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Influence of titanium and zirconia modified surfaces for rapid healing on adhesion and biofilm formation of *Staphylococcus epidermidis*

Marcel F. Kunrath[a,c,*], Marina S.G. Monteiro[b], Saurabh Gupta[d], Roberto Hubler[c], Sílvia D. de Oliveira[b]

[a] Pontifical Catholic University of Rio Grande Do Sul (PUCRS), School of Health and Life Sciences, Dentistry Department, Av. Ipiranga, P.O. Box 6681, 90619-900, Porto Alegre, RS, Brazil
[b] Pontifical Catholic University of Rio Grande Do Sul (PUCRS), School of Health and Life Sciences, Immunology and Microbiology Laboratory, Av. Ipiranga, P.O. Box 6681, 90619-900, Porto Alegre, Brazil
[c] Pontifical Catholic University of Rio Grande Do Sul (PUCRS), Materials and Nanoscience Laboratory, P.O. Box 1429, 90619-900, Porto Alegre, Brazil
[d] International Academy of Ceramic Implantology, Silver Spring, MD 20901, USA

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ABSTRACT

Objective: Surface alterations have been employed to enhance the osseointegration process in biomedical implants. However, these modifications may influence bacterial adhesion in different ways. Therefore, this study developed five different surfaces and evaluated the *Staphylococcus epidermidis* growth in early (1 h) and late (24 h) contact.
Design: The Titanium (Ti) and Zirconia (Zr) surfaces were divided in five groups and characterized concerning your morphology, roughness, wettability and chemical surface composition. Then, were evaluated regarding bacterial adhesion and biofilm formation/thickness, viability and morphology.
Results: Different topographies were manufactured resulting in a variety of combinations of surface properties. High roughness showed significantly higher bacterial adhesion in 1 h, while high hydrophilicity revealed greater bacterial proliferation in 24 h. Morphological changes were not found visually, however the viability test showed some cell membrane damage in the Ti micro and nano groups.
Conclusions: Finally, surface distinct properties influence the growth of *S. epidermidis* independent of the based-material. Furthermore, some surface properties require precautions for use in contaminated sites according to the increased adhesion of *S. epidermidis* presented when in contact.

1. Introduction

Biocompatible materials that replace body hard tissues have been the subject of numerous researches concerning their biological and biomechanical properties (Ananth et al., 2015; Cross, Thakur, Jalili, Detamore, & Gaharwar, 2016; Tosiriwatanapong & Singhatanadgit, 2018). Pure Titanium (Ti) and Zirconia (Zr) are widely used in dental implants, oral prostheses and orthopedic implants/prostheses (Ananth et al., 2015; Osman & Swain, 2015). The surfaces of both materials can be modified in terms of morphology, roughness, surface energy and wettability by different treatments (Henningsen et al., 2018; Schünemann et al., 2019). These surface treatments directly influence interaction, adhesion of osteoprogenitor cells, anti-inflammatory responses and antibacterial activity (Bosshardt, Chappuis, & Buser, 2017; Hirano et al., 2015; Kunrath, Leal, Hubler, de Oliveira, & Teixeira, 2019; Ma et al., 2014).

Most studies show better osteogenic cells interaction on rougher surfaces for both Ti and Zr (Hirano et al., 2015; Kuo et al., 2017). However, higher roughness surfaces also directly support the bacterial colonization (Al-Ahmad et al., 2016; Roehling et al., 2017). As the surface treatment methods of Ti and Zr present wide variation and different approaches are employed because of their different chemical compositions and hardness of materials (Civantos et al., 2017; Schünemann et al., 2019), the adhesion of important bacteria in initial infection processes with the use of these biomaterials is not fully understood.

Surface nanotechnology is rapidly evolving and showing promising results when applied in both Ti and Zr (Kulkarni et al., 2015;

* Corresponding author at: Dentistry Department, School of Health and Life Sciences, Pontifical Catholic University of Rio Grande do Sul (PUCRS), P.O. Box 1429, 90619-900, Porto Alegre, Brazil.
E-mail address: marcelfkunrath@gmail.com (M.F. Kunrath).

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Table 1
Groups division and surface treatments.

Group	Surface Treatments	References
Ti Macro	Machined: Only cleaned	(Kunrath, dos Santos et al., 2020)
Ti Micro	Double acid etched: with a solution of hydrochloric acid and 70 % diluted sulfuric acid for half hour at 98 °C.	Modified from (Kunrath, dos Santos et al., 2020)
Ti Nano	Anodized: in an electrolytic solution composed of ethylene glycol, 0.5 % NH_4F, 10 % DI H_2O with controlled voltage (40 V) for 1 h followed by a heat treatment (300 °C).	(Kunrath, Penha, & Ng, 2020)
Zr Macro	Machined: Only cleaned	–
Zr Micro	Particle Blasting: airbone particle abrasion with 50 – 100 μm Al_2O_3 particles.	–

Schünemann et al., 2019). Nanometer-scale surface changes reveal differentiated topographic morphologies with also diverse cellular responses (Faghihi et al., 2007), especially due to the nanoscale interaction of the cell membrane along the nanotextured surface (Faghihi et al., 2007). Meantime, the majority of all nanometric technology coupled with changes in wettability, roughness and possibilities of bioactive surfaces creates huge challenges in bacterial adhesion and proliferation, which could avoid biomaterial contamination in the initial stages after insertion into an organism (Kunrath et al., 2019).

Staphylococcus epidermidis is considered a transient member of the oral microbiota, but it has been detected as a prevalent species in healthy patients with implants and in patients with peri-implantitis (O'Connor et al., 2018). In fact, *S. epidermidis* is one of the most common pathogens found in body-implanted biomaterial infections (Arciola, Campoccia, & Montanaro, 2018; Oliveira et al., 2018), and ability to form biofilm is considered its major virulence factor (Arciola et al., 2018). The bacterial adhesion process usually occurs by chemical linkage between the bacterial surface and the external layer of the biomaterial, evolving into biofilm formation. The biofilm formation by *S. epidermidis* include the expression of the polysaccharide intercellular adhesin and the release of extracellular DNA derived from bacterial autolysis and from dead host cells (Arciola et al., 2018). In *S. epidermidis*, the β- subclass of phenol- soluble modulins contributes to biofilm structuring and leads to the formation of characteristic water channels observed in mature biofilms (Le, Dastgheyb, Ho, & Otto, 2014), beyond to be involved in biofilm dispersal, together with proteases and nucleases (Arciola et al., 2018). The most important regulatory system in *S. epidermidis* biofilm is the accessory gene regulator (agr) quorum sensing system, since it is involved in the regulation of the initial attachment, aggregation, maturation and dispersion steps, and, simultaneously regulates the expression of other virulence factors (Le & Otto, 2015).

Presence of biofilm in the early stages of surgery is reported as one of the major factors responsible for loss of implantable biomaterials (Quirynen, De Soete, & Van Steenberghe, 2002). To control possible infections, understanding the ability of *S. epidermidis* to colonize these biomaterials in the early stages is of paramount importance. However, *in vivo* approaches, it is possible to occur interaction among more than one bacterium, making it more difficult to visualize and analyze the responses of isolated bacterium. Thus, the use of *in vitro* methodologies becomes essential to observe the isolated behavior of bacteria. Adherence of *S. epidermidis* to different types of biomaterials used in surgeries can be affected by slightest change in roughness, although, other surface properties could influence adhesion as well (Al-Ahmad et al., 2013; Yoda et al., 2014). Cao et al., 2018 reported that different surface nanomorphologies distinctly affect bacterial adhesion in a short and extended period, proving that the minimal change in topography and surface property lead to interference in the colonization of *S. epidermidis*. To the best of our knowledge, no other studies have evaluated differences in macro, micro and nano-topography scale, surface morphology changes and hydrophilicity simultaneously, in relation to colonization of *S. epidermidis* on Ti and Zr surfaces. Therefore, this study aimed to investigate the physicochemical properties of materials, as well as the ability of *S. epidermidis* to adhere and colonize a range of surfaces with different properties that are similar to those found in commercial implants.

2. Materials and methods

2.1. Materials

Pure titanium grade II plate (TitanioBrasil, São Paulo, Brazil) was prepared into 45 discs (1 mm thick x 8 mm diameter). The samples were cleaned with 70 % ethanol using different sandpapers manually for a clean burr-free surface. Zr plate (ZrO2-TPZ, Coho Biomedical Technology Co., Taiwan) was prepared into 30 discs (1 mm thick x 6 mm diameter). To initiate the Ti and Zr texturing, all samples were placed in acetone for 5 min and washed with deionized water (DI).

2.2. Surfaces treatments

To demonstrate the variety of surface properties currently found on the implant market, Ti and Zr samples were divided into five groups focusing on different final surface properties found in market, as can be seen in Table 1. Ti samples were treated with three distinct methodologies: Ti Macro - machined, Ti Micro - double acid etched and Ti Nano - anodization. Zr surfaces were modified by two treatments: Zr Macro - machined and Zr Micro - particle blasting. After the treatments, samples were sterilized with an autoclave at 125 °C for 30 min and wrapped in sterilization paper for subsequent biological assays.

2.3. Surfaces characterization

Surfaces morphologies and elemental analysis were investigated (n = 3 per group) using a scanning electron microscope (SEM, Inspect F50, Prague, Czech Republic) with an energy dispersive X-ray spectrometry (EDS, Prague, Czech Republic). To roughness evaluation (n = 3 per group), an Atomic Force Microscope (AFM, Dimension Icon, Bruker, Massachusetts, USA) and the NanoScopeAnalysis® software were used. Four parameters were investigated (Sa = average surface roughness, Sq = root square, Ssk = skewness of height distribution, Sku = kurtosis of height distribution) using a cut-off value of 30 μm. Wettability was evaluated (n = 3 per group) by the sessile drop method using a Goniometer - Contact Angle Measure (Phoenix 300, SEO, Kosekdong, Korea) equipped with DI water and the software (Surfaceware8, version 10.11, Korea).

2.4. S. epidermidis adhesion

S. epidermidis ATCC 35984 was grown overnight in Brain Heart Infusion (BHI) (Oxoid Ltd., Basingstoke, Hampshire, England) broth at 37 °C. Afterwards, the culture was centrifuged at 8000 rpm for 10 min, and pellet was resuspended in 0.85 % saline solution to obtain 10^7 colony forming units (cfu)/mL. A 20 μL-aliquot of this inoculum was dropped onto a sterilized microscope slide enclosed inside a Petri dish and the samples (n = 4 per group) to be tested were placed face-down onto the suspension, according to Narendrakumar et al., 2015. Bacterial attachment was allowed by incubation at room temperature for 1 h.

After that, samples were gently washed thrice with 10 mL of phosphate-buffered saline (PBS) to remove loosely bound bacteria. 10 mL of sterile PBS were added to the samples that were incubated in an ultrasonic water bath (Ultrasonic Cleaner 1400A, Unique, Indaiatuba, Brazil) for 10 min to detach the bacteria adhered to the surfaces. Sonicated material was vortexed and diluted until 10^{-6} in 0.85 % saline solution. 10 µL-aliquots of each dilution, in triplicate, were dropped on BHI agar and incubated at 37 °C for 24 h to determine the number of the adhered cells.

2.5. S. epidermidis biofilm formation

100 µL-aliquots of an overnight culture of *S. epidermidis* ATCC 35984, corresponding to a 10^7 cfu/mL, were added to 24-wells plates containing the samples (n = 4 per group) immersed in 1 mL of BHI broth, and incubated at 37 °C for 24 h to form biofilm. Afterwards, samples were gently removed and added to a sterile microtube for washing twice with PBS for removing non-adherent cells. 1 mL of PBS was added to each sample, biofilm was disrupted in an ultrasonic water bath, and diluted until 10^{-6} in 0.85 % saline solution. Number of cells forming biofilm was measured by drop plating aliquots of 10 µL on BHI agar surface, in triplicate, and incubating at 37 °C for 24 h.

2.6. S. epidermidis morphology and viability on different surfaces

Cell morphology and distribution on different surfaces were investigated by electron microscopy after adhesion and biofilm formation assays. The samples after 1 h and 24 h under culture were gently washed three times with 10 mL of PBS to remove loosely bound bacteria, and immediately fixed by immersion in 2.5 % glutaraldehyde and 0.1 M phosphate buffer (pH 7.2–7.4) for one week. Then, samples were prepared with critical point methodology and sputtered with gold for SEM analysis.

Bacterial viability on different surfaces was analyzed in a qualitative (n = 2 per group) way through confocal laser scanning microscope (Zeiss, LSM 5 Exciter, Germany). A 24 h biofilm was formed in each sample and non-adherent cells were removed as described in the section above. The samples were placed in a new 24-well plate and stained with a solution of the Live/Dead® BacLight™ Bacterial Viability kit, where 3 µL of SYTO®9 and propidium iodine were added to 1 mL of PBS. Then, 150 µL of the staining solution were gently added to each sample, which was incubated for 15 min in the dark. Suspensions were then aspirated and PBS was added to fully immerse the sample. Samples were then visualized by a confocal laser scanning microscope with a 40x water dipping lens. The viable bacteria were stained green and dead bacteria were stained red.

2.7. Statistical analysis

Data on continuous variables were presented as means ± standard deviation (SD). For continuous data (roughness parameters - Sa/Sq; adhesion; biofilm formation and thickness), comparisons between groups were employed using Student's *t*-test. Then, one-way ANOVA followed by post hoc testing (Tukey HSD), when necessary, was performed to analyze the results. Analyzes were conducted using (SPSS, version 20, USA), considering the significance level at 5% ($p < 0.05$).

3. Results

3.1. Surfaces characterization

Treatments were employed to develop five different surface topographies, in macro, micro and anodic scale, as can be seen in Fig. 1. The elemental composition analyzed by EDS showed the purity of Ti and Zr materials without unexpected impurities (Table 2). The Ti Nano group showed an oxygen peak, as can be seen in the graph (Fig. 1c), due to the presence of a TiO_2 nanotube layer (Fig. 2a), where the O_2 molecule can be stored internally, proving the surface nanomorphology and a more reactive surface area from this surface treatment.

In terms of roughness (Table 3), the Ti micro group revealed significantly major roughness parameters in comparison with all groups, being followed by the Ti nano and Zr micro groups, which showed higher roughness than Ti and Zr machined groups. Regarding spatial parameters, Ti micro presented the most expressive results, due to its more aggressive surface treatment involving acid attacks. Furthermore, the three-dimensional graphics generated (Fig. 2b) showed the five different topographies in terms of roughness, corroborating the images acquired by SEM, where the morphological differences are visualized.

Regarding wettability, Ti nano was the only surface that presented hydrophilic characteristics due to its surface treatment while the samples from the other groups presented more similar hydrophobic characteristics (Table 3).

3.2. S. epidermidis adhesion

At the early stages (1 h), microtexturization (Ti micro and Zr micro) provided a significant higher adhesion to *S. epidermidis* (Fig. 3b), when compared with all other surface treatments. Ti machined and Ti nano showed lower bacterial adhesion compared to microtexturization; however, Ti machined and Ti nano showed higher bacterial adhesion, when compared with Zr machined. Taking into account results regarding roughness, it is possible to suppose that higher roughness had more influence in early adhesion of *S. epidermidis* than other properties.

Comparing the base materials evaluated (Ti and Zr) alone, no significant difference was found in the bacterial adhesion after 1 h, revealing that both materials offer similar conditions regarding early bacterial contact.

3.3. S. epidermidis biofilm

All groups showed a high rate of colonization and biofilm formation after 24 h. A biofilm with a thin thickness covered all surfaces without the possibility of visualizing the surface characteristics of each group as can be seen in Fig. 4a. Measurements of the biofilm thickness formed using a lateral view of the samples showed no significant difference between the groups (Fig. 5 a/b), suggesting a similar growth in height of the bacteria.

Regarding biofilm counting, Ti Nano, a hydrophilic surface, provided the higher biofilm formation (Fig. 4b) in comparison with all other surfaces. Likewise, Ti micro, Zr macro, and Zr micro supported a higher biofilm formation, when compared with Ti machined. Hydrophilicity seems to influence in biofilm formation when the surface was immersed in the culture, followed by the roughness properties that showed importance in biofilm formation under immersion.

As in the adhesion assessment, the base materials *per se* did not influence in the biofilm formation, suggesting that the properties of surfaces acquired by subsequent treatments can present more interference than the base material employed.

3.4. S. epidermidis morphology and viability

Qualitative SEM analysis showed no visible differences in *S. epidermidis* morphology in either surface group, either 1 h or 24 h (Figs. 3a, 4 a, 5 a). Images showed cells with intact morphology; however, viability analysis by confocal microscopy (Fig. 6) suggested a higher number of dead cells in Ti nano surfaces, indicating a harmful effect of these surface structures on the bacterial cell membrane at the proposed time.

4. Discussion

Osseointegration capacity and biocompatibility are the major

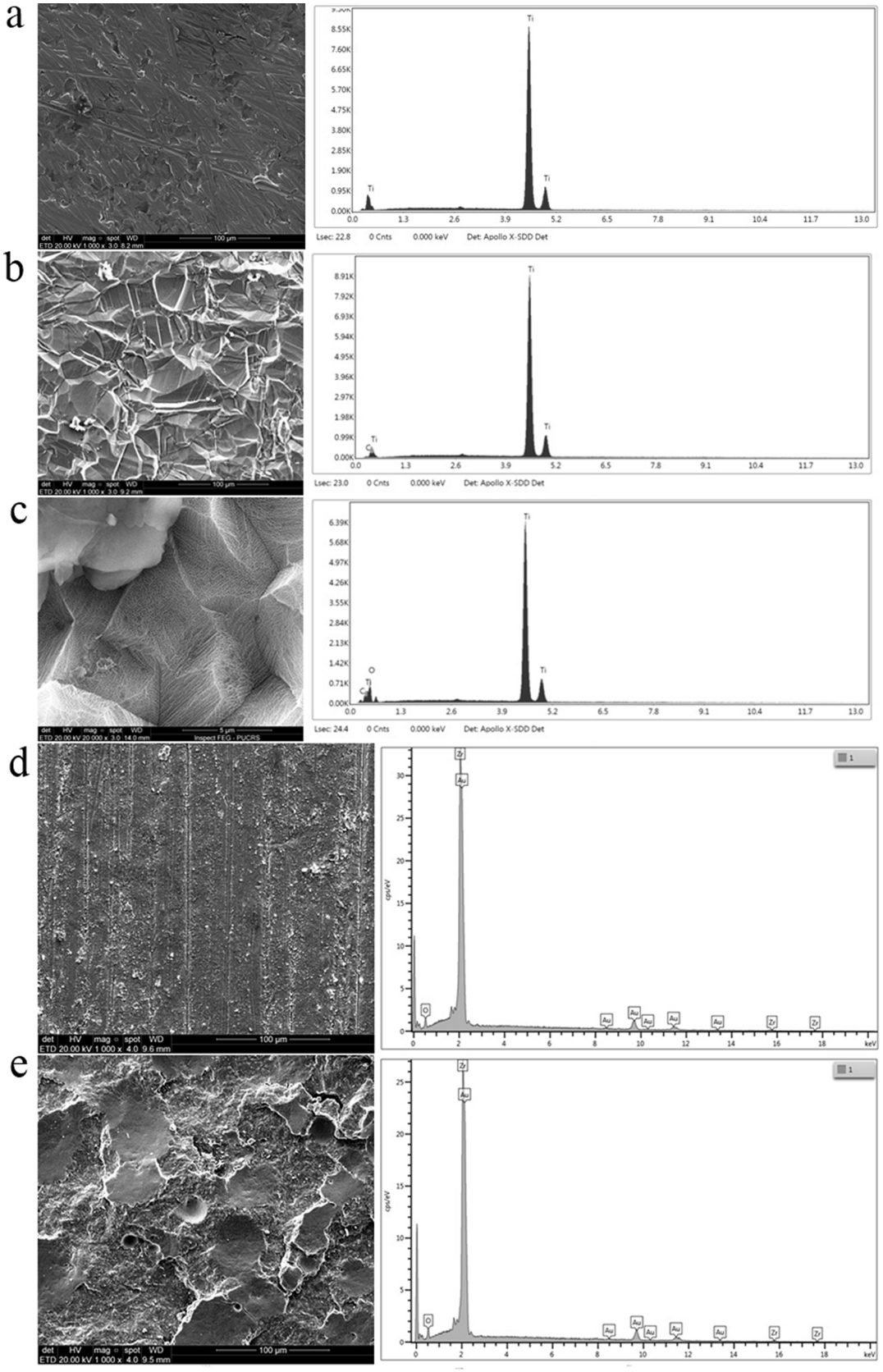

Fig. 1. Morphology and elemental analyses of each group (a- Ti machined, b- Ti micro, c- Ti nano, D- Zr machined and e- Zr micro) showing high peaks of Titanium in a, b and c and high peaks of Zirconia in d and e.

Table 2
Surfaces elemental compositions (%).

Groups	Atomic composition (%)			
	Titanium (Ti)	Oxygen (O)	Carbon (C)	Zirconium (Zr)
Ti Machined	99.1 %	–	0.9 %	–
Ti Micro	93.93 %	–	6.07 %	–
Ti Nano	65.94 %	31.66 %	2.40 %	–
Zr Machined	–	16.89 %	–	83.11 %
Zr Micro	–	18.25 %	–	81.75 %

differential of Ti and Zr. Researchers have modified the surfaces of these biomaterials seeking the best surface features to promote cell adhesion and accelerated bone healing (Civantos et al., 2017; Gupta, Noumbissi, & Kunrath, 2020; Schünemann et al., 2019). Moreover, topographies at different scales have been reported as influencing the inflammatory response to implant surfaces. Ma et al. (2014) revealed a significant role of surface nanostructuring in relation to the inflammatory response mediated by macrophages. Their study provides knowledge *in vitro* and *in vivo* about the positive polarization of macrophages when in contact with TiO_2 nanotubes (30 nm), inducing a positive immune regulatory process for healing (Ma et al., 2014).

The increase in surface roughness has been reported as improving the surface area and bone cell adhesion (Andrukhov et al., 2016). In addition, surface nanomorphology is investigated for its potential to interact at nanoscale with cells and bacteria revealing antibacterial properties (Cao et al., 2018; Faghihi et al., 2007), and hydrophilicity is reported by accelerating the process of cell spreading and adhesion (Kopf, Ruch, Berner, Spencer, & Maniura-Weber, 2015) as well as of proteins adsorption (Fabre et al., 2018). Therefore, in order to support the choices for their use in biomedical implants, materials with distinct surface characteristics (morphology, roughness, chemical composition, and wettability) matching the options currently available in the implant market were developed, characterized and analyzed concerning colonization of *S. epidermidis*. In addition to the well-recognized role of this bacterium as an important contaminant of prosthetics, catheters and several other implanted devices (Arciola et al., 2018; Oliveira et al., 2018), *S. epidermidis* was described as prevalent in patients with peri-implantitis, even overtaking *Staphylococcus aureus*. Actually, these authors reported that *S. epidermidis* was also more prevalent than *S. aureus* in orally healthy patients with and without implants and in patients with periodontal disease (O'Connor et al., 2018).

Usually, local implant infections occur at the first hour of biomaterial contacting with the body, period when the body's defenses are still in migration and formation (de Oliveira, Rosowski, & Huttenlocher, 2016), and, subsequently along exposure to a contaminated environment (Pye, Lockhart, Dawson, Murray, & Smith, 2009). Longer periods end up providing a more critical analysis of biofilm comprising linkage among cells and extracellular matrix above the surface, rather than investigating the interaction of the surface interface of the biomaterial and bacterial cell. Thus, this investigation aimed to analyze early adhesion and biofilm formation by *S. epidermidis*, evaluating two time points (1 h and 24 h), which allowed us to observe that features of surfaces may differently influence on bacterial growth over time.

The *S. epidermidis* showed different growth levels when in contact with the diverse surfaces evaluated, and more intensely under culture for 24 h in immersion. When the contact of the sample with bacteria was only superficial for a short period of time (1 h), surface roughness demonstrated to improve bacterial adhesion, as can be seen in Fig. 3a. Bacterial adhesion has been supported by high roughness in different biomedical materials (Wu, Zitelli, TenHuisen, Yu, & Libera, 2011; Wu, Zhang, Liu, Suo, & Li, 2018; Yoda et al., 2014). Minimal changes in roughness measurements proved to be sufficient to increase the adhesion of *S. epidermidis* bacteria to Ti alloy surfaces (Yoda et al., 2014); however, only one roughness parameter (Ra) was analyzed in this study. On the other hand, roughness variations measured in a similar way did not influence the adhesion of *Escherichia coli* growing for 8 h, and greater adhesion was observed only in the rougher samples after 12 h of growth (Chen, Ding, & Chen, 2016). Surface roughness has been described as a major feature that implies the adhesion of a range of both gram-negative and gram-positive bacteria, mainly due to irregularities

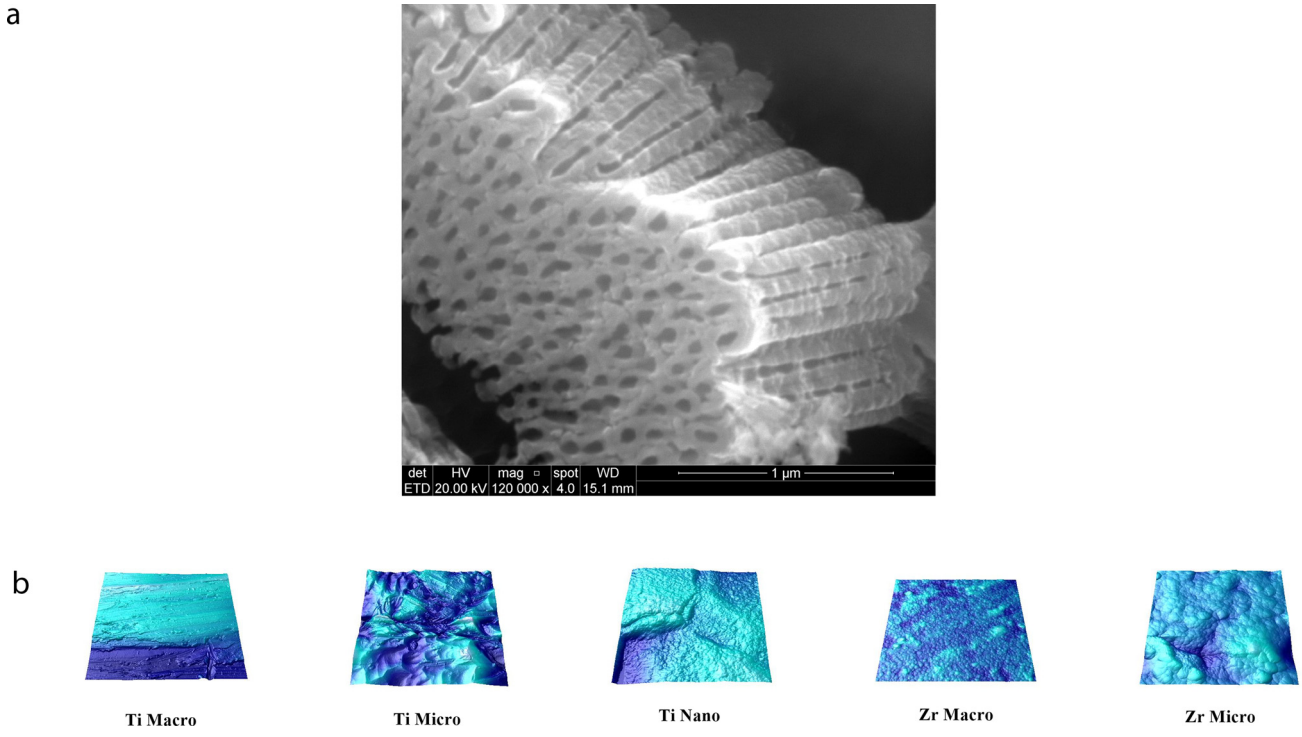

Fig. 2. High resolution microscopy image of Ti nano group in lateral view showing TiO_2 nanotubes(a). Three-dimensional images showing the surface topographies, using a cut-off value of 30 µm (b).

Table 3
Roughness measurements and wettability analysis.

Groups	3D surface roughness parameters				Contact angle (wettability measurement)
	Sa (μm)	Sq (μm)	Sku (dimensionless)	Ssk (dimensionless)	
Ti Macro	0.12 ± 0.01	0.14 ± 0.01	6.98	0.16	61° ± 4
Ti Micro	2.67 ± 0.20 *	2.78 ± 0.21 *	22.20	−1.07	89° ± 4.5
Ti Nano	1.07 ± 0.09 **	1.14 ± 0.10 **	15.80	−0.37	12° ± 1
Zr Macro	0.08 ± 0.00	0.11 ± 0.01	7.01	0.38	65° ± 3
Zr Micro	0.60 ± 0.05 **	0.80 ± 0.06 **	12.10	−1.05	78° ± 3.5

Notes: Data was expressed as mean and standard deviations.
* $p < 0.05$ against all other groups.
** $p < 0.05$ against Ti Macro and Zr Macro groups.

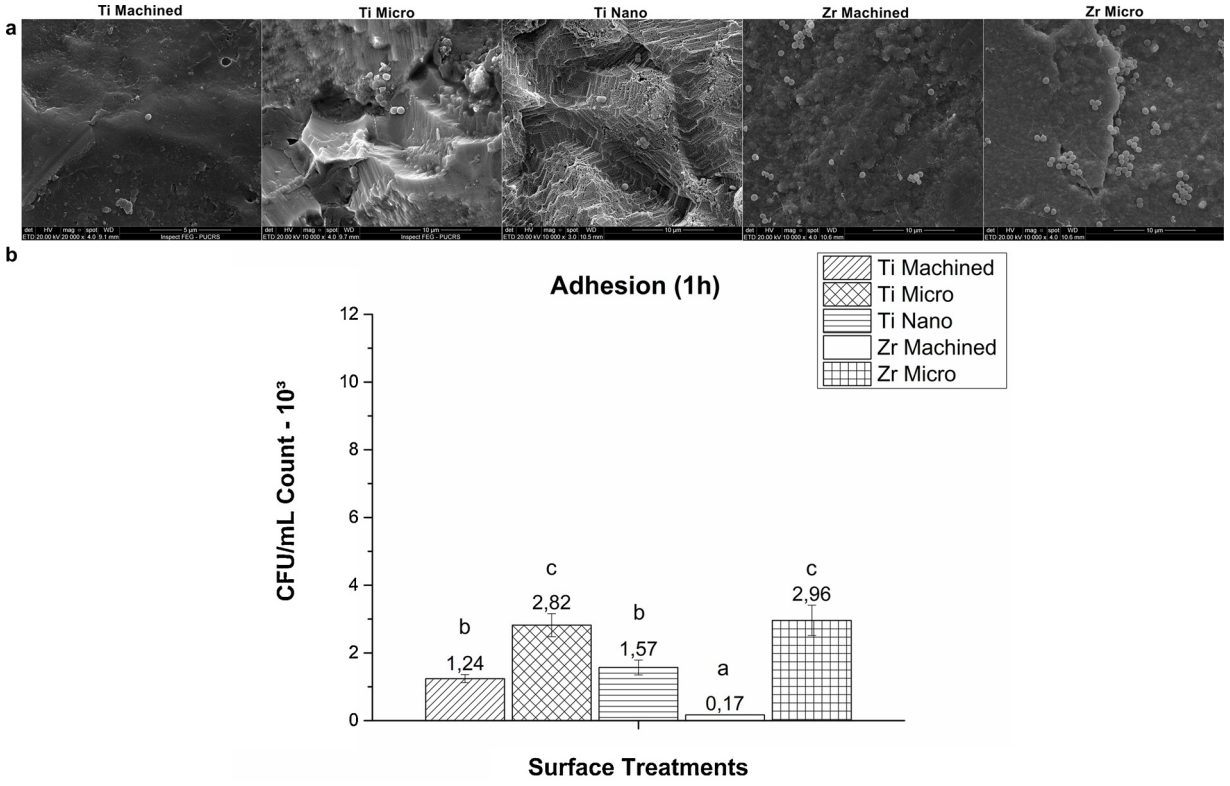

Fig. 3. Qualitative adhesion acquired by SEM (a) - 10.000x magnification; Bacterial counts after exposure of surfaces to *Staphylococcus epidermidis* culture for 1 h (b). Columns with different letters indicate counts that are significantly different from each other ($p < 0.05$).

and increased surface area (Bohinc et al., 2016). Nevertheless, these authors also consider that the extracellular characteristics of bacteria may generate different levels of adhesion.

Hydrophilicity has been reported as an advantageous property due to the promotion of osseointegration (Kopf et al., 2015; Wei et al., 2009). Conversely, hydrophilicity seems to present the disadvantage to favor bacterial colonization when we evaluated bacterial growth by immersion of the samples during 24 h. *S. epidermidis* and *S. aureus* demonstrate multiple mechanisms in their extracellular matrix that combine for adhesion in hydrophilic biomaterials (Arciola et al., 2018).

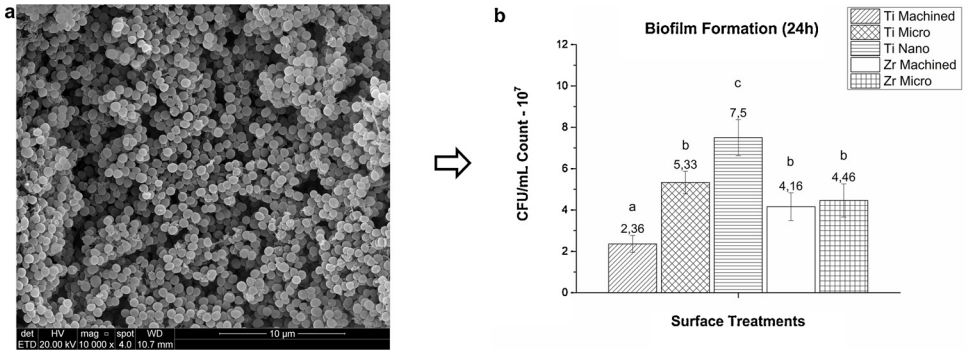

Fig. 4. Qualitative adhesion acquired by SEM - 10.000x magnification - showing the biofilm formation covering all surfaces characteristics (a); Bacterial counts after exposure of surfaces to *Staphylococcus epidermidis* culture for 24 h (b). Columns with different letters indicate counts that are significantly different from each other ($p < 0.05$).

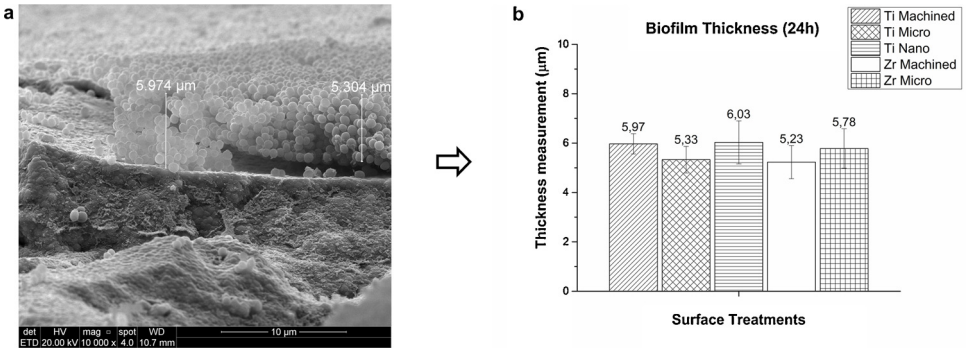

Fig. 5. Biofilm thickness measurements using SEM - 10.000x magnification, without statistical significance.

In agreement with our study, Wang et al. showed that titanium surfaces with superhydrophobic characteristics have anti-adhesion properties in bacteria such as *Streptococcus mutans*, while surfaces with hydrophilic characteristics revealed greater bacterial adhesion (Wang, Weng, Chen, Chen, & Wang, 2020), highlighting a great concern when hydrophilic implant is inserted in a contaminated site.

On the other hand, machined surfaces provided the lowest bacterial adhesion and proliferation in both materials, showing the interesting difficulty of interaction of these bacteria with smoother surfaces, as also reported by other previous studies (Pilz et al., 2018; Yoda et al., 2014). However, smooth surfaces are known to be weaker in interacting with osteogenic cells than modified surfaces (Hirano et al., 2015; Kuo et al., 2017), providing a duel of surface properties for researchers to investigate and improve this technology.

Morphological alterations in *S. epidermidis* have been observed when it was exposed to nanostructured surfaces for 48 h and six days (Cao et al., 2018). By contrast, we couldn't detect such changes in any studied surfaces; however, we evaluated bacterial growth for only 1 h, suggesting that the short period may be not enough to induce morphological changes. Nevertheless, we observed cell membrane damage and cell death on our nanostructured surface, as also previously described (Cao et al., 2018; Truong et al., 2015).

Finally, concerning to the substrates applied, it was demonstrated in the same materials, Ti and Zr, with modified surfaces, that Zr supported a less thick bacterial biofilm after 72 h; however, mass and metabolism of biofilm formed in Zr samples were not significantly different when compared to those grew on Ti (Roehling et al., 2017). Otherwise, our results did not reveal any difference caused by the variation of the material, suggesting similarity of antibacterial responses in the first contacts, probably due to the culture time up to 24 h.

5. Conclusion

Ti and Zr were similar in antibacterial properties at the first contact stages; however, *S. epidermidis* demonstrated a significant different ability to grow according to the varied surface properties proposed in this study. Higher surface roughness and higher hydrophilicity seem to promote greater bacterial adhesion in early periods, as well as nano-texturization suggests to present a harmful effect on the bacterial cell membrane. Further investigations are needed with extended time and co-cultures to clarify all bacterial involvement on the current surfaces properties as hydrophilicity and high roughness, nevertheless, caution is suggested using implantable devices with these characteristics in contaminated sites.

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CRediT authorship contribution statement

Marcel F. Kunrath: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Supervision. **Marina S.G. Monteiro:** Formal analysis, Investigation, Writing - original draft. **Saurabh Gupta:** Writing - original draft, Methodology. **Roberto Hubler:** Methodology, Formal analysis, Investigation, Writing - original draft. **Sílvia D. de Oliveira:** Methodology, Formal analysis, Investigation, Writing - original draft, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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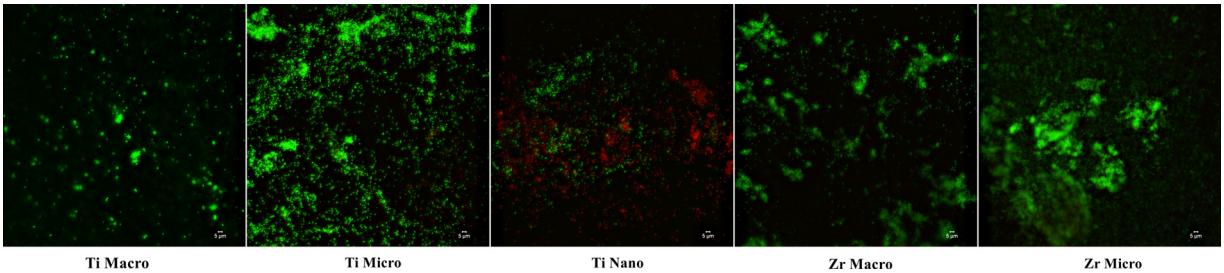

Fig. 6. Viability observation with LIVE/DEAD staining - (viable bacteria in green and dead in red). Confocal images with 40x magnification (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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