

In vitro biological response of medical grade titanium. Comparative study of four surface treatments

PURPOSE

To determine the changes in the *in vitro* biological response of Ti6A14V with the application of 4 surface treatments by measuring surface free energy, fibronectin absorption and organisation, and the fibroblast proliferation.

MATERIALS AND METHODS

Sample Preparation

Ti6A14V ELI discs measuring 2 mm in thickness by 10 mm in diameter were used for later utilisation in all the tests described in this report. The preparation of each of the samples studied is described below.

a) Polished surface (P).

The samples were polished by the successive application of silicon carbide papers. Afterwards, they were polished with Υ -alumina.

b) Shot-Blasted Surface (S)

The samples were treated with a shot-blasting process using particles of white fused aluminum oxide.

c) Anodised Surface (P/A)

First the samples were polished according to the procedure described in section a). Then, the samples were anodised by connecting them to the positive pole (anode) of a continuous current supply and submerging them in an electrolyte.

d) Biomimetic Advanced Surface (BAS)

The discs were treated according to the procedure described in section b) and then anodised according to section c) taking into consideration the increase in real area produced by the shot-blasting treatment. In the study, it is known as S/A (BAS)

Document prepared by the **scientific committee of AVINENT Implant System S.L.** ⁽¹⁾ with the collaboration of **CREB** (Centre de Recerca en Enginyeria Biomèdica) ⁽²⁾

⁽¹⁾ **Scientific committee of AVINENT Implant System S.L.**, A. Cortina, C. Vendrell, E. Falcó, J. Serra

⁽²⁾ **CREB**: A. Mestre



Figure 1. Jeol JSM-640 Electron Microscope

After applying the corresponding surface treatment, each sample was first washed in acetone and then with ethanol in ultrasound for 10 minutes.

Scanning Electron Microscopy

The JSM-640 scanning electron microscope (fig.1) (Jeol, Japan) was used to obtain images of the prepared surfaces applying an acceleration potential of 20kV. The EDS detector (Energy Dispersive X-Ray Spectroscopy), integrated within the microscope, was also used to semi-quantitatively analyse each of the surfaces studied.

In vitro biological response of medical grade titanium. //

Comparative study of four surface treatments

Interferometry

The roughness of the treated surfaces was measured with the use of a WYCO NT 1100 interferometer microscope (Veeco, USA) in VSI (Vertical Scanning Interferometry) mode (fig. 2). The surface parameters obtained are summarised in Table 1.



Figure 2. WYCO NT 1100 interferometer microscope

SYMBOL	DEFINITION	FORMULA
R _a	Mean value of the profile's roughness	$R_a = \frac{1}{NM} \sum_{i=1}^N \sum_{j=1}^N Z_{ij} $
R _q	Root mean square of the profile's irregularities	$R_q = \sqrt{\frac{1}{NM} \sum_{i=1}^N \sum_{j=1}^N Z^2(x_i, y_j)}$
R _t	Maximum distance between the highest peak and the lowest valley	$R_t = R_v + R_p$

Table 1. Description of the surface parameters obtained with optical interferometry.

Contact Angle and Surface Free Energy Test

The contact angle was measured using Data Physics OCA 15 equipment (DataPhysics Instruments GmbH, Germany) and with the help of SCA 20 software. The sessile drop method was used with a 2 μl volume of liquid. The liquids used were ultra pure water and diiodomethane, the measurements being taken in an atmosphere saturated with the vapour of the liquid being used at room temperature. The surface tension values of the polar and dispersive component of the two liquids used are shown in Table 2.

LIQUID	DISPERSIVE COMPONENT (γ^d , in mN/m)	POLAR COMPONENT (γ^p , in mN/m)
Ultra pure water ¹	21.8	51
Diiodomethane ²	50.8	0

Table 2. Surface tension values of the polar and dispersive component of the liquids used

The surface free energy measurement was made using the model proposed by Owens-Wendt-Rabel-Kaelble (OWRK), which utilised the contact angles obtained with a polar liquid and a non-polar liquid.

$$\cos\theta = 2\sqrt{\gamma_S^D} \left(\frac{\sqrt{\gamma_L^D}}{\gamma_L} \right) + 2\sqrt{\gamma_S^P} \left(\frac{\sqrt{\gamma_L^P}}{\gamma_L} \right) - 1$$

Equation 1. Generalised Owens-Wendt model for calculation of the polar and dispersive components of the surface free energy of a solid. Being: θ is the contact angle between the liquid and the solid; γ_L and γ_S are the surface tension of the liquid and surface energy of the solid respectively; and super indexes D and P refer to the dispersive and polar components respectively.

In vitro biological response of medical grade titanium. Comparative study of four surface treatments

Fibronectin Adsorption

A fibronectin (FN) solution was obtained from human plasma using column chromatography (Millipore, USA). Sucrose gelatine was used as the stationary phase and 4M urea solution as the mobile phase.

The fractions obtained were adjusted to a pH of 9 with 0.1 M sodium bicarbonate. The fibronectin was marked with the addition of 10 μ l of a 10 mg/ml FITC solution (fluorescein isothiocyanate) in DMSO (dimethyl sulfoxide) for each ml of protein/urea/sodium bicarbonate solution, and then incubated at room temperature for 2 hours.

Later, the FITC-marked fibronectin was separated from the urea and unmarked protein by size-exclusion chromatography and its concentration was determined using UV/Vis spectroscopy.

The marked fibronectin solution was diluted in PBS (phosphate buffered saline) at a concentration of 20 μ g/ml. 100 μ l of this solution was deposited on each of the discs P, S, P/A and S/A (BAS) and was incubated at 37°C for 30 minutes.

Sodium dodecyl sulfate was used, diluted to 10% in water and with 0.2 M NaOH, to recover the adsorbed fibronectin during a 24-hour incubation at room temperature. Each experiment was repeated 6 times.

The amount of FITC-FN was semi-quantitatively measured using spectrofluorimetry utilising an excitation and transmission wavelength of 494 and 518 nm, respectively. A 10% SDS solution was used as a target.

Fixation of the adsorbed fibronectin

In order to determine the possible overall differences in the organisation of fibronectin on surfaces P, S, P/A, and S/A (BAS), the same procedure described in the previous section was used to adsorb the FITC-FN(2.5) and it was fixed by means of incubation with 3% paraformaldehyde for 10 minutes and was washed with a solution of 20nM glycine in PBS.

A confocal microscope (TCP SP5, Leica, Germany) with a 40X objective was used to observe the fixed FITC-FN.

Proliferation of Fibroblasts

A primary fibroblast culture from rat peritoneal fibroblasts (RPFb) was used as a cell line. The cells were maintained in T75 culture flasks in a humidified incubator at 37°C in a 5% CO₂ atmosphere for four days. 10,000 cells were deposited on each of the samples after being sterilised with gamma radiation at 25 kGy, and on TCPS (tissue culture polystyrene), which was used as a control surface. Reagent WST was added after 4 hours, 24 hours, 3 days and 7 days in each of the culture wells at a 1:10 ratio and it was incubated for 1 hour at 37° in a 5% CO₂ atmosphere. 100 µl was then taken from each well and the absorbance was measured using a 450 nm wavelength spectrophotometer.

In vitro biological response of medical grade titanium. Comparative study of four surface treatments

RESULTS AND CONCLUSIONS

Scanning Electron Microscopy

The polished samples (P) had a completely smooth surface with the presence of some typical point defects (polish marks) (fig. 3). The shot-blasted samples (S) had an irregular surface with sharp edges and shiny areas that correspond to the presence of alumina particles encrusted in the metal. The anodised surface (A) had a homogenous topography formed by pores of approximately 2-4 microns in diameter. The S/A samples with BAS surface had surface micromorphology of significant roughness with the presence of an alumina particle embedded in the metal combined with the presence of pores due to the anodising process. It was observed that the edges of the micro-irregularities of the S/A (BAS) samples had been smoothed by the anodising process as opposed to sample (S) where they were sharper.

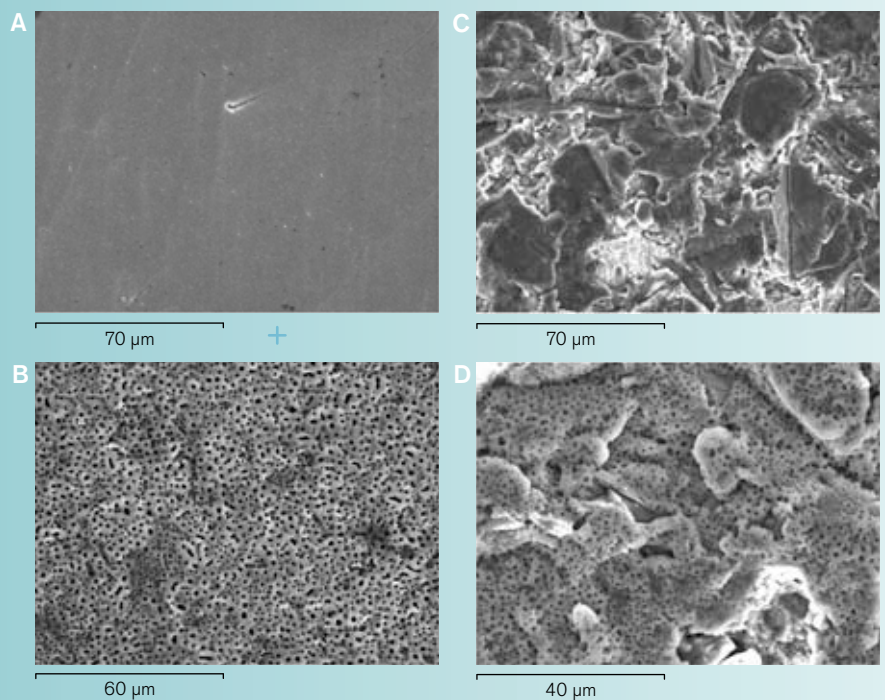


Figure 3. Electron microscopy images of the surfaces studied. A) Polished B) Anodized C) Shot-blasted and D) BAS.

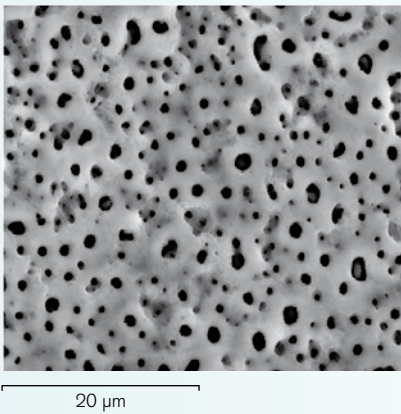


Figure 4. Close-up of the porosity of the Biomimetic Advanced Surface (BAS).

The polished samples had a chemical composition corresponding to Ti6Al4V alloy with inconsistencies due to the analysis technique used. The P/A and S/A anodised samples had a similar composition of Ca and P in addition to the presence of sodium, present in various types of the electrolyte. The high level of oxygen in the anodised samples is due to oxidation of the material and, mainly, to the formation of titanium oxides. The shot-blasted samples had a higher amount of aluminum than the polished samples due to the presence of encrusted alumina particles in the surface.

Interferometry

As already demonstrated by the electron microscopy images, the plasma-chemical anodising treatment increases the roughness of the smooth samples. However, there are no relevant differences in the surface parameters studied between the shot-blasted samples (S) and the S/A (BAS) samples (Table 3). As expected, the shot-blasting treatment significantly increases roughness.

Beck et. al. demonstrated similar behaviour after applying chemical plasma anodising to the smooth and rough surfaces obtained by projection of glass particles. [3]

	P	P/A	S	S/A
R_a	0.19 ± 0.04	0.68 ± 0.08	2.93 ± 0.08	2.89 ± 0.07
R_q	0.24 ± 0.05	0.83 ± 0.10	3.73 ± 0.08	3.68 ± 0.09
R_t	2.55 ± 0.71	7.27 ± 0.64	32.9 ± 1.41	31.5 ± 2.19

Table 3. Topographic parameters obtained

In vitro biological response of medical grade titanium. Comparative study of four surface treatments

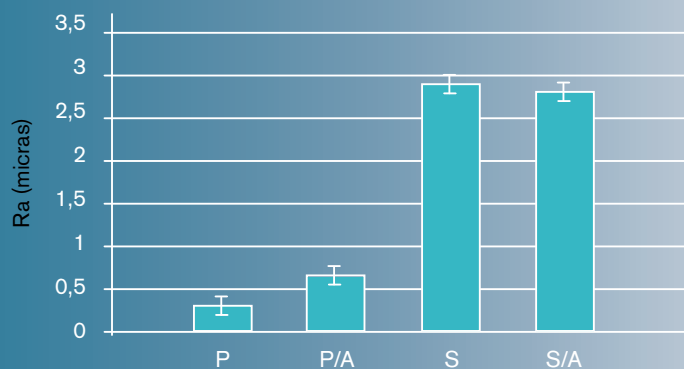


Figure 5. Graphical representation of the Ra values obtained. Notice that the anodizing treatment did not cause significant variations in the Ra parameter of the shot-blasted samples

Measurement of Contact Angle and Surface Free Energy

The model proposed by Owens-Wendt was used to obtain the surface free energy values for each of the four surfaces studied. This model requires the measurement of the contact angle with a polar liquid and a non-polar liquid. Therefore, the contact angle for each surface was measured with water (polar liquid) and diiodomethane (the polar part is considered to be negligible).

Table 4 shows the values of the parameters obtained for each of the surfaces.

Sample	Contact angles (°)		Surface energy (mN/m)		
	Θ water	Θ diiodomethane	γ^p_s	γ^d_s	γ_s
P	21.01 ± 4.29	32.54 ± 5.39	28.74	47.13	75.87
P/A	12.72 ± 3.83	21.40 ± 6.75	30.04	49.11	79.15
S	44.37 ± 8.37	45.58 ± 5.08	19.23	44.33	63.56
S/A	0	15.01 ± 7.47	30.99	49.95	80.94

Table 4. Surface physicochemical parameters.

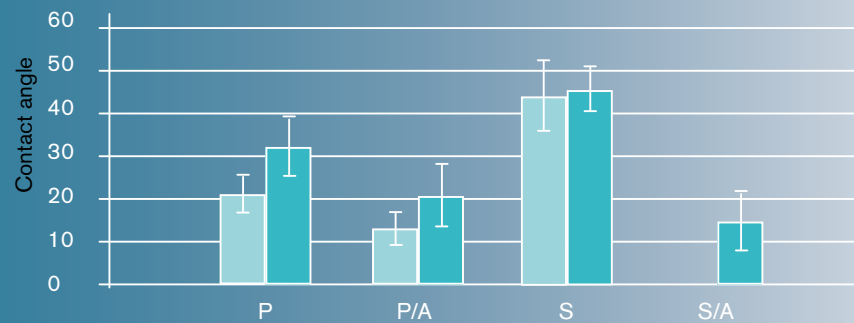


Figure 6. Mean contact angle values

As can be seen, plasma-chemical anodising reduces the contact angle (increases the hydrophilicity) of the smooth and shot-blasted surfaces. This behaviour was produced with both liquids used. The S/A (BAS) samples also demonstrated a 0° contact angle (complete wetting) when water was used for the measurement. Shot-blasting increased the contact angle for the non-anodised surface (P and S) but increased hydrophilicity on the anodised surfaces (P/A and S/A (BAS)). (fig. 6).

Plasma-chemical anodising increased the surface energy as much in the smooth samples as in the shot-blasted samples, especially in the polar component. The increase in surface energy was greater in the shot-blasted samples. (fig. 7)



Figure 7. Surface energy values obtained

In vitro biological response of medical grade titanium. Comparative study of four surface treatments

Various studies exist that specify a preference for the use of surfaces with an increased surface energy for endosseous implants because an increase in hydrophilicity improves interaction between the implant and the biological medium, producing an increase in cellular dissemination and in the cellular coat. Ericksson et al. performed *in vivo* studies where they related the hydrophilicity of an implant and its osteointegration capacity. As such, the hydrophilicity of a surface is one of the factors that determines the biocompatibility of a biomaterial and is directly dependent on the surface energy.

Organisation of the absorbed fibronectin (FITC-FN)

Observation of the FITC-FN absorbed in surfaces P, P/A, S, and S/A (BAS) was performed using confocal microscopy (fig. 8).

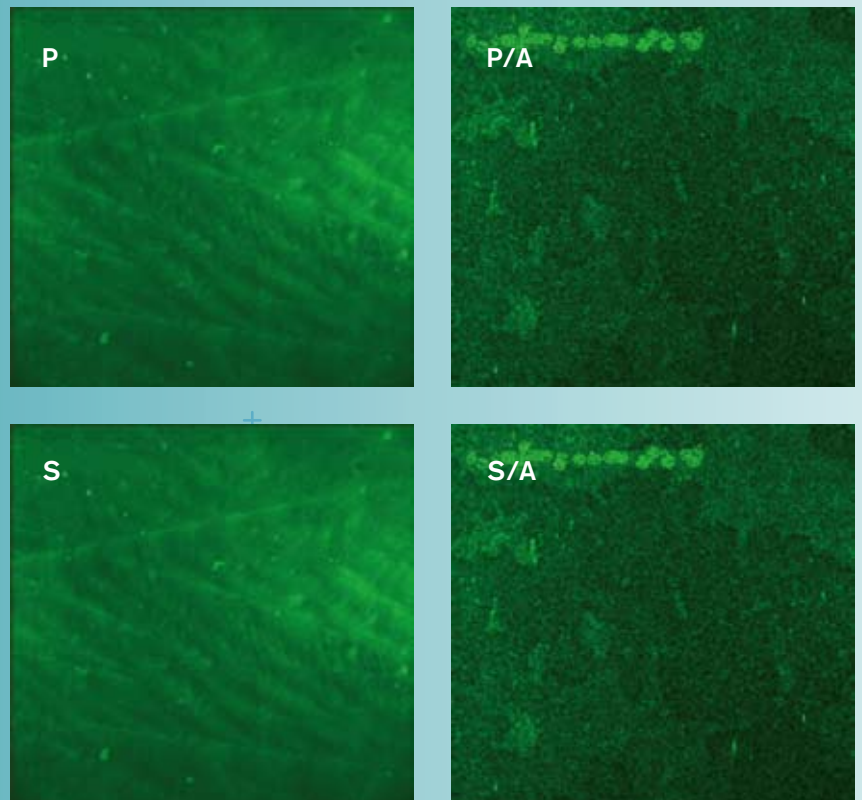


Figure 8. FITC-FN organization in samples P, P/A, S y S/A (BAS). Images from confocal microscopy at 40x.

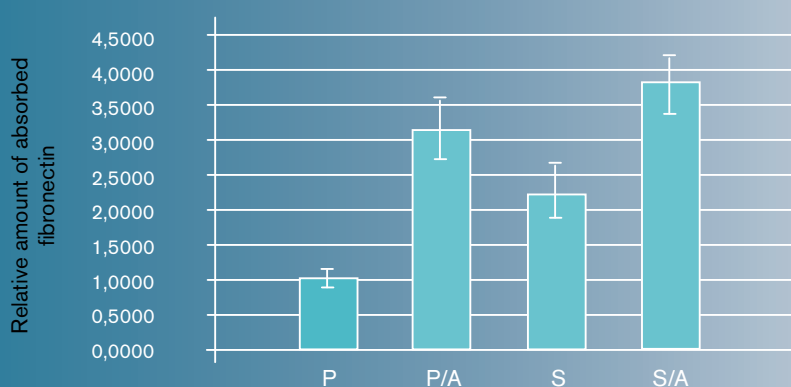


Figure 9. Amount of FITC-FN adsorbed relative to the P samples.

Surfaces P/A, S and S/A (BAS) had a higher intensity than the P samples indicating higher FITC-FN adsorption. The S samples demonstrated a heterogeneous FITC-FN distribution with points of increased intensity, indicating a higher accumulation of fibronectin, and slightly dark points where the adsorption was less favourable. The S/A (BAS) samples demonstrated a more heterogeneous intensity than the samples that were only shot-blasted despite having a similar roughness, but with important differences in the surface free energy (80.94 and 63.56 mN/m, respectively).

The quantitative results of the amount of FITC-FN adsorbed are shown in Figure 9. It is observed that the plasma-chemical anodising treatment increases the amount of protein adsorbed when comparing samples P/A and S/A (BAS) with samples P and S. The shot-blasting process doubled the amount of protein adsorbed with respect to the P samples. It should be taken into account that the shot-blasting treatment increases the real area of the samples as much as anodising and, as such, the P/A, S and S/A (BAS) samples offer a greater available surface for adsorption.

In vitro biological response of medical grade titanium. Comparative study of four surface treatments

Proliferation of Fibroblasts

An increase of fibroblastic cell proliferation was seen with time in all P, P/A, S and S/A (BAS) surfaces studied, with the speed of proliferation of these cells being the only difference between them.

REFERENCES

- [1] Erbil Y. Surface tension of polymers. In; *CRC Handbook of surface and colloid chemistry*. Boca Raton. FL: CRC Press: 1997, p. 292.
- [2] Vanoss CJ, Lu L,, Chaudhury MK, Good RJ. Estimation of the polar parameters of the surface tension of liquids by contact-angle measurements on gels. *J Colloid Interf Sci* 1989;128:333-9.
- [3] Beck U, Lange R, Neumann H-G. *Micro-plasma textured Ti-implant surfaces*. *Biomol Eng*. In press 2006.
- [4] G. Zhao, Z. Schwartz, M. Wieland, F. Rupp, J. Geis-Gerstorfer, D. L. Cochran, B. D. Boyan. *High surface energy enhances cell response to titanium substrate microstructure*. Published online 27 May 2005 in Wiley InterScience (www.interscience.wiley.com).
- [5] Schrader ME. *On adhesion of biological substances to low-energy solid-surfaces*. *J Colloid Interface Sci* 1982;20:773-784.
- [6] Schakenraad JM, Busscher HJ, Wildevuur CR, Arends J. *The Influence of substratum surface free energy on growth and spreading of human fibroblasts in the presence and absence of serum proteins*. *J Biomed Mater Res* 1986, 20:773-784.
- [7] Baier RE, Meyer AE, Natiella JR, Natiella RR, Carter JM. *Surface properties determine bioadhesive outcomes : methods and results*. *J Biomed Mater Res* 1984;18:327-355.

[8] Hurbert TA, Ratner BD, Schakenraad JM, Schoen FJ. *Some background concepts. In Ratner B, Hoffman A, Schoen F, Lemons J, editors. Biomaterials Science. An introduction to materials in medicine.* New York. Academic Press; 1996. p 133-164.

[9] Kilpadi DV, Lemons JE. *Surface energy characterisation of unalloyed titanium implants.* J Biomed Mater Res 1994;28:1419-1425.

[10] Eriksson C, Nygren H, Ohlson K. *Implantation of hydrophilic and hydrophobic titanium discs in rat tibia: cellular reactions on the surface during the first 3 weeks in bone.* Biomaterials 2004;25:4759-4766.