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Effect of Laser-Induced Microtopographic Pattern on Biofilm Formation

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Abstract

This work aims to study the effect of two different types of micron-sized structures on biofilm formation. Nanosecond pulsed laser (Nd: YAG) is used to fabricate groove and pits pattern on the biomedical Grade-5 Ti-6Al-4V alloy. Field emission scanning electron microscopy (FE-SEM) is used to characterize the structured surfaces. The surface wettability of plain and structured surfaces is measured by sessile drop method using goniometer. Two different types of bacteria; *Escherichia coli (E.coli)* and *Staphylococcus aureus (S. aureus)* are cultured on the structured and plain sample for 48 hrs. The attachment of bacterial cell on all samples are observed using FE-SEM. The number of bacteria on each sample is estimated using crystal violet binding assay. The result shows that the bacteria behave differently on a different kind of surface topography. Bacteria are randomly distributed and closely attached to each other on plain samples resulting into formation of biofilm. However, on groove sample, bacteria are oriented along the groove direction while on pit structured sample, bacteria get crowded inside the pits. The bacteria do not form a biofilm on both the structured samples. The number of bacteria is found to be more on plain samples compared to structured sample. This study can be useful in the biomedical application for metallic implants in order to minimize the attachment of bacteria during and after the process of implantation.

Keywords: Laser Beam Machining, Topography, Surface Wettability, Bacterial Adhesion, Biofilm.

1. INTRODUCTION

Nowadays the biomaterials have gained importance due to the high demand of implants for aging population. Among many biomaterials, advancement in metallic biomaterials has made possible to produce better and long-lasting implants. In the category of metallic implants, the use of titanium and its alloy increased due to their superior biocompatibility, the low value of Young Modulus, light in weight and better corrosion resistance compared to other metallic implants [1]. Titanium and its alloys are used in dental and orthopedic implants, replacement of parts in hip and knee joints, bone fixation parts etc. [1,2]. However, despite the significant development in biomaterials, implant failure is still a problem in biomedical field [3]. Infection is the major cause of implant failure [3]. It is mainly due to the presence of bacteria in and outside of the patient's body. These bacteria forms biofilms, the biofilms are structured communities of bacterial cells that adhere to one another on the abiotic surface and produce extracellular polymeric substances which protect them from the external environment [4]. The formation of biofilm on implant surface results into poor osseointegration and high resistance to antibiotics [4,5].The initial stage of bacterial attachment to the surface is very complex as bacterial cell attachment depends on many factors like the chemistry of surface, charge on the surface, surface topography, wettability etc. [5]. The earlier approach was the use of biocides. In this approach, coatings were deposited on implant surface that releases antimicrobials or kills bacteria by direct contact [6]. However extensive use of biocide could lead to damage of mammalian cells or provide resistance to antibiotics [6,7]. Recently researchers are focusing on the latest strategies of development of anti-adhesive material against bacteria. In this approach, the risk of biofilm formation can be minimized by reducing or delaying the first step of bacterial cell attachment.

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By fabricating micron or sub-micron sized topography with defined feature dimensions specially selected according to the size of the bacterial cell can restrict the attachment and movement of bacterial cell [8]. The characteristics of superhydrophobic surfaces can be useful to make anti-adhesive surfaces [9,10]. In the present work, the author investigated the effect of two different microtopographic patterns produced by nanosecond laser on bacterial behavior and subsequent biofilm formation.

2. EXPERIMENTAL PROCEDURE

2.1 Materials and Methods

Medical grade Ti-6Al-4V alloy was cut into square samples of 12 mm × 12 mm × 2 mm dimensions using wire cut electro-discharge machining. Initially, samples were mechanically polished using silicon carbide (SiC) waterproof grit papers of sizes 80, 220, 320, 600, 800, 1000 and 1200 followed by first and second grade of alumina polishing with a particle size of 1 µm and 0.5 µm respectively. Samples were ultrasonically cleaned in ethanol and deionized water for 15 minutes in order to remove any alumina particle attached to sample surface. After cleaning, the sample surface was completely dried by blowing hot air over the samples. Micro-grooves and pits structures were fabricated on the Ti-6Al-4V sample using nanosecond Nd:YAG laser. The parameters; wavelength (1064 nm), pulse energy (25 mJ), pulse duration (20 ns) and the scanning speed (100 mm/sec) were kept constant for both the patterned surfaces. Field emission scanning electron microscopy (FE-SEM) was used to characterize the laser-induced micro-topographic pattern. In order to study the effect of a change in surface topography on surface wettability, the sessile drop test was carried out using goniometer (DSA25, KRUSS). Deionized water droplet volume of 3 µL was used with a micrometric syringe to dispense on samples.

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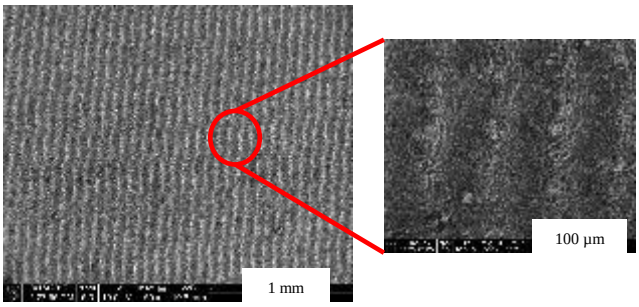
2.2 Bacterial Culture

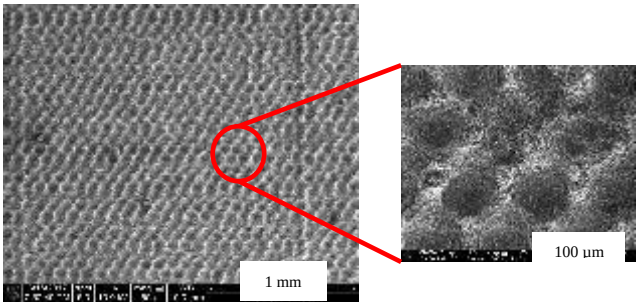
Plain and structured samples were ultrasonically clean in ethanol and deionized water so as to remove dust from the sample surface. Then, samples were put in 12-well plates, sterilized under UV light in ethanol for 10 minutes and washed five times with phosphate buffered saline (PBS). Two types of bacterial cell; *E. coli* (gram-negative) and *S. aureus* (gram-positive) were chosen to test against plain and structured surfaces. Initially, the primary culture of DH5α strain of *E. coli* and *S. aureus* allowed to grow overnight in Luria Bertani Broth at 37 ^{0}C in a shaking incubator. A secondary culture was set up by adding 10 % of the primary culture at 37 ^{0}C in a non-shaker incubator. The culture was allowed to grow till it reached to an optical density (OD) of 0.6 measured at 600 nm wavelength using a spectrophotometer. All samples were completely submerged in the culture for 48 hours [11]. After 48 hours, the samples were gently washed with PBS and fixed with 4% paraformaldehyde for 15 minutes. After 15 minutes, samples were blown with air and then coated with 5 nm gold thickness using DC sputter coater (Q150 RS, Quorum Technologies) in order to avoid charging during SEM imaging.

2.3 Quantification of Bacteria

Crystal violet binding assay technique was used to quantify the bacteria from all surfaces [12]. Another set of samples were submerged in the culture of both the type of bacterial cells for 48 hrs. After 48 hours, samples were washed five times with 1.0 mL PBS in order to remove non-adhere bacteria. Then, the remaining adhered bacteria were stained with 1.0 mL of 50 % filtered crystal of violet diluted in PBS for five minutes. The excess stain was washed off several times with PBS until the observation of a non-colored solution. Then, the dye bound to adherent cells was resolubilized with 700 µL of absolute ethanol for 20 minutes. Then, 100 µL of colored solution from each specimen was transferred to 96-well plates and the absorbance (OD) was measured at a wavelength of 600 nm using a spectrophotometer. The mean value of optical density was shown in plots with corresponding standard deviation. Two tail t-Test was done with (confidence level 95%) assumption of unequal variation between samples to confirm the consistency of results. If $p < 0.05$, the difference between results was considered significant and shown with asterisks sign.



(a) Linear grooves



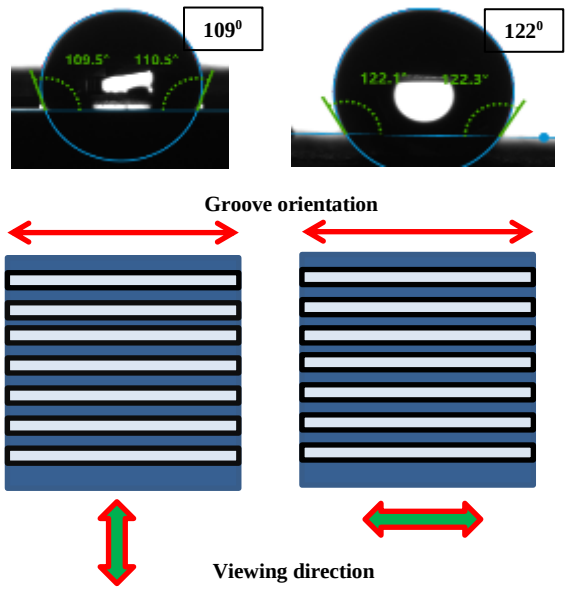
(b) Pit structures

Fig. 1. FE-SEM images of two different types of structures

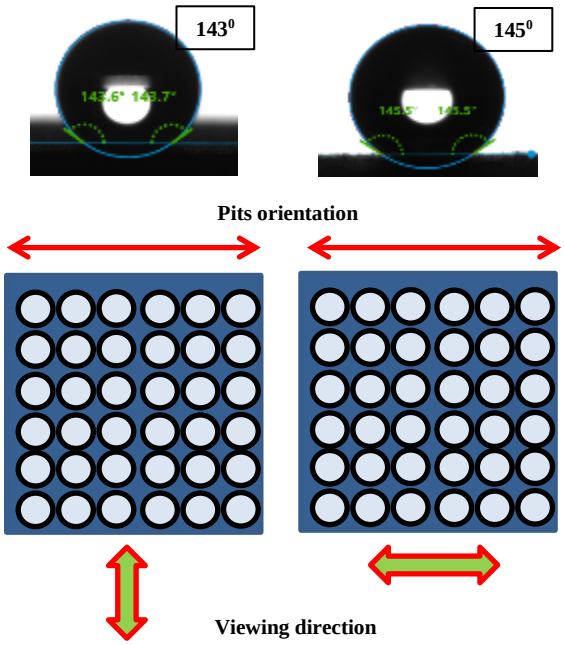
3. RESULTS AND DISCUSSION

Nanosecond laser process was used to fabricate groove and pits pattern of 40 µm spacing between structure on Ti-6Al-4V sample. The structured patterns were characterized using FE-SEM as can be seen from Fig. 1. Due to ablation phenomenon of the laser process, the spacing between structure slightly deviates from desired spacing. It has been proved that the surface wettability depends on the topography of surface [9,10]. Therefore, in order to test the wettability of structured Ti-6Al-4V samples, the sessile drop test was carried out using goniometer. The water droplet contact angle (WDCA) on both the structured surfaces was shown in Fig. 2. It was observed that in case of linear grooves, there is a reduction in WDCA (109^0), especially when viewed perpendicular to the groove orientation direction. This might be due to spreading of water droplet along the groove axis. The water droplet tries to enter into a groove and spreads along the axial direction of the groove. However, WDCA (122^0) is higher when viewed along the groove orientation direction. In case of pits structured surface, there is not much difference in WDCA when viewed either perpendicular (143^0) or parallel (145^0) with the orientation of pits. The reason for the same is no variation in the distribution of pits in both the directions. WDCA of 67^0 is observed on the plain Ti-6Al-4V surface. Overall the WDCA on pits structure is higher than the grooved surface. This could be due to the more entrapped air inside the pit structure compared to groove structures. Therefore in order to alter the wettability of surface or to achieve near-superhydrophobic or superhydrophobic surface, the pits or dimple like structures can be more effective.

After wettability testing, all the three samples (plain, groove, and pit) were taken for bacterial testing and the steps given in section 2.2 were followed. *E. coli* and *S. aureus* were tested against plain and structured samples. The qualitative analysis of biofilm was done with the help of FE-SEM images. Fig. 3 shows the representative images of both the types of bacteria on all three samples and shown with red circles. The attachment of both types of bacteria seems to be more on plain samples. Bacteria stick to each other and formed biofilm within 48 hrs. as can be seen in Fig. 3(a). This could be due to the more secretion of extrapolymeric substance on plain (hydrophilic) surface. On the plain surface, *S. aureus* formed thicker biofilm compared to *E. coli* because *S. aureus* requires a low surface area for its attachment due to its spherical shape.



(a) WDCA on groove structured



(b) WDCA on pit structured

Fig. 2. WDCA on different structured Ti-6Al-4V surface

On grooved sample, both bacteria are spread along the groove axis (Fig. 3(b)). Few bacteria are adhered to the grooved surface but not stick to each other as firmly as seen on the plain sample. The same results are observed on pits structured sample as shown in Fig. 3(c), however, the bacteria fallen inside the pits and gets crowded but still did not form a biofilm. The reason behind this could be that the bacteria might have taken a long time to cross or break air interface present between structures compared to the grooved surface.

The result discussed so far was qualitative in nature. In order to quantify the number of bacteria on the complete surface of samples, crystal violet binding assay technique was used. The number of bacteria on each sample was quantified in terms of optical density (OD) value of resolubilized dye. The higher value of OD indicates the more bacteria adhered on the sample. The OD value was measured at a wavelength of 600 nm using a spectrophotometer.

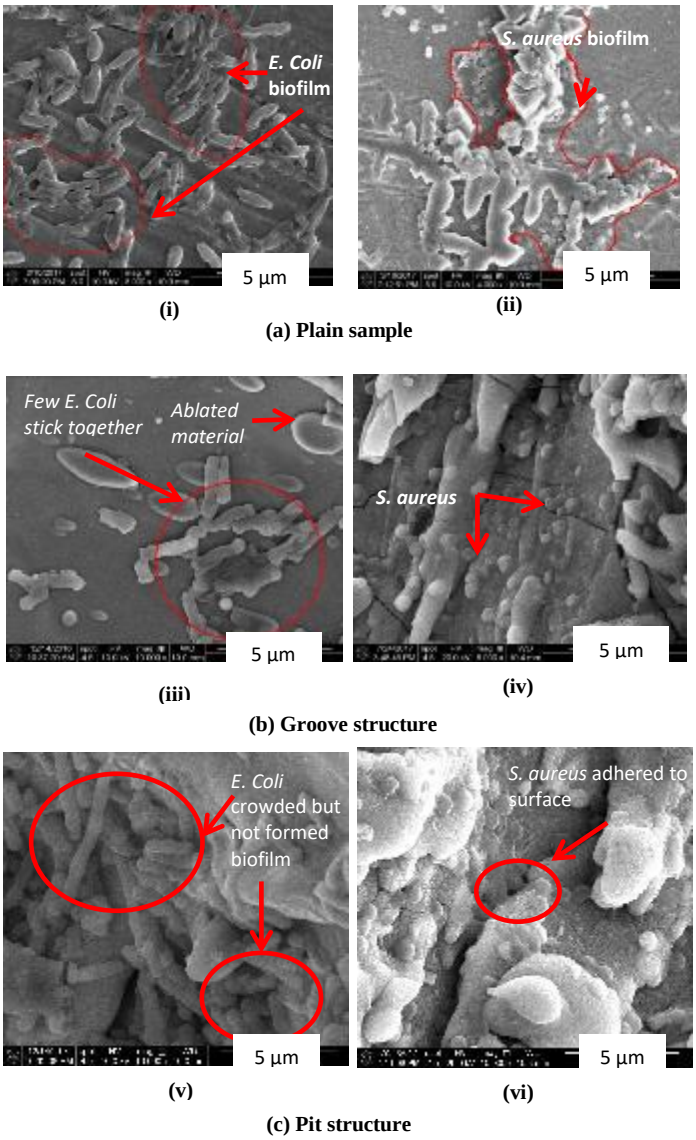


Fig. 3. Representative SEM images of *E. coli* and *S. aureus* tested on (a) plain (b) groove structure (c) pit structure Ti-6Al-4V surface

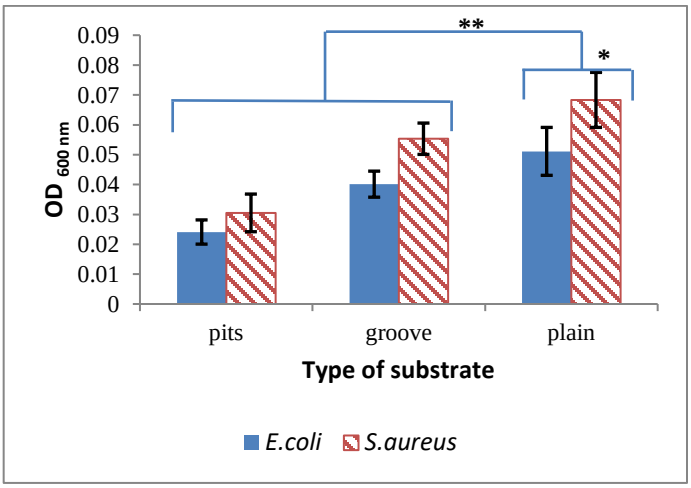


Fig. 4. Optical density (OD_{600nm}) of resolubilized crystal violet dye collected from cultured samples

Fig. 4 shows OD value for resolubilized crystal violet dye collected from all three samples with their respective standard deviation. Plain (67^0) sample has shown the higher value of OD compared to structured samples. Moreover, the OD value for *S. aureus* (OD = 0.068333) is significantly greater than OD value of *E. coli* (OD = 0.0511) on the plain sample. The difference between these OD value is statistically significant as shown by a single asterisk (*) sign. This quantitative result supports the qualitative observation that the biofilm form by *S. aureus* is denser as compared to *E. coli* on the plain sample. The pit structured (WDCA= 145^0) shown quite better adhesion resistance (OD for *E. coli* = 0.0241, OD for *S. aureus* =0.0305) to both bacterial cells compares to groove structured (OD for *E. coli*= 0.04122, OD for *S. aureus* = 0.055333) as can be seen from Fig. 4. The difference between OD value for the structured and plain surface was statistically significant shown by double asterisk (**) sign. Hence, the near superhydrophobic surface has shown good anti-adhesion property against bacterial cells. However, this approach assessed only the ability of a surface to resist bacterial attachment to the surface. This approach may be extended for the longer duration of time to observe the capability of a surface to resist further biofilm formation.

4. CONCLUSION

Microtopographic patterns were fabricated by nanosecond laser on the Ti-6Al-4V surface. The change in surface topography alters the wettability of titanium surface. Pit structures selection can be a good option compared to linear groove structure in order to get a less wettable surface. The attachment of bacterial cells depends on the wettability of surfaces. Bacteria adhered more in number on hydrophilic surfaces which results in the formation of biofilm whereas fewer bacteria were able to attach on near superhydrophobic surfaces and not form a biofilm. Moreover, the type of cell also has influenced on its adhesion as *S. aureus* requires a very small area for its attachment compare to *E. coli* that results into more biofilm formation. This approach of altering topography may help in reducing the risk of infections associated with implants.

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