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N Chik[1], W S Wan Md Zain[16], A J Mohamad[2], M Z Sidek[3], W H Wan Ibrahim[1], A Reif[4], J H Rakebrandt[4], W Pfleging[4] and X Liu[5]

[1]Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, Pahang, Malaysia
[2]Faculty of Electrical and Electronics Engineering, Universiti Malaysia Pahang, Pahang, Malaysia
[3]Faculty of Mechanical Engineering, Universiti Malaysia Pahang, Pahang, Malaysia
[4]Institute for Applied Materials, Karlsruhe Institute Technology, 76344 Eggenstein-Leopoldshafen, Germany
[5]School of Engineering, University of Warwick, Coventry, CV4 7AL, UK

n.atiqahchik@gmail.com

Abstract. Bacterial adhesion has become a significant problem in many industries causing billions of dollars for its complicated removal treatment and maintenance. In this study, metal surfaces undergone treatment with ultrafast laser with varies power. The microstructure produced on its original surfaces were expected to prevent the adhesion of *Escherichia coli* (*E. coli*) ATCC 8739 and *Staphylococcus aureus* (*S. aureus*) ATCC 6838. The laser treatment was performed at 380 fs pulse duration, 515 µm central wavelength and a repetition rate of 200 kHz. Stainless steel AISI 316L was treated with an average laser power of 0.04 W (SS-0.04) and 0.11 W (SS-0.11), while Grade 5 titanium alloy was tested with high laser power 0.11 W (T-0.11). The adhesion was observed after 16 hours and the number of adhering bacteria was counted per cm^2. The result achieved shows that, increasing the average laser power is leading to an enhanced *S. aureus* adhesion while *E. coli* adhesion is reduced which is due to the hydrophobicity interaction and difference in surface texture. Meanwhile, the laser treatment showed significant reduction of the bacterial adhesion on its surface compared to the polished surfaces. Thus, ultrafast laser texturing can be suggested as a promising method to reduce the bacterial adhesion, which reduced the adhesion of >80% for *E. coli* and >20% for *S. aureus*.

1. Introduction

Bacteria are ubiquitous and easily adhere to the exposed surfaces. This natural phenomenon creates problems to many industries such as contamination in medical devices, food industry, pharmacy sector, marine science and bio-fouling or bio-corrosion of industrial equipment [1,2]. The severity of the adhesion occurs when adhered bacteria form biofilm, which is an assemblage of microbial cells that is irreversibly and often embedded in a matrix of extracellular polymeric substances (EPS) [3]. Biofilm and eradication of the pathogen can be extremely difficult to be removed, time-consuming and expensive [3]. Thus, many studies have been performed in order to investigate the factors that contributed to the bacterial adhesion and also all the possible methods for avoiding the formation of biofilm on certain surfaces [1]. The adhesion of bacteria on material surfaces is governed by many factors, including bacterial characteristic (e.g., hydrophobicity, surface charge), surface properties (e.g., roughness, wettability) and environment condition (e.g., pH, temperature) which involve the

physicochemical and also the molecular interactions [4,5]. The net interaction forces between a cell and a flat surface in the adhesion process are well described by DVLO (Derjaguin, Verwey, Landau and Overbeek) theory [5]. Meanwhile, studies had emphasized that surface roughness and wettability affects the rate of bacterial adhesion [1,4], but, some researchers also report that the final finishing process for the metal surfaces had significant contribution and influenced the bacterial adhesion [6]. Reports on the polished surfaces with respect to bacterial adhesion are countless and established, but limited reports were found on the effect of laser treated surface especially with respect to ultrafast laser radiation. The laser treatment process was carried out in order to realize laser-induced periodical surface structure (LIPSS) on the metal surfaces by controlling the average laser power [7].

In this study, the bacterial characteristics such as surface hydrophobicity and grams type were studied where they were included as factors that affect the rate of adhesion on the metallic surfaces. Besides that, titanium and stainless steel have been tested due to its vast usage as medical implant materials and in industrial purposes [1,8]. Therefore, we investigated the adhesion rate of *E. coli* and *S. aureus* on the nano-textured surface (LIPSS) produced by ultrafast laser ablation, aiming at assessing the potential of using laser materials processing for producing surfaces with low bacterial adhesion rate which can help both, medical and industrial sectors to overcome their problems, while exploring the effect of the average laser power to the rate of bacterial adhesion on the metal surfaces.

2. Methods

2.1. Bacteria and growth condition

The bacterial strains used for this study were *E. coli* ATCC 8739 and *S. aureus* ATCC 6838, obtained from culture collection of Central Laboratory, Universiti Malaysia Pahang. For long term preservation, the bacterial cultures were maintained in Luria Bertani (LB) broth with 20% (v/v) of glycerol, in - 80 °C freezer. Prior to the experiment, the stock culture was plated on LB agar at 30 °C for 24 hours. Single loopful of bacterial cells was transferred to a 250 mL shake flask containing 50 mL of LB broth for inoculation, followed by incubation at 30 °C, 150 rpm for 18 hours. Then, the inoculums were harvested by centrifugation at 5000 rpm x g for 5 min. The absorbance of the bacterial suspension (cell pellets and phosphate buffer saline (PBS)) for both cultures were adjusted to ~1.0 M (10^6 bacterial/ml) before adhesion test was started.

2.2. Metal surfaces treatment

In this study stainless steel AISI 316L and Grade 5 titanium alloy were used. The surface of both metals were treated using laser ablation and polishing technique. The metals used in this study were cut into small pieces (1 cm x 1 cm x 3 mm). Laser treatment was performed at Karlsruhe Institute of Technology (KIT, Germany) using a micromachining workstation (PS450-TO, Optec, Belgium) equipped with an ultrafast fiber laser (Tangerine, Amplitude Systemes, France) operating with an average power of 35 W. Laser surface texturing was carried out under ambient air with a central wavelength of 515 μm, a laser pulse duration of 380 fs and a repetition rate of 200 kHz. The scanning speed of the laser beam onto the metals was maintained at 20 mm/s while the average laser power was varied. Stainless steel has been exposed to the laser beam by applying an average laser power of 0.04 W (SS-0.04) and 0.11 W (SS-0.11). While titanium only undergone processing with laser power of 0.11 W (T-0.11). The laser beam for LIPSS generation was guided through a beam expander (2-fold) and the scan head (Newson Engineering BV, Belgium) was used together with an f-theta lens with a focal length of 100 mm [7]. After the laser treatment, the achieved surface structure was characterized by scanning electron microscopy (SEM) (Carl Zeiss, SEM EVO 50). Polishing technique also has been applied to both metals. Silicon carbide (SiC) papers with grit 180 and 1500 were used to polish the stainless steel and titanium by using 6-in. grinder-polisher (Adolph and Buehler, Chicago). All metal undergone polishing technique were labelled as P-SS for stainless steel and P-T for titanium.

2.3. Bacterial characterization

Gram staining method, bacterial size analyzer and bacterial adherence to hydrocarbon (BATH) technique were carried out for the bacterial characterization. Bacterial smear from 12 hours culture was covered with crystal violet for 1 minute, gram's iodine also for 1 minute and then, followed by

decolourization using 95% of ethyl alcohol. Lastly, safranin was added for 1 minute, washed with distilled water and examined under light microscope. Bacteria were viewed using SEM at 100x magnification and the sizes were measured using ImageJ software. Values were taken as average from 5 bacteria images. Prior to the test, procedures that included centrifugation of bacterial cell (15 ml) at 5000 rpm x g for 5 min, cell washing with sodium chloride (NaCl) solution twice, re-suspension the bacterial suspension in 15 ml of PBS (0.1 M, pH 7.4) solution were performed . The samples then were fixed in 2% of glutaraldehyde (1 hour, 4 °C), centrifuged at 5000 rpm x g for 5 minutes and were dehydrated in an ethanol series; 30%, 50%, 70%, 80%, 90%, 100% (each step for 10 minutes excepting 100% ethanol treatment was for 30 minutes).

Surface hydrophobicity of bacterial cells has been determined by using BATH technique. During BATH technique, several steps included centrifugation of bacterial cell and cell washing process as described in bacterial size analyzer test were performed and the absorption (A_0) was adjusted until ~1.0 (106 bacteria/ml) using UV-Vis spectrophotometer (Hitachi, U-1800 spectrophotometer). Then, 4 ml of bacterial suspension were vigorously mixed with 1 ml of n-hexane, n-hexadecane and xylene separately for 2 min. The water and organics phase were allowed to separate and after 15 min the absorbance (A) aqueous phase was measured [1,9]. The percentage of bacteria hydrophobicity was measured by the following equation (1):

$$\% \text{ of hydrophobicity} = \left(1 - \left(\frac{A}{A_0}\right)\right) \text{ x } 100 \quad (1)$$

2.4. Bacterial adhesion test

Adhesion test was carried out in a glass container (6 cm x 9 cm x 7 cm) containing a baby cradle-like holder for holding the metal slide (2.5 cm x 7.6 cm x 0.1 cm) in the upright-vertical position. The metals slide that carry stainless steel or titanium was suspended in 70 ml of bacterial solution (absorbance adjusted to ~1.0 M) and shake at 70 rpm for 16 hours (laser structured metals) and 4 hours (polished metals). Samples were taken and dried in the incubator at 30 °C overnight and then were examined under the fluorescence microscope (Olympus FluoView Ver. 1.3). Prior to viewing, adhered bacteria were stained with Syto9 dye for 5 min, followed by flushing with generous amount of distilled water. ImageJ was used to calculate the number of bacterial adhere on metal per cm^2 based on the image captured by the fluorescence microscope.

3. Results and discussion

3.1. Bacterial characteristics

Based on the gram staining image (Figure 1a and 1b) *E. coli* ATCC 8739 was stained red, categorized as gram negative bacteria, while *S. aureus* ATCC 6838 was stained purple and is a gram positive bacteria. This staining response is based on the chemical and structural makeup of the cell wall of bacteria. Besides that, some proteins associated with the cell wall and the cell membrane of the bacterial are responsible for bacterial cell-surface hydrophobicity where hydrophobicity can influence the rate of bacterial adhesion [10] on a metal surface. The size of the bacteria were determined from the SEM images (Figure 1c and 1d) of the free cell (non-adhered bacteria), taking as average from 100 of bacteria that were selected randomly, with the help of the ImageJ software. The length of *E. coli* ranges between 0.98 μm – 2.24 μm, while *S. aureus* was between 0.41 μm – 0.73 μm. The non-adhering cell showed that the *E. coli* and *S. aureus*, are rod and spherical shape, respectively. The surface hydrophobicity for *E. coli* and *S. aureus* were determined using different types of hydrocarbon which were hexane, hexadecane and xylene. Cells are classified as highly hydrophobic when the hydrophobicity is greater than 70%, moderate at 50% - 70% and subsequently hydrophilic when it is lower than 50% [1]. Referring to Table 1, *E. coli* has hydrophilic surface as the percentage of hydrophobicity for all the hydrocarbons is varied between 4.42 % - 23.43 %. In contrast, *S. aureus* shows high affinity towards hydrocarbon because of its high hydrophobic surface (>80%) due to the presence of the protein constituents (e.g., fibrinogen, fibronectin) at the cell surface (peptidoglycan) [10].

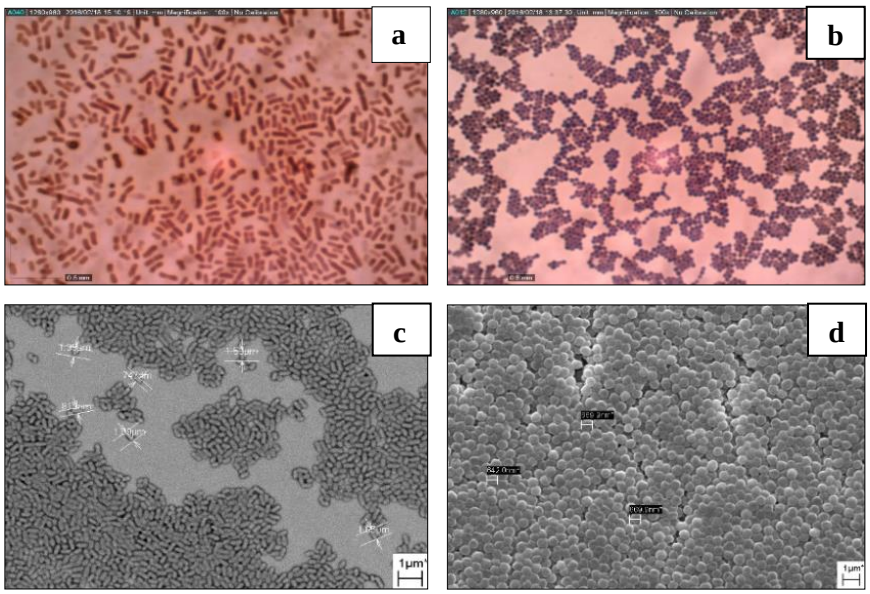

Figure 1. a) Gram stained of *E. coli* ATCC 8739 (red colour), b) gram stained of *S. aureus* (purple/violet colour) viewed under light microscope with 100x magnification, c) shape of *E. coli* under SEM at 4000x magnification, d) shape of *S. aureus* from SEM images at 4000x magnification.

Table 1. Average of bacterial cell hydrophobicity at exponential phase (12^{th} hour) determined with hexane, hexadecane and xylene.

Hydrocarbon	**% Hydrophobicity**	
	***Escherichia coli* ATCC 8739**	***Staphylococcus aureus* ATCC 6838**
Hexane	4.42 ± 0.86	92.57 ± 1.20
Hexadecane	11.90 ± 4.75	75.18 ± 7.41
Xylene	23.43 ± 0.87	88.78 ± 1.51

3.2. Assessment of bacterial adhesion

Stainless steel AISI 316L and Grade 5 titanium alloy have been treated by ultrafast laser radiation at different average laser power (0.04 W and 0.11 W). SEM images of the surface textures after the laser treatment are presented in Figure 2. The laser treatment produced a so-called LIPSS texture. The difference between surfaces treated at different average laser power was the additional appearance of irregular nano-sized metallic particles which were produced during the ablation especially for an average laser power of 0.11 W. Various sizes of metal particles were attached on the ripple structures (Figure 2b and 2c). This creates additional submicron structures that might add to increase active surface area and roughness to the original surfaces. All metals that have been laser processed were used in the adhesion test and studied under fluorescence microscope (Figure 3) for calculating the number of bacterial adhered per cm^2. Prior to the viewing process, the samples were stained by SYTO9 dye which diffuses into the cells and fluoresces upon binding nucleic acids in green fluorescence [2].

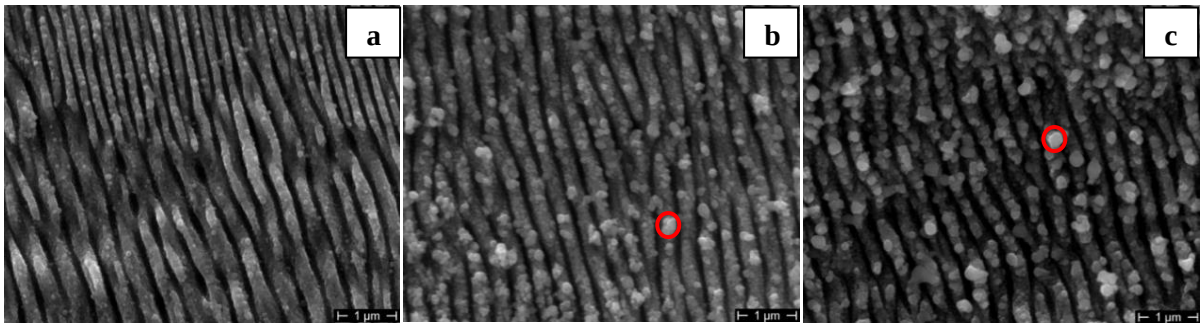

Figure 2. SEM images of the surface texture with 5000x magnification, a) SS-0.04 b) SS-0.11 c) T-0.11.The circled area highlighted nano-sized of irregular grains formed on top of the LIPSS.

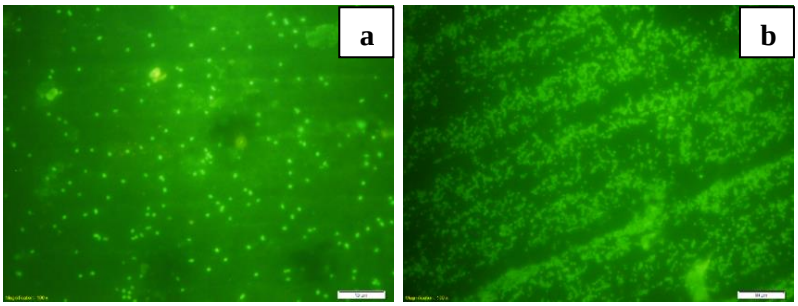

Figure 3. Fluorescence image of *E. coli* and *S. aureus* after 16 hours adhesion at 100x magnification, a) *E. coli* on T-0.11 and b) *S. aureus* on SS-0.11.

The SS-0.04 and SS-0.11 were subjected to bacterial adhesion for 16 hours. The results in Figure 4a indicate that the adhesion of *S. aureus* was doubled from 396 x 10^5/cm^2 to 703 x 10^5/cm^2 for surfaces which were laser structured with higher average laser power. In contrary, the adhesion of *E. coli* was decreased from 20 x 10^5/cm^2 down to 7 x 10^5/cm^2, contributed to 65% reduction of adhesion on surface structured with high average power. Stainless steel is a hydrophobic surface [11] which attracts *S. aureus* and repel *E. coli* due to hydrophobic and hydrophilic interaction. Hydrophobic bacteria (*S. aureus*) showed higher affinity towards hydrophobic surface, while the hydrophilic bacteria (*E. coli*) will repel from hydrophobic surface [4]. Besides that, the surface with additional nano-sized particles (Figure 2b and 2c) will increase the surface roughness [12] and presumably had contributed to increase the hydrophobicity which can attract the hydrophobic bacteria. Thus, increased the adhesion rate of *S.aureus* to stainless steel 10-100 times higher than *E.coli* (Figure 4a) which varies accordingly to the effect of laser power. Meanwhile, the different surface texture with and without nano-sized particles, reduced the adhesion of *E. coli* on stainless steel. It is assumed that the irregular metallic particles prevent the attachment of *E. coli* and also limit the contact surface between *E. coli* and the original surface. Therefore, the adhesion of *E. coli* on stainless steel can be reduced by increasing the average power during laser structuring. Referring to the Figure 4b, 43 x 10^5/cm^2 of *E. coli* adhered on T-0.11 which is 6 times higher than on SS-0.11 (7 x 10^5/cm^2). It is expected that the degree of titanium hydrophobicity in this study is lower than the stainless-steel hydrophobicity which is supported by Ludecke et al. [13]. Ludecke et al., stated that all titanium surfaces were moderately hydrophilic. Thus, *E. coli* which found to have hydrophilic cell surface in this study was adhered more on the titanium surface. SEM images of *E. coli* and *S. aureus* on the metallic surfaces were shown in Figure 5. Most of the bacteria were attached on top of the submicron-sized particles, and the size of bacteria were much larger compared to the gap between neighbouring nano-ripples, which seem to prevent the adhesion on surfaces without nano-particles. The adhesion of bacteria on the laser structured surfaces was further compared to the polished metals treated with SiC paper (180 and 1500 grit). Figure 6 shows that the laser structured surfaces recorded lower adhesion for both *E. coli* (>80%) and *S. aureus* (>10%) compared to polished surfaces. Herein, the result suggests that ultrafast laser material processing can

be used to reduce the bacterial adhesion as the periodicity of the LIPSS structure is smaller than bacterial size (Figure 5), which can inhibit the penetration of bacteria to the original surfaces. Moreover, the presence of the additional nano-particles on the LIPSS pattern, reduced the contact area between the bacteria and the metal surface, thus can either limits or weaken the strength of adhesion [8].

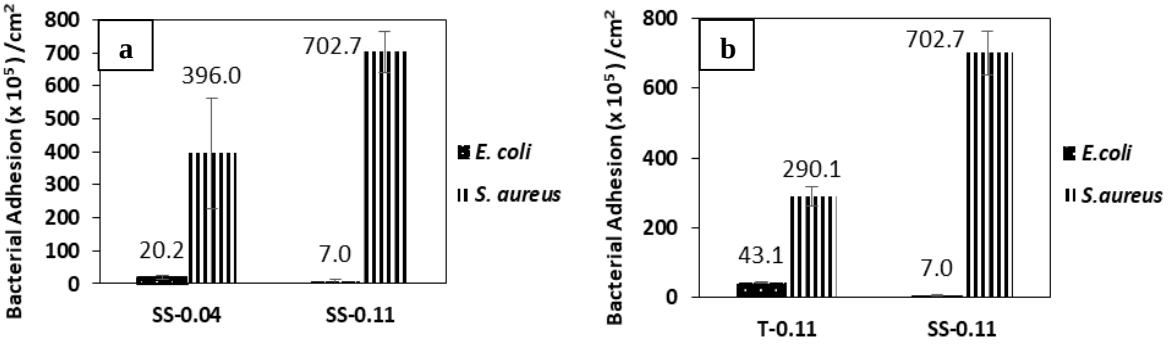

Figure 4. The total of bacterial adhesion (x10^5)/cm^2 on a) SS-0.04 and SS-0.11 b) T-0.11 and SS-0.11.

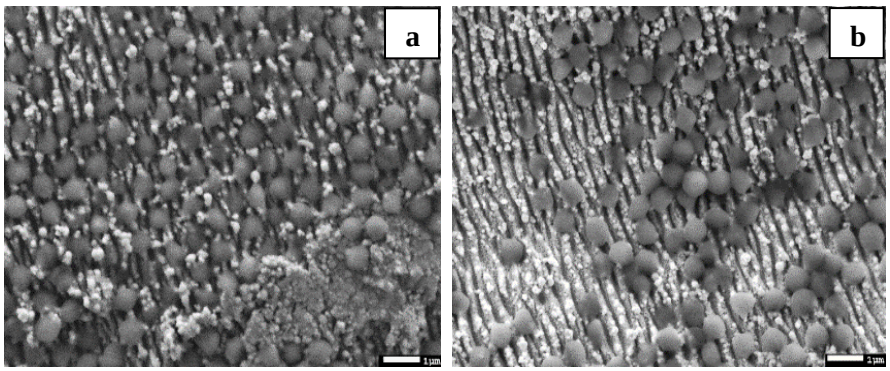

Figure 5. SEM images of bacterial on laser treated surface with 10000x magnification a) *E. coli* on T-0.11 b) *S.aureus* on SS-0.11.

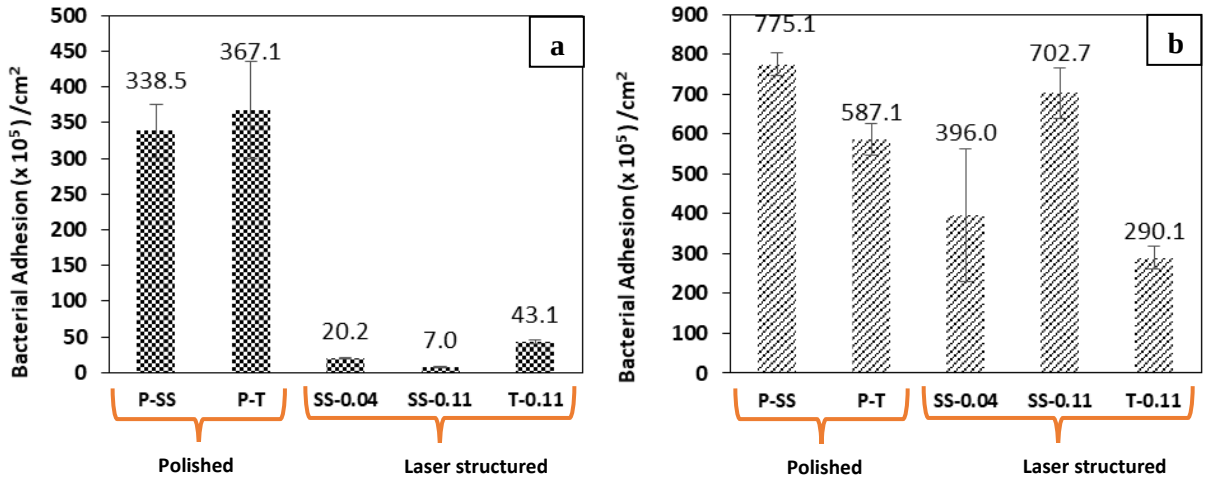

Figure 6. Bacterial adhesion on polished and laser structured surface, a) *E. coli* b) *S.aureus*.

4. Conclusion

Increasing the average laser power from 0.04 W to 0.11 W resulted in two different types of LIPSS structure. The type of LIPSS structure with and without nano-particles has a significant impact on the bacteria adhesion which varies differently for both *E. coli* and *S. aureus*. LIPSS with nano-particles led to an increased adherence of *S. aureus* on AISI 316L stainless steel by 77%, while the adhesion of *E. coli* was reduced by 65%. On the other hand, *E. coli* was observed adhered 6 times higher on Grade 5 titanium alloy compare on AISI 316L stainless steel. Presumably the different of surface hydrophobicity with the existence of laser generated nano-particles on the metal surfaces (at higher average laser power) was the governing factor that affected the rate of adhesion for both *E. coli* and *S. aureus* on these surfaces. Overall findings showed that, all the metals that undergone laser treatment had lower bacteria compared to polished metallic surfaces where *E. coli* showed >80% of reduction and *S. aureus* >10% for both stainless steel and titanium.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant (MSCA-RISE) agreement number 644971. In addition, the support for laser materials processing by the Karlsruhe Nano Micro Facility, a Helmholtz research infrastructure at KIT, is gratefully acknowledged.

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