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In vitro study of platelet behaviour on titanium surface modified by plasma

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Abstract

Biomedical devices introduced in the human body interact initially with blood cells, their success being dependent on the result of this interaction. This work aimed to obtain optimum biocompatibility and hemocompatibility of the titanium by plasma treatment. For this, discs of commercially pure titanium (CP) were subjected to plasma nitriding using different mixture of nitrogen and hydrogen; the effect of addition of hydrogen to the nitriding plasma was investigated. Before and after treatment, samples were evaluated in terms of topography and wettability using atomic force microscopy and sessile drop tests, respectively. The titanium biological response was evaluated *in vitro* through the application of platelet-rich plasma (PRP) on the surfaces modified and analysis of their behaviour from the point of view of surface tension and cell adhesion. Surface properties, such as roughness and wettability, were sensitive to the hydrogen/nitrogen ratio in the nitriding plasma, suggesting a strategy for producing different surfaces of biomedical devices. Results showed to be possible to obtain surfaces with different response to the adhesion of platelets, covering different applications.

Keywords: Biocompatibility; hemocompatibility; titanium; plasma treatment; biological response.

1. Introduction

Biocompatibility and hemocompatibility are important properties to ensure success of an artificial implant biomedical since initially it will interact with blood cells. Blood is a mixture of plasma, various types of cells and platelets. Adsorption of plasma proteins is one of the first events that occur when surfaces of implants are exposed to the contact with blood [1], followed by others process such as adherence, activation and proliferation of platelets [2]. Activated platelets attract others platelets forming aggregates; thus, activations are characterized by morphological changes and by the presence of aggregates [3].

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Commercially pure titanium (CP-Ti) is an important material used as artificial implant due to its characteristics of biocompatibility, corrosion resistance and high strength-to-weight ratio [4,5]. However, the surface characteristics desired to medical devices depend on the application. For example, to ensure bone-biomaterial interface integrity it is desired a good surface wettability. Unlike, a low wettability of the blood is required to avoid adhesion and activation of platelets on surfaces of stents and valves. Such opposite conditions indicate the need for surface modification after manufacture of biomaterials in order to direct them for specific applications [6]. Modification of the surface without adversely changing the characteristics of biocompatibility of the biomaterial is a challenge which motivated the present work. The purpose of this investigation was to study CP-Ti with the surface modified by nitriding plasma

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and the influence of the addition of hydrogen in the nitriding atmosphere on that modification, with respect to the behaviour of platelets *in vitro*.

2. Experimental Methods

Discs of commercially pure titanium (CP-Ti) were subjected to a nitriding atmosphere of plasma and the effect of addition of hydrogen to the plasma was investigated. Seven different experimental groups, including polished samples used as the control, were studied.
Discharge plasma was produced in a vacuum at a fixed pressure of 1.0 mbar while the temperature and time of tests were maintained at 450°C and 1 hour, respectively. A flow of 5 sccm of Argon, and varying proportions of nitrogen and hydrogen, as shown in Table 1, was maintained during the plasma treatment.

Table 1. Gaseous flow used in the plasma treatment.

$_{H2}A_{N2}$	Flow (sccm)		
	Ar	H_2	N_2
control	-	-	-
$_{15}A_0$	5	15	0
$_{12}A_3$	5	12	3
$_9A_6$	5	9	6
$_6A_9$	5	6	9
$_3A_{12}$	5	3	12
$_0A_{15}$	5	0	15

Before and after the plasma treatment, the samples were evaluated in terms of topography and wettability. Atomic force microscopy (AFM) was used to characterize the surface topography utilizing the contact mode and images with areas of 10x10 μm^2 were obtained. These results enable to calculate the roughness parameters (Ra, Rp, Rz). Wettability was evaluated by the sessile drop tests, depositing 10 µL of distilled water, glycerol or components of the blood on the surfaces of the samples, control and treated. Surface energy and interfacial tension were determined through a contact angle using methods of calculating based on the work of Huang *et al.* [7].
The biological response was evaluated by *in vitro* test through the application of platelet-rich plasma (PRP) on the titanium modified surfaces. Dilution was made to guarantee that $8x10^7$ platelets were present in 1 mL of plasma. Analysis of their behaviour from the point of view of adherence of platelets was investigated trough images by scanning electronic microscopy (SEM).

3. Results

Figs. 1 and 2 present the roughness and contact angle of the titanium surface before (control) and after the plasma treatment with different flows of nitrogen (N_2) and hydrogen (H_2). Plasma treatment resulted in rougher surfaces and sharp peaks, confirmed by the Ra value and by the Rp/Rz higher than 0.5, respectively, as can be seen in Fig. 1. Rp/Rz ratio indicates the surface shape; values higher than 0.5 refer to sharp peaks and values lower than 0.5 indicate round peaks and, according Zhu *et al.* [8], round peaks promote the scattering of liquids.

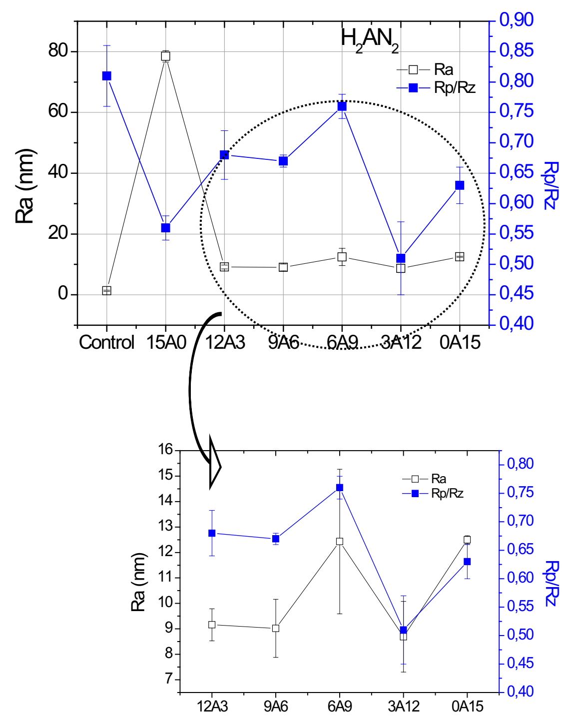

Fig. 1. Roughness parameters before and after plasma treatment obtained by AFM.

$_3A_{12}$ sample presented Rp/Rz = 0.51, thus on the threshold between round and sharp peaks. Roughness Ra of the sample treated with only H_2 ($_{15}A_0$) presented the higher values (Ra≅78 nm), probably in this case prevailed the etching caused by hydrogen, corroborated by reduction in the Rp/Rz. However, roughness increased smoothly when nitrogen was added in the plasma atmosphere, indicating the possible deposition of TiN, overlapped to etching.
All the treated surfaces showed a reduction in wettability for liquids, water and glycerol, as observed by contact angle values presented in Fig. 2, highlighting the sample treated only with hydrogen which showed the highest hydrophobic character.

These results suggest a correlation between roughness Ra and contact angle, as the sample that showed higher roughness Ra also showed the larger contact angle (sample $_{15}A_0$) and the increase in the roughness was accompanied by an increment of the contact angle in others samples.

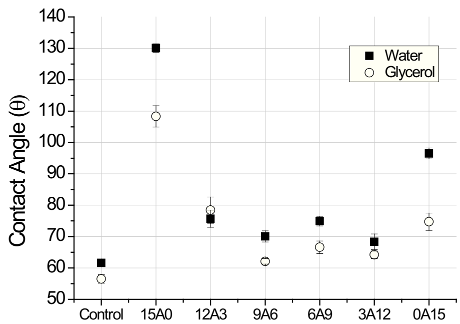

Fig. 2. Contact angle results for a sessile drop of water and glycerol.

The interfacial tension of the samples, control and treated, in water, blood, fibrinogen and albumin are shown in Fig. 3. Interfacial tension increased to all samples after plasma treatment and higher results were identified to $_0A_{15}$ and $_{15}A_0$ which refer to samples treated only with H_2 + Ar and N_2 + Ar, respectively.

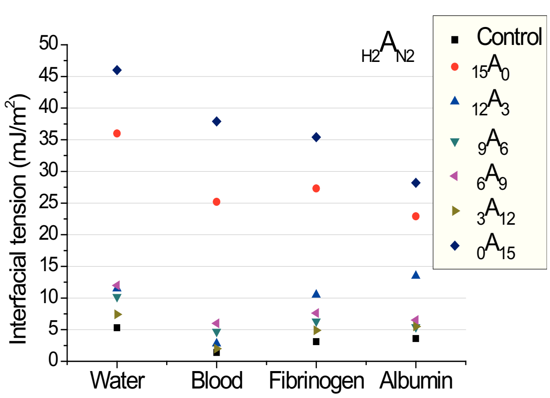

Fig. 3. Interfacial tension of the samples in contact with water, blood, fibrinogen and albumin.

$_3A_{12}$ sample presented lower interfacial tension with water, blood, fibrinogen and albumin than other samples treated. Wen *et al.* [9] comments the relation between interfacial tension and protein adsorption, where, according them, the high interfacial tension would cause multilayer adsorption of protein. They studied the biological response of the films of DLC doped with Hydrogen and Nitrogen using DC-MFCVA at RT and found that the doping of N or H element would decrease interfacial tension of DLC films; especially the effect was evident when N and H elements were added together. $_{12}A_3$ sample presented greater interfacial tension with albumin than with fibrinogen. However, $_{15}A_0$, $_0A_{15}$ and $_6A_9$ samples showed the inversed response of interfacial tension where higher values were found to fibrinogen in relation to albumin. These results can be associated to topography of the surface, since these samples presented higher roughness Ra as seen in Fig. 1. Others samples presented approximate values of interfacial tension to two proteins (albumin and fibrinogen). The relation between interfacial energy, the kind and amount of protein adsorbed and adherence of platelet to biomaterials is mentioned by Topala [1].

According to Kowk *et al.* [10], the platelet adhesion test is a simple approach to evaluate the biocompatibility of a material. Good surface trombogenicity is associated with small quantity and less morphological changes of the adherent platelets. In all surfaces analysed, platelets adherent were identified with formation of pseudopodia, indicating platelet activation; however, some presented higher activation than others (Fig. 4). Wetter *et al.* [11] utilized the morphology to classify the platelet activation, using four classes: (a) discoid or round, (b) dendritic or nearly pseudopodial, (c) intermediary pseudopodial and (d) full spreading. Different stages of platelet activation were observed in all samples. In the control sample were observed discoid, dendritic and intermediary pseudopodial platelets, besides small platelets aggregation (Fig. 4 a)). The $_{15}A_0$ sample presented higher amount of adherent platelets isolated and round with less activated state (discoid and dendritic forms) and without the formation of platelet aggregates (Fig. 4 b)). Possibly the high value of roughness (Ra) of this surface resulted in a large amount of platelets adhered; however, the platelets were not activated which can be associated with the high hydrophobicity and interfacial tension presented for this surface. Furthermore, this sample presented a surface covered by precipitates of residual titanium oxides not reduced by hydrogen.

Fig. 4 c) presents a SEM image of the $_{12}A_3$ sample where platelets with discoid and dendritic shape can be observed besides aggregation. This sample showed greater interfacial tension with albumin than with fibrinogen, which could indicate a preference for adsorption of albumin, suggesting retardation in the adhesion and activation of platelets. However, high amount of platelets and morphological changes were found, maybe due to the increase of the interfacial tension, for both albumin and fibrinogen.

Sample $_6A_9$ presented a large quantity of platelets adhered to the surface besides the higher activation of platelets among all samples; four classes of indication of activation were identified (discoid, dendritic, intermediary pseudopodial and full spreading). This sample also showed higher interfacial tension with fibrinogen than with albumin and higher Rp/Rz among the treated samples, indicating a surface with sharper peaks. These characteristics can indicate a tendency to promote the platelets activation.

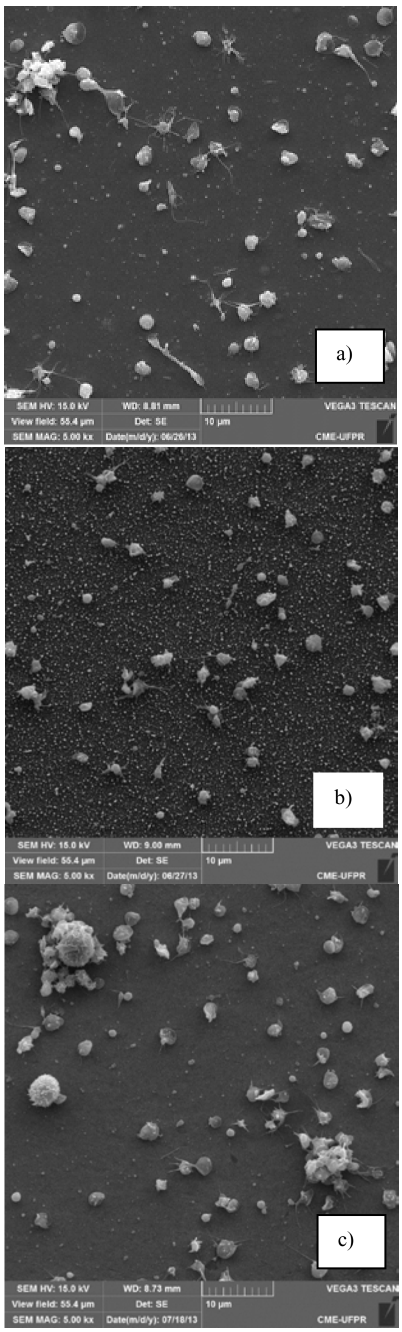

Fig 4. Morphology and amount of platelets on samples of titanium untreated and treated observed by SEM: a) Control, b) ${}_{15}A_0$ and c) ${}_{12}A_3$.

Sample ${}_{3}A_{12}$ showed less platelets adhered and less platelets activation than other samples treated with hydrogen, nitrogen and argon together as can be seen in the SEM images (Fig. 5 b)). This results can be associated with the interfacial tension and the surface topography presented by this sample, as ${}_{3}A_{12}$ presented lower interfacial tension with water, fibrinogen and albumin than other samples treated, and also showed less sharp peaks observed by Rp/Rz. Samples with lower Rp/Rz presented lower platelets activation (${}_{15}A_0$, ${}_{3}A_{12}$ and ${}_{0}A_{15}$), suggesting inhibition of platelet activation in surfaces with less sharp peaks.

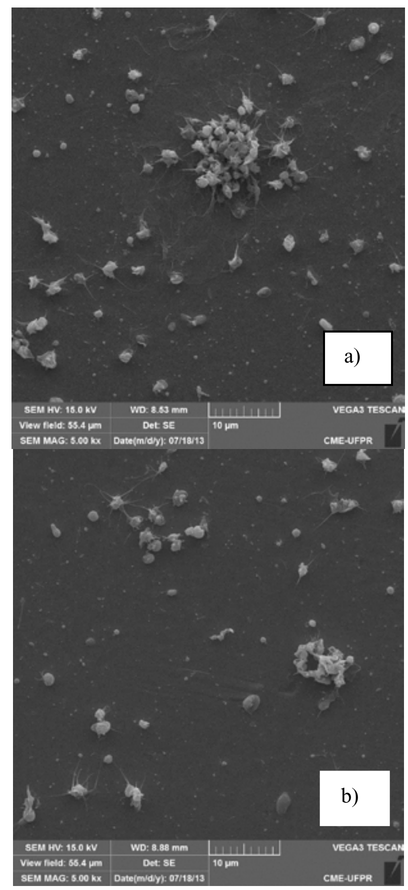

Fig 5. Morphology and amount of platelets on samples of titanium treated observed by SEM: a) ${}_{6}A_9$ and b) ${}_{3}A_{12}$.

Biological response was evaluated *in vitro* through the application of platelet-rich plasma (PRP) on titanium surfaces treated and untreated by plasma. Results of amount of platelets isolated are presented in Fig. 6. It can be observed a large variation in the amount of platelets in relation to the control condition. Sample ${}_{15}A_0$ presented higher amount of isolated platelets; however, they were not activated, as observed by SEM image (Fig. 4 b)).

Analysing the influence of the use of hydrogen, nitrogen and argon mixture in the nitriding plasma for treatment of the CP-Titanium, the results suggest a relation between interfacial tension and amount of platelets on the surface of the treated samples, as presented in Fig. 7. These results corroborate the literature results [1].

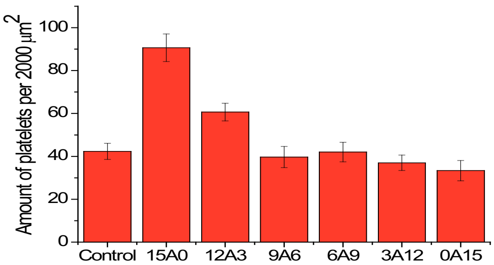

Fig. 6. Amount of platelets on titanium surfaces after tests *in vitro* through application of platelet-rich plasma (PRP).

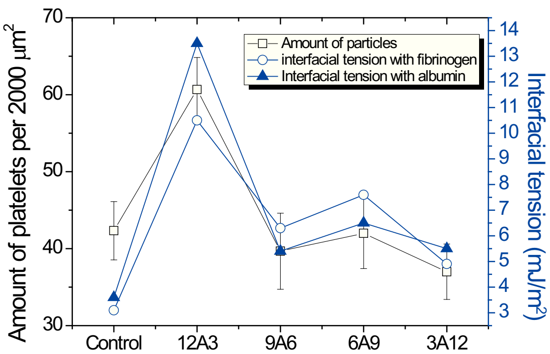

Fig. 7. Interfacial tension and amount of platelets of samples treated using hydrogen and nitrogen together.

4. Conclusions

CP-Titanium was modified by nitriding plasma treatment using different proportions of hydrogen and nitrogen as work gases. Results showed to be possible to obtain surfaces with different characteristics, covering different applications, depending only on the mixture of H_2 and N_2 in the plasma. It was found that the $_{15}A_0$ and $_3A_{12}$ conditions presented the desired behaviour for applications requiring low wettability to avoid activation of platelets, for example to be applied in stents. However, to an application that requires good activation of platelets, such as in the osseointegration required for implants, it was observed that the $_{12}A_3$ and $_6A_9$ conditions are ideal, as identified by the higher activation of the platelets found in these conditions.

It was also identified that surface morphology can influence platelets activation, as surfaces with peaks less sharp, observed by low Rp/Rz ratio, showed lower platelets activation (samples $_{15}A_0$, $_3A_{12}$ and $_0A_{15}$).

Process parameters are difficult to relate to the characteristics of platelet adhesion/activation; however, the latter can be correlated with the interfacial tension properties. A direct relation between interfacial tension and amount of platelets on the surfaces was observed in conditions where the plasma treatments were developed with mixtures of H_2+N_2+Ar. The conditions with higher interfacial tension showed greater amounts of particles while, for the conditions with lower interfacial tension, minor amount of particles were found.

References

[1] I. Topala, Plasma Chem. Plasma Process. 28(4) (2008) 535.
[2] H. Yan *et al.*, Appl. Surf. Sci. 255 (2008) 257.
[3] U. Cvelbar *et al.*, Surf. Coat. Technol. 211 (2012) 200.
[4] N. Lin, X. Huang, J. Zou, X. Zhang, L. Qin, A. Fan, B. Tang, Surf. Coat. Technol. 209 (2012) 212.
[5] S. Farè, N. Lecis, M. Vedani, A. Silipigni, P. Favoino, Surf. Coat. Technol. 206 (2012) 2287.
[6] M.A.M. da Silva *et al.*, Surf. Coat. Technol. 235 (2013) 447.
[7] N. Huang *et al.*, Biomaterials 24 (2003) 13.
[8] X. Zhu, J. Chen, L. Scheideler, R. Reichl, J. Geis-Gerstorfer, Biomaterials 25 (2004) 4087.
[9] F. Wen *et al.*, Appl. Surf. Sci. 255 (2008) 469.
[10] S. Kowk *et al.*, J. Biomed. Mater. Res., Part A 70A (2004) 107.
[11] C.S. Wetter *et al.*, Immunol. Cell Biol. 86, (2008) 466.