak density via nano adhesion points. This new understanding will help towards the design of materials surfaces for controlling microbial adhesion.


## 1. Introduction

Microbial adhesion to natural and synthetic materials surfaces is a key issue e.g. in food industry, sewage treatment and most importantly in the biomedical field. The current development and progress in nanoscale structuring of materials surfaces to control microbial adhesion requires an advanced understanding of the microbe-material-interaction [1].

On the macro and micro scale it has been shown that topographic surface features of materials significantly affect the initial adhesion of microorganisms [2]. In particular, the impact of surface roughnesses on microbial adhesion has been extensively studied [3–8]. The findings have established the common knowledge that as a rule of thumb microbial adhesion increases with increasing surface roughness. Researchers assume that microbes adherent to macro- or micrometer rough surfaces are protected against abrasion from shear stress [7–11]. In addition, materials with surface structures of the size of the microbial cells may provide a maximum contact area between microbe and material and, thus, promote adhesion [12–15]. Consequently, one current strategy for reducing microbial adhesion on materials' surfaces is preparing surfaces as smooth as possible.

Recently, nanostructured materials gained interest because of their effect on microbial adhesion [16–24]. In particular, titanium as most often used material in the biomedical field was in the focus of several studies. Singh *et al.* [20] observed for example a significantly increased adhesion of *Escherichia coli* and *Pseudomonas aeruginosa* on supersonic cluster beam deposited titanium thin films with a surface nanoroughness of 21.7 nm compared

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http://dx.doi.org/10.1016/j.colsurfb.2016.05.049
0927-7765/

to 16.2 nm. The authors assume that differences in the wettability of the surfaces may have caused this increase. Ivanova *et al.* [17,18] reported contradicting results. They found that the surface coverage of magnetron sputtered titanium thin films with *Staphylococcus aureus* was reduced with increasing surface roughness from 0.2 nm to 0.5 nm and 0.7 nm to 1.6 nm, respectively. These authors, as well, assumed that a change in surface wettability at least partly caused the differences in microbial adhesion. Summarizing, to date there is no consensus in the current literature on how nanoroughness affects microbial adhesion and results are contradictory. Moreover, standard roughness values (such as the average roughness or the root mean square roughness) as used in these studies for description of the surface topography are vertical surface parameters not considering the spatial/horizontal distribution of structure elements e.g. grains/peaks and valleys on the surface. To better understand the effect of surface nanoroughness on microbial adhesion it is, thus, important to include additional surface parameters, such as peak-to-peak distance or peak density in the analysis.

To fill this gap in knowledge, the aim of the current study was [i] to investigate the microbial adhesion kinetics of *S. aureus* and *E. coli* as a function of titanium surface nano-roughness in particular considering next to vertical also spatial surface parameters, [ii] to elucidate the unknown nanostructure of the microbe-titanium-interface and [iii] to propose a qualitative model of microbial adhesion to materials surfaces as a function of nanoroughness and time.

In a previous study, we found that with increasing stochastic nanoroughness of physical vapor deposited titanium thin films from 2 nm to 6 nm, the distance between the topographical surface peaks increased threefold [21]. Thus, with increasing roughness the relative number of titanium surface peaks, i.e., the peak density, decreased. Based on these results and based on a previous simulation study [25], we hypothesize that these surface peaks may be the points of first contact between the microbial cell and the titanium surface during initial stage adhesion. Accordingly, with increasing surface nanoroughness, less points for first contact are available between the microbial cell and the material which might result in reduced adhesion. The hypothesis tested in the present study was, therefore, that with increasing surface nanoroughness and decreasing peak density, microbial adhesion is reduced.

## 2. Materials and methods

### 2.1. Titanium thin film preparation

Titanium thin films (Ti-TF) were deposited on glass slides (diameter 15 mm; Borofloat® B33; Jena 4H Engineering GmbH, Jena, Germany) using the PVD method as described elsewhere [26]. Titanium surface roughness was adjusted by varying the deposition rate and the film thickness with 0.1 nm/s and 100 nm (sample group A), 0.5 nm/s and 200 nm (sample group B), 0.5 nm/s and 500 nm (sample group C), and 1.0 nm/s and 500 nm (sample group D). Samples were sterilized in the autoclave for 20 min at 121 °C before investigating microbial adhesion.

### 2.2. Titanium surface characterization

An atomic force microscope (AFM, Dimension 3100, Digital Instruments, Santa Barbara, CA, USA), equipped with a standard silicon tip (tip diameter < 5 nm) was used to characterize the topography of the Ti-TFs surfaces. The AFM was operated in tapping mode with a scan rate of 2 Hz, an image resolution of 512 × 512 points and a scan size of 1 μm × 1 μm.

AFM image processing and calculation of titanium surface parameters were performed with Gwyddion 2.25, a free SPM data analysis software [27] according to the protocol described elsewhere [21]. The roughness parameters were calculated according to the standard ISO 4287-1997. For the calculation of the average peak-to-peak distances and the peak densities of the titanium surfaces, the coordinates of the local maxima of the AFM height images were estimated using ImageJ (National Institutes of Health NIH, Bethesda, Maryland, USA). Based on a Delaunay triangulation algorithm, the distances between the neighboring maxima were calculated using MATLAB (MathWorks, Natick, Massachusetts, USA).

The contact angle measurements were performed with the dynamic drop method using double-distilled water drops (advancing angles: increase of drop volume from 5 μL to 7 μL with a rate of 10 μL/min) (DSA 10Mk2 drop shape analysis system, Krüss, Hamburg, Germany) on n = 3 replicates of each Ti-TF surface. 10 images of each increasing drop on the materials surfaces were recorded by a camera and analyzed using the software supplied by the manufacturer according to the circle fitting method.

### 2.3. Microbial strains

*S. aureus* and *E. coli* both common pathogens relevant e.g. in the biomedical field were used as test organisms in this study. The microbial strains *E. coli* EC042, producing the green fluorescent protein (GFPuv) [28], and *S. aureus* HG003, producing the red fluorescent protein mCherry [29], were used for microbial adhesion tests. Preparation of the fluorescent strains based on transformation is described in the supplemental material (Text S1).

### 2.4. Cultivation of the microorganisms and adhesion test

A recently described in vitro device [26] was used to investigate microbial adhesion as a function of the titanium surface nanoroughness. This in vitro system allows for the investigation of microbial adhesion on materials surfaces at reproducible and constant conditions throughout the experiment. *E. coli* and *S. aureus* were each cultivated in a separate continuous culture (chemostat) which was used for inoculation of a biofilm reactor (non-constant depth film fermenter; nCDFF) containing the materials samples. A complete description of the system can be found elsewhere [26]. Experimental details of the cultivation of the microorganisms are provided as supplemental material (Text S2).

Based on the results of pre-experiments in culture well plates (data not shown), the sampling time points were set to 1 h, 3 h, 5 h, 7 h, 9 h and 11 h, respectively, with n = 3 replicates for analysis with CLSM. For cross-sectioning of adherent cells and high resolution SEM, additional Ti-TFs were sampled after 3 h and 9 h, respectively, with n = 2 replicates.

### 2.5. Confocal laser scanning microscopy

Using confocal laser scanning microscopy (CLSM), the adhesion kinetics of *E. coli* and *S. aureus* were investigated as a function of the nanoroughness of physical vapor deposited titanium thin films. Samples for CLSM imaging were prepared as described previously [26]. A confocal laser scanning microscope (Zeiss LSM 510 Meta, Carl Zeiss MicroImaging, Jena, Germany) equipped with an Argon laser (488 nm) and Helium-Neon laser (633 nm) and a 63 × NA 1.4 oil immersion lens objective (Zeiss PLANAPOCHROMAT®) was used for fluorescence imaging of the microorgansims. The materials' surface coverage with the microbes was calculated based on five single plane CLSM images per sample for each sampling time point using the free software bioImage_L v.2.1 [30]. For image analysis, a factor of 0.03 was applied for noise reduction.