Advanced surface treatment techniques counteract biofilm-associated infections on dental implants

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Abstract

Topography and surface chemistry can significantly affect biofilm formation on dental implants. Recently, the γ-TiAl alloy was considered as the most reliable candidates for the preparation of dental implants because of its excellent mechanical strength, chemical stability and biocompatibility. The emphasis of this study lies in the effects of high-speed milling assisted the minimum quantity of lubrication (HSM-MQL), micro-current wire electrical discharge machining (mWEDM), Er,Cr:YSGG laser and sandblasting/large-grit/acid-etching (SLA) treatments on surface morphology, topography, chemical composition, wettability and biofilm-associated infections on the surface of each group. The surface-treated samples were analyzed using a scanning electron microscope (SEM), SEM surface reconstruction, energy dispersive x-ray spectroscopy (EDS) and water contact angle measuring system. SEM and topography images of mWEDM and laser-treated surfaces showed more irregular surfaces compared to SLA and HSM-MQL surfaces. Results showed that mWEDM and laser-treated surfaces revealed hydrophobic behavior. A significant decrease of biofilm formation was observed on mWEDM treated surface due to the hydrophobicity and existence of the copper element in the recast layer chemical composition. Moreover, EDS confirmed that the zirconium, silicon, and fluorine elements were decorated onto the SLA treated γ-TiAl surface that can have a direct effect on the anti-bacterial activity.

1. Introduction

Implants have been commonly used in biomedical engineering such as orthopedic and dentistry fields. Dental implants are placed in an oral microbial environment of commensal bacteria and potentially pathogenic microorganisms or pathobionts [1]. Implant failure because of reasons such as peri-implantitis may have significant health implications and impose extra treatment and painful surgeries on the patient. Peri-implantitis is an inflammatory process with bacterial etiology that is characterized by the loss of supporting bone and the inflammation of surrounding mucosa. Inflammation around the implant can be caused by numerous microbes including a combination of anaerobic gram-negative bacteria, such as *Actinobacillus actinomycetemcomitans* (AA), *Eikenella corrodens* (EC), *Porphyromonas gingivalis* (PG) [2]. Dental implants require acceptable mechanical properties due to exposure to stresses and fatigue cycles, which only metallic materials withstand these conditions. Titanium (Ti) and its alloys, particularly Ti-6Al-4V, have extensive use in implant fabrication [3]. The biocompatibility of Ti and its alloys originates from the formation of a 4-6 nm thick layer of titanium oxide (TiO2) on the implant surface. This passive oxide layer exhibits high stability, low chemical permeability and good corrosion resistance in biological media [4].
Researchers have attempted to improve the functional conditions of implants by changing their chemical composition. Other motivations behind these attempts have been to address the possible release of toxic and allergenic vanadium ions [5], the low wear resistance of Ti-6Al-4V [6], low oxidation and corrosion resistance [7]. This has led to the investigation of various V-free alloys as alternatives to conventional implants. A new generation of V-free alloys for biomedical applications, known as TiAl intermetallic alloys, has shown particularly favorable biocompatibility properties [8]. Having low density (3.8 g cm$^{-3}$), high hardness (450 HV), acceptable mechanical strength (up to 1000 MPa), desirable oxidation properties, proper corrosion resistance and wear resistance, γ-TiAl alloys are a suitable alternative for Ti-6Al-4V and commercially pure Ti [8]. The in-vitro corrosion behavior of γ-TiAl was first examined by Escudero [8]. It was found that γ-TiAl has a higher corrosion resistance than α2- and β-Ti alloys [8]. It was also observed that γ-TiAl alloy has a slower corrosion rate in biological media than Ti-6Al-4V [9]. In the first attempts to establish the biocompatibility of γ-TiAl, an animal model was used to measure the biocompatibility of the Ti-48Al-2Cr-2Nb intermetallic compound and significant osseointegration and osteoconductivity were reported [10]. Ti-based implants have some amounts of aluminum (Al), which may exacerbate Alzheimer’s disease and neuro-inflammation [5]. The high Al content in γ-TiAl alloy (33.34%) may be a source of concern in this regard, but given the intermetallic nature of this alloy, there is a strong bond between Al and Ti at the atomic level, which significantly reduces the likelihood of release of Al in the biological environment [11, 12]. In addition, surface reactions such as oxidation reduce the chance of Al being released into physiological environments [13].

Surface properties of Ti implants are known as key factors for biofilm formation [14]. Indeed, surface chemistry and functional groups on the surface also influence bacterial adhesion [15]. Decreased bacterial colonization on TiO$_2$ coatings is observed even though these surfaces promote osteoblast adhesion and differentiation [14]. Various surface modification methods such as plasma electrolytic oxidation (PEO) [16], chemical vapor deposition (CVD) [17], thermal annealing [18], and sol-gel [19] have been applied to prevent biofilm formation on the Ti implants surfaces. It has also been shown that bacterial adhesion is influenced by the topography and roughness of the TiO$_2$ coating present on the implant surface. Furthermore, it is commonly accepted that surface wettability and surface plays a role in the bacterial adhesion [20].

Previous studies that have investigated the biological properties and biocompatibility of γ-TiAl have shown that it can serve as a good alternative to conventional implant materials [21]. Improved biocompatibility is known to be associated with increased bacterial growth, especially in dental implants commonly [22]. Many efforts have been made to increase biocompatibility while decreasing the growth of periodontal bacteria [23]. Among different surface treatments of dental implants, those effect on surface topography, including high speed milling assisted minimum quantity of lubrication (HSM-MQL), micro-current wire electrical discharge machining (mWEDM), erbium chromium-doped yttrium scandium gallium garnet (Er:Cr:YSGG) laser and sandblasting/large-grit/acid-etching (SLA), have attracted more attention, since these methods are able to control the biocompatibility and enhance the mechanical functionality. Recent research activities revealed that HSM-MQL surface treatment has a positive influence on the fatigue properties of metallic biomaterials [20]. The fatigue resistance of metallic dental implants is important, because they are subjected to cyclic loading during use [6]. It has been reported that, mWEDM surface treatment produces a fissured surface structure with deep, shallow and sharp edged surface flaws, which enhance the surface bioactivity [3]. Moreover, studies have shown that the laser treatment of Ti dental implant leads to an increased surface area in the micron range, thus resulting in an improved bonding strength between bone tissue and implant [6, 21]. The SLA technique combines the advantages of sandblasting and acid-etching techniques to impart macro-roughness and micro pits to the dental implant surface [20]. These hierarchical structures can enlarge the contact area of Ti dental implant, which favors the adhesion, proliferation and differentiation of osteoblasts [20, 23]. To the best of authors’ knowledge, effect of these surface treatment techniques on the response of periodontal bacteria to surface γ-TiAl is still not reported. Therefore, this study investigated the effect of HSM-MQL, mWEDM, Er:Cr:YSGG laser and SLA surface treatments on surface morphology, topography, chemical composition, wettability and the growth of AA and EC bacteria on these treated surfaces. We hope the present study can provide new insight for the surface treatment of Ti-based dental implants for clinical applications.

2. Materials and methods

2.1. Preparation of specimens

The γ-TiAl specimens were used in this study were prepared by vacuum casting method with a chemical composition of Ti-48Al-2Cr-2Nb (at%). To eliminate micro-pores and other casting defects, the specimens were subjected to HIP operation in Argon atmosphere at the temperature of 1250 °C and pressure of 150 MPa for 4 h. The specimens were then heat-treated at 1380 °C for 1 h to reach the desired mechanical properties and a uniform structure. The hardness of the samples after these steps was measured to about 320 HV$_{0.015}$. The specimens were subjected to surface modification by high-speed milling assisted minimum quantity of
lubrication (HSM-MQL), micro-current wire electrical discharge machining (mWEDM), Er,Cr:YSGG laser and sandblasting/large-grit/acid-etching (SLA). The identification codes for different surface treatments are presented as H, W, L, and S respectively. Prior to surface treatments, the surface layers of the heat-treated samples were completely removed by sandpaper and then the discs were ultrasonically cleaned in ethanol, rinsed with de-ionized (DI) water and dried in an autoclave at 120 °C for 15 min.

2.1.1. HSM-MQL surface treatment
The parameters for HSM-MQL surface treatment were chosen based on previous research that explained in detail in [24].

2.1.2. mWEDM surface treatment
In this method, the surface treatment was performed by a discharge of electric current in the presence of deionized water acting as the dielectric. The wire electrode was a brass wire of 200 μm in diameter. The mWEDM settings, which were chosen based on previous studies on Ti alloys as well as pilot studies [25], are given in table 1. The dielectric fluid used was deionized water with the addition of Ti powder with an approximate grain size of 2-5 μm in a concentration 2 g.l⁻¹. After performing the mWEDM process, the specimens were washed with deionized water and dried in air.

2.1.3. Er,Cr:YSGG laser surface treatment
After reviewing the past studies on the effects of Er,Cr:YSGG laser and conducting a pilot study, the laser settings were chosen in such a way as to minimize the defects and maximize the TiO₂ formation on the surface of γ-TiAl implants [26]. Er,Cr:YSGG laser with wavelength 2780 nm, using the gold handpiece with MZ10 Tip in non-contact and ‘H’ mode, power of 1.5-2 Watt, frequency 30 Hz, 50% water and 50% air for each square centimeters (Waterlase iPlus; Biolase Technology Inc., San Clemente, USA) was used. The other laser settings are given in table 2. The laser tip was MZ quartz with the fiber diameter of 600 μm with a distance of 1 mm between the tip of laser and surface samples.

2.1.4. SLA surface treatment
The polished γ-TiAl specimens were sandblasted under a pressure of 4.5 bars using SiO₂ and ZrO₂ particles with an average particle size of 120 to 250 μm. Subsequently, the specimens were ultrasonically cleaned in an alkaline solution, washed in distilled water and pickled with 3 vol% HF (48 wt%, Sigma-Aldrich) and 97 vol% HNO₃ (70 wt%, Sigma-Aldrich) and dried in the autoclave for 20 min at 120 °C.

2.2. Surface characterizations
The surface morphologies were observed by Scanning Electron Microscopy (SEM, Zeiss, Oberkochen, Germany). In addition, cross-sections of each surface-treated specimen were prepared by a cold mounting, cutting with a precision cutter, grinding with 400, 800, 1500 and 2400 grit SiC paper and polishing with 9 μm diamond suspension and, lastly, etching with Kroll’s reagent for 90 seconds at ambient temperature before viewing in the SEM. The chemical composition of the surfaces and cross-sections was analyzed by energy

Table 1. Parameters of mWEDM used for surface treatment of samples.

| Parameters          | Unit     |
|---------------------|----------|
| Pulse on Time (Ton) | 1 μs     |
| Pulse off Time (Toff)| 50 μs   |
| Input Power (Pi)    | 0.50 A fine pulse (m.c⁻¹ units) |
| Wire Tension (Tw)   | 5 kg     |
| Servo Voltage (Vs)  | 10 V     |
| Temperature of the dielectric | 25 °C |
| Conductivity of the dielectric | 20 mho |
| Wire type           | 0.20 mm diameter brass |
| Wire feed (Fw)      | 5 m.min⁻¹ |

Table 2. Parameters of Er,Cr:YSGG Laser used for surface treatment of samples.

| Energy | Spot size | Fluence | Power density | Total time | Pulse duration |
|--------|-----------|---------|---------------|------------|----------------|
| 60 mj  | 0.6 mm    | 35.4 J.cm⁻² | 707 W.cm⁻²    | 45 s       | 50 μs (H mode) |

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dispersive x-ray spectrometer (EDS, Oxford Instruments X-Max detector with INCA energy 300 software, UK) attached to the SEM. All SEM images and EDS data were acquired with a 20 kV beam, at magnifications 1000 ×. To evaluate the surface topography of surface-treated γ-TiAl specimens, 3D surface modeling (3DSM, Carl Zeiss microscopy) and scanning probe image processor (SPIP 6.7.8) equipped with image metrology software (Hørsholm, Denmark) were used.

The water contact angles on the sample surfaces were measured with distilled water using the sessile drop method (DSA30, Krüss, Germany), coupled with a camera-based contact angle analyzer (Image Metrology, Hørsholm, Denmark). The volume of each sessile drop was 5 μL (i.e. controlled using a micro-liter syringe) and three points were measured for each sample. The results were expressed as mean values and standard deviations. Contact angle measurements were performed under room temperature in air.

2.3. Microbiological studies
Before Microbiological studies, γ-TiAl specimens were sterilized with isopropanol 70% for 2 h with gentle shaking after which the samples were washed 5 times with DI water. The biofilm layer on the specimens was studied for Actinobacillus actinomycetemcomitans (AA) and Eikenella corrodens (EC). For this purpose, a suspension of $3 \times 10^5$ CFU.ml$^{-1}$ in Tryptic Soy Broth (TSB, Oxoid, Milan, Italy) plus 0.5% of D-glucose in Tryptic Soy Agar (TSA; Oxoid) was prepared. The bacterial suspensions were uniformly spread in sterilized cultivation plates (IWAKI microplate, Bibby Scientific Limited, Staffordshire, UK).

Two implant discs were placed in each well. Biofilm was produced by 48 h of incubation at 35 °C. Fresh TSB medium was added 24 h after the start of incubation (1.5 ml/well). Unstable bacterial cells were washed with phosphate-buffered saline (PBS). Then, the bacteria grown on the surface of the titanium aluminate were placed on sterile slides. To determine the viable cell count (CFU), disks were placed in 1.5 ml sterile tubes (each disc in one tube) containing 500 μl of TSB plus 0.1% Tween 20 as recovery media. To facilitate biofilm decomposition, each tube was stirred for 30 s and centrifuged for 30 min at 30 kHz and 300 W.

After second cycle (30-s centrifuging), dilutions of each sample were prepared in a sterile solution and 100 ml of each dilution was placed on TSA and incubated at 35 °C for 24 h. Finally, the bacteria of each sample were counted. For each sample, all tests were repeated three times and the mean value was reported. After counting the bacterial colonies, their number was compared with the number of colonies in the control group.

Figure 1. SEM micrographs of (a) HSM-MQL-, (b) mWEDM-, (c) laser- and (d) SLA-treated γ-TiAl.
2.4. Data presentation and statistical analysis
The values are expressed as mean values and standard deviations of triplicate experiments. Statistical analysis of differences between the experimental groups was performed using One-way ANOVA with Tukey’s multiple-comparisons test and results were considered statistically significant if \( p < 0.05 \). Calculations were performed using the SPSS 22 (SPSS, Chicago, IL, USA).

3. Results and discussion
3.1. Surface characterizations
The SEM micrographs of the surface of all samples obtained with different surface treatment are displayed in figure 1. The SEM image of Sample H exhibited a flat surface, smoothly scratched with unidirectional grooves and tiny scratches left during the HSM-MQL operation (figure 1(a)). Conversely, after mWEDM surface treatment, the surface morphology was completely transformed. The top surface of mWEDMed \( \gamma \)-TiAl consists...
of the crater, rippled surface, a number of cavities and overlapping craters similar to what has been previously reported by others [27, 28]. In WEDM method, individual wire electrical discharges cause the removal of micro-particles of the material. Material removal in WEDM is by means of spark erosion [29]. Discharged material particles are subsequently carried away from the cut area by a flushing jet of dielectric liquid. In this way, a great number of craters are formed on the surface [27]. Moreover, micro-cracks are present on mWEDM modified surfaces, which were likely formed due to thermal stresses [28]. The surface morphology formed on the Sample L (figure 1(c)) is quite non-uniform and accumulated fluencies a fast-melting and solidification process occurring on the surface. In the Er, Cr:YSGG laser surface treatment, the substrate melted from the impact of the pulsed laser [26]. In addition, nanoscale wrinkles emerged on the surface of the micron-sized structures. As seen in figure 1(d), the SLA process produced the fine 1–5 μm micro-pits superimposed on the rough-blasted surface. The micro-pit structure of the Sample S resulted from pickling via the HNO3/HF solution. Moreover, fewer and smaller micro-cutting or ploughing tracks were observed due to the high velocity of abrasive SiO2 and ZrO2 particles.

The topographical characteristics of the surfaces are related to the physical properties of the surface, such as roughness. In this study, the topography was examined by noncontact 3D optical microscopy. The 3D and 2D optical interferometry images for treated surfaces are presented in figure 2. No image is shown here for polished γ-TiAl substrate since it was nanometrically smooth. It can be observed the topography changed due to different surface treatments. Sample H presented less irregularity with some peaks and valleys than was achieved with other samples. Samples after mWEDM and laser treatment exhibit a more diversified surface topography with sharp peaks compared with other samples [26, 27]. It is worth adding that the interaction between the acid and γ-TiAl substrate after sandblasting caused a slight smoothing of the surface. To easier compare the changes in the surface roughness, the mean values with standard deviations of the Ra were calculated from 2 to 4 images. The Ra values follow the same trend of the microscopic topographic average peak-to-valley waviness and were 0.53 ± 0.14 μm in Sample H, and were 1.82 ± 0.31, 2.13 ± 0.25 and 1.33 ± 0.17 μm in Samples W, L, and S, respectively. It is widely found that surface roughness enhances osteoblast differentiation and affects growth factor production by increasing the physical contact area, which provides increased primary mechanical stability [30].

Figure 3 shows cross-sectional SEM-BSD images of the surface-treated γ-TiAl substrate. The observations at higher magnifications in Sample H (figure 3(b)) reveal the presence of well-defined 2 μm thick, a continuous deformed layer representing work hardening and plastic deformation [24, 31]. It has been reported, work hardening generally improves the mechanical properties of this area [32]. Additionally, lack of the micro-cracks or delaminations emphasizes beneficial features of HSM-MQL technique. Figure 3(b) details sample cross-sectional micrographs for Sample W, where the γ-TiAl substrate is covered by a thin recast layer. In this layer a
lot of delaminations were visible, directed from the surface of the recast layer to the substrate. In addition, more micro-cracks and micro-void were seen in the recast layer. Micro-cracks, when evident, terminated within the formed layer and did not penetrate into the substrate. It can be seen from figure 3(c) that the laser-treated γ-TiAl exhibited the thin and separated melted zone (4-6 μm thick). The rapid heating and melting accompanied by the high pressure of Er,Cr:YSGG caused the liquid metal spilled from the central part of the melted area towards the periphery [26, 33]. In addition, the surface roughness was increased by an effect of overlapping of subsequent melted areas [33]. The formation of micro-pits with a flat edge during SLA treatment is shown in figure 3(d). Combined with the surface SEM image in figure 1(d), the formation of a layer consisting of valley superimposed on micro-pits is observed [34].

Chemical analysis of the surface layer was performed using EDS analysis. EDS analysis of the surface of the Sample H did not reveal any change in the chemical composition of the deformed layer during HSM-MQL method. The cross-section EDS point spectra and line-scanning profiles of Sample W are displayed in figures 4(a) and (b), respectively. Chemical analysis of the recast layer (Region A) and subsurface area under the recast layer (Region B) indicated the presence of Ti, Al, O, Cu, Nb, and Cr. The presence of Cu in the recast layer is due to ion transfer from the brass wire electrode which has been previously reported by other researchers [24, 27, 28]. The presence of the oxygen for oxides produced by mWEDM as compared with the original γ-TiAl substrate can be attributed to preferential oxidation of Ti which is thermodynamically favored [35]. Nb and Cr are γ-TiAl alloy elements. The cross-section EDS line-scanning profiles suggested that the recast layer was approximately 5.5 μm, as indicated by the peak width of Cu and O elements. One can also observe some oxide dispersion in the recast layer [36]. These results show that greater incorporation of Cu contributes to a significant reduction of Al intensity in the recast layer.
EDS spectra of the chemical compositions of the laser-treated $\gamma$-TiAl are shown in figure 5(a). The content of Ti decreases from 60.3% for the substrate (Region B) to 41.2% at the melted-zone (Region B), while the content of O correspondingly increases to 40.8%. Presence of oxygen indicates possible $\gamma$-TiAl alloy oxidation during Er, Cr:YSGG laser processing. Previous laser modifications of Ti and its alloys emphasized the possibility of forming relatively thick oxides layers which are also beneficial in terms of bio-stability [37]. The elemental line-scan profile of the Sample L is shown in figure 5(b). A significant decrease in the Al concentration of the alloy was observed at the interface of the substrate and melted-zone. In addition, the melted-zone was well adherent to the substrate in the interface, while some pore or crack was observed in the melted layer.

The EDS analysis for Sample S (not shown) demonstrated that Ti (41.6 wt%), O (21.9 wt%), Al (12.2 wt%), F (10.1 wt%), Nb (4.9 wt%), Zr (4.3 wt%), Si (2.9 wt%), and Cr (2.1 wt%) were found to be present on the modified SLA surface. The abundance of O is due to the stable native TiO$_2$ layer that forms on the $\gamma$-TiAl substrate [38]. The existence of Zr and Si elements was probably because of the retention of zirconia and silica abrasive particles on the substrate during the sandblasting [39, 40]. Adventitious F element was most likely present due to the acid etching by HNO$_3$/HF solution and attaching fluorine ions to the sandblasted surface [41]. EDS mapping was performed to study the elemental distribution of the Sample S as shown in figure 6.

For any new surface, the wettability is measured to analyze its surface energetics and interaction of the liquid molecules with a solid surface [42]. Figure 7 shows the drop shape and contact angle measurements. The images were captured immediately after placing the drop on the surface. In the case of Sample H and S, the contact angle was measured 78.2° and 86.5°, respectively and these surfaces presented the hydrophilic behavior ($\theta < 90^\circ$).
However, the wettability analysis of the L and W surfaces revealed that the mWEDM and laser-treated surfaces were more hydrophobic, exhibiting contact angle of 97.5° and 108.1°, respectively. This increase in the contact angle can also be related to the decreased surface energy of the mWEDM and laser-treated samples [42]. Moreover, this hydrophobicity behavior agrees with studies reporting that WEDM treated surfaces show higher contact angles [43]. According to Ra and contact angle values (figures 2 and 7), the surface wettability decreases by increasing the surface roughness. This is due to the highly expanded surface topography in combination with heterogeneous surface morphology, which could entrap the air between the rough asperities, prevent the water intrudes into the spaces and therefore increase the hydrophobicity of the surface according to the Cassie-Baxter model, which the water droplet is suspended on an interface made of solid and air trapped in the rough asperities [42, 44]. The apparent contact angle is governed by the Cassie–Baxter equation (equation (1)):

\[
\cos \theta'^E = r_s \phi_s \cos \theta_E (1 - \phi_s) \cos \theta_A
\]

where \( r_s \) is the surface roughness, \( \phi_s \) is the areal fraction of the liquid-air at the bottom of the droplet, \( \theta_E \) is the equilibrium contact angle with a solid surface, and \( \theta_A \) is the equilibrium contact angle with air. This means that the air trapped in the gaps can produce a large contact angle, preventing the penetration of water droplets into the surface. According to equation (1), the contact angle increases with the surface roughness for the hydrophobic surface [42].

Figure 6. Elemental mapping using EDS analysis on the SLA-treated γ-TiAl.

Figure 7. Water contact angle of (a) HSM-MQL-, (b) mWEDM-, (c) laser- and (d) SLA-treated γ-TiAl.
Changes of the water contact angle over time are presented in figure 8. The water droplet contact angle decreases gradually with time in all surface treated samples. The reduction rate of contact angle in Samples H, L and S were quick for the first 20 s, thereafter the rate was slowly decreased. In comparison, on the surface of Sample W, the contact angle was still up to 90°, which indicates good surface hydrophobic stability. Moreover, the reduction rate of the contact angle in Sample W was dramatically slow during the 180 s contact time. It has been reported, the decrease of the water contact angle value was due to the absorption of the water droplets by the surfaces of the materials and a decrease in the volume of the water droplets over time. Surface wettability is an important parameter that can be used to assess the possibility that a surface could exhibit anti-bacterial behavior.

3.2. Microbiological studies

Successful dental implant integration relies on a balance of wettability with anti-bacterial properties [1–3]. The results of the microbial biofilm analysis are shown in figure 9. In general, significantly more AA bacterium formed on the treated surfaces compared with EC. However, similar formation trend was achieved for both of them. The topography is an important factor in biofilm adhesion, for example, the interaction of bacteria with two surfaces of identical chemistry, but differing topography can result in significantly different densities of adherent bacteria in-vitro [45]. It seems that the formed grooves HSM-MQL surface treatment (figure 2) provide a good environment for the growth of bacteria. This observation is in line with Giulio et al’s report [46]. From figure 7(a), it could be observed that the water permeation through the coating can be high due to the coating’s increased hydrophilicity. The hydrophilic surface of the Sample H resulted in a greater bacteria response in comparison to hydrophobic surfaces such as Sample W and L [20, 45]. A hydrophobic coating separates the liquid from its surface, thus reducing the number of attached proteins and bacteria [20]. The physiological role of implant surface chemistry is important in determining the success of implant osseointegration and inhibition of biofilm formation [47]. As seen in figure 8, biofilm formation on the Sample W surface was generally lower than to the Sample H despite having higher roughness values. This illustrates the significance of the chemical composition of Sample W surface for promoting bacterial adhesion. As previously noted, EDS measurements also confirm the presence of Cu in the mWEDM treated surface (figure 4). Cu has been proven to have good anti-bacterial and anti-infection capabilities, enhancing alkaline phosphatase activity, and increasing the bone apposition rate in the early phases of osteogenesis [48, 49]. It is believed that the heavy metal ions such as Cu^{2+} can kill bacteria by destroying their proteins [50]. Kuppusami et al [51] demonstrated that CrN/Cu coatings containing 15.1 wt% copper had a durable antibacterial effect, which copper ions were responsible for destroying the cell walls of the bacteria and inhibiting their growth. Hadidi et al [52] reported HA-Cu nanocomposite coatings produced by the electrophoretic deposition method on the Ti6Al4V substrates and suggested that the coating containing 5 wt% copper expressed both a
powerful anti-bacterial and anti-infection activities and good cytocompatibility. In this study, the surfaces of the W and L sample could be classified as 'rough' surfaces while that of the Sample S was designated as 'moderately rough'. It has been reported, moderately rough surfaces displaying stronger bone responses than rough ones. As seen in figure 8, the SLA treated surface showed higher anti-bacterial efficiency compared with the laser-treated surface. As stated previously, zirconium, silicon and fluorine enriched layer formed during the sandblasting and the reaction with HNO₃/HF solution. It has been clearly demonstrated in recent years that zirconium [53], silicon [54] and fluorine [55] incorporated surfaces that can have strong anti-bacterial and anti-infection effects on biofilm development on implant surfaces and improve the biological capability. Fluoride has been known for its bactericidal effect by the disruption in metabolism because of the inhibition of bacterial enzymes and membrane function [55, 56]. Recently, silicon and fluoride ions have been reported to enhance the incorporation of newly formed collagen into the bone matrix and increase the rate of seeding of apatite crystals [57, 58]. Nurhaerani et al [55] found that plasma-based fluorine ion implantation into stainless steel provided surface anti-bacterial and anti-infection activities and reduced the attachment of S. mutans bacterium. The results of percentage change of two micro-organisms in different surface treated samples compared with Sample S, as seen in table 3. Again, it can be observed the biofilm growth in Sample W reduced significantly.

Table 3. Comparison of percentage change of biofilm growth on the surface of the different samples toward Sample S, as the control sample.

| Samples | Percent changes of growth AA bacterium | Percent changes of growth EC bacterium |
|---------|--------------------------------------|--------------------------------------|
| H       | 190.69%                              | 5485.45%                            |
| W       | −56.03%                              | −73.15%                             |
| L       | 108.139%                             | 208.11%                             |

Figure 9. Results obtained from counting AA and EC bacteria on the different surfaces. The data are expressed as mean values ± standard deviation of 4 independent experiments (n = 3). Asterisk (*) denotes significant difference at p < 0.05 compared to the control sample (Sample S).

4. Conclusion

In this study, we examined four different surface treatments and tried to find out whether the surface roughness, wettability and chemical composition of the each treated samples could be an effect on biofilm formation. The
mWEDM and laser-treated surfaces showed some irregularities with sharp peaks and valleys and these surfaces exhibited the water contact angles greater than 90°. However, the water contact angles on the HSM-MQL-treated surfaces were smaller than the other samples. The surface wettability and chemical composition were independently affected by biofilm formation. This study affirms the antibacterial capacity of mWEDM surface-treated against AA and EC periodontal bacteria due to the hydrophobicity behavior and presence of copper ion in the top recast layer. Moreover, incorporation of zirconium, silicon and fluorine elements add antibacterial properties to the SLA sample, resulting in significant reductions of AA and EC biofilms. Finally, this study advances that either the mWEDM and laser surface treatment techniques could lead to broad implant applications of Ti alloys by promoting antibacterial activity.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This study was approved by a panel from the Tehran University of Medical Sciences Ethical Committee (Ethics code: IR.TUMS.DENTISTRY.REC.1397.4921).

Informed consent

Informed consent was obtained from all individual participants included in the study.

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