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ABSTRACT
Surface treatments are designed to promote modified implant surfaces with positive interactions with the surrounding living tissues. However, the inadvertent early contact of these surfaces with oral fluids during surgery may lead to undesired conditions affecting osseointegration. This study aimed to investigate the possible alterations in the physico-chemical properties of modified-surfaces caused by early saliva exposure. Titanium (Ti) surfaces were exposed to three different samples of human saliva and later analyzed for protein adhesion, physico-chemical surface alterations, and osteogenic cell-viability. The results indicated that surface roughness was the most significant factor influencing saliva protein adsorption; moreover, hydrophilic surfaces had critically lost their characteristics after contact with saliva. Decreased cell viability was observed in cultures after contact with saliva. Early contact with saliva might negatively influence modified surface properties and local cell viability. Careful surgical insertion of implants with hydrophilic surfaces is recommended, particularly in sites where saliva interaction is prone to occur.

Introduction

Texturizations of titanium (Ti) surfaces are designed to promote faster osseointegration and also stable soft tissue relations around biomedical implants (Dai et al. 2016; Spriano et al. 2018; Kunrath and Hübler 2019). Alterations in surface roughness, morphology, and hydrophilicity are designed to increase cellular adhesion and growth on Ti surfaces (Dai et al. 2016; Zhukova et al. 2017; Spriano et al. 2018; Kunrath and Hübler 2019). Nanomorphology and superhydrophilicity are surface characteristics showing promising preliminary results when considering the promotion of bone cell adhesion and proliferation (Zhukova et al. 2017; Spriano et al. 2018; Kunrath and Hübler 2019; Vishnu et al. 2019; Lin et al. 2020; Kunrath et al. 2020a).

However, some of these designed physico-chemical properties may be affected by local factors during implant insertion. For instance, surface hydrophilicity might be negatively affected by contact with O_2 (oxidization), and nanotextured surfaces might be damaged or significantly altered during implant surgery.

To minimize these problems, some alternatives such as keeping the implant immersed in specific fluids or stored in O_2-free environments have been proposed (Choi et al. 2019; Milleret et al. 2019). Nonetheless, the possible consequences of saliva contamination on textured Ti surfaces during implant surgical insertion have been rarely discussed, mainly concerning possible significant alterations in Ti surface properties.

Saliva has been said to be the human body fluid presenting the most diverse composition in terms of proteins, minerals, bacteria and dead cells (Carpenter 2013; Roblegg et al. 2019). Saliva presents higher viscosity, heterogeneous composition, lubrication properties and density when compared with water (Roblegg et al. 2019). Investigations on the adhesion process of *Streptococcus oralis* on titanium surfaces with smooth and intermediate surface roughness suggested increased bacterial adhesion on saliva-exposed rougher surfaces, along with alterations in the chemical adhesion properties of the textured surface itself (Dorkhan et al. 2012). Additionally, other studies have demonstrated the possible negative influence of saliva protein adsorption on the physico-chemical

Table 1. Summarized description of the surface treatments.

Groups	Methodology applied	Reference
Macro	Machined: only polished with sandpappers and cleaned in 70% ethanol.	Kunrath et al. 2020b
Micro	Double acid-attacked: using a solution of hydrochloric acid and 70% diluted sulfuric acid for 30 min at 98 °C.	Modified from Kunrath et al. 2020b
Nano	Anodization: using an electrolytic solution composed of ethylene glycol, 0.5% NH_4F, and 10% DI H_2O. The controlled voltage applied was 40 V for 1 h, followed by a reactive plasma of Ar/O2 for 5 min under vacuum to activate hydrophilic features.	Kunrath et al. 2020c

characteristics of titanium textured surfaces (Pantaroto et al. 2019; Jinno et al. 2019; Hirota et al. 2019). Also, other investigations have pointed to the significant changes that saliva protein adsorption may generate on the surface properties of bioceramic implants (Wang et al. 2012). These investigations suggested that the possible influence of these proteins on the early processes of cell surface adhesion is still poorly understood and needs further investigation.

Alterations in the hydrophilicity of titanium surfaces could adversely affect the osseointegration process, as it is considered to be relevant for early cell adhesion and protein synthesis (Boyan et al. 2017; Choi et al. 2019). The degree of adsorption of saliva proteins or other impurities on micro- or nano-textured surfaces could be influenced by surface morphology, roughness and other characteristics, which could later determine the viability of the osseointegration process (Rösch et al. 2017; Penha et al. 2018).

As the possible effects of saliva adsorption and its influence on the surface properties of titanium are not fully understood, the present study aimed to evaluate the influence of saliva protein on the surface properties of titanium with distinct morphologies, roughness and hydrophilicity, by assessing the possible alterations in their physico-chemical properties and the consequences on cell viability rates. The null hypothesis proposed here was that saliva contamination on modified surfaces will not induce significant differences in terms of cell viability and protein adhesion or physico-chemical surface characteristics.

Materials and methods

Preparation of surface samples

A titanium grade II plate (TitanioBrasil®, São Paulo, Brazil) was milled to generate 60 discs (1 mm thick; 6 mm Ø), that where manually polished with sandpaper, cleaned in 70% ethanol and dried (Kunrath et al. 2020b). Then, samples were divided into three different groups and the following surface treatments were applied: Macro (machined), Micro (double acid-etching) (Kunrath et al. 2020b) and Nano (double acid-etching + anodization) with enhanced hydrophilic properties by reactive plasma with Ar/O_2 for 5 min after anodization, as previously described (Kunrath et al. 2020c) (Table 1).

Surface characterization

To investigate surface morphology and chemical composition (n = 3), scanning electron microscopy (SEM, Inspect F50, Prague, Czech Republic) with energy dispersive X-ray spectrometry (EDS, Oxford, UK) and transmission electron microscopy (TEM, Tecnai G2 T20, Prague, Czech Republic) were applied. To evaluate roughness (n = 3), atomic force microscopy (AFM, Dimension Icon, Bruker®, Massachusetts, USA) along with specific analysis software (NanoScopeAnalysis® software) was used. Roughness parameters were analyzed using a cut-off value of 30 μm as suggested (Kunrath et al. 2020b). Wettability properties (n = 6) were measured by the sessile drop method using a goniometer (Phoenix 300, SEO, Kosekdong, Korea) with deionized water and computer software (Surfaceware8, version 10.11, Korea). The crystalline phase (n = 3) of each resultant modified surface was characterized by X-ray diffraction (XRD; XRD-7000, Shimadzu).

Saliva collection, ethics statements and protein assays

Saliva samples were collected from 3 healthy volunteers without any active carious lesions or history of periodontal disease (2 males and 1 female, 31 to 43 years old). Donors consented to saliva sample collection and analysis by signing an informed consent approved by the Pontifical Catholic University of Rio Grande do Sul Research Ethics Committee (protocol n°: 7467) and was performed in accordance with the Helsinki Declaration of 1975, as revised in 2013. Prior
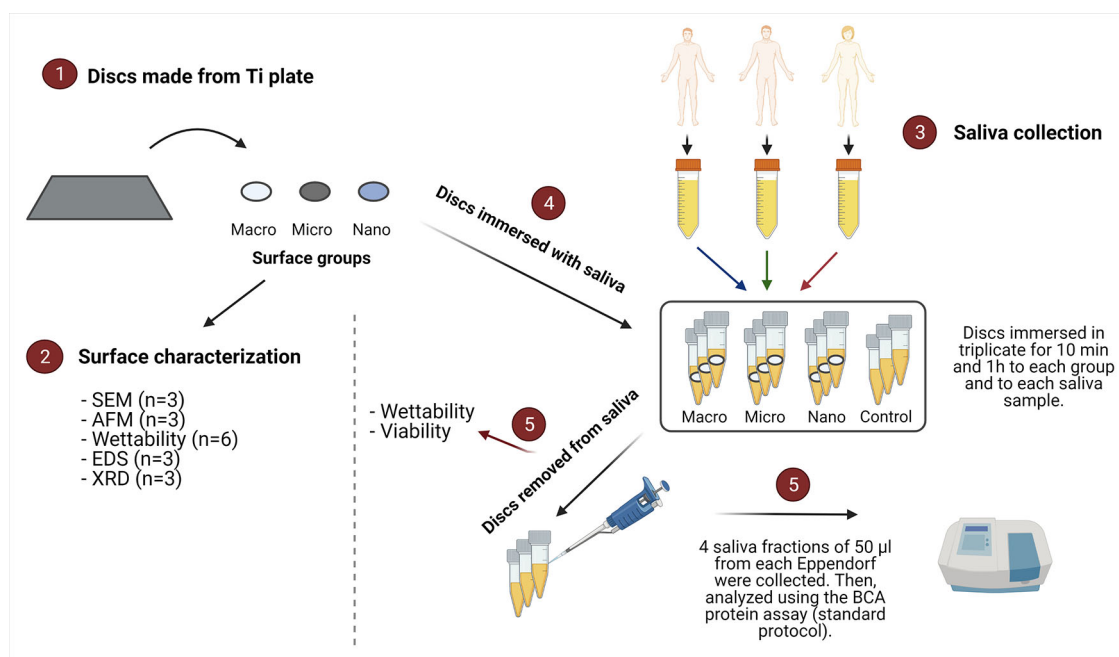
Figure 1. The methodology applied to surface characterization and protein saliva quantification.

to the saliva protein adhesion assay, a collection of unstimulated whole saliva (WS) was performed as previously described (Rosa et al. 2016). Briefly, sublingual saliva was collected with two cotton rolls placed under the volunteer's tongue for 2 min. The cotton rolls were then transferred to a 15-ml sterile tube with a 1-ml pipette tip for saliva collection after centrifugation at 10,000 g/4 °C for 10 min (Rosa et al. 2016).

Protein quantification

To investigate saliva protein adhesion to each tested surface group (n = 3 surface replicates per donor), the disks were inserted in Eppendorf tubes and immersed in 200 μl of saliva for 10 min and 1 h to totally cover all the samples investigated. The Eppendorf tubes were constantly agitated in all directions (5-min periods) in order to avoid protein accumulation at the bottom of the tubes. Afterwards, the disks were carefully removed and the remaining saliva was collected in four fractions (50 μl each) and subjected to the bicinchoninic acid (BCA) protein assay (BCA Protein Assay Kit, Pierce, Bonn, Germany) analysis. Total protein quantification was performed using bovine serum albumin (BSA) as standard (Figure 1). The measured protein binding on each sample was verified by comparing the total protein concentration value found in the control group against the value found in the groups with immersed samples. A group containing only saliva (n = 9) (without samples) was used as a control. Moreover, when the samples were

removed from the Eppendorf tubes, no additional procedures to detach weak or unbounded saliva on the samples were performed. Only the remaining saliva in the tube was analyzed in order to maintain a similar environment resembling the clinical insertion of a dental implant, where all levels of proteins might be present in the oral environment in case of implant surface-saliva interaction (adhered or detached).

Surface properties after saliva contact

After contact with saliva for 10 min, the samples (n = 3) were again subjected to wettability tests, having their contact angles measured by computer software (Surfaceware8, version 10.11, Korea) and the results compared against those previously obtained. To perform surface morphological evaluation and elemental analysis, the different groups were analyzed with a scanning electron microscope (SEM) with energy dispersive X-ray spectrometry (EDS, Oxford, UK) and investigated for possible surface impurities and their composition.

Biological assays after saliva contact

In order to evaluate the different cell viabilities between groups after saliva contact, an osteogenic cell culture analysis was performed utilizing murine pre-osteoblast cell line MC3T3-E1(Sigma Aldrich, St Louis, MO, USA). Cells were cultivated in a culture medium following a previously reported methodology
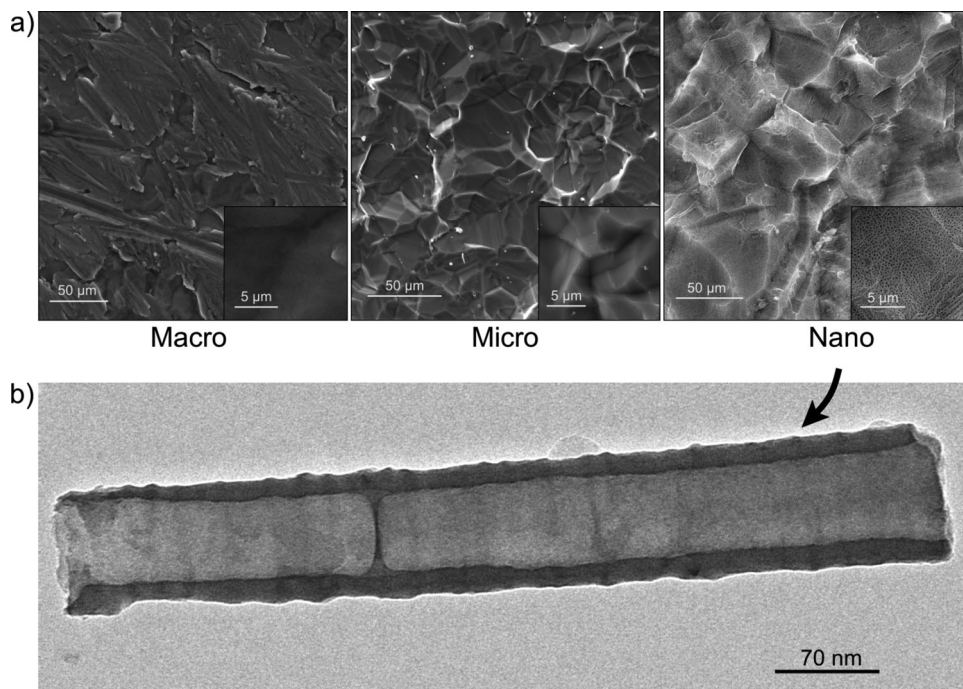
Figure 2. (a) SEM images of surface and morphology of different texturization treatments at low magnification (50 μm) and high magnification (5 μm). (b) TEM images of the Nano group revealing its nanotube structures and approximate diameter (70 nm).

(Zhukova et al. 2017). After reaching confluence, cells were detached from culture vessels by pronase incubation. All surface samples were subjected to a sterilization process with an autoclave (30 min, 125 °C) before the experiments.

Three samples of each group were embedded in saliva simulating early contact and immediately removed for cell culture. Control samples without saliva (n = 3) were used for comparison. Then, osteogenic cells were seeded on each surface and evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetra-zolium bromide assay (MTT, Sigma-Aldrich, St Louis, MO, USA) methodology after culture for 24 h. The absorbance was measured with a spectrophotometric microplate reader (Bio-Rad 600, California, USA) at a wavelength of 490 nm to assess cell viability.

Statistical analysis

Data were presented as means ± standard deviation (SD). For continuous data (roughness, viability, protein adhesion and wettability), comparisons between groups were made applying the student's t-test. One-way ANOVA followed by *post hoc* testing (Tukey HSD), was used in further comparisons. Physicochemical analyses were performed in triplicate. For saliva protein quantification, G*Power 3.17 software was used in sample calculation and to achieve a minimal significance applying an effect size of 0.35 and a probability error of 0.05%. Triplicates were used for each saliva sample and for each surface group at two specific times. Additionally, four saliva collections from every single sample were analyzed for protein quantification. OriginPro 9.0 (OriginLab, Northampton, MA, USA) was applied in statistical analyses and significant differences were characterized at 5% ($p < 0.05$).

Results

Surface characterization

Three different surface treatments were designed to represent commercially available implant surfaces. The macro-, micro- and nano-scale texturizations obtained ware shown in Figure 2a and b, including a generated TiO_2 nanotube surface by anodization (Nano), depicted by transmission electron microscopy in Figure 2b. TEM images revealed TiO_2 nanotubes with an average diameter of 70 ± 2 nm. Additionally, the atomic composition of distinct surfaces showed varying atomic percentages, generated due to differences in the methods involved in each proposed surface treatment. Furthermore, no impurity was detected by EDS (Table 2). The roughness analysis demonstrated a significantly higher quantification in Ra (two-dimensional parameter) and Sq (three-dimensional parameter) for the Micro group (double acid-etching),

being $1{,}671 \pm 41$ nm and $1{,}789 \pm 187$ nm respectively, followed by the Nano group (anodization) being 871 ± 51 nm and 945 ± 55 nm, respectively (Figure 3a). A further 3-D-dimensional parameters of roughness were analyzed as reported in Supplementary Table S1.

Regarding wettability, only the Nano group revealed hydrophilic characteristics, presenting an average contact angle of $13° \pm 1.5°$, as seen in Figure 3c, while the Macro and Micro groups presented contact angles of $75° \pm 3°$ and $83° \pm 2.5°$ respectively. Alterations in the crystalline phase of the Nano group were observed by X-ray diffraction, revealing the presence of the crystalline anatase phase, represented by the peaks: $2\theta = 25{,}38°$, $48{,}07°$, $55{,}10°$ (Figure 3b – red arrows). The combination of crystalline phase alterations plus modifications in the atomic structure of the TiO_2 nanotube layer after plasma-treated promoted higher surface hydrophilicity.

Table 2. Atomic percentages (%) of the different surface treatments.

Groups	Titanium	Oxygen	Carbon
Macro	98.9%	–	1.1%
Micro	92%	–	8%
Nano	60.9%	38%	1.1%

Saliva protein adsorption

According to measurements of the protein concentration using the BCA methodology, it was possible to observe that all groups had some saliva protein adhesion at both the proposed specific periods. The Micro group showed statistical significance regarding surface protein adhesion compared with the control group at 1 h (saliva sample 1: reducing the quantification from $128\,\mu g\ \mu l^{-1}$ [control] to $92\,\mu g\ \mu l^{-1}$ [micro]; saliva sample 2: reducing the quantification from $134\,\mu g\ \mu l^{-1}$ [control] to $88\,\mu g\ \mu l^{-1}$ [micro]; saliva sample 3: reducing the quantification from $133\,\mu g\ \mu l^{-1}$ [control] to $92\,\mu g\ \mu l^{-1}$ [micro]) and in one sample at 10 min (saliva sample 3: reducing the quantification from $132\,\mu g\ \mu l^{-1}$ [control] to $102\,\mu g\ \mu l^{-1}$ [micro]), suggesting the influence of higher surface roughness on protein adhesion ($p < 0.05$) (see Table 3). The Macro and Nano groups did not present significant adsorption results in all samples; however, a tendency to lower mean adsorption values might be observed in all samples compared with the control.

Alterations in surface properties

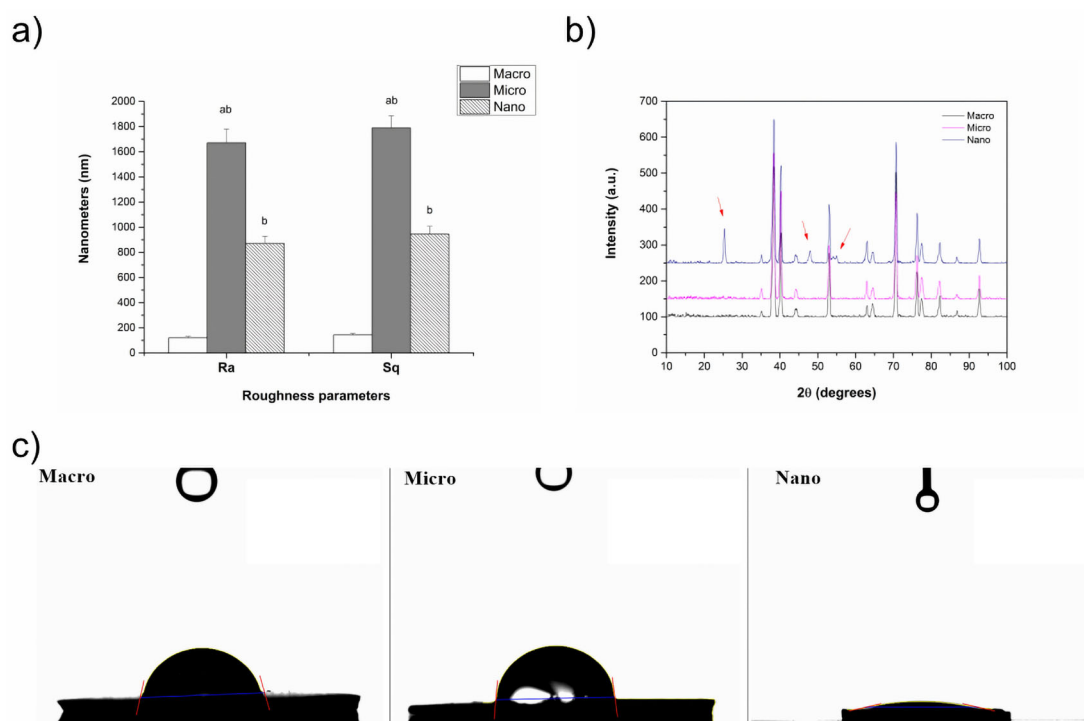No surface morphological alterations were found by SEM on the different surfaces after saliva

Figure 3. (a) Measurements of roughness parameters (Ra and Sq) in different surface treatments. Different letters indicate statistically significant differences ($p < 0.05$) among groups. (b) XRD spectrum from different surface treatments; red arrows show the alteration in the crystalline phase to anatase only in the Nano group. (c) surface wettability properties from all groups.

Table 3. Saliva protein quantification measured by BCA methodology in three different saliva samples and at two different times.

Saliva samples / Time of interaction	Protein quantification (µg µl-1)			
	Control (without samples)	Macro	Micro	Nano
Saliva sample 1 (10 min)	126 ± 4 µg µl^{-1}	116 ± 4 µg µl^{-1}	111 ± 3 µg µl^{-1}	110 ± 2 µg µl^{-1}
Saliva sample 1 (1 h)	$128 \pm 4,5$ µg µl^{-1}	119 ± 4 µg µl-1	92 ± 3 µg µl-1*	$112 \pm 2,5$ µg µl-1
Saliva sample 2 (10 min)	136 ± 4 µg µl^{-1}	128 ± 3 µg µl-1	$112 \pm 6,5$ µg µl-1	110 ± 4 µg µl-1
Saliva sample 2 (1 h)	134 ± 5 µg µl^{-1}	$124 \pm 4,5$ µg µl-1	88 ± 2 µg µl-1*	110 ± 3 µg µl-1
Saliva sample 3 (10 min)	$132 \pm 2,5$ µg µl^{-1}	127 ± 4 µg µl-1	102 ± 1 µg µl-1*	112 ± 4 µg µl-1
Saliva sample 3 (1 h)	133 ± 3 µg µl^{-1}	124 ± 3 µg µl-1	92 ± 2 µg µl-1*	110 ± 5 µg µl-1

* - Asterisks represent statistical significance compared with the control group ($p < 0.05$).
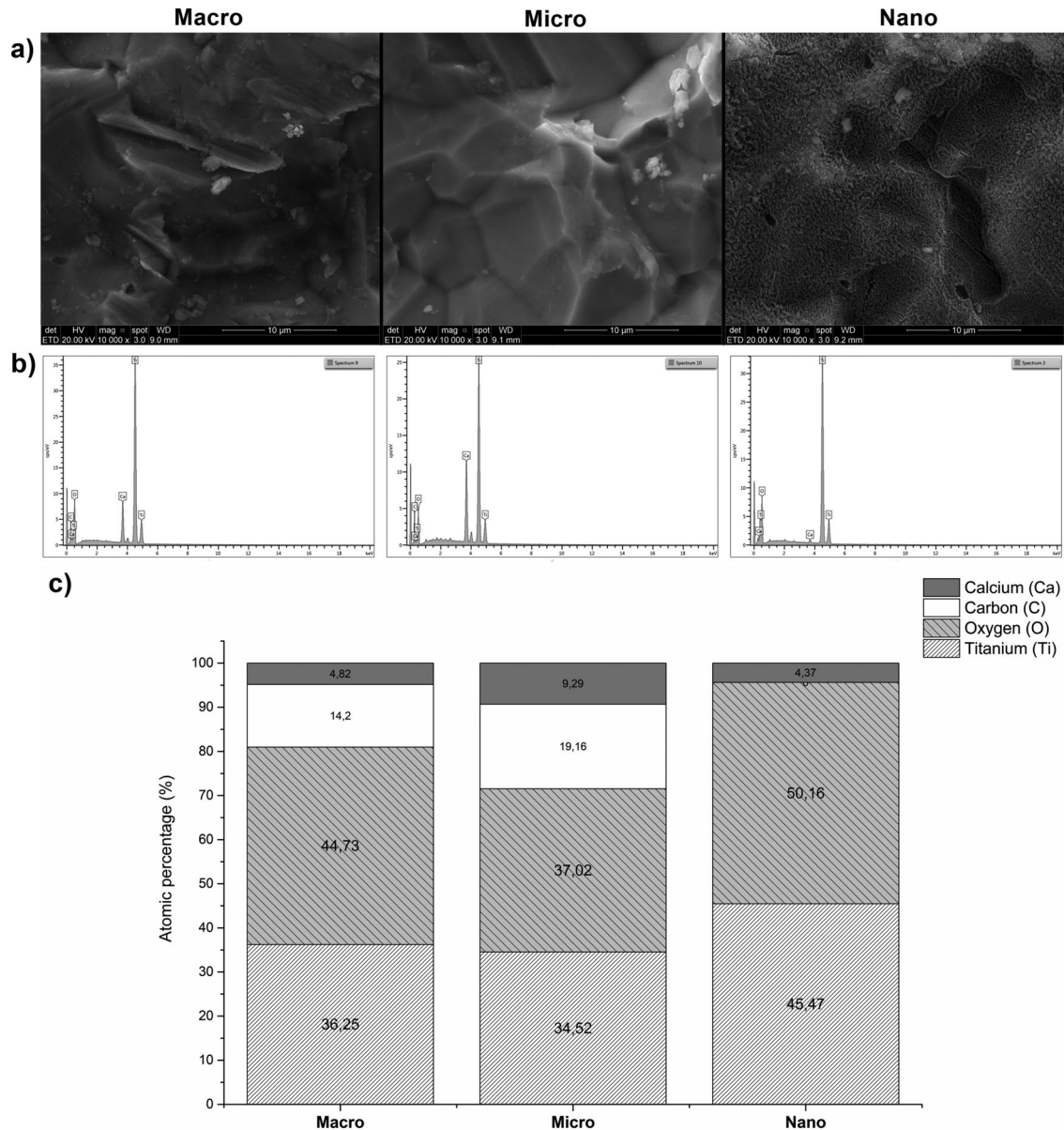

Figure 4. Visualization of the surface morphology of the three groups after saliva contact. (a) Spectrum and percentage findings by EDS of verified atomic elements on the surfaces at the selected points (red stars), indicating adhesion of impurities after contact with saliva (b, c).
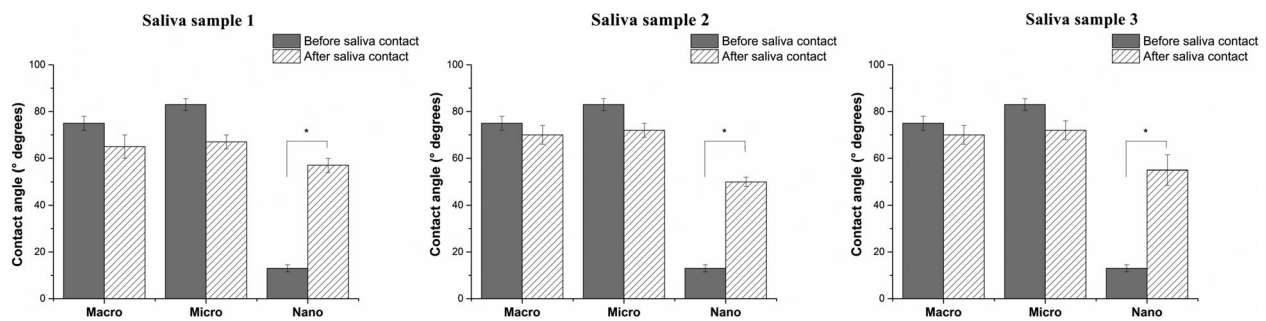
Figure 5. Wettability contact angles between surface treatments before and after saliva contact for each studied group. Asterisks indicate statistical significance for intra-group comparisons ($p < 0.05$).
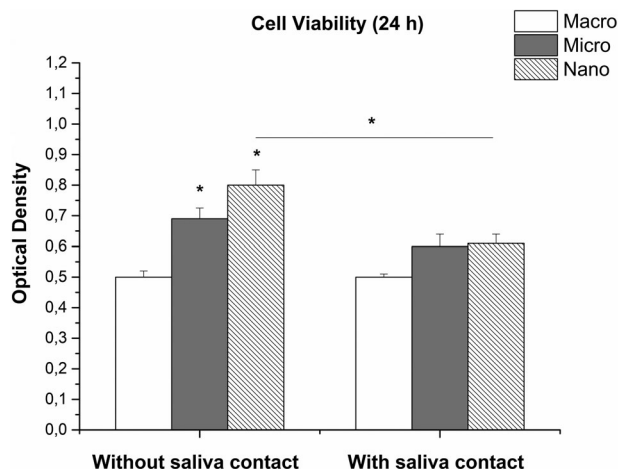

Figure 6. Cell viability analysis (MTT) with and without contact with saliva after cell culyure for 24-h. Asterisks represent statistical significance ($p < 0.05$) between groups.

contamination (Figure 4a), although adhered impurities were verified in all groups. Calcium (Ca) predominated as the impurity present by EDS, being 4.82%, 9.29% and 4.37% for the Macro, Micro and Nano groups, respectively. Other scattered impurities could be verified and did not critically alter the surface morphology of any of the groups, suggesting a negligible influence on their properties.

A significant change in hydrophilicity of the Nano group was verified after saliva exposure in all samples. ($p < 0.05$) In the Nano group, the interaction with saliva generated an increase in contact angle from 13° ±1.5° to 57° ±3° in the "saliva 1" samples; 13° ±1.5° to 50° ±2 in "saliva 2" samples and 13° ±1.5° to 56° ±6,5 in "saliva 3" samples. Figure 5 shows the decreased hydrophilicity after saliva exposure for all groups studied.

Biological assay

The cell viability (MTT) was verified by applying an osteogenic cell lineage cultured for 24 h on surfaces with and without saliva exposure. Figure 6 shows the significantly greater cell viability when not subjected to saliva contact for the Micro and Nano compared with the Macro group. On the other hand, following saliva exposure, cell viability was significantly affected in all groups ($p > 0.05$), suggesting that changes resulting from saliva protein adsorption might negatively influence cell viability.

Discussion

Important characteristics when considering faster osseointegration of titanium implants are the surface modifications obtained with specific physico-chemical treatments, promoting alterations in surface morphology, roughness and wettability (Wennerberg et al. 2014; Shibata and Tanimoto 2015; Dai et al. 2016; Zhukova et al. 2017). This study developed and investigated three different surfaces with distinct characteristics: machined (Macro: low roughness and non-hydrophilic), double acid-etched (Micro: high roughness and non-hydrophilic) and a nanotextured anodized surface (Nano: high hydrophilicity and nanoporous topography). When considering the clinical application of modified surfaces for implants, all resultant titanium surface characteristics and properties are supposed to reach the surgical site unaltered, to provide the necessary substratum for bone cell adhesion and protein synthesis, thus favoring the tissue healing response. However, as saliva might be considered a frequent component in the oral surgery environment, its undesired presence in close contact with the implant surface might induce undesired biological effects. Saliva is found in all healthy individuals, usually contaminated by bacteria or mixed with blood during oral surgery (Motamayel et al. 2018). So far, few attempts have been made to investigate the possible consequences on the bone tissue healing response regarding saliva contamination of titanium implant surfaces.

In the present investigation, a specific protocol to collect human saliva was applied, including filtered cells and bacteria from a depuration process (Rosa et al. 2016), promoting a standard protocol to store and maintain the integrity of human saliva proteins for research purposes. Non-filtered saliva requires direct application in experimentation without previous storage, to maintain the integrity of its constituents such as cells and bacteria among other substances. This specific protocol was considered applicable for the present analysis, as the results indicated that this filtered saliva was able to significantly alter the surface properties analyzed. The results verified here showed that even these cleaned and filtered saliva samples were able to cause important modifications in surface hydrophilicity, and possibly influence the tissue healing response as suggested by cell viability tests.

Regarding changes in surface wettability, it is well-known that high hydrophilicity is an important surface feature when considering the promotion of faster cell viability, proliferation and spreading (Wennerberg et al. 2014; Boyan et al. 2017; El Chaar et al. 2019; Gao et al. 2020). This surface property has been reported to directly influence bone cell healing speed around implants (Boyan et al. 2017; El Chaar et al. 2019; Gao et al. 2020). Distinct chemical processes have been proposed to improve surface wettability, such as the application of plasma, high-intensity lights and heat treatment, among others (Henningsen et al. 2018; Todea et al. 2019; Kunrath et al. 2020c). In the present investigation, the Nano group was manufactured by an anodizing process followed by a reactive plasma treatment with Ar/O_2, resulting in improved surface hydrophilicity. However, after contact with saliva, this property was substantially affected, as the Nano group presented contact angle results not compatible with typical hydrophilic surfaces. Even so, the Nano group continued with the lowest contact angle (wettability) after saliva contamination, still presenting slightly lower wettability values compared with the other surfaces. The Macro and Micro groups revealed a minimum decrease in wettability after saliva exposure, probably influenced by the new chemical bonds caused by the saliva/liquid/surface interaction.

Surface roughness seems to be an important factor regarding the adherence of saliva proteins, as shown by the decreased protein content results in the saliva-exposed samples with higher roughness. The influence of this property on cell and bacterial adhesion has been reported in the literature (Alla et al. 2011; Bigerelle et al. 2011; Lin et al. 2013). However, few reports have addressed the interactive mechanisms involved in cell and bacterial growth behavior on modified surfaces contaminated with saliva.

Surfaces presenting a nanoporous topography similar to the Nano group have been said to present advantages in terms of cell adhesion and migration for hard/soft tissues, (Ehlert et al. 2020; Gulati et al. 2020) as well as antibacterial properties (Kunrath et al. 2020d). Furthermore, the results indicated that this nanotopography has not promoted saliva protein retention when compared with surfaces with higher roughness. Similarly, the crystalline anatase structure seen on the hydrophilic nanotextured surface after reactive plasma treatment (Kunrath et al. 2020c) has not been shown to enhance the adhesion of saliva proteins. Meanwhile, it is also known that reactive treatments could alter the physico-chemical interaction between implant functionalized surfaces and living tissues, which was demonstrated in other investigations applying different polarization on titanium surfaces (Gittens et al. 2013; Sun et al. 2018).

Specific patient and/or local conditions may significantly contribute to implant contamination with saliva during surgery, such as hypersalivation, restricted access limiting saliva control caused by clinical devices in guided surgeries and/or intraoperative complexities in patients with special needs (Lustig et al. 2002; Romero-Pérez et al. 2014). In this study, an intentional saliva exposure was proposed to evaluate its possible influence on the implant surface properties and cell response. The results indicated significant exposure-related modifications in surface properties specifically designed for faster cell proliferation and adhesion. Decreased cell viability in saliva-contaminated samples with higher surface roughness, along with significant alterations in hydrophilicity of nano-textured surfaces were observed. Considering the limitations of this *in vitro* study, the results shown here do not endorse the concept of osseointegration impairment of saliva-contaminated titanium surfaces. Usually, *in vitro* simulations cannot be directly translate to clinical reality, for instance, the time of saliva exposure applied here (10 min/1 h) can be considered prolonged when comparing with possible saliva contact during clinical insertion of an implant. Moreover, the friction against the bone during implant insertion might disperse or even intensify saliva protein adherence on its surface. Thus, further *in vitro* studies applying distinct models of osteogenic cell cultures, along with *in vivo* preclinical investigations are required to evaluate the possible short, mid and long-term consequences of implant saliva exposure on

osseointegration. Additionally, further investigations to confirm these surface modification tendencies when exposing modified surfaces for implants to unfiltered human saliva with more diverse pathogenic bacteria might be suggested.

Conclusions

When in contact with modified implant surfaces, saliva and its proteins have been shown to negatively influence titanium surface properties designed to enhance the bone healing response. Saliva proteins adhered to all the tested surface, regardless of the treatment received and the exposure period. Roughness was shown to be the most influential surface property regarding saliva protein adhesion. Surface morphology revealed no significant changes after saliva interaction, whereas hydrophilicity was critically altered upon saliva contamination, particularly on hydrophilic surfaces.

Lower cell viability results were observed in all saliva-exposed groups regardless of the surface treatment received, indicating a negative influence of saliva contamination on early cell behavior. Although the limitations of the present *in vitro* study do not allow direct translation to clinical reality, the results reported here might suggest the need for extra careful surgical handling of implants during clinical procedures when rougher, hydrophilic surfaces are used, to avoid possible contamination and consequent impairment of their surface properties.

Disclosure statement

No potential conflict of interest was reported by the authors.

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