Live endothelial cells on plasma-nitried and oxidized titanium: An approach for evaluating biocompatibility

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ABSTRACT

We evaluated the effects of titanium plasma nitriding and oxidation on live endothelial cell viscoelasticity. For this, mechanically polished titanium surfaces and two surfaces treated by planar cathode discharge in nitriding (36N2 and 24H2) and oxidant (36O2 and 24H2). Surfaces were characterized regarding wettability, roughness and chemical composition. Rabbit aortic endothelial cells (RAECs) were cultured on the titanium surfaces. Cell morphology, viability and viscoelasticity were evaluated by scanning electron microscopy (SEM), methyl thiazolyl tetrazolium (MTT) assay and atomic force microscopy (AFM), respectively. Grazing Incidence X-ray Diffraction confirmed the presence of TiN0.26 on the surface (grazing angle theta 1°) of the nitrided samples, decreasing with depth. On the oxidized surface had the formation of TiO3 on the material surface (Theta 1°) and in the deeper layers was noted, with a marked presence of Ti (Theta 3°). Both plasma treatments increased surface roughness and they are hydrophilic (angle<90°). However, oxidation led to a more hydrophilic titanium surface (66.59° ± 3.65 vs. 76.88° ± 2.68; p = 0.001) due to titanium oxide films in their stoichiometric varieties (Ti3O, TiO2, Ti6O), especially Ti3O. Despite focal adhesion on the surfaces, viability was different after 24 h, as cell viability on the oxidized surface was higher than on the nitrided surface (9.1 × 10 3 vs. 4.5 × 103 cells; p < 0.05). This can be explained by analyzing the viscoelastic property of the cellular cytoskeleton (nuclear and peripheral) by AFM. Surface oxidation significantly increased RAECs viscoelasticity at cell periphery, in comparison to the nucleus (2.36 ± 0.3 vs. 1.5 ± 0.4; p < 0.05), and to the RAECs periphery in contact with nitrided surfaces (1.36 ± 0.7; p < 0.05) and polished surfaces (1.55 ± 0.6; p < 0.05). Taken together, our results have shown that titanium plasma treatment directly increased cell viscoelasticity via surface oxidation, and this mechanobiological property subsequently increased biocompatibility.

1. Introduction

Restenosis is one of the major complications in patients using intravascular devices. Its main cause is a vascular endothelium dysfunction, leading to uncontrolled antithrombotic activity, resulting in blood flow blockage [1]. Although the use of new stent generations has reduced restenosis rates, patient risks are still imminent [2]. Consequently, the field of bioengineering has sought biomaterials and surface modification methods that guarantee patient quality of life [3,4].

Titanium is one of the most recommended biomaterials for biomedical applications, used in pumps and cardiac devices [5,6]. This metal is biocompatible, presents adequate hardness and is corrosion resistant. In this context, surface roughness and wettability are important parameters concerning cell integration processes, such as osseointegration [7]. However, to promote tissue repair in addition to bone integration, angiogenesis using endothelial cells (EC) is required [8]. This process can trigger the activation of cell adhesion proteins (integrins), as well as rapid adhesion, proliferation and development of normal endothelial
function \([9,10]\). Interestingly, titanium surface modifications, such as by argon plasma and \(\mathrm{Cl}_2/\mathrm{Ar}\) plasma, allow for improvements in cellular response and the prevention of restenosis of intra-stents comprising this metal \([11,12]\).

Plasma processing aiming at surface modifications has been known and applied for over 20 years to improve physical and chemical properties of several materials \([5]\). This is considered a low cost and safe method, as it does not use environmental chemicals and/or pollutants \([13]\). In addition, it has displayed good results regarding cell behavior, as well as antibacterial potential \([7,14]\). Usually, evaluations concerning cell adhesion, proliferation and viability on titanium surfaces are performed. However, alterations in the mechanical properties of different cell regions under the influence of the applied biomaterial are commonly neglected by researchers.

The interactions between cells or biological tissues and “outside the body” physical forces lead to functional implications, which can be characterized by mechanical properties \([15]\). For example, external mechanical forces stimulate cells to react through mechanosensitivity (the cellular sensory ability to perceive the material); mechanotransduction (intracellular biochemical alterations), and mechanoresponse (integration of cellular signals for cell motility and contractility modifications) \([16]\). As cells respond to mechanical environmental changes promoted by cytoskeleton reorganization (morphology and motility), this in turn can directly modify cell stiffness and viscoelastic properties \([17–20]\). Alterations in viscoelasticity occur heterogeneously in the cellular environment, and can be quantified in a liquid environment through atomic force microscopy (AFM) \([21]\). Because it is a non-invasive method, it is considered a promising tool to perform diagnoses in the medical clinical practice, such as in assessing cardiovascular disease risks \([22]\). Although pathological changes may be detectable through cell biomechanics relative to the applied material \([15]\), no studies considering this property as a tool to evaluate biocompatibility of plasma-treated metal materials are available.

In this context, the aim of this study was to investigate the effect of plasma nitridding and oxidation of titanium surfaces on live endothelial cell behavior and mechanobiology.

2. Material and methods

2.1. Preparation and characterization of titanium disks

II-grade Ti disks with pre-established dimensions (diameter: 19 mm, thickness: 3 mm) supplied by Metallum Brazil were used. All surfaces were previously sanded with silicon carbide grit with granulometry ranging from 80 to 2000 MESH. Polishing was performed using a colloidal silica and hydrogen peroxide solution on an OP-CHEM polishing cloth for 30 min. For the preparation of nitrided surface (SN) and oxidized surface (SO) disks, the disks were precleaned to remove contaminants, through ion bombardment of 2.0 sccm of argon and 2 sccm of hydrogen at 200 °C, at 1.5 mbar, 448 V and 0.1A current for 30 min. The disks were then conditioned in a vacuum plasma reactor for the plasma treatment under a nitrogen atmosphere (36N₂ and 24H₂) for SN and SO (36O₂ and 24H₂) for SO in a 200 × 300 mm hermetic cylindrical chamber (diameter and height), under 1 mbar pressure, at 450 °C for 1 h.

2.2. Surface characterization

The surface nanotopography analysis was based on roughness parameters (\(R_p, R_z\) and \(R_p/R_z\)) obtained using an Atomic Force Microscope (model SPM 9700, Shimadzu). Wettability was determined through the sessile drop method \([23]\), which consists in measuring the contact angle formed by a 20 μL drop of deionized water pipetted onto the surfaces by means of a video camera image capture of the goniometer using the Softens software. Grazing incidence X-ray diffraction (GIXRD) was employed in order to obtain diffraction patterns at different depths, varying the incidence angle from 1° to 3°. This allowed for the assessment of the crystalline phase profiles \([23]\).

2.3. Rabbit aorta cells (RAECs) culture

The established lineage of rabbit aortic endothelial cells (RAECs) was purchased from the São Paulo Federal University Cell Bank. Cells were expanded in HAM-F12 medium, supplemented with fetal bovine serum (10%), and penicillin/streptomycin antibiotics (100 U/mL, 100 μg/mL, respectively). Subsequently, they were incubated at 37 °C in a 5% CO₂ chamber, with culture medium exchange every 48 h. After reaching confluence (80–90%), RAECs were subjected to the assessed surfaces.

2.4. Cellular morphology

Endothelial cells were cultured on the surfaces for 4 h for morphological evaluations. The disks were washed with phosphate buffer solution (PBS), fixed with 2.5% glutaraldehyde in PBS, pH 7.2, and post-fixed with 1% osmium tetroxide in the same buffer. Samples were then dehydrated in an increasing ethanol series and metalized with gold (Q Plus Series, Quorum Technologies Ltd., Laughton, England). Images were captured by a Scanning Electron Microscope (SEM) (SEM-SSX 550 Superscan, Shimadzu Corporation, Tokyo, Japan).

2.5. Cell viability

RAECs (2 × 10³ cells/disk) were cultured on the surfaces for 24 h, followed by the addition of 1 mL of 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyl-tetrazoliumbromide (MTT, Invitrogen, Life Technologies, Carlsbad, CA, USA) diluted in the culture medium. After 3 h of incubation, the formazan crystals produced by the reduction of MTT were dissolved by the addition of 1 mL of ethanol P.A. (ACS reagent ≥ 99.5%) in each well for 15 min under constant stirring. Then, 100 μL of each well were transferred to 96-well culture plates and quantified by absorbance spectrophotometry at 570 nm on a microplate reader (Quant MKX200, BioTek Instruments, Winooski, VT, USA). Absorbance values were used to determine the percentage of viable cells based on a standard curve obtained by culturing three different RAEC cell concentrations, in triplicate, in a 24-well plate.

2.6. Atomic force microscopy (AFM)

An MFP-3D-SA AFM system (Asylum Research) with BL-TR400PB cantilevers (Olympus) was used. The analyses were performed in tapping mode with nominal spring constant of 0.09 N/m and nominal tip radius of 42 nm (±12 nm). The cantilever was mounted on the idrive force excitation for direct cantilever excitation, passing an alternating force excitation through the V-shaped Cantilever. After calibration, the cantilever was directed in the frequency bending mode (6–8 kHz) away from the indentation can be recorded as a force function, which is proportional to the cantilever deflection. Therefore, force curves are the result of set of forces versus indentation, where the greater the force curve slope, the greater the surface rigidity. It is also possible to calculate the elastic property of the surfaces, applying the theoretical Hertz model (1882) to analyze the force curves. This model relates the force of a load \(F\) with an indentation \(\delta\) according to the following expression:

\[
F = \frac{2}{\pi} \times \frac{E}{1-\nu^2} \times \delta^2 \times \tan \alpha
\]

Where \(E\) is Young's elastic modulus, \(\alpha\) is the opening angle of the recoil cone and \(\nu\) is Poisson's constant of the sample, considered herein
as 0.5. Thus, two parameters are extracted from each force curve, the Young modulus of the sample and the contact point. The force-indentation curves were obtained and analyzed using the IGOR Pro Software 6.37.

2.7. Mechanical cell properties

A total of 5 × 10^4 cells/disk were cultured in HAM-F12 medium and incubated at 37 °C in a 5% CO2 chamber. After 24 h, the disks were washed twice with PBS and plated with Tyrode buffer (NaCl 119 mM; KCl 5 mM; CaCl2 2 mM; MgCl2 2 mM; Glucose 6 g/L;pH 7.4) at 37 °C. A total of 32 force curves were analyzed for each cell (n = 6), 16 for cell nuclei and 16 for peripheral cell regions for each type of treatment. The AFM methodology was applied in dynamic mode, allowing to obtain viscosity loss module (E"), and the elastic storage module (E'atog), from which viscoelasticity is calculated (E"/E'atog) [24].

2.8. Statistical analyses

The experiments were performed in replicate for each surface. A One-Way ANOVA test followed by multiple comparative tests was applied for surface roughness and wettability. The MTT and mechanical property data were submitted to an analysis of variance (ANOVA) followed by a post-hoc student's t-test. All analyses were performed assuming p < 0.05.

3. Results

3.1. Surface characterization

The material roughness profiles are displayed in Fig. 1(A–F). Plasma nitriding increase the topography more than oxidized (Fig. 1C–D). The 3D AFM images of nitride surface reveal regular peaks (Fig. 1D and F), significantly increasing roughness compared to the oxidized samples (Table 1). The plasma treatment significantly increased roughness in both treated surfaces compared to the polished surface, with the highest values observed for the nitrided surfaces. The mean roughness (Ra) of the nitrided titanium surface was higher (27.7 ± 1.3 nm, p < 0.05) than that of the oxidized (10.3 ± 1.1 nm, p < 0.05) and polished (1.3 ± 0.2 nm, p < 0.05) surfaces. Although the peak height (Rp) and maximum profile height (Rz) were significantly higher in the nitrided surfaces (140.1 ± 23.5, 220.4 ± 18.9, respectively) compared to the other surfaces, no Rp/Rz ratio variation was observed.

In addition, the contact angle of the nitrided surface was higher when compared to the polished (76.88° ± 2.68 versus 67.83° ± 4.7; p = 0.001) (Fig. 2) and oxidized (66.59° ± 3.65°; p = 0.001) surfaces. Thus, nitriding resulted in a more hydrophobic titanium surface, while plasma oxidation improved surface hydrophilicity.

GIXRD standards for the untreated, oxidized and nitrided samples are shown in Fig. 3. The surface of untreated titanium (Fig. 3A) displays a predominant Ti phase. However, the TiO2 phase, which spontaneously forms on the titanium surface, appears mainly for 1°, which is the lowest level, indicating a thinner material. In the case of plasma oxidized samples, Ti3O, Ti5O and TiO2 oxides are present. As the intensity of the TiO2 peak for the three incidence angles is very close, this phase is characterized as uniformly distributed along the depth profile scanned by the incident radiation. This is the same phase present in untreated Ti, which forms spontaneously at room temperature according to the literature. The Ti3O oxide was also observed, displaying greater intensity at a 1° angle, indicating it is a thin film on the surface formed during the plasma oxidation process. In addition, the TiO phase is also observed, which is apparently a transition phase between Ti5O and the Ti substrate. The TiN0.26 phase appears in the plasma-nitrided surface, also presenting peaks with reduced intensity according to increasing incidence angle, indicating a thin film.
thrombi [31]. Furthermore, the lower the Rp/Rz ratio, the lower the contact angle, thus raising hydrophilic potential [14].

The three produced surfaces presented a contact angle of <90°, thus classified as hydrophilic surfaces [32]. However, the oxidation treatment resulted in a statistically significant reduction in the contact angle per sessile drop of the polished, nitrided and oxidized plasma surfaces. (a-b) p < 0.001.

Table 1
Roughness parameters of the titanium surfaces.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Ra (nm)</th>
<th>Rp (nm)</th>
<th>Rz (nm)</th>
<th>Rp/Rz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polished</td>
<td>1.3 ± 0.2a</td>
<td>6.8 ± 0.2a</td>
<td>9.9 ± 0.9a</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Nitrided</td>
<td>27.7 ± 1.3b</td>
<td>140.1 ± 23.5b</td>
<td>220.4 ± 18.9b</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Oxidized</td>
<td>10.3 ± 1.1c</td>
<td>63.0 ± 13.0c</td>
<td>95.9 ± 21.8c</td>
<td>0.7 ± 0.6</td>
</tr>
</tbody>
</table>

(a,b,c) (p < 0.001).

Fig. 1. Titanium nanotopography differences between three conditions treatment by AFM imaging. (A–B) Polished titanium surface is flat. (C–D) Plasma nitride titanium surface has homogenous roughness. (E–F) Oxidized titanium surface has heterogeneous roughness. Area = 50 × 50 μm. Left and right panels represent the same AFM image in 2D and 3D dimensions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Contact angle per sessile drop of the polished, nitrided and oxidized plasma surfaces. (a-b) p < 0.001.
angle of the oxidized surface in relation to the nitride surface, while no difference was observed in relation to the polished surface. The increases hydrophilicity of the oxidized surface is due to the formation of the titanium oxide film in its stoichiometric varieties (Ti$_3$O, TiO$_2$, Ti$_6$O), especially Ti$_3$O, as it is more superficial, as demonstrated in the GIXRD surface analyses. These titanium oxide forms increase titanium surface hydrophilicity [33]. On the oxidized surface, Ti$^{4+}$ cation reduction to the Ti$^{3+}$ state was observed, generating oxygen vacancies. Water molecules can occupy the oxygen spaces, producing chemically adsorbed –OH groups, which increases Van der Waals forces and hydrogen bonding interactions between H$_2$O and –OH enhancing hydrophilic properties as a result [34]. The smaller contact angle on the polished surface is also related to the presence of TiO$_2$ resulting from the natural oxidation of the Ti surface [35].

Under the conditions employed in the present study (450 °C for 1 h), the plasma, nitriding and oxidation treatments applied to Ti were able to bombard the surface with nitrogen and oxygen ions, respectively. Thus, some Ti atoms were removed when reacting with N and O from the plasma and became available on the material surface, in the form of nitrides and oxides [36,37]; however, the films produced by the treatments were very thin, with magnitudes in the order of nanometers, estimated by the difference in peak intensity for different incidence
angles in GIXRD, which could not be perceived using conventional X-ray diffraction analysis (Bragg - Brentano configuration). Therefore, the GIXRD technique was applied, which, due to the small depth of beam penetration in the deposited layer, provided information on the treatment diffusion profile. This result is important concerning cell bio-compatibility, as cell interaction occurs directly with the most superficial layer of the material, and not the deepest.

Physical and chemical titanium properties, such as crystalline structure, roughness and wettability, are also considered external cellular microenvironments [38,39]. Therefore, these biomaterial variables can act together by stimulating or inhibiting normal or pathological physiological cell responses [40]. Therefore, our work revealed that the microenvironment produced through plasma nitriding and oxidation led to endothelial cell adhesion after the first 4 h after incubation on both the nitrided and oxidized surfaces. The oxidized surface, presenting lower average roughness and regular topography, stimulated the increase of endothelial cell viability when compared to nitrided surface. Thus, the findings reported herein confirm that endothelial cells, present in artery and vein endothelium, display a preference for surfaces presenting a lesser roughness degree. Some reports indicate that osteoblastic cells on titanium surfaces present greater roughness. However, fibroblast and epithelial cells have been reported as demonstrating a preference for smoother surfaces [41,42]. In addition, it is likely that the surface roughness of nitrided titanium inhibited intercellular endothelial cell contact [43], which are known to be signal transduction sites affecting cell migration and repairing, and in vitro and in vivo endothelium regeneration processes [44,45]. Thus, the oxidized surface reported herein may offer better physiological conditions for angiogenesis in the post-implantation period in the cicatricial phase.

Nevertheless, this focal adhesion aspect is not sufficient to confirm biocompatibility improvement, as adhesion is closely related to endothelial cell interactions with cell surface morphology. In turn, cellular morphology depends on cytoskeleton distribution [46]. An intrinsic mechanical property of the cell's cytoskeleton, present in the nucleus and periphery (cytoplasm and plasma membrane) regions, is viscoelasticity [47]. Cytoskeleton conformational changes depend on the interaction between proteins and focal extension organization, resulting in viscoelastic processes [21]. AFM was used to investigate cell morphology response stimulated by plasma surface treatment. Titanium plasma nitriding did not significantly increase the viscoelasticity of endothelial cell peripheries when comparing the polished and oxidized surfaces. On the nitrided surfaces, cells adhered and emitted small cytoplasmic projections (philopodia). This implies that the endothelial cells, when in contact with the external microenvironment of the nitrided titanium, become more rigid in their periphery, i.e. less viscoelastic, as an increase in extracellular matrix synthesis (EMS) is noted, in an attempt to improve focal adhesion strength [25]. However, this matrix stiffens the cell and reduces surface spreading [48]. This high EMS production is due to nuclear cytoskeleton activity (viscoelastic) in order to produce more matrix, inhibiting the β1 integrin expression and VEGF (Vascular Endothelial Growth Factor) internalization, hindering cell viability and proliferation on the material surface [48]. In vivo, this stiffness would imply in vascular dysfunction, with risk of restenosis after intravascular biomaterial implantation [49,50]. Endothelial cells are the tunica intima of arterial vessels, as in the coronary arteries of the heart; they require a paved external microenvironment resistant to pressure variations without displaying structural and functional impairment.

Fig. 5. RAECs viability (n° cells) by MTT after 24 h of culture on the polished, nitrided and oxidized Ti surfaces. (a, b) p < 0.05.

Fig. 6. Different live RAECs regions by AFM imaging. (A–D) Polished titanium surface; Area = 40 × 40 μm. (B–E) Plasma nitrided titanium surface; Area = 60 × 60 μm. (C–F) Oxidized titanium surface; Area = 60 × 60 μm; Yellow: cell nucleus. Top and bottom panels represent the same AFM image in 2D and 3D dimensions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
However, the oxidized surface significantly increased cellular periphery viscoelasticity in relation to the nitrided surface. The cells are on a smoother microenvironment, similar to the normal physiological environment of blood vessels (paved epithelium, known as endothelium). The nucleus viscoelasticity in cells with carcinogenic potential influences cell morphology, where decreased viscoelasticity stimulates spreading cells, presenting more flattened form and longitudinal extensions [51]. In the present study, normal endothelial cells, although displaying a more spread out form (flattened) with several prolongations, presented the highest nuclear viscoelasticity values, possibly due to the influence of the adhesion substrates, in this case the polished and oxidized surfaces. Lower fibroblast nucleus viscoelasticity has been reported as triggering nuclear deformation and fibronectin cell adhesion inhibition, decreased the focal adhesion

Fig. 7. Viscoelastic RAECs properties on different titanium surfaces. (A) Deflection of the cantilever in the nuclear and peripheral RAEC regions on titanium surfaces. (B) Viscoelasticity properties of the nuclear and peripheral RAEC regions on different titanium surfaces. (a+b) Cellular periphery viscoelasticity on the different surfaces; (p < 0.05) (*) nucleus vs. periphery viscoelasticity on the oxidized surface; (p < 0.05). Representative force-indentation curve on the polished titanium surface, nucleus (C) and cellular periphery (D). Representative force-indentation curve on the titanium nitride surface, nucleus (E) and cellular periphery (F). Representative force-indentation curve on the oxidized titanium surface, nucleus (G) and cellular periphery (H).
strength [52]. The nitrided surface induced the lowest nuclear viscoelasticity reduction among the assessed surfaces, which may justify the decreased viability of these cells at 24 h. The oxidized surface presented higher proliferation of these cells. Therefore, it is possible that the increase of the elastic modulus of the cytoskeleton of the nuclear region prevented these nuclear deformations [53]. Another hypothesis includes a strengthening of the lamina lattice that hardens the isolated nuclei, as a strategy to protect chromatin against excessive mechanical stress [54]. Taken together, we postulated that it is a plasma oxidation treatment as one of the greatest potentials for application of titanium intravascular devices. Whereas, the oxidized surface stimulated a focal adhesion, increased endothelial cell viability, kept the cell morphology and increased the viscoelasticity of the endothelial cells.

5. Conclusions

The microenvironment influences endothelial cells in a differential manner. Titanium surface plasma nitriding and oxidation promoted adhesion and ensured RAECs viability, although cellular adhesion on surfaces is not enough to affirm that cells are physiologically normal, they may be in physiological distress to survive, whereas the different surfaces modified cellular viscoelastic properties. AFM is an alternative to analyze the viscoelasticity of living cells and their implications for the biocompatibility of plasma-treated materials. Concerning titanium nitriding, a greater force curve slope observed in the AFM analyses implies in higher cell stiffness on the metal surface. Thus, cellular proliferation on this implant surface is more difficult, while endothelial cells softened and displayed potential cellular adhesiveness in plasma titanium oxidation. Both are adaptive mechanisms to increased cell proliferation rates. These findings suggest that oxidized surfaces display significant potential application in the manufacturing of metallic endoprostheses, as slow endothelial cell proliferation prevents cardiovascular ischemia, common in patients with intravascular stents, which would justify its choice for in vivo tests concerning intravascular devices. This AFM method offers insights into physical principles that can become a potential tool to understand the complex biological mechanisms of biocompatibility of endothelial cells on plasma-nitrided titanium.

Credit authorship contribution statement


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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