Physical characterization and biological tests of bioactive titanium surfaces prepared by short-time micro-arc oxidation in green electrolyte

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Abstract

Titanium (Ti) and its alloys are the most used biomaterials in dental and orthopedic implant applications. However, despite the good performance of these materials, implants may fail; therefore, several surface modification methodologies have been developed to increase the bioactivity of the metal surface, accelerating the osseointegration process while promoting improved corrosion performance. In this work, the production of a TiO2 coating on titanium through a short-time micro-arc oxidation (MAO) in a green electrolyte (obtained by a mixed solution of K3PO4 and Ca(CH3COO)2.H2O) is proposed, aiming at obtaining a porous oxide layer with Ca and P incorporation through an environmentally friendly experimental condition. The morphology, chemical composition, crystalline structure, wettability, hardness and bioactivity of the modified Ti surfaces were characterized. The MAO process at 250 V for 1 min in the green electrolyte solution allowed the production of a highly porous oxide surface in the anatase crystalline phase, with effective Ca and P incorporation. Pre-osteoblastic cells were used in in-vitro assays to analyze viability, adhesion, proliferation and ability to perform extracellular matrix mineralization on the Ti surfaces (polished and MAO-treated Ti). The MAO-treated Ti surface exhibited better results in the bioactivity tests, presenting more calcium phosphate precipitates. This surface also presented higher hardness, lower hydrophilicity and better performance in biological tests than the polished surface. The here-reported MAO-treated Ti surface is promising for dental implants, especially in patients having poor bone quality that requires greater stimulation for osteogenesis.

1. Introduction

With the advancement of medicine, many countries are experiencing an aging population with increasing problems related to oral health [1, 2]. A systematic analysis by the Global Burden of Disease Study 2016 allowed the estimation that half of the world population, about 3.6 billion people, suffers from some type of oral disease. Dental cavities and periodontal diseases are the main cause of tooth loss [3]. Another problem that also corroborates oral health injuries is dental trauma. Its prevalence is estimated to exceed those of cavities and periodontal diseases in certain populations [4] and about one billion people sustain some traumatic dental injury [5]. In this scenario, the worldwide market for dental implants is expanding [6].

Titanium and its alloys, being biocompatible and presenting excellent corrosion resistance and good mechanical properties, are the most used biomaterials for the manufacture of dental and orthopedic implants [7, 8]. Ti presents a high corrosion resistance because it reacts spontaneously with oxygen to form a nanometric (2 to 7 nm), stable, non-porous oxide layer on its surface [9, 10]. In the manufacture of dental implants, crowns,
bridges and overdenture, commercially pure titanium (Ti cp) is the material of choice. However, Ti cp exhibits a low wear resistance, which limits its use in orthopedic implants [11].

Despite the good performance of Ti and its alloys, the human body is a hostile environment for implants; these materials experience wear in vivo, which can induce an inflammatory response that results in serious complications and affects the lifetime of the materials [7, 12]. Additionally, titanium is a bioinert material, therefore being unable to directly bond to bone [12]. For this reason, many techniques and treatments have been used individually or together to modify titanium implant surfaces in order to select some characteristics of interest, such as wettability, roughness and biocompatibility. These techniques include: 3D printing, laser surface texturing, micromachining, mechanical polishing, sandblasting, ionic implantation, chemical etching and several coating techniques, such as chemical or physical vapor deposition, plasma spraying, thermal oxidation and anodization [13, 14]. Among them, anodization has gained attention because, through the manipulation of the experimental parameters, it allows a greater control of the thickness, composition, morphology and structure of the titanium oxide film [15, 16].

When anodization is performed at low potential differences, a thin oxide film is formed, which can or cannot exhibit coloration and does not show many imperfections in morphology [17]. On the other hand, if this process is carried out at high potential differences (more positive than the one needed to cause oxide rupture) there is intense O₂ evolution, and sparks are often observed over the oxide surface; this process is referred to as microarc oxidation (MAO), also known as plasma electrolytic oxidation. MAO-obtained films are generally thick (a few micrometers) and efficient in protecting the metal from corrosion and wear [18]. They are also adherent, rough, and highly porous, providing a good mechanical fixation because the bone cells can grow into this porous surface. Compared to other coating techniques, MAO is considered a simple, versatile, and cost-effective technique, which allows the use of non-toxic, environmentally friendly electrolytes. Besides that, MAO is very useful when coating metallic implants with complex geometries [16, 18, 19].

The predominant material property that improves the biocompatibility of an anodic Ti surface is not yet well defined, although it is believed that characteristics of the topmost surface of the oxide layer, such as hydrophilicity, composition, structure and morphology (roughness or porosity) are responsible for driving the cell response on an implant [16, 20–22]. In general, it has been shown that both morphology and surface composition play a decisive role in the implant performance, because they modulate surface interaction by cells and microorganisms. Micrometric-scale roughness is believed to promote interconnection between cells; nanotopography is also important in protein adsorption, osteoblast adhesion and, consequently, osseointegration speed, and in antimicrobial activity [23].

Regarding the chemical composition of the implant surface, when elements that are natively present in the bone such as calcium (Ca) and phosphorus (P) are incorporated in the Ti oxide film, there is an increase in protein adsorption speed leading to an early biological fixation of the implant [24–27]. This is of great interest because it can avoid aseptic loosening, which is one of the major causes of implant failure [28]. This complication usually occurs when complete osseointegration between the peri-implant bone and the implant surface does not occur [29]. Moreover, rapid osseointegration promotes implant sealing, reducing the likelihood of bacterial invasion [15].

Soares et al. [25] produced titanium oxide nanotubes on Ti substrates via anodization in the presence of Ca and P and concluded that these elements improved the adhesion, proliferation and differentiation of adipose-derived stem cells, compared to unmodified titanium. In addition, the modified surface also presented higher hydrophobicity, protein adsorption and calcium mineralization. It is already known that MAO also enables the insertion of Ca and P in the oxide film when they are present in the electrolyte [30]. Ribeiro et al. [31] used MAO to obtain a nanostructured bioactive titanium oxide film doped with these elements, in which a nanometric calcium-rich amorphous layer was formed on the top surface. Then fibroblast cells showed higher affinity for these areas. Felgueiras et al. [22] reported similar results in assays with MG63 osteoblast-like cells, when treated surfaces enhanced osteoblast attachment and differentiation (ALP production and mineralization).

Although anodization provides promising results, acids or strong bases at high concentrations are employed as electrolytes in most procedures [16, 32], making problematic the handling and the segregation and waste treatment processes. To circumvent this problem, many studies on anodization have been conducted in greener electrolytes that are Ca and P precursors that provide greater bioactivity to the titanium surface. The main Ca precursors are calcium acetate and calcium chloride; the P precursors are commonly sodium phosphate and sodium dihydrogen phosphate. Advantageously, the same electrolyte can be a precursor of both elements, as in the case of calcium glycerophosphate and calcium dihydrogen phosphate. Moreover, the MAO time usually ranges from 3 to 60 min, and the applied potential difference varies from 250 to 500 V [30].

Therefore, the aim of this work was to prepare and characterize porous TiO₂ coatings with Ca and P incorporation, obtained by employing a short-time micro-arc oxidation along with an alternative non-toxic, green electrolyte that does not require any disposal treatment. Calcium acetate hydrate and potassium phosphate were chosen as the alternative green and less expensive Ca and P precursors, respectively. The
morphology, chemical composition, crystalline structure, wettability, hardness and bioactivity of the modified Ti surfaces were characterized. Further, in vitro assays were done with pre-osteoblastic MC3T3-E1 cells to analyze viability, adhesion, proliferation, and ability to perform extracellular matrix mineralization on both polished and MAO-treated Ti surfaces.

2. Material and methods

2.1. Preparation of the MAO coatings
Disks (12.6 mm diameter) of a 2.0 mm thick Ti cp plate (Titanews, Brazil; purity >99 mass%) were used as samples. The disks were mechanically polished (320 grit) and then embedded in polyester resin (the area in contact with the electrolyte was 125 mm²). Then the surface of each Ti disk was ground to 600 grit finish using a silicon carbide paper, thoroughly rinsed with deionized water and air dried. A platinum plate (Johnson Matthey) was used as counter electrode. The green MAO electrolyte was obtained by preparing a mixed solution of 0.167 mol L⁻¹ calcium acetate dihydrate (Synth) and 0.100 mol L⁻¹ potassium phosphate (Sigma Aldrich), when a suspension was formed due to the low solubility of calcium phosphates. MAO was performed at room temperature (about 23 °C) using a DC power supply (Tectrol, Brazil) at 250 V for different anodization times (1, 5 and 10 min) while magnetically stirring the electrolyte.

2.2. Surface characterization and contact angle studies
The Vickers microhardness of the Ti surfaces was evaluated at room temperature using a Shimadzu HMV-2000 tester equipped with a Vickers pyramid indenter at a load of 25 g to 100 g and a dwell time of 10 s. The Vickers hardness value (VHN) was calculated by dividing the indentation force by the surface of the imprint observed at the microscope. The measurements were performed at ten points randomly distributed on the surface of each disk. The average of two replications was taken as the VHN value.

The morphology of the untreated and MAO-treated Ti samples was observed by scanning electron microscopy—SEM (Philips XL–30 FEG microscope). For the MAO coatings, two specimens and three points of vision fields for each were used to measure the mean pore size with the ImageJ software. In addition, energy dispersive x-ray spectrometry (EDX) allowed the evaluation of the mean elemental composition of the MAO coatings. In order to estimate the oxide film thickness of a representative MAO-treated sample, a cross-section SEM micrograph was further analyzed. For this, the specimens were mounted in an acrylic resin, cut to reveal the cross-section and fine polished with a 0.3 μm alumina solution. The crystalline phase of the samples was identified by X-ray diffraction (XRD-6000, Shimadzu) analysis using Cu-Kα radiation at 30 kV and 30 mA; the scan range was 20°–80° and the scanning speed was 2° min⁻¹ at 0.02° scan steps. The average roughness (Ra) and area of the surface were measured using a confocal laser scanning microscope (CLSM, LEXT OLS 4000, Olympus); six measurements were done on each sample.

Contact angle measurements were performed with a goniometer (Optical contact angle meter CAM 101, KSV Instruments) via the sessile drop method using Milli-Q water at room temperature. Water contact angles were measured at 10 and 90 s after dispensing sessile water droplets (5 μl) on the sample surface with the aid of a syringe needle. Experiments were performed in five samples of each surface treatment, six measurements per sample, at different positions.

2.3. In-vitro bioactivity tests
In-vitro bioactivity tests were conducted to analyze the ability of apatite formation or calcium phosphate precipitation on the sample surface. This test was adapted from the ISO 23317:2014 protocol. In a plastic vial, the samples were immersed in 15 ml of a simulated body fluid (SBF) solution at 37 °C, without stirring. The SBF solution was renewed every 24 h to maintain the appropriate ionic concentration. After 7 and 14 days, the samples were taken out, rinsed with deionized water and then dried at 60 °C before characterization by SEM and XRD analyses.

2.4. Biological tests
2.4.1. Cell culture
Pre-osteoblastic MC3T3-E1 cells (BCJR, Brazil) were incubated in an alpha-minimum essential medium (αMEM, Gibco, Thermo Fisher Scientific), with 10% fetal bovine serum—FBS (Gibco), 100 U ml⁻¹ penicillin (Gibco), 100 μg ml⁻¹ streptomycin (Gibco), 50 μg ml⁻¹ ascorbic acid (Sigma Aldrich) and 10 mmol L⁻¹ β-glycerophosphate (Sigma Aldrich), and maintained in a humidified atmosphere of 5% CO₂ at 37 °C. The cell culture medium was refreshed every 3 days until the cells reached 80%–90% confluency. Then, the cells were detached from the culture flasks with a trypsin-EDTA solution (Gibco) and transferred to the titanium disks at a density of 2 × 10⁵ cells mL⁻¹.
2.4.2. Cell viability and proliferation
Cell viability/proliferation was evaluated on both polished and anodized titanium disks using an alamarBlue® kit (Life Technologies, USA), which contains a nontoxic blue dye (resazurin) that indicates the accumulative metabolic activity. This depends on the cell viability and on the number of cells in the culture. Viable cells with active metabolism reduce resazurin to resorufin, a reddish and fluorescent compound. Therefore, the number of viable cells can be related to the amount of dye reduction, being expressed as a percentage of reduction of alamarBlue®, according to the manufacturer’s protocol.

For these assays, cells were seeded on the surfaces of treated and control Ti disks, placed in 24 well plates, and cultured for 1, 3, 7, 10, 14 and 21 days in the previously described medium and conditions, with addition of 50 μg ml⁻¹ ascorbic acid (Sigma-Aldrich) and 10 mmol L⁻¹ β-glycerophosphate (Sigma-Aldrich). At the end of each culture period, the cells seeded on the discs were incubated for 4 h, at 37 °C, with 500 μl of the working solution (α-MEM with 10% FBS, 1% P/S (penicillin/streptomycin) and 10% alamarBlue®). One well having only the working solution was used as the negative control. Afterward, 150 μl aliquots from each sample were transferred to a 96 well plate and read on a spectrophotometer at the wavelengths of 570 and 600 nm.

2.4.3. Cell morphology and spreading
Cell morphology and spreading were evaluated by SEM and direct fluorescence microscopy. MC3T3-E1 cells seeded on the surfaces of titanium discs were cultured for 1, 3, 7, 14 days.

For the SEM analysis after each culture time, the samples were washed with a phosphate-buffered saline (PBS) solution, fixed with 2% glutaraldehyde in 0.1 mol l⁻¹ cacodylate buffer, washed again with the PBS solution, followed by dehydration in a graded series of alcoholic solutions (25, 50, 70, 95, and 100% V/V), and then dried in a vacuum desiccator. Finally, the dried samples were sputter-coated with gold, and random images were registered for each sample using a Philips XL-30 FEG microscope.

For the fluorescence analyses, phalloidin conjugated with the Alexa Fluor® 488 dye (Molecular Probes) was used, which signals the actin cytoskeleton, and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes) for nuclear labeling. Image acquisition of the cells was performed in an inverted fluorescence microscope (Evos FL, AMG Micro).

2.4.4. Mineralized nodule formation
The alizarin red test was performed to quantify the formation of mineral nodules on different surfaces by cells cultured over the Ti sample. The presence of mineral nodules indicates cell differentiation into osteoblasts and suggests matrix mineralization. For this, calcium deposited on pre-osteoblast cell membranes was stained with alizarin red after 21 days of cell cultivation. The cells attached to the Ti discs were washed twice with the PBS solution at 37 °C and fixed with 1 ml of 70% (V/V) ethanol at 4 °C for 1 h. After, the samples were washed twice with the PBS solution and twice with deionized water, and then stained with 1 ml of an alizarin red solution (40 mmol L⁻¹, pH 4.2, Sigma-Aldrich) at room temperature for 5 min, in the absence of light and under gentle shaking (VDRL Shaker, Biomixer). Non-adherent dye was aspirated, and each Ti disk was then washed several times with deionized water to remove excess stain. These samples were then photographed, and the alizarin red of each specimen was dissolved by the addition of 500 μl of a 10% cetylpyridinium chloride solution (Sigma-Aldrich) for 15 min under stirring at room temperature. Finally, three 100 μl aliquots of these solutions were collected, transferred to a 96 well plate and the absorbance was read at 550 nm in a microplate reader (Power Wave XS, BioTek Instruments, USA). Three measurements were performed for each sample.

2.5. Statistical analysis
Two-way analysis of variance (ANOVA) within a 95% confidence interval (α = 0.05) was used to determine the variability of the results obtained in the cell viability and proliferation assays, as well as the mineralized nodule formation assays. The significance was regarded at p value <0.05.

3. Results and discussion
3.1. Surface characterization
The macroscopic appearance and SEM micrographs of the polished (Pol) and 1 min MAO-treated Ti surfaces can be observed in figure 1. From the SEM micrograph of the Pol sample (figure 1(a)), it is possible to perceive a uniformly marked surface with the typical grooves left by the abrasive particles of the surface treatment. In contrast, a rough and porous oxide layer was formed by anodic oxidation on the MAO-treated sample (figure 1(b)); here, the pores are homogeneously distributed over the entire surface. Additionally, figure 1(c) shows that the distribution of pore diameter for this sample (anodized for 1 min) ranged between 0.1 and 2.0 μm, with an average diameter of (0.65 ± 0.40) μm. The oxide layer morphology of the MAO-treated
samples obtained at 5 and 10 min of anodization was similar to the one shown in this figure and thus is not
presented here.

Although it is common to vary the MAO time to change the thickness, roughness and other characteristics of
titanium films, there is an implicit time threshold after which the oxide film can no longer be thickened
regardless of the increase in the procedure time \[30\]. This happens because, after this threshold, the oxide film
begins to suffer so many electrical and mechanical breakdowns that the rate of oxide formation is only able to
reconstruct the failures imposed on the film \[33–35\]. Given that no morphological variations were found for the
Ti samples treated at different MAO times (1, 5 and 10 min) and considering that due to the breakdown-
reconstruction phenomenon the film thickness may be very close for the analyzed samples, the MAO-treated
sample obtained at the shortest time (1 min) was selected for further characterizations.

As mentioned hereinbefore, the composition of an oxide layer plays an important role in its
biocompatibility. Figure 1 (d) presents the EDX spectrum of the MAO-treated sample, confirming Ca and P
incorporation in the oxide coating. From the EDX elemental mappings shown in figure 2, it can be inferred that
Ti, O, P and Ca were uniformly distributed throughout the coating surface. Clearly, the alternative non-toxic,
green electrolyte provided excellent results as P and Ca precursor. This is an auspicious result, as the presence of
these elements seems to improve cell adhesion, proliferation and differentiation \[30\]. By employing a modified
MAO technique (anodic and cathodic pulse voltages), Ponomarev et al \[26\] also reported a homogeneous
insertion of Ca and P in the titanium oxide, although using a voltage of 350 V, which is considerably higher than
the one used in this work. Moreover, Zhang et al \[27\], using another variation of the MAO technique (applied
current of 100 mA cm\(^{-2}\) at 600 Hz for 10 min), were able to uniformly insert Ca and P into the Ti surface,
although the porosity of this surface was not uniform.

Furthermore, semiquantitative analysis of the EDX data revealed a Ca/P atomic ratio of 2.22 ± 0.28 for the
MAO-treated sample. Bone tissue is composed of calcium phosphates, mainly hydroxyapatite (HAp) in its
crystalline form having a Ca/P atomic ratio of 1.67 \[36\]. Wu et al \[37\] investigated the influence of increasing
Ca/P ratio on the alkaline phosphatase activity, which is an indicator of cell metabolism. They found ratios
between 0.40 and 2.21 and that alkaline phosphatase increased for the higher ratios. In addition, Marques et al
\[38\] produced MAO-treated Ti surfaces with Ca and P incorporation and obtained Ca/P ratios in the range of
1.70 to 2.08. They concluded that higher ratios provided increased wear and corrosion resistance to the

Figure 1. SEM micrographs of polished (a) and MAO-treated (b) Ti samples and their respective photographs (inserts); (c) pore
diameter distribution for the MAO-treated Ti samples; (d) EDX spectrum of the MAO-treated Ti sample. The MAO treatment was
performed at 250 V for 1 min in the green electrolyte obtained by a mixed solution of 0.1 mol l\(^{-1}\) K\(_3\)PO\(_4\) and 0.167 mol l\(^{-1}\) Ca
(CH\(_3\)COO)\(_2\)H\(_2\)O, kept at 23°C.

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biomaterial. Therefore, in the present study, the high Ca/P ratio observed for the MAO-treated samples may provide interesting properties to the obtained coating.

Cross-sectional analyses of SEM images of the MAO coatings were performed to gather information concerning their morphology and thickness. For a MAO-treated sample anodized at 250 V for 1 min, the average oxide film thickness, calculated from measurements at four distinct points (as indicated in the micrograph shown in figure 3), was $(3.20 \pm 0.44) \mu m$. Generally, oxides grown by MAO in Ca and P precursor electrolytes present thickness between 1 and 6 $\mu m$ [39, 40]. Furthermore, as highlighted in the micrograph shown in figure 3, the MAO-formed titanium oxide coating is composed of two layers: the first one, adjacent to the metallic substrate, is compact with few failures, while the second one is thicker and contains a high number of pores. This bilayer structure is characteristic of Ti oxides formed by MAO processes, as reported in other works [41–43].

As mentioned earlier, MAO is a technique known to increase the substrate roughness due to the in-depth growth of a porous oxide coating. The greater the surface roughness of the implant material, the greater is its direct contact area with bone tissue, consequently improving cell attachment [44]. Hence, average surface roughness ($R_a$) and height ($S_a$) values were calculated from confocal microscopy images (figure 4) for the Pol and MAO-treated samples—see table 1. The $R_a$ value obtained for the Pol sample was $(0.20 \pm 0.03) \mu m$, while that for the MAO-treated sample was $(0.47 \pm 0.12) \mu m$. Therefore, the 1 min MAO treatment significantly
increased the surface roughness. Another important factor about this parameter is that similar values were obtained for the different MAO-treated samples, indicating surface homogeneity despite the varying treatment times. An additional parameter calculated from the images shown in figure 4 was the average surface area, which, as expected, presented significantly higher values for the MAO-treated sample (see table 1).

Vickers microhardness data are also presented in table 1. The MAO-treated sample showed higher values compared to the Pol specimen. This increased surface hardness is beneficial for dental implants, as it leads to a higher material resistance and, accordingly, improves its durability [45, 46].

Another characteristic of micro-arc oxidation on Ti is the formation of titanium oxides with crystalline structures (rutile, anatase and brookite). Among them, anatase is possibly the one of greatest interest in the medical field, because it promotes antimicrobial activity under ultraviolet radiation [47, 48] and enhances cell response [31, 49]. Figure 5 shows the X-ray diffraction patterns for the Pol and MAO-treated samples. The diffractogram for the Pol sample consisted of the characteristic pure titanium peaks ($2\theta = 35.1^\circ$, $38.4^\circ$, $40.2^\circ$, $53.0^\circ$, $62.95^\circ$, $70.66^\circ$, $76.2^\circ$ and $77.3^\circ$), according to the JCPDS 44–1294 file. On the other hand, in addition to these peaks, the MAO-treated sample presented other peaks characteristic of crystalline TiO$_2$ in the anatase phase ($2\theta = 25.3^\circ$, $36.9^\circ$, $37.8^\circ$, $48.0^\circ$, $53.9^\circ$ and $55.1^\circ$), according to the JCPDS 21–1272 file.

These XRD results are promising because, as already mentioned, the anatase phase under certain conditions promotes antimicrobial activity [47]. In addition, it has been reported that this phase can attract, more efficiently than other Ti oxide phases, calcium and phosphate ions from the physiological environment to form a hydroxyapatite-like coating. Besides, anatase can also improve the osteogenic properties due to lattice match and superposition of hydrogen bonding groups in this crystal phase [50, 51].

Additionally to the surface crystallinity and roughness, the surface wettability was also found to be another important aspect that improves the bioactivity of titanium surfaces; the wettability of a solid surface can be characterized by the contact angle of a liquid [52]. Thus, the surface wettability of the MAO-treated Ti samples was also investigated in this work in comparison with the just polished samples—see figure 6. After 10 s of dispensing sessile water droplets on the sample surface, contact-angle values of $(77.5 \pm 1.7)^\circ$ and $(107.6 \pm 8.1)^\circ$ were registered for the Pol and MAO-treated samples, respectively, indicating that MAO increased the
Figure 5. XRD patterns of the polished (Pol) and MAO-treated titanium samples. For the MAO treatment conditions, see figure 1.

Figure 6. Contact angles of polished (Pol) and MAO-treated Ti samples after different times of dispensing water droplets on the surfaces (indicated in the figures). For the MAO treatment conditions, see figure 1.
hydrophobicity of the surface, making it less wettable. Figure 6 also reveals that, after 90 s of dispensing sessile water droplets on the sample surface, the contact angle for both specimens had no significant changes. Implant hydrophilic surfaces were reported to enhance early osteogenic cell responses and, consequently, early osteointegration. This can possibly extend the long-term success rate of the implant [53]. However, although cell proteins have greater affinity for hydrophilic surfaces, they can also interact with hydrophobic surfaces due to their unfolding process [54]. Additionally, in contrast with hydrophilic surfaces, hydrophobic surfaces can inhibit bacterial growth because bacteria cannot adhere to the surface [55].

3.2. In-vitro bioactivity tests
The ability of an implant to induce apatite formation in a physiological medium plays a crucial role in osseointegration. In-vitro tests were performed in a SBF solution to allow the analysis of calcium phosphate precipitate formation on the titanium surfaces after different immersion times. Generally, better bioactive materials are able to induce greater precipitation [56]. As shown in figure 7, after 7 days of immersion in the SBF solution, almost all polished Ti samples retained a bare surface with few spherical clusters. In contrast, calcium phosphate, consisting of subtle rice-grain structures seen at high magnification, precipitated over the entire surface of the anodized sample (see inset in the SEM micrographs of the MAO-treated samples). Again, the 1 min MAO-treated surface seems to be a better bioactive surface. After 14 days of immersion, there was no apparent variation in the surface morphology compared to 7 days, for both the polished and MAO-treated samples.

3.3. Biological tests
3.3.1. Cell viability and proliferation
The viability and proliferation of the MC3T3-E1 cells cultured on the Pol and MAO-treated surfaces were determined by using the alamarBlue® assay up to 21 days. In this assay, viable cells reduce the non-fluorescent blue-colored resazurin dye to the fluorescent reddish resorufin compound. Therefore, there is a direct correlation between the reduction of the dye present in the osteogenic medium and the density of living cells, i.e. this test is indicative of cell growth and metabolic activity [25, 57]. As can be inferred from the data in figure 8, both tested samples presented a significant intra-group increase of alamarBlue® reduction during the assay, indicating that they provide good conditions for cell viability, promoting cell proliferation over time. This result indicates that the anodization treatment did not cause any toxic effects to MC3T3-E1 cells, as well as that the morphology of the samples did not interfere with the cell adhesion mechanism. Besides, there was no significant inter-group difference within a 95% confidence interval (p < 0.05, two-way Anova) along the analyzed period.

![Figure 7: SEM micrographs of polished (Pol) and MAO-treated titanium surfaces after immersion in SBF solution for 7 and 14 days. For the MAO treatment conditions, see figure 1.](image-url)
3.3.2. Cell morphology and spreading

SEM and direct fluorescence microscopy were used to evaluate the MC3T3-E1 cells morphology and spreading on titanium samples at 1, 3 and 7 days of culture. For the fluorescence microscopy cell evaluation, DAPI and FITC-conjugated phalloidin were used. DAPI (4′,6-diamidino-2-phenylindole) is a blue-fluorescent nucleus stain because it binds to the adenine-thymine-rich regions of the DNA. Fluorescein phalloidin dyes the cytoskeletal actin fiber; it binds F-actin with a high-selectivity conjugated to the fluorescent green dye, fluorescein (FITC). As can be inferred from the confocal images shown in figure 9, both surfaces promoted substantial cell growth over time; therefore, the Ti surface modified by MAO supports cell proliferation. Furthermore, as can be seen in figure 9, even as early as after only 1 day, the cells were homogeneously distributed and widely spread on the surface of the samples. The Pol surfaces have more broad-shaped, or polygonal-shaped cells, whereas the cells on the MAO-treated surfaces exhibit more multipolar spindle osteoblastic morphologies with numerous elongated lamellipodia, sometimes overlapping their neighboring ones. Round cells were rarely detectable. After 3 days, cells on both surfaces proliferated readily, spread out and kept their shapes. In this period, all cells were already sub-confluent and adhered tightly to the substrates. In addition, they were interconnected by filopodia and lamellipodia. By the 7th day, most of the cells were in confluence, forming a more homogeneous cell layer on the titanium surfaces. However, the morphology of cells...
covering the Pol surface still had the polygonal shape and those covering the anodized samples kept the spindle shape, demonstrating a more advanced stage of development.

The SEM images (see figure 10) showed that pre-osteoblastic cells presented the same morphology, adhesion and proliferation behavior on both samples, as in the fluorescence analysis. As can be seen from the lower magnification SEM micrographs of figure 10, both the Pol and MAO-treated samples exhibited a significant number of cells after the 1st day of cultivation, but still possible to be identified separately. An interesting fact is that there is more contact between cells proliferated on the MAO-treated sample. After 3 days of cultivation, the number of cells increased, but total confluence was reached after 7 days. Regarding cell morphology, the cells on the Pol sample appeared to be more flat-shaped, i.e. having a lower volume than those on the MAO-treated sample. Therefore, from the observation of the pre-osteoblast behavior in both SEM and fluorescence microscopy results, it is believed that cell differentiation was facilitated on the anodized surfaces.

Figure 10. SEM micrographs for the morphology and viability analyses of MC3T3-E1 cells cultured on polished (Pol) and MAO-treated Ti surfaces at 1, 3 and 7 days. For the MAO treatment conditions, see figure 1.

Figure 11. Extracellular matrix mineralized nodules stained by alizarin red, generated by MC3T3-E1 cells cultured for 21 days on polished (Pol) and MAO-treated Ti samples (no significant discrepancy, p < 0.05, two-way Anova). For the MAO treatment conditions, see figure 1.
3.3.3. Mineralized nodule formation

The production of calcified nodules by the MC3T3-E1 cells was analyzed after 21 days of incubation by staining extracellular calcium deposits using alizarin red. Mineralization is an important late marker of osteoblastic differentiation and an indicator of successful in-vitro bone formation [58]. As illustrated in figure 11, no calcification of cells appeared over the entire surface, for both samples. Although visually the osteoblasts grown on the MAO-treated samples had displayed the highest level of mineralization compared to that on the Pol samples, there was no statistical difference between the two groups (p < 0.05, two-way, ANOVA). Collectively, these results demonstrate that the MAO treatment of the Ti substrates improves osteoblast-like behavior in terms of differentiation and mineralization.

4. Conclusions

A bioactive micro-porous TiO2 coating containing Ca and P in the oxide layer was obtained by MAO at 250 V for only 1 min, which is a short anodization time compared to others in the literature. The proposed green electrolyte, obtained by a mixed solution of K3PO4 and Ca(CH3COO)2·H2O, provided effective result as a phosphorus and calcium precursor. XRD analyses showed that the TiO2 oxide formed on the anodized sample surface is the anatase crystalline phase. This is auspicious, since anatase can be more efficient than other titanium oxide phases in the nucleation and growth of the hydroxyapatite that forms the bone. In addition, MC3T3-E1 cells cultured on the MAO-treated Ti surfaces exhibited enhanced differentiation and mineralization compared to those on polished Ti. Therefore, the here-reported MAO-treated Ti surface is promising for use as an implantable material, being especially useful in patients having poor bone quality that requires greater stimulation for osteogenesis.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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