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Bone cell adhesion on ion implanted titanium alloys

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Abstract

The authors have previously reported than ion implantation can have a significant effect on osseointegration of an implant, specially when the latter is introduced in areas of poorer bone density. These results indicate that this process is particularly suited for implant devices introduced in elderly patients or in those regions that have a poor quality of bone. The aim of this work is directed to study osteoblast adhesion on Ti alloy surfaces with different ion implantation treatments, so osseoconductive properties of several surfaces can be assessed.

Polished discs of Ti–6Al–4V and Ti CP GR1 titanium alloy have been prepared and ion implanted with different species and parameters (dose and energy). Afterwards, the samples have been sterilized by UV light, inoculated with 1.5×10^5 human bone cells and incubated during 4 h at 37 C and 5% CO_2 atmosphere. Then, once fixed and rinsed, image analysis has been used to quantify the number of cells attached to the Ti discs. On a second round of tests, cell proliferation tests have been conducted during 24, 48, 144 and 192 h, respectively. Furthermore, surface analysis techniques (e.g. AFM) have been applied to learn about the qualitative behavior, i.e. morphology, of the attached cells.

Cell attachment has shown to be highly sensitive to ion implantation parameters. Although some quantitative differences have been observed, the more significant differences were qualitative. AFM analysis has shown that the star-shaped bone cells attached spread more and occupied larger surfaces like in osseointegration prone surfaces, most probably due to extracellular matrix synthesized around them, while other surfaces showed mainly large and narrow shaped or round shaped bone cells often with great cellular nucleus in the middle of the cells and little extracellular matrix around. So, ion implanted surfaces that facilitate osseointegration have been identified, in terms of initial bone cell attachment quality, where although the number of attached cells were not necessarily always larger, they tended to occupy wider areas with healthier cells.

Keywords: Ion implantation; Titanium alloys; Osteoblast; Cell culture; Cell proliferation; Implant

1. Introduction

The tissue integration of a biomaterial is a key factor in determining how well the implant materials commonly used in bone surgery or reconstruction are incorporated into the human body. The biocompatibility of a biomaterial is highly related to the behavior of the cells in contact and in particular the cell adhesion to its surface.

The surface characteristics of a material, including its topography and physical and chemical properties at a micro and nano-scale, play an important role in osteoblast adhesion on biomaterials [1]. Therefore, the attachment, adhesion and spreading of osteoblasts form a first phase of the interactions between cells and the material and will affect the cells' capacity to proliferate and get established in contact with the surface and ultimately generate bone tissue around the implant.

In the case of orthopedic and dental implants, the formation of a strong mechanical interface is of paramount importance to guarantee a long functionality. This implies a good joint between the surface of the implant material and the bone tissue without any fibrous tissue interface. The target in many cases is to ensure a good mechanically bonded implant even in areas of poor bone quality and, additionally, to shorten patient treatment times, by achieving shorter integration times. This objective responds to the wish of maintaining a healthy quality of life in addition to aesthetical

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aspects in a population with increased life expectancy. As an example only in Europe, one million people underwent a dental implant procedure in the year 2000, only a fraction of the people who present edentulism problems. This low implant penetration rate in the population is mainly due to cost and the lengthy implant treatment time. The latter would be enhanced significantly by engineering the cellular attachment to the implants surface.

A complete understanding of osteoblast adhesion on materials is therefore essential to engineer and optimize the bone–biomaterial interface, especially in the case of materials that have been designed to be osteoinductive. Different surface engineering processes have been applied to biomaterials to alter their surface properties, aiming at enhancing attachment of the cells. These include modification of the surface roughness by mechanical blasting [2] and acid etching [3] or altering the surface chemistry through plasma based sterilizing treatments [4], forming oxide layers by processes such as electrochemical anodization [5], and finally surface modification and coating deposition [6,7].

Ion based surface modification processes, including direct ion beam and plasma source ion implantation, are also good candidates to influence osteoblast–material interactions, since these can tailor the topography, surface chemistry and surface energy of a biomaterial. Ion implantation and related beam processing methods are today widely recognized as commercial surface treatments for orthopedic implants and for example are related to the reduction of polyethylene wear debris in articulated bearing surfaces such as hip joints and knees [8]. A number of studies have been carried out on the application of ion implantation to influence cell attachment to surfaces. For example, ion implantation from gaseous precursors has shown to improve the bone integration of dental implants [9,10]. Implantation of Na ions into titanium have been carried out to form sodium titanate at the surface and subsequently induce the precipitation of hydroxyapatite from the body fluid, which in turn would enhance osseointegration [11]. Additionally, implantation of Ca ions has also been carried out successfully to facilitate the osseointegration of titanium alloys [12].

In the case of ion implantation there is an existing background of in vivo and in vitro tests carried out by the authors [9,10] that have shown good results concerning the osseointegration of the surface treated material. To optimise the treatment conditions concerning cellular attachment and to gain a clear understanding on the effects of the treatment on cell adhesion, the authors have carried out in the present work cell adhesion and proliferation tests.

2. Experimental procedure

2.1. Sample preparation and ion implantation procedure

Two sets of experiments were carried out, in the first one two ion implantation treatments, designated as Type A and Type B, were evaluated for cell attachment in contact with bone cells for a fixed period of time. Secondly, cell proliferation tests were conducted with the selected treatment from the earlier study (Type A), investigating cell adhesion as a function of time.

In the first experiment, 16 samples of mirror polished titanium disks were prepared for each of the two treatments and the control material. Table 1 summarises the different material and surface treatment combinations that were applied in the cell attachment and proliferation tests. Two titanium alloys were used, a Ti–6Al–4V and a commercial pure Ti grade Ti CP GR1. The samples were 15 mm diameter discs cut from bars in the case of Ti–6Al–4V and 15×15 mm square samples in the case of Ti CP GR1 with 1 mm in thickness, polished from one side to a surface finish of 0.01 μm Ra. Ion implantation was carried out on the polished side of discs in a Danfysik high-current implanter Model 1090. All titanium discs were ultrasonically degreased and cleaned prior to ion implantation treatments, which were performed using a Chordis ion source at doses from 0.5 to 5×10^{17} ions/cm^2 and energies in the range of 40 to 100 KeV, using gaseous precursors [13]. The treatments were performed at low temperature (<170 °C) and at a vacuum better than 4×10^{-4} Pa.

Once that the cell attachment tests were completed and evaluated, the second group of assays concerning the study of cell proliferation was performed on Ti–6Al–4V samples with the Type A ion implantation process. In this case, 12 samples of the control unimplanted titanium alloy and another 12 specimens of the ion implanted titanium alloy were evaluated (see Table 1).

2.2. Cell culture

Cell adhesion and proliferation tests were determined with hFOB 1.19 human bone cells (ATCC, CRL-11372). Before performing these assays, the cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-

Table 1
Material and surface treatment combinations used in the cell attachment and proliferation tests

Test type	No. of samples	Sample ref.	Material	Surface treatment
Cell attachment	16	A (A1 to A16)	Ti–6Al–4V alloy	Type A ion implantation
	16	B (B1 to B16)	Ti–6Al–4V alloy	Type B ion implantation
	16	C (C1 to C16)	Ti–6Al–4V alloy	Unimplanted (control)
	16	D (D1 to D16)	Ti CP GR1	Type B ion implantation
	16	E (E1 to E16)	Ti CP GR1	Unimplanted (control)
Cell proliferation	12	Control Ti	Ti–6Al–4V alloy	Unimplanted (control)
	12	Treated Ti	Ti–6Al–4V alloy	Type A ion implantation

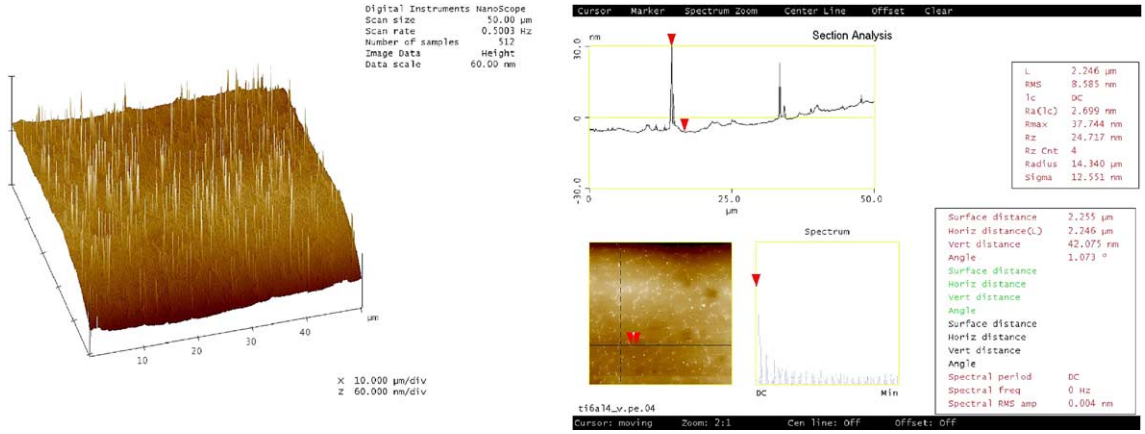

Fig. 1. AFM analysis of unimplanted Ti–6Al–4V surface (Sample ref. C).

12 Ham. (DMEM-F12; Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin–streptomycin (Sigma) and 0.5% geneticin (Invitrogen) under standard cell-culture conditions until they reached the confluent state.

To carry out the cell attachment tests, 16 samples of each type of surface-modified Ti and control Ti discs, which were sterilized by UV light and placed individually into six-well plate, were inoculated with 1.5×10^5 human bone-cells and incubated during 4h at 37 °C, 5% CO_2. After rinsing and fixing, an Image Analysis System (OMNIMET) was used to quantify the number of attached cells for each titanium sample. Subsequently, surface analysis techniques (SEM and AFM) were used to evaluate the morphology of the cells attached to the different surface modified discs.

In the case of the subsequent cell proliferation tests, human bone cells were seeded at a concentration of 2.5×10^4 cell/well onto control and treated titanium specimens (12 samples at each time and surface), which were previously sterilized by UV light and placed individually into six-well plates. These cells in contact with the Ti discs were incubated during 24, 48, 144 and 192 h at 35 °C and 5% CO_2. At those times, the cells were washed and were collected by trypsinisation with 0.25% trypsin–EDTA solution (Sigma). The collected cells were stained with 7-Amino-Actinomycin D (7 AAD) (Beckton & Dickinson) and the number of cells/cm^2 was quantified using a flow cytometry method (*TruCOUNT Tubes*, Beckton & Dickinson).

3. Results and discussion

Ion implantation did not change significantly the surface roughness of the polished samples at a micro-scale, though the changes were significant at nano-scale as revealed by Atomic Force Microscope images (AFM) in Figs. 1 and 2. The difference in surface roughness in both samples is evident in these 3D images. Profile roughness measurements have registered Ra 2.70 nm on the untreated sample (Fig. 1), while this value rises to Ra 3.73 nm after ion implantation due to the sputtering process (Fig. 2). Therefore any effect of the ion treatments on cellular attachment can be interpreted mainly as a consequence of the induced surface chemistry changes on the material together with topography changes at nano-scale. A number of studies have demonstrated that the surface topography of a material is an important parameter

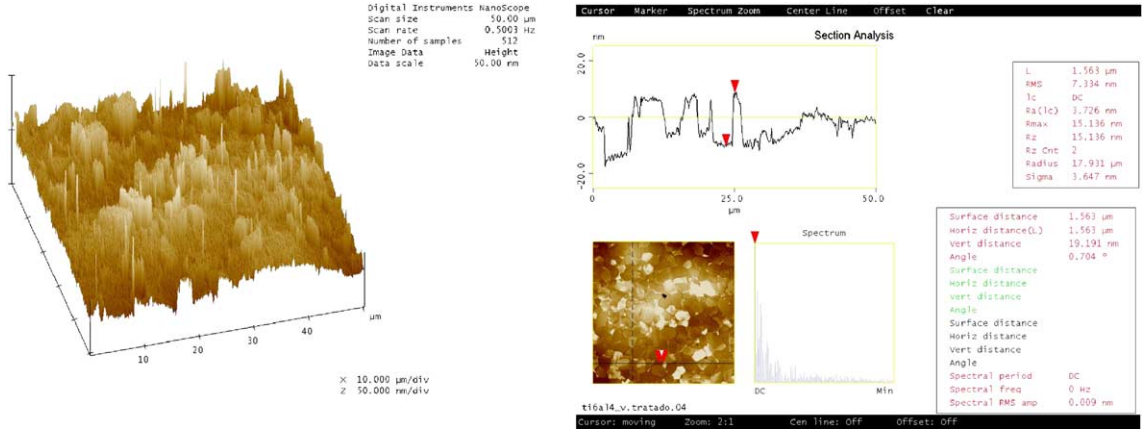

Fig. 2. AFM analysis of Type A ion implanted Ti–6Al–4V surface (Sample ref. A).

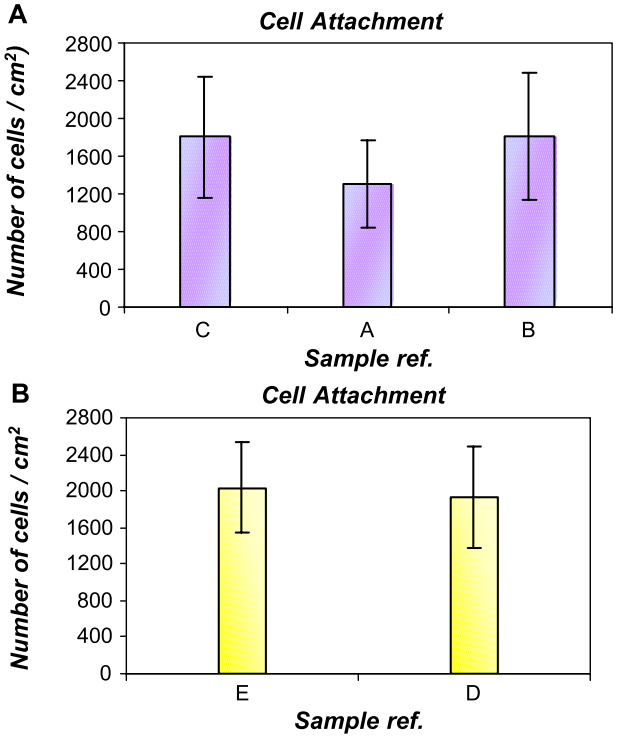

Fig. 3. (A) Cell attachment of control specimen (Sample C) and ion implanted specimens, made of Ti–6Al–4V alloy. Sample A corresponds to Type A Ion Implantation and Sample B to Type B Ion Implantation. (B) Cell attachment on control, unimplanted specimens (Sample E) and ion implanted specimens (Sample Ref. D), made of Ti CP GR1 alloy. Sample ref. D corresponds to Type B ion implantation.

to be considered; hence, any change in surface roughness induced by a coating or surface modification process should also be taken into consideration. However, most of the studies have concentrated on the effect at the macro-roughness range, neglecting any effect by the surface treatments at a nano-scale [2].

The quantitative results obtained with the Image System Analysis from the cell attachment tests are presented in Fig. 3A and B for the Ti–6Al–4V and Ti CP GR1 alloys, respectively. Cell attachment to Ti–6Al–4V-Type A Ion Implantation discs (Sample ref. A in Fig. 3A) was slightly reduced by 27% compared with control discs (unimplanted Ti–6Al–4V, Sample ref. C). In contrast, cell adhesion to Ti–6Al–4V-Type B Ion Implantation discs was not significantly altered (only very slightly reduced by about 1%) compared with the control sample (Sample C). Differences between both cases are not statistically significant according to Student's *t*-test.

Fig. 3B shows the cell attachment results on the Ti CP GR1 alloy, also in this case the cell attachment induced by Type B ion implantation (Sample D) was only reduced by 5% compared with control discs (Sample E). Cell attachment on the Type B implanted surfaces is very similar to that of the control, unimplanted materials, while Type A ion implantation appears to inhibit cell attachment. These results seem to be similar to those obtained in other studies on the effect of cellular attachment of a range of ion implantation treatments on titanium alloys.

Fig. 4 presents cell morphologies of untreated or control and ion implanted titanium samples observed with the aid of the AFM. These micrographs showed that star-shaped cells attached to ion implanted Ti–6Al–4V discs (Sample ref. A, ion implantation Type A) were more spread and had a tendency to occupy a larger surface area compared with the control and Type B implanted Ti–6Al–4V alloy (see Fig. 3). On the contrary, on the surface of Ti–6Al–4V-C discs the cells were mainly large and narrow shaped and cells with round shape.

Fig. 5 shows the growth ratio of the cells on the samples used in the cell proliferation assays. Although the initial ratio of osteoblast growth in untreated samples is greater than in treated samples, at the end of this testing time (i.e. 192 h) the growth ratio on ion implanted titanium discs is statistically greater than untreated samples (see bar with an asterisk in Fig. 5).

Depending on ion implanted species and processing variables, the effect on the behavior of cells cultured on the surface varies significantly. In the cellular adhesion test, it is possible to conclude that the number of adherent cells is not directly related to the form of material interaction. That is, morphological differences have been observed in the test tube where less adhered cells have been found, since the

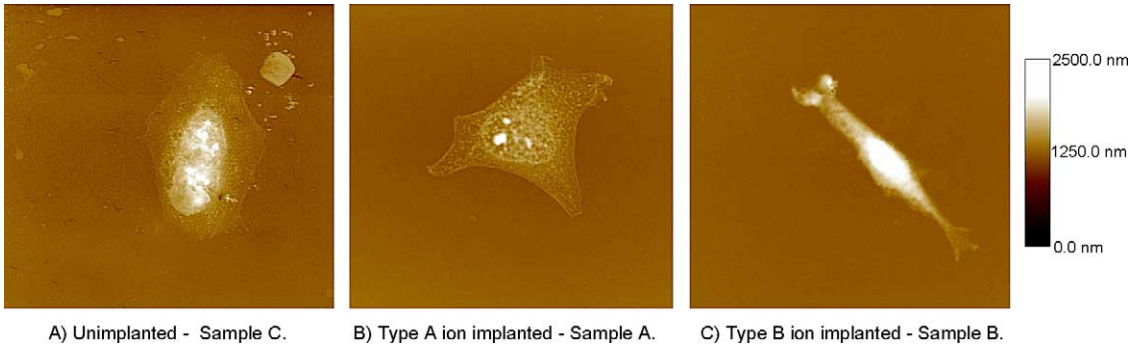

Fig. 4. Characteristic cell morphologies on Ti–6Al–4V material as seen from the AFM. (A) Unimplanted, control Sample C. (B) Type A ion implanted, Sample ref. A. (C) Type B ion implanted, Sample ref. B.

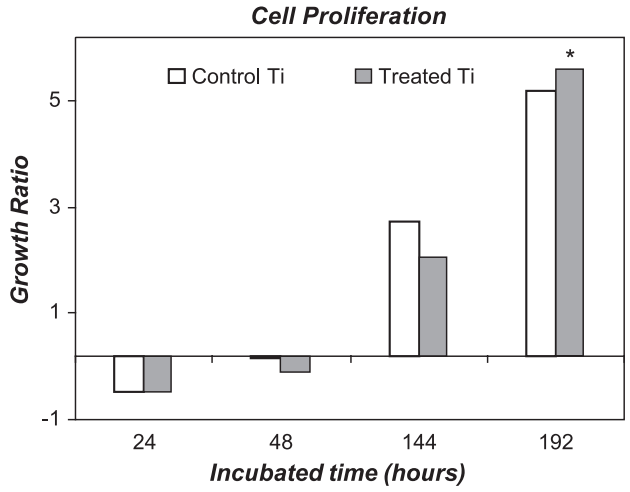

Fig. 5. Cell proliferation test results on the untreated, control Ti–6Al–4V, and the Type C implantation treated Ti alloy.

cells tend to occupy a greater surface than in untreated material. Although the number of adherent cells on Ti–6Al–4V discs is superior to the adherent ones on discs of Ti–6Al–4V with an ion implantation treatment, the latter cells adhere in better conditions. When the cells adhere in more favorable conditions begin to synthesize more extracellular matrix in this case [14], increasing the possibility of a greater bone integration.

On the other hand, the cellular attachment test is carried out in a very short time range (4 h), and is not sufficient to observe great differences in the behavior of these cells in contact with different surface treated materials. To observe more significant differences between adhered cells for the different material options, a cellular proliferation test is generally conducted. Analysing the results of the proliferation tests one can conclude that at short-term cells proliferate less in the surface modified titanium discs in comparison with the untreated discs (see Fig. 5). Nevertheless, in the long term, the ion implanted titanium is increasingly becoming more friendly to the osteoblast cells; since, as Fig. 5 depicts, there is a greater proliferation ratio in comparison to the titanium surface without treatment. It is evident that even 196 h do not seem sufficient to appreciate the differences that were observed in "in vivo" studies [9,10] and it becomes necessary to continue this model for superior temporal ranges or to appreciate subtle metabolic changes (structural proteins, apoptosis, etc.).

4. Conclusions

The following conclusions can be obtained from the present study.

- The ion implantation treatments offered a different result in the cell adhesion tests. While Type B implantation treatment offered a similar result to that obtained on control specimens, Type A ion implantation appeared to lower cell attachment by about a 27%.
- In the cell proliferation tests, although the initial ratio of osteoblast growth in untreated samples is greater than in treated samples, at the end of this testing time (i.e. 192 h) the growth ratio on the ion implanted titanium discs is statistically greater than on untreated, control samples. Longer proliferation tests would yield more significant differences for the ion implanted surfaces.
- Although there are no significant differences in the cell adhesion degree, the morphology of the attached cells shows different levels of polarization and reaction to the surface depending on the ion implantation treatment that has been applied. The redistribution of charges, nano-roughness modification and surface energy that are induced by ion implantation produce more spread and star-shaped cells, which are much more flattened.
- The morphological differences that have been observed in the cells attached on the (Type A) ion implanted titanium surfaces indicate that these adhere in better conditions to the surface, increasing the possibility of a greater bone integration.
- Ultimately, the difference in the cell behavior on the titanium surface is due to the changes originated by the ion implantation treatment both in the physical–chemical surface properties and topography, which is modified at nano-scale providing better anchorage points to the cells.

Acknowledgments

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