Wettability and osteoblastic cell adhesion on ultrapolished commercially pure titanium surfaces: the role of the oxidation and pollution states

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The oxidation state of the surfaces of titanium-based biomaterials strongly depends on their previous history. This factor affects the titanium wettability and it probably conditions the success of the implanted biomaterials. However, the separate role of the pollution and oxidation states of metallic titanium surfaces remains still controversial. To elucidate this, it is required to standardize the initial surface state of titanium in terms of roughness and surface chemistry and then, to monitor its wettability after the corresponding treatment. In this work, we studied finely polished surfaces of commercially pure titanium (cpTi) which were subjected to cleaning surface treatments. XPS was used to characterize the surface chemistry and the oxide film thickness. The contact angle hysteresis in underwater conditions was measured with the growing/shrinking captive bubble method, which allowed for mimicking the real conditions of implantable devices. The water wettability of smooth cpTi surfaces was stabilized with weak thermal oxidation (230ºC, 30 min). The osteoblastic cell response of the stabilized and non-stabilized cpTi surfaces was analyzed. Although the oxidation and pollution states were also stabilized and normalized, no correlation was observed between the stable response in wettability of titanium and its cell adhesion.

Keywords: Titanium, Oxidation, Wettability, Captive bubble, Cell adhesion.

Introduction

In implantology, it is a usual task to correlate biological performance and wettability of titanium surfaces in order to discriminate surface treatments for improving the osteoconductivity [1-3]. However, the full understanding of cell spreading, the impact of sterilization on the titanium surface properties before cell culturing and the meaningful interpretation of the wettability data of titanium oxide surfaces condition the biological response-wettability correlation.

The link between the early stages of osseointegration and physico-chemical adhesion (wettability) remains controversial [4]. As an example, Vlacic-Zischke et al. [5] found that the osteogenic differentiation increased with increasing hydrophilicity. On the other hand, Gittens et al. [6] found that the increase in roughness of strong hydrophobic titanium surfaces enhanced the maturation of osteoblast-like cells.

Furthermore, Iwasa et al. [7] found no correlation between the hydrophilic status and the protein adsorption or cell attachment capacities of titanium surfaces. In other respects, the surface sterilization previous to every cell culture may change remarkably the wettability properties of titanium [2, 3, 8-10]. All these factors must be considered when the correlation between cell growth and wettability is examined for titanium surfaces subjected to different treatments.

Wettability measurements enable to characterize the affinity of a surface towards a liquid [11]. Usually, the wettability of titanium surfaces is evaluated by measuring the static contact angle of sessile drops placed over the initially dry surface [3, 6, 8, 12]. However, titanium implants are always immersed in body fluids. Besides, the "intrinsic" contact angle of the oxide surface formed on metallic titanium is still controversial because it depends on the oxide crystallinity and the presence of adventitious contaminants [11, 13]. This confirms that the wettability of titanium surfaces is strongly dictated by their previous history. The water contact angles of metallic titanium surfaces are typically below 10º when measured immediately (<5 min) after UV/ozone cleaning [14]. However, for a titania thin film (amorphous), the water contact angle was 80º prior and 6º after UV irradiation overnight [15].

The oxidation state of titanium surfaces is a critical factor for wettability because titanium develops, due to the air exposition, an uncontrolled and stable oxide layer of varying nanometer-thickness and stoichiometry [16, 17]. This protective oxide layer promotes osseointegration [17]. In order to establish a controlled starting point, several authors used plasma ashing/etching to remove the surface pollutants and the native oxide layer [18-20]. This way, a highly hydrophilic surface is obtained although it always recovers the initial wettability. Lin et al. and Mills and Crow [18] explained the origin of the hydrophilization of plasma-treated titanium in terms of the formation of

hydroxyl surface groups attached to the crystal structure of titanium. The hydroxyl groups are less stable than the oxygen atoms and they are replaced by oxygen, recovering the initial wettability of titanium. However, another explanation refers to the removal of adventitious hydrocarbon compounds formed over the titanium surface, because it is well-known that clean oxidized metals are rapidly polluted upon exposure to ambient air [11]. This high rate of pollution may be caused by the existence of numerous active surface sites generated during the plasma treatment [11]. This way, after an etching treatment, titanium is inevitably contaminated, recovering the initial wettability. Another treatment used to stabilize the oxidation state of titanium surfaces is heating in air [9]. At high temperature, titanium oxidation is accelerated but below 275 ºC it reaches a steady state at short times (~20 min) and the crystalline structure of metallic titanium remains unchanged [21].

In this study, we standardized the states of pollution and oxidation of commercially pure titanium (cpTi) surfaces to examine the correlation between wettability and cell response. The surface roughness of the cpTi surfaces was removed above micrometer scale. Different surface treatments based on weak thermal oxidation and plasma ashing were applied to the ultrapolished cpTi surfaces. Contact angle measurements in underwater conditions were performed to mimic the real conditions of titanium implants immersed in body fluids. The impact of each treatment was studied in terms of contact angle hysteresis, surface chemistry and cell response.

Materials and methods

Sample preparation

Commercial pure ASTM grade II titanium ingots (Manfredi) were cut into discs (1.6-cm diameter, 1.6-mm thickness). A hole of 1 mm diameter was drilled at the center

of each sample. Although the roughness improves the mechanical retention of the cells over the titanium surfaces [6, 12], implant surfaces with nanometer-scale roughness may help to understand the direct cell-surface interactions [4]. In this work, the titanium samples were finely polished with a grinder/polishing machine (Beta Grinder Polisher, Buehler). After polishing, the samples revealed very low values of roughness over an area of $1\mu m^2$ ($R_a, R_q < 1\,nm$), measured with an atomic force microscope (see Section Topography). The freshly polished samples were degreased [11] in ultrasonic baths of acetone, soapy water, ethanol and distilled water and finally, MilliQ water. Next, the samples were dried with N_2 gas. The titanium samples after cleaning were referred to as Control in this study.

A radio frequency plasma device (KX1050 Plasma Asher, Emitech) with argon gas was used for ashing/etching treatment of the Control samples. Argon gas was used to avoid metal oxidation [11]. The plasma treatments were performed for 15 min at a power of 25 W and a gas flow rate of 15 ml/min. The plasma-treated samples were referred to as Ar Plasma.

The titanium samples were also heated in an oven. The samples were heated below 275 ºC to avoid crystalline change, but providing enough time to obtain a steady state of titanium oxidation (> 20 min). We explored a wide range of temperatures and times but the optimal treatment was reached at 230 ºC for 30 min. The temperature of 230 ºC was enough high to reach rapidly (30 min) a steady state of titanium oxidation [21]. This treatment was referred to as Heating.

The samples were stored before and after each treatment in Petri dishes to mitigate their pollution. During the heating treatment, the samples were placed inside the oven in a sealed dish. All treatments were applied just before each analysis to avoid eventual aging of the samples.

Wettability

The captive bubble method was used to examine the titanium wettability in underwater conditions, which are more realistic for a biomaterial. The low-rate dynamic contact angle technique was applied, as described by Montes Ruiz-Cabello et al. [22], with a micro-injector (ML500, Hamilton). This technique consisted in growing and shrinking an air bubble through a small hole drilled in the titanium sample immersed in MilliQ water with a maximum bubble volume of 80µl and at a constant flow rate of 1µl/s. From the plot of contact angle in terms of bubble contact radius, the advancing contact angle (θ_{adv}) was averaged over the maximum stable values of contact angle and the receding contact angle (θ_{rec}) over the minimum stable values of contact angle. The difference between these two contact angles is the contact angle hysteresis $H= \theta_{adv}- \theta_{rec}$.

We used fresh MilliQ water in all experiments. The wettability of all proposed treatments was examined at room temperature and by duplicate.

Surface chemistry

Eventual changes in the titanium crystalline structure were explored with X-Ray diffraction (XRD). XRD spectra were taken with a Philips PW 3710 Diffractometer, 2θ range, CuKα radiation (λ=1.54Å). Absorbance plots as a function of the 2θ angle were compared with other works [23]. This technique has a depth resolution of several micrometers [24], which provides bulk crystallographic analysis of the titanium surfaces. The surface chemistry was also quantified by X-Photoelectron spectroscopy (XPS) with an Axis Ultra-DLD, Kratos. The maximum depth resolution of the XPS device was lower than 10 nm [25]. The thickness of titanium oxide layer of each sample was also estimated from the XPS spectra [25, 26].

Topography

Height and phase images of the titanium samples were acquired with AFM (MultiMode Scanning Probe Microscope, Nanoscope IV, Veeco) in tapping mode, over an area of $10\mu m^2$. The phase images intended to identify possible heterogeneities in the surface composition. The arithmetic mean of height deviations respect to the central plane (R_a) and the standard deviation of heights (R_q) were averaged over two different regions on each sample studied.

Cell response

Cell culture

The human MG-63 osteosarcoma cell line was purchased from American Type Cultures Collection (ATCC) and maintained as described in reference [27] in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Gibco Cell Culture Products) with 100 IU/ml penicillin (Lab Roger), 50µg/ml gentamicin (Braum Medical), 2.5µg/ml amphotericin B (Sigma), 1% glutamine (Sigma), and 2% HEPES (Sigma), supplemented with 10% Foetal Bovine Serum (FBS, Gibco). The cultures were kept at 37ºC in a humidified atmosphere (95% of air and water vapor and 5% of CO_2). Cells were detached from the culture flasks with a solution of 0.05% trypsin (Sigma) and 0.02% ethylene diamine tetra-acetic acid (Sigma) and then, they were washed and redispersed in complete culture medium with 10% FBS.

Cell adhesion

The osteoblasts obtained were inoculated onto the titanium samples at 10^4 cell/ml in a 24-well plate (Falcon, Becton Dickinson Labware) following the ratio of 2 ml/well. The samples were kept at 37º C in a humidified atmosphere of 95% air and water vapor and 5% CO_2. The assays were performed at 24 h and 48 h for each surface. After the culture

time, the medium was replaced with DMEM containing 0.5 mg/ml 3(4,5-dimethyl-thiazoyl-2-yl)2,5 diphenyl-tetrazolium bromide (MTT, Sigma) and incubated for 4 h. Cellular reduction of the MTT tetrazolium ring resulted in the formation of a dark-purple water-insoluble deposit of formazan crystals. After incubation, the medium was aspirated and dimethyl sulfoxide (Sigma) was added to dissolve the formazan crystals. The number of adhered cells over the titanium surfaces was determined with a spectrophotometer (Sunrise). The absorbance was measured at λ=570 nm. Only the Control samples were subjected to autoclave sterilization because the Ar Plasma and Heating treatments actually sterilized the titanium surfaces [3].

Statistical analysis

All cell culture trials were conducted by triplicate. The results were statistically described by analysis of variance and of significant differences between measurements with the Student's t-test (software Libreoffice). Statistical significance level was set at $p < 0.05$.

Results

Wettability results for the Control and Ar Plasma samples are shown in Figure 1. It is worth pointing out that the contact angles measured over the Control and Ar Plasma samples were strongly dependent on the selected sample. This is reflected in the high scattering of values. However, we were able to stabilize the wettability response of the Control and Ar Plasma samples by applying the Heating treatment. The samples subjected to Heating treatment presented the same contact angle hysteresis within the errors, and well-defined advancing and receding contact angles. Otherwise, the Ar Plasma samples presented zero hysteresis, within the errors.

The results of Figure 1 reveal that the Control samples are not a good starting point in terms of reproducibility and that the Ar Plasma treatment decreases the contact angle hysteresis, but with very low contact angles and with a very high variability between samples subjected to the same Ar Plasma treatment. However, both Control + Heating and Ar Plasma + Heating combined treatments produced a very similar wettability response.

XRD analysis of the treatments studied (see Figure 2) revealed peaks of titanium identified with the planes [100], [101] and [110], which point out rutile rather than anatase [23]. All samples gave similar results due to the depth resolution of the XRD technique (see Section Surface chemistry). This confirms that the crystal structure of titanium remained unaltered after each treatment.

From the XPS spectra (see Figure 3), the atomic percentages of each surface chemical compound were evaluated. The main species of the titanium surfaces were oxygen O-1s, titanium Ti-2p and carbon C-1s (adventitious contamination). Two detailed analysis were separately performed with the O-1s spectrum and the Ti-2p spectrum. The deconvolution of the O-1s spectrum provided the relative percentages of physisorbed water on the surface, hydroxide, hydrated or defective oxygen, organic oxygen and TiO_2 [26, 28]. Otherwise, the deconvolution of the Ti-2p spectrum provided the relative percentages of the different oxidation states of titanium including the metallic titanium [26, 28-30]. In addition, the thickness of the oxide layer was estimated by averaging the values extracted from the peaks TiO_2 2p3/2 and TiO_2 2p1/2 [25, 31]. The XPS results are summarized in Table 1. The Ar Plasma samples presented the lowest percentage of adventitious C-1s and the Control samples were the most polluted. The Heating treatment reached similar values of carbon signal regardless of the Control and Ar Plasma samples. The atomic percentage of O-1s that forms TiO_2 revealed high

levels of TiO_2 (>65% of the O-1s signal) on all the studied surfaces. The amount of physisorbed water was negligible (≤1% of the O-1s signal) regardless of the sample. Although the main specie of the oxide layer was TiO_2 (>90% of the Ti-2p signal), small traces of TiO, Ti_2O_3 and Ti were present on the treated cpTi surfaces. The TiO_2 thickness values were greater than 10 nm for the Control and Ar Plasma + Heating samples. Finally, the Ar Plasma samples revealed the thinnest TiO2 layer (6.6±0.7 nm).

AFM images of a Control sample, the same sample subjected to Ar Plasma and next subjected to Heating treatment are shown in Figure 4. The phase images of these cases (not shown) did not significantly change with the treatments. The roughness values for the Control sample subjected to each treatment are shown in Table 2. The Ar Plasma treatment decreased the roughness of the Control sample although the roughness was recovered with the Heating treatment.

The cell adhesion on the different titanium surfaces is plotted in Figure 5 for the two culture times: 24 h and 48 h. All cases improved significantly the cell adhesion on the Control samples after the two culture times. At 24 h, there were no significant differences between Control + Heating and Ar Plasma + Heating. At 48 h, there were no significant differences between Ar Plasma and Ar Plasma + Heating. Moreover, Control and Ar Plasma + Heating samples separately presented no significant difference as the culture time. The highest cell adhesion was reached for Control + Heating samples at 48 h. All significant differences were considered with a confidence level of 95% ($p < 0.05$).

Discussion

The Heating treatment was applied to the Control and Ar Plasma samples to stabilize the titanium surfaces in terms of wettability. After the Heating treatment, the wettability was stable and independent of the previous states of the titanium surface.

The hysteresis values in Figure 1 allow classifying the smooth titanium surfaces obtained after the treatments into three types:

- Highly hydrophilic surfaces with low contact angles (≤20º) and zero hysteresis (H~0º): Ar Plasma samples.

- Hydrophilic surfaces with advancing and receding contact angles between ~80º and ~40º respectively, high scattering of values and remarkable hysteresis H~42º: Control samples.
- Near-hydrophobic surfaces with maximum advancing contact angle (~90º) and H~30º: Control + Heating and Ar Plasma + Heating samples.

The highly hydrophilic titanium surfaces reproduced in this study with the Ar Plasma treatment are consistent with the low contact angles reported in literature [18]. Considering the adventitious hydrocarbon elimination as well as the surface hydroxylation, the hydrophilic character of titanium is explained by one or the other mechanism, separately. The wettability stabilization of all samples subjected to the Heating treatment might be explained in terms of the temperature required to reach a reproducible oxidation state due to the removal of surface hydroxyl groups or chemisorbed water but without changing the crystalline structure of titanium [9]. This was assured because the temperature value was below 275 ºC, reported as limit value [21]. Moreover, the Heating treatment was enough fast to reduce the possibility of significant pollution. In the case of the Ar Plasma samples, the surface oxide could be likely unstable but it was rapidly stabilized with the Heating treatment. Furthermore, on the Control samples, the Heating treatment also fixed the oxidation state of titanium to similar levels.

The XRD spectra revealed no difference in the crystalline structure of the titanium bulk after each treatment. Thus, the Ar Plasma and Heating treatments only affected the first nanometers of depth of the titanium surfaces.

From the XPS data, the Ar Plasma treatment was a suitable treatment for cleaning the titanium surface due to its ashing/etching effect whereas the Control samples were the most polluted. Instead, the Heating treatment normalized the pollution state for both Control and Ar Plasma samples. The heating seems to pollute the highly clean titanium surface after Ar Plasma treatment, and to volatilize partially the pollutants of the Control samples. When the titanium samples showed lower C-1s percentage, they revealed higher Ti-2p and O-1s signals, because the titanium oxide arose out at the surface. On the other hand, all the samples presented high levels of TiO_2 on the surface (>70%). The rest of oxygen O-1s could form non-stoichiometric oxides, hydroxyl groups and water (TiO_x, -OH and H_2O). These results are verified with the estimated thickness of the oxide layer (see Table 1). We assumed that the Control samples were significantly covered by a thick oxide layer because they were stored in air for days [32]. The Control samples subjected to the Heating treatment did not improve the amount of TiO_2 as expected, because the Control samples were already oxidized. However, the Ar Plasma treatment revealed the highest TiO_2 content due to the removal of water and other oxygen compounds. Also, this treatment decreased the oxide layer due to the etching effect. The sample recovered the oxide thickness when it was subjected to the Heating treatment, increasing further the TiO_2 percentage, possibly due to the controlled oxidation using the oxygen available from other forms. These results reinforce the hypothesis that the Heating treatment produced a stabilized oxidation state of the titanium surfaces.

The roughness parameters in Table 2 and the height images in Figure 4 reinforce the hypothesis that the Ar Plasma treatment removed partially the oxide layer and the most of pollutants, smoothing the titanium surface. According to this hypothesis, since the Heating treatment increased the oxidation of the surface, the heated samples recovered or even exceeded their initial roughness.

From Figure 5, we found that all treatments improved the cell adhesion over the Control samples, regardless of the culture time. This might be explained by the autoclave sterilization of the Control samples, which could mitigate at some extent the cell response [3, 8].

If we consider the controversial hypothesis that the cell aggregates spread as a viscous liquid [33, 34], the spreading of cells over a surface points out to the advancing contact angle as ruling parameter. But the mobility of the cells during spreading involves the detachment of certain parts of the cells and thus, the receding contact angle should describe better this behavior. Finally, the contact angle hysteresis might be related to the roll-off behavior of the cells as they are globally moving in a given direction on the surface. Following this mechanical reasoning, the Ar Plasma samples with low contact angle and null hysteresis would be more suitable for cell spreading because the cells might freely move on the surface of the Ar Plasma samples. However, the moderate early cell response (24 h) disagrees with the former hypothesis and suggests that there are other parameters to consider.

On the other hand, we expected similar values of cell growth for the Control + Heating and Ar Plasma + Heating samples since the Heating treatment stabilized, and even normalized, both titanium surfaces in terms of oxidation, pollution and wettability response. However, at 48 h the Control + Heating samples reached the greatest cell growth, followed by the Ar Plasma and Ar Plasma + Heating treatments. This points out

that the stabilized cpTi surfaces evolved in different way at long times during the cell culture although the oxidation and pollution levels were similar. It should be noticed that there is no way to decouple the effects of metal oxidation and surface pollution during the cell adhesion. There are multiple factors that difficult the understanding of cell behavior on titanium surfaces: the biological variability, the interactions of the culture medium with the surface and the complex cell-surface interactions. However, we confirm that the cell response is strongly dependent on the previous cleaning of the titanium surfaces.

Conclusions

The wettability of nanometer-scale rough titanium surfaces was successfully measured in underwater conditions with the captive bubble method. This method enables to examine reliably the wettability (contact angle hysteresis) of titanium surfaces for bioadhesive applications. We propose the Ar Plasma + Heating combined treatment as a new cleaning route of titanium surfaces with stable wettability. The XRD results confirmed that this treatment did not alter the titanium bulk and the XPS results validated the surface cleanliness and TiO_2 layer formation. In addition, the AFM topographies and roughness parameters suggested that the Ar Plasma treatment removed pollutants and part of the oxide layer. However, this layer was recovered with the Heating treatment. Finally, the cell cultures revealed that all treatments improved the Control samples, subjected to autoclave sterilization, regardless of the culture time and that the Control + Heating treatment was the most osteoconductive after 48 h. This proves a different time evolution of the stabilized cpTi surfaces with very similar properties, although by different ways, in biologically active media. In this case, the two-way correlation between wettability and cell adhesion might be misleading. We recommend examining carefully the oxidation/pollution state and the wettability of the

titanium surfaces just before the cell assays to obtain meaningful interpretations of the correlation between cell adhesion and surface treatment.

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Table 1. Atomic percentages of the main chemical species on the treated titanium surfaces, percentages of species with oxygen relative to O 1s signal, percentages of species with titanium relative to Ti 2p signal and thickness of the oxide layer.

Signal (Binding energy, eV**)**	**Control (%)**	**Control+ Heating (%)**	**Ar Plasma (%)**	**Ar Plasma + Heating (%)**
Ti 2p (459)	17.9	20.5	26.3	21.4
O 1s (530)	45.0	48.9	57.5	49.6
C 1s (285)	37.1	30.7	16.2	29.0
XPS fitting from O 1s spectra (% relative to O 1s signal)				
TiO_2 (530.5)	69	68	73	74
O 1s (531.3)*	30	32	27	26
H_2O (534.3)	1	0	0	0
XPS fitting from Ti 2p spectra (% relative to Ti 2p signal)				
TiO_2 (459.1-2p3/2 and 464.8-2p1/2**)**	99.6	94.0	91.6	90.7
Ti_2O_3 (458.0-2p3/2 and 463.6-2p1/2)	0.2	3.3	2.7	3.3
TiO (456.7-2p3/2 and 460.5-2p1/2)	0.0	1.7	2.7	5.7
Ti (454.2-2p3/2 and 460.2-2p1/2)	0.2	1.0	3.1	0.3
Thickness of the TiO_2 layer (nm)				
	>10	8.8±0.7	6.6±0.7	>10

* Hydroxide, hydrated or defective oxygen, organic oxygen

Table 2. Roughness of titanium surfaces measured with AFM over 10μm^2 scansize.

	R_q (nm)	R_a (nm)
Control	7.1±.1	5.5±1.3
Control + Ar Plasma	5±3	3.8±2.4
Control + Ar Plasma + Heating	6.9±1.6	5.5±1.4

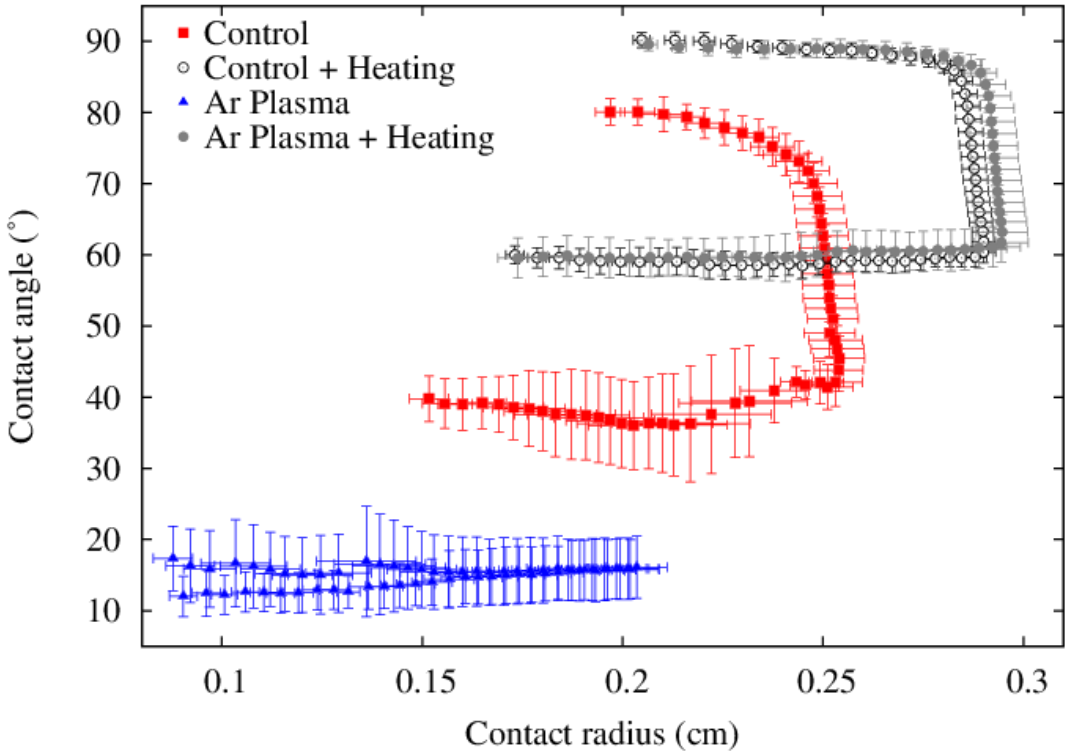

Figure 1. Contact angle as a function of contact radius of shrinking and growing captive bubbles in MilliQ water for smooth cpTi surfaces subjected to different treatments.

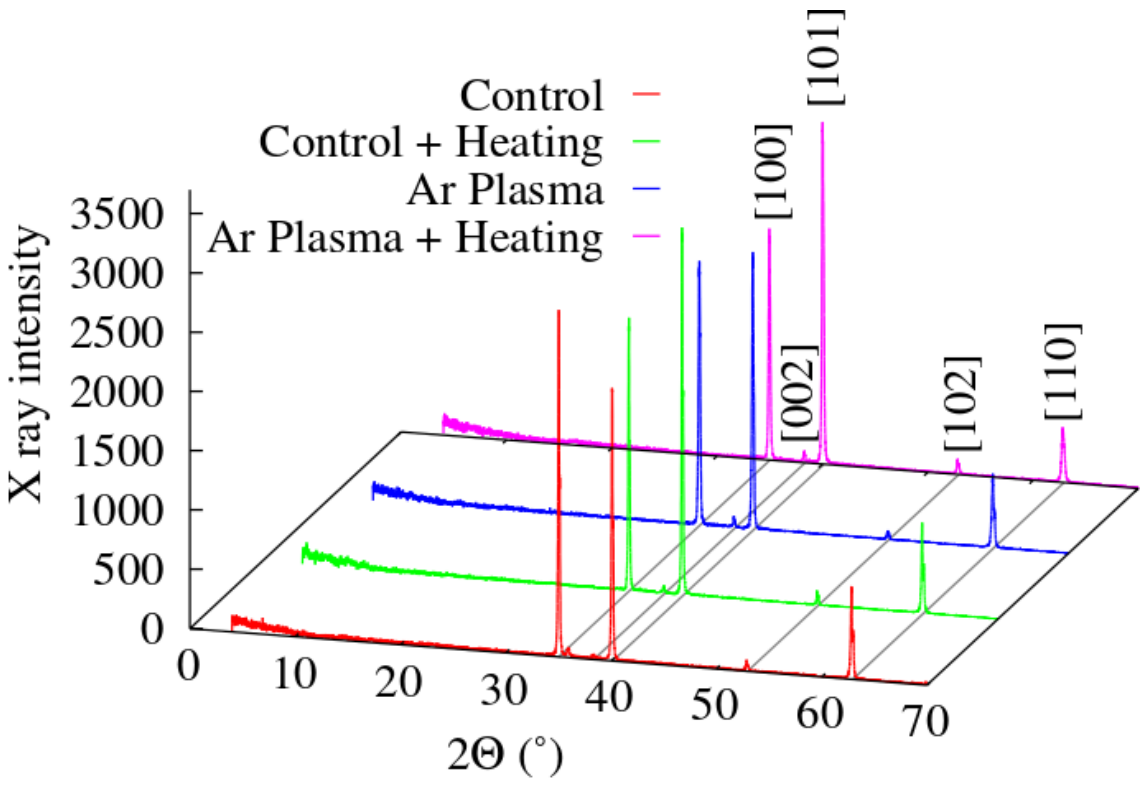

Figure 2. XRD spectra of the smooth cpTi surfaces subjected to different treatments. It should be noticed that there is no significant difference between treatments.

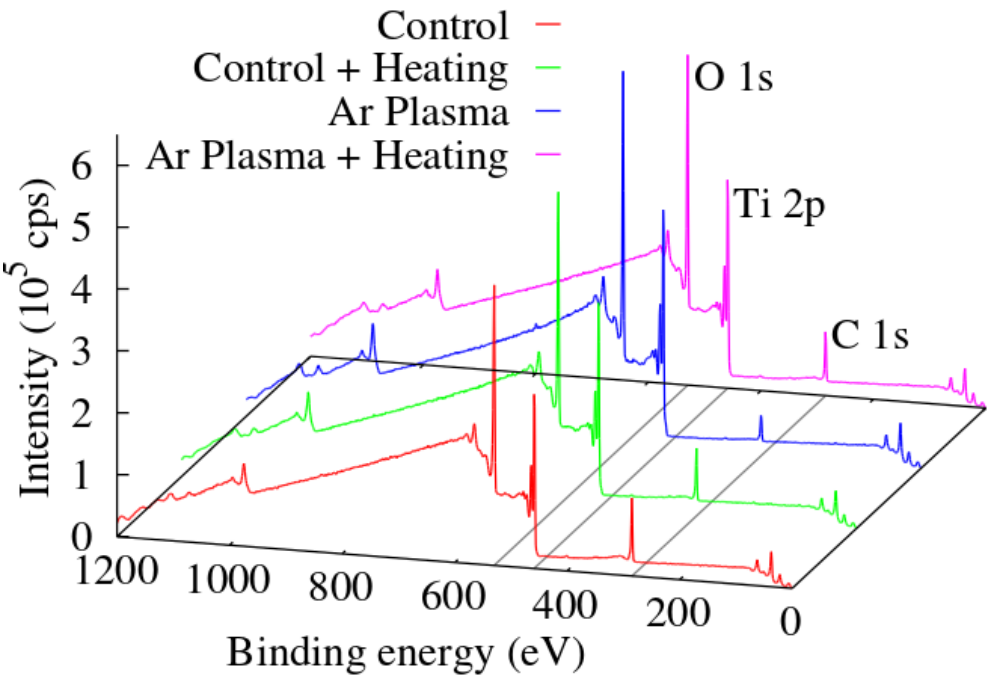

Figure 3. XPS spectra of the smooth cpTi surfaces subjected to different treatments.

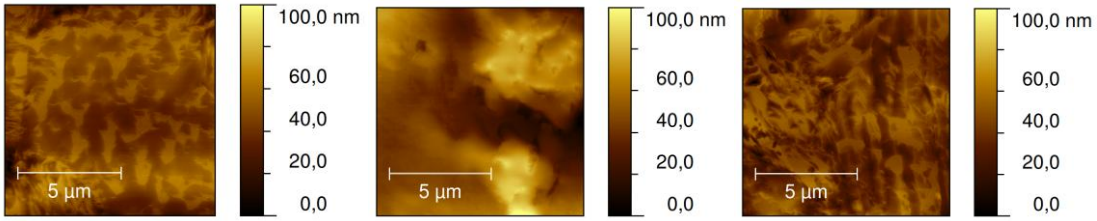

Figure 4. AFM height images of (a) Control sample, (b) the same sample after Ar Plasma and (c) finally after Heating treatment. All scansizes were 10µm$^{2.}$.

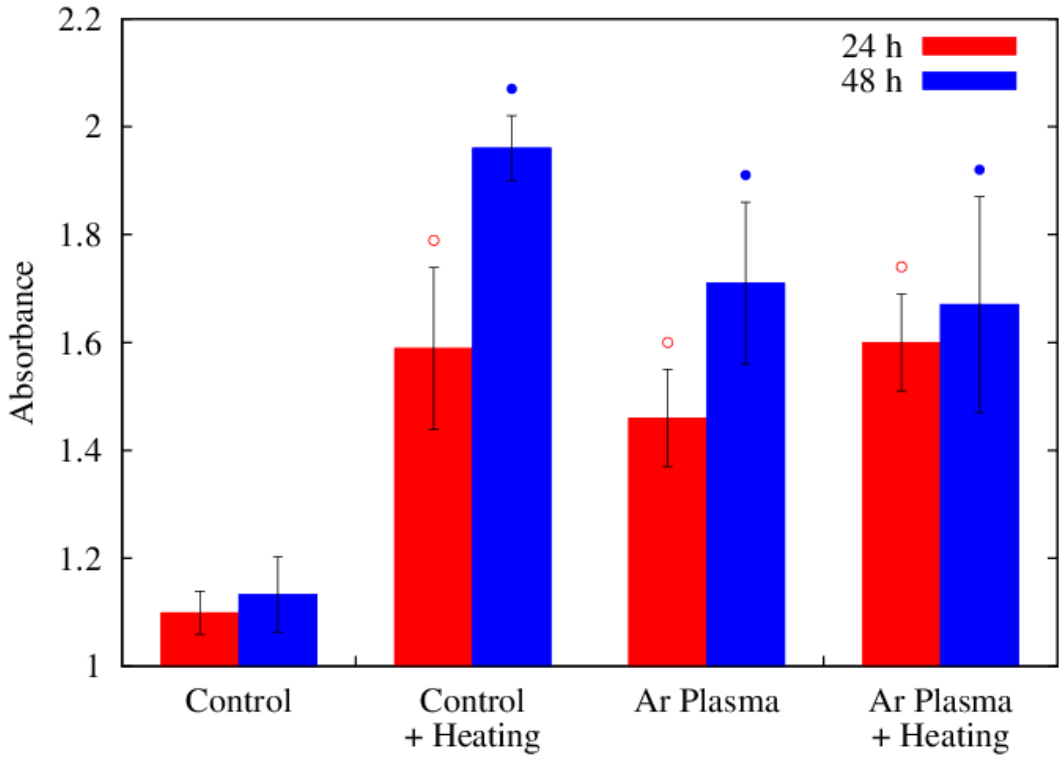

Figure 5. Cell adhesion for the different treatments performed on titanium surfaces. The cell culture times were 24 h and 48 h. Circle symbol: Significantly different at $p < 0.05$ vs. Control 24 h. Bullet symbol: Significantly different at $p < 0.05$ vs. Control 48 h.