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Evaluation of the haemocompatibility of TiO_2 coatings obtained by anodic oxidation of Ti-6Al-4V

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Abstract

The blood compatibility (haemocompatibility) is a requirement in materials used in prosthetic heart valves. No standardized methods are available to measure haemocompatibility in solid materials. However, a material could be considered haemocompatible if its surface does not modify the normal activation of the coagulation pathway and produces no damage in the blood components. In this work, the haemocompatibility of coatings obtained by anodic oxidation of Ti-6Al-4V (with H_2SO_4 1M and 40 to 70 V) with and without heat treatment (1h for 500ºC), is evaluated and compared with the haemocompatibility of the Ti-6Al-4V substrate and pyrolytic carbon. Standard clinical trials for clothing: Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) were adapted to evaluate the haemocompatibility of solid materials. Besides, the interaction between blood platelets and solid material were performed through an analysis of interfacial tension ($\gamma_{i,j}$), this parameter could indicate the degree of preferential adsorption of each protein of the blood plasma. The ratio between albumin-material and fibrinogen-material interfacial tension, ($\gamma_{s,Alb}/\ \gamma_{s,Fib}$), is an important parameter used to predict the platelets adhesion and the trend at the formation of cloth. The results of the clothing trials indicate that the tested surfaces would be haemocompatibles due to the values of PT and APTT obtained which were in range of the normal ones. In addition, the measured values of $\gamma_{s,Alb}/\ \gamma_{s,Fib}$ indicate that all the tested surfaces are more haemocompatible than pyrolitic carbon. The recrystalized films by heat treatment which produce some rutile crystals are the most haemocompatibles.

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*Keywords:*Hemocompatibility; Prosthetic heart valve; TiO_2 coatings; Anodic oxidation

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1. Introduction

A key requirement for materials used in prosthetic mechanical heart valve is the property of haemocompatibility. However there are no standard methods to measure and quantify it. A perfect haemocompatible material must be innocuous when immersed in blood. The material surface should not alter the normal activation of the coagulation system and produce any damage in the blood components [Schaldach (1993)].

Blood coagulation is produced by cascade of complex reactions which may be activated by contact (extrinsic pathway) or tissue damage (intrinsic pathway), and in either case it ends with the formation of a fibrin clot. Fibrinogen is a blood plasma protein, which transforms in fibrin by the action of the trombine enzyme, forming three dimensional networks that may trap and connect blood cells to form a clot [Schaldach (1993)]. A third body surface present in the blood system may activate the coagulation process control the coagulation process.

In principle, a foreign material and blood interact in a reciprocate way [Schaldach (1993)]. The blood response to foreign material will depend on the properties of the surface material and blood components. The first step of interaction is selective protein adsorption on surface material. The next steps will depend on the composition of proteins film [Ruckenstein and Gourisankar (1984)].

The haemocompatibility of TiO_2 coatings, produced by anodic oxidation of Ti-6Al-4V substrates, is evaluated. Results of standard coagulation clinical tests of film materials, with and without heat treatment, are presented. The selected standard tests are: Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT). PT test permits the determination of the consumption of factors of the extrinsic pathway of the coagulation chain. APTT test determines the consumption of factors of the intrinsic way of the coagulation chain. These tests indicate if the presence of materials alters the normal coagulation process [Duboscq et. al. (2003)].

In addition, the interaction between the main blood plasma proteins and the materials is also analyzed, measuring the interfacial tension. This magnitude predicts the selective adsorption of specific proteins from the blood plasma [Kwok et. al. (2005), Zhang et. al. (2009)]. This first step determines the particular evolution of platelet adhesion on the protein film and clotting activation [Horbett et. al. (1996), Paul and Sharma (1981)].

Three of the proteins present in human blood plasma are: albumine (Alb), fibrinogen (Fib) and γ-globuline (Glo) [Paul and Sharma (1981)].It is known that the selective adsorption of albumine passivates the surface of the material and reduces platelet adhesion. Also, the selective adsorption of γ-globuline or fibrinogen, favor platelet activation and blood coagulation. Therefore, an important parameter to predict platelet adhesion and activation and the tendency for blood coagulation in a surface material is the ratio between albumin-material and fibrinogen-material interfacial tension, $\gamma_{s,Alb}/\gamma_{s,Fib}$. A large value will indicate that material is more haemocompatible than another with a smaller value [Yan et. al. (2008)].

On the other hand, a large value of interfacial tension between the surface material and blood ($\gamma_{s,blood}$) indicates a higher tendency for adhesion and anchorage of plasma proteins onto the surface, modifying considerably the native configuration of the protein. On the contrary, a low value of $\gamma_{S,blood}$ indicates that the adsorbed proteins experience less distortion with respect to the native configuration [Shao et. al. (2010)]. The conformational configuration of proteins in blood plays an important role on platelet adhesion and activation, and therefore, the subsequent possibility of cloth formation. This is due to the fact that the changes in configuration induce the exposition of micro sites, available for bonding of platelets with the fibrinogen molecule [Tomikawa et. al. (1980), Tanaka et. al. (2000)].

Another aspect for long term haemocompatibility is the relation between the blood-material interfacial tension ($\gamma_{S,blood}$) and blood cell-plasma interfacial tension ($\gamma_{cell,blood}$~ 1-3 mN/m) [Ruckenstein and Gourisankar (1984)]. The blood cells are naturally highly compatible with the blood and, their interfaces are mechanically stable. A comparable value of blood-material interfacial tension will produce a long term haemocompatibility [Ruckenstein and Gourisankar (1984), Kwok (2005)].

This work shows results of standard coagulation tests for TiO_2 films, produced by anodic oxidation with different thickness and structure. In addition, it is made the comparison with pyrolitic carbon and Ti-6Al-4V, both materials used at present in heart valves [Butany et. al. (2003)]. The study includes results of interfacial tension determinations and the predicted behavior.

2. Experimental procedure

2.1. Film preparation

2.1.1 Substrate conditioning. The substrates used for oxidation were flat samples of Ti-6Al-4V alloys with a surface area of 1 x 2 cm^2 and 0.2 cm thick. They were polished with abrasive SiC papers with decreasing granulometry (from # 120 up to # 1500), with diamond paste (1 μm) lubricated with ethylenglicol, finishing with 4:1 mix of colloidal silica (Mastermet- Buehler®) and hydrogen peroxide. The mirror surfaces were then cleaned with water and detergent, rinsed with alcohol and hot air dried. One of the tested substrates was not coated (TiG5).

2.1.2 Anodic oxidation. Oxidation of the samples was carried out at room temperature (25 ºC) in a beaker glass containing a 1 M H_2SO_4 solution. A DC electric current was applied between Pt cathode and Ti-6Al-4V anode, separated each other by 5 cm. The applied voltages were 40 V, 60 V and 70 V. Immediately after oxidation, the oxidized samples were rinsed with demineralized water and dried with hot air.

2.1.3 Heat treatment. Some samples, oxidized at 40 V and 60 V, were subject to thermal cycle by heating treated of 10 ºC/min, soaked at 500 ºC during 1 h, and cooled to room temperature inside the furnace. The different steps of the processes, performed in six samples, the film thickness and the resulting structure are listed in Table 1. The thickness and structure were previously obtained by X-ray reflectometry and diffraction, respectively [Vera et. al. (2010), Vera (2013)].

Table 1. Oxidation voltage, heat treatment, thickness and structure of five samples.

Sample	Oxidation voltage [V]	Heat treatment	Thickness [nm]	Structure
TiG5	-	-	2-10	Rutile
S1-V40	40	no	92	Amorphous
S1-V60	60	no	132	Amorphous
S1-V70	70	no	168	Anatase
S1-V40-T500	40	1h 500 ºC	100	Anatase and Rutile
S1-V60-T500	60	1h 500 ºC	140	Anatase and Rutile

2.2. Coagulation tests

Platelet Poor Plasma (PPP) was prepared by centrifuging 20 ml (during 15 min) of blood from four healthy adults (5 ml of each one). 2 ml PPP was put into contact with each sample at 37 °C (during 10 min) in a sterile beaker. One beaker containing only PPP incubated in the same conditions was used as control (blank).

ProthrombinTime (PT) tests were performed by adding 100 μl of PT reagent (Calcium Tromboplastine, Neoplastin Plus®) to 50 μl PPP in a test tube. This addition triggers the cascade reactions resulting in the formation of a fibrin cloth. The time taken to start coagulation is measured with a clotting tester (Stago Start 4®).

The Activated Partial Thromboplastin Time (APTT) measurements were carried out by adding 50 μl of a mix containing cefaline (platelet substitute) and a suspension of caolin (activator) in 50 μl PPP. The whole mix was incubated during 3 min at 37 ºC which produced the activation by contact of factors XII and XI. After this, 50 μl of calcium chloride (STA®) was added to activate the endogen system of fibrin formation. The time from the addition of calcium chloride until coagulation was measured with a clotting tester (Stago Start 4®).

2.3. Wettability and interfacial tension measurement

2.3.1 Contact Angle Measurement. The contact angle (θ) was measured by the sessile drop method using a goniometer built for this purpose in our lab [Schuster et. al. (2013)]. The measurement technique consisted on taking pictures of the drop and processing the image with a free software, Image J.

The contact angle of four liquids on each material was determined. The four liquids are; demineralized water, ethilenglicol, glycerol and di-methylsulfoxide. The contact angle value for each drop was the average between the left and right side angle. The process was applied for five drops of each liquid and the contact angle for each was the average value obtained for each liquid. Before each test the sample surface was washed with detergent, rinsed with demineralized water and ethyl alcohol and dried with hot air.

2.3.2 Surface Tension. The surface tension (γ_i) of each sample was calculated using the theoretical model of Owens and Wendt. This considers that the surface tension of solid (γ_S) and liquid (γ_L) depends on intermolecular interactions within each substance [Owens and Wendt (1969)]:

$$\gamma_L = \gamma_L^d + \gamma_L^p \tag{1}$$

$$\gamma_S = \gamma_S^d + \gamma_S^p \tag{2}$$

were γ_S^d and γ_L^d are the dispersion components of surface tension of solids and liquids respectively (related to London interactions). γs^p and γ_L^p are the polar component of surface tension of solids and liquids (related to hydrogen bonds) respectively.

The relationship with contact angle is given by Equation (3):

$$\gamma_L(1+\cos\theta) = 2\sqrt{\gamma_S^d.\gamma_L^d} + 2\sqrt{\gamma_S^p.\gamma_L^p} \tag{3}$$

The values of γ_L^d, γ_L^p for each liquid were obtained from the literature [Hallab et. al. (2001), Huang et. al. (2003)]. γ_L was calculated using Equation (1), where γ_S^d and γ_S^p are unknown values, which are necessary to determine the surface tension of the samples (γ_S).

Linearization of Equation (3) is obtained by rearranging [Clint (2001)]:

$$\frac{0.5\gamma_L(1+\cos\theta)}{\sqrt{\gamma_L^d}} = \sqrt{\gamma_S^p}\left(\frac{\gamma_L^p}{\gamma_L^d}\right)^{1/2} + \sqrt{\gamma_S^d} \tag{4}$$

Values of γ_S^d and γ_S^p were obtained by linear regression, using the measured values of contact angles of the four liquids and plotting $\frac{0.5\gamma_L(1+\cos\theta)}{\sqrt{\gamma_L^d}}$ vs. $\left(\frac{\gamma_L^p}{\gamma_L^d}\right)^{1/2}$. Detailed calculations are found in reference [Schuster (2013)].

2.3.3 Interfacial Tension. The interfacial tension, $\gamma_{S,j}$, between two condensed phases, S and j, was calculated using the following Equation (5) [Schuster et. al. (2013)]:

$$\gamma_{S,j} = \left(\sqrt{\gamma_S^d} - \sqrt{\gamma_j^d}\right)^2 + \left(\sqrt{\gamma_S^p} - \sqrt{\gamma_j^p}\right)^2 + \Delta_{Sj} \tag{5}$$

were γ_S^d and γ_S^p are dispersion and polar components of surface tension of material samples, calculated previously. γ_j^d and γ_j^p are dispersion and polar components of surface tension of plasma proteins and blood, taken from the literature [Sharma (1984), Agathopoulos and Nikolopoulos (1995)] and listed in Table 2. The term Δ_{Sj} accounts for the contribution of the ion covalent interaction. This ion-covalent contribution could be neglected,

considering that Van der Waals interaction is dominant. The values of α and β for the plasmatic proteins and blood were taken from the literature and listed in Table 2.

Table 2. Values γ_j^d y γ_j^p for plasma proteins [Sharma (1984), Agathopoulos and Nikolopoulos (1995)].

Biological substance	γ_j^d [mN/m]	γ_j^p [mN/m]
Fibrinogen	24,721	40,272
γ-Globuline	29,463	35,534
Albumine	31,382	33,617
Blood	10,890	36,000

3. Results and Discussion

3.1. Coagulation Test Results

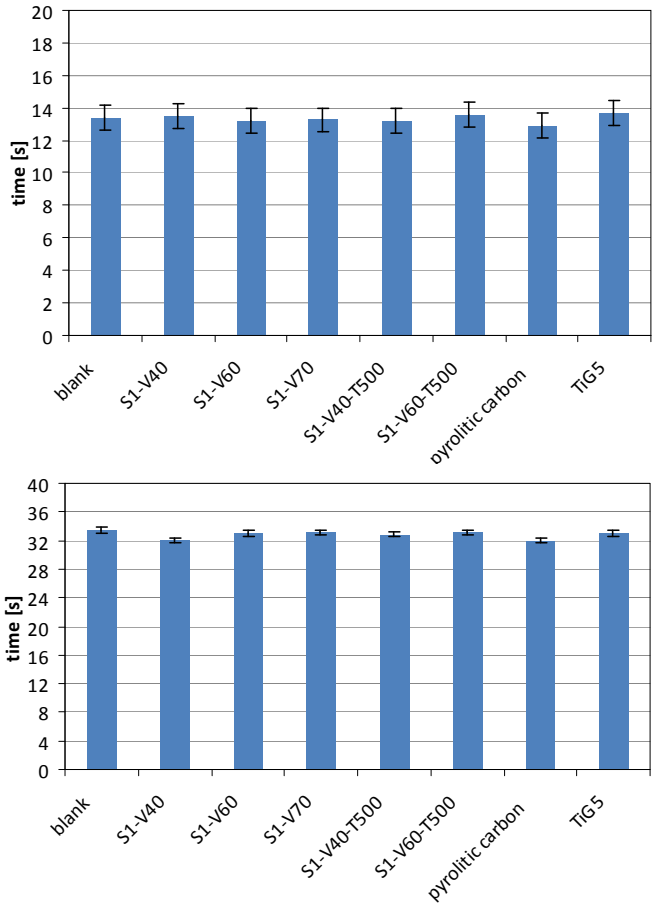

Fig. 1. Results of the global coagulation tests (a) PT; (b) APTT.

The results of the PT and APTT tests are shown in Figures 1 (a) and (b) respectively. Both figures show the results correspond to the six samples listed in Table 1, including pyrolitic carbon, standard haemocompatible

material used in heart valves [Butany et. al. (2003)]. In addition, results were compared with those of the blank which consist of PPP without incubated samples.

It is observed that there is no significant difference in activation time among the blank and seven samples tested. These results indicate that there was no autocatalytic activation of any coagulation factor due to the presence of the materials, which could produce a reduction in time. In addition, the materials did not consume nor alter the activation factors or the fibrinogen which would increase the time.

It may then be concluded that the seven tested materials have the same degree of haemocompatibility, because there is no significant difference between coagulation time of PPP without samples (blank) and PPP with samples. It shows that the tested materials do not alter the normal coagulation time evaluated by TP and APTT tests.

3.2. Interfacial Tension

The most basic behavior is the wettability different materials surfaces. The degree of wettability is determined by measuring the contact angle of water drops onto the material surface as explained above. The results are shown in Table 3 for the five oxidized surfaces, TiG5 and pyrolitic carbon. The results clearly show that the wettability decreases in the following order S1V60 ≈ S1V70 > S1V40 > S1-V60-T500 > S1-V40-T500. This indicates that those films heat treated are the most hydrophobic, compared to pyrolitic carbon, but less than TiG5.

Table 3. Contact angle values of water on surface materials.

Sample	Contact Angle [º]
TiG5	51.4 ± 1.1
Pyrolitic carbon	65.7 ± 1.3
S1-V40	66.3 ± 0.5
S1-V60	76.5 ± 1.3
S1-V70	76.3 ± 1.7
S1-V40-T500	61.0 ± 0.5
S1-V60-T500	65.6 ± 0.5

The surface tension γ_S for the five oxidized samples, TiG5 and pyrolitic carbon were calculated using the contact angles measured for the four liquids (demineralized water, ethilenglicol, glycerol and di-methyl sulfoxide) as described in Section 2.3.

Table 4. Surface tension and polar and dispersive components.

Sample	γ_S^d	γ_S^p	γ_S
TiG5	16,9	29,9	46,8
Pyrolitic carbon	23,8	14,9	38,7
S1-V40	23,7	15,2	38,9
S1-V60	25,1	8,4	33,5
S1-V70	23,2	9,3	32,5
S1-V40-T500	19,7	21,0	40,7
S1-V60-T500	20,4	17,2	37,6

The values of each component of surface tension; the polar, γ_S^p, and dispersive, γ_S^d, components were calculated (Table 4). It is observed that the highest tension corresponds to the TiG5 surface with a larger polar component. This could be associated with the presence of OH^- in the surface introduced by the polishing process. The substances employed, colloidal silicon and hydrogen peroxide, have been reported to leave OH^-, O_2 and H_2O on surface. The adsorbed water, for instance, may form reaction products from the colloid and the Ti or TiO_2 [Tanaka et. al. (2007)]

like $Ti(H_2O)_6^{3+}$. This specie in the presence of the OH^- ions may contribute to form a polar surface, providing bio- and haemocompatibility to the TiG5 surface [Tanaka et. al. (2007)].

The coated samples, subject to heat treatment increase the value of the polar component of the surface tension with respect to the un-treated samples. This could be associated with TiO_2 crystals (anatase and rutile) produced by heat treatment. These phases improve the haemocompatibility of the surface. A similar effect may be expected in the case of the TiG5 surface in which the rutile structure may form since it is the stable structure. Moreover, according to values of Table 4, γ_S increases when the coating thickness increases.

Table 5. Interfacial tension between the material surfaces and blood components.

Sample	$\gamma_{S,Fib}$ [mN/m]	$\gamma_{S,Glo}$[mN/m]	$\gamma_{S,Alb}$ [mN/m]	$\gamma_{S,blood}$ [mN/m]	$\gamma_{S,Alb}/\gamma_{S,Fib}$
TiG5	1.50	2.00	2.30	0.90	1.53
Pyrolitic carbon	6.20	4.70	4.30	7.10	0.69
S1-V40	6.01	4.57	4.15	6.90	0.69
S1-V60	11.83	9.51	8.71	12.48	0.74
S1-V70	10.90	8.85	8.17	11.03	0.75
S1-V40-T500	3.39	2.87	2.82	3.31	0.83
S1-V60-T500	5.03	4.12	3.90	4.89	0.78

It has been stated before that the coagulation chain is a complex process of activation of the different blood components. In this case it is important to know how each component interacts with each surface. In particular, it is important to know the interfacial tension between the material surface and the blood components [Ruckenstein and Gourisankar (1984)]. The values calculated for fibrinogen, γ-globuine, albumine and blood are listed in Table 5 for the five coated samples, TiG5 and pyrolitic carbon. In addition, the relative values between albumine-material and fibrinogen-material interfacial tension ($\gamma_{s,Alb}/\gamma_{s,Fib}$) are listed in the last column in Table 5. It is observed that all the samples coated with TiO_2 (heat treated and un-treated), present a value of $\gamma_{s,Alb}/\gamma_{s,Fib}$ larger than pyrolitic carbon. This indicates that pyrolitic carbon will adsorb relatively more fibrinogen than the TiO_2 coated surfaces, and therefore, will promote the coagulation process in carbon in a stronger way than in the TiO_2 coated surfaces [Wang et. al. (2000)].

It is observed that TiG5 exhibits the highest interfacial tension is with albumine ($\gamma_{S,Alb}$), indicating that this will be the preferred blood component for adsorption. This favorable result of TiG5 may be attributed to the presence of chemical species, produced by the polishing process and to presence the natural film of TiO_2 with the rutile structure [Bakir (2012)]. In all the other surfaces the highest interfacial tension is for fibrinogen ($\gamma_{S,Fib}$) indicating a preferred adsorption of this blood component. The relative larger adsorption of albumine in TiG5 and fibrinogen in pyrolitic carbon was quantified experimentally by Dion et. al. [Dion et. al. (1993)].

The relative value of interfacial tension ($\gamma_{S,Alb}/\gamma_{S,Fib}$) increases with the thickness of the un-treated TiO_2 films (last column of Table 5), which increases with the applied voltage in agreement with other reports [Bakir (2012)].

The heat treated films present higher values of relative interfacial tension ($\gamma_{S,Alb}/\gamma_{S,Fib}$) than the corresponding un-treated ones which is attributed to the higher degree of crystallization of the films in the rutile structure. The observation of un-treated sample S1-V70 supports this thesis. Here, anatase crystal structure is the unique phase and resulted in a lower relative ratio than the other un-treated samples which were mainly amorphous. This conclusion is in agreement with other reports showing similar results [Bakir (2012), Huang et. al. (1994), Maitz et al. (2008)].

Considering now the effect on the behavior of the different surfaces with the coagulation process, it may be observed that the crystalline surfaces like S1-V40-T500 and S1-V40-T500 have the interfacial tension with the plasma protein much smaller than for the other surfaces like S1-V40, S1-V60 and S1-V70. The last would produce a low denaturalization of the adsorbed proteins. This will avoid adhesion and activation of the platelets, reducing the probability of formation of clots.

It may also be noted that heat treatment also reduces the interfacial tension between blood and material surface ($\gamma_{S,blood}$) (Table 5) to values suggested in the literature (1-3 mN/m). This magnitude warranties good mechanical stability of the interface, and contributes to haemocompatibility founded in *in vitro* experiments [Huang et. al. (2003), Wang et. al. (2000)].

The fundamental basis for the better values of interfacial tensions between crystalline samples and all the used plasma proteins is related to the band gap (E_g) of TiO_2, which depends on the degree of crystallinity. For instance, for rutile the band gap is 3 eV and for anatase is 3.2 eV, values larger than the band gap of fibrinogen (1.8 eV) [Bakir (2012)]. This difference will inhibit the electron transfer and therefore reduce the structural distortion of the fibrinogen molecule. On the contrary, the non-crystalline oxides may provide with small density of electron states available for accepting electrons from the fibrinogen. This induces an increase structural distortions and activation of the coagulation mechanism [Bakir (2012)].

4. Conclusions

The haemocompatibility of TiO_2 coatings obtained by anodic oxidation, with and without heat treatment, was determined by *in vitro* tests and the results compared to those obtained for uncoated TiG5 and pyrolitic carbon surfaces. In addition, the interfacial tension of the different material surfaces and blood components were determined in order to predict the possibility of adsorption and denaturalization of fibrinogen, which is the activator of fibrin cloth formation.

The test results of coagulation indicate that all the coatings were haemocompatible, since the normal extrinsic and intrinsic parameters of the coagulation chain were not affected.

The values of relative interfacial tensions between all the material surfaces tested with albumine and fibrinogen ($\gamma_{S,Alb}/\gamma_{S,Fib}$) showed better haemocompatibility than pyrolitic carbon.

The heat treated TiO_2 surfaces exhibit reduction in interfacial tension with the plasma proteins, predicting their smaller de-naturalization when adsorbed. Also the smaller interfacial tension with blood (similar to those between the cells and blood) permits to predict a long term compatibility and mechanical stability of interfaces in the presence of blood.

Among the tested coatings, the heat treated and crystallized as rutile phase, would be the most haemocompatible, due to their electronic band gap configuration.

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