Surface Modifications of Biomaterials vs. Biological Behaviour

by

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Publications

This thesis is based on the publications numbered below from 1 to 8. In all the publications the author was responsible for all the experimental work except in paper 1 where Dr. Carlos Sá was responsible for the X-ray photo-electron spectrometry analysis and Dr. H. Ali for the samples' N⁺-ion implantation.

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Foreword

Producing and improving material characteristics to achieve a perfect integration with the human body is one of the main targets in biomaterials research. Joint prostheses, due to the high mechanical solicitations involved, have not only to fulfil biocompatibility but also corrosion requirements. Surface modification of metals, by means of ion implantation and sputter coating, was used in this work to produce surfaces that are expected to satisfy the requirements for a good joint-prosthesis performance.

Chapters 2 and 3 of this thesis were developed as an integral part of project BRITE/EURAM n° 0477, "Comparison of Surface Modifications by Ion Implantation and Cold Plasma Assisted Treatments as an Alternative to Other Coatings".

In chapter 2 the influence of the ion-implantation process on the corrosion characteristics is studied. 316 L stainless steel, Ti-6Al-4V and Ti-5Al-2.5Fe were nitrogen-ion implanted with $10^{15}$, $10^{16}$ and $10^{17}$ ions/cm$^2$. The ion-implanted materials were electrochemically characterized by potentiodynamic techniques in a simulated body fluid. Surface analysis techniques were used to study the material's surfaces before and after the corrosion tests. This study allowed the chemical characterization of the surfaces and the way in which they were influenced by the ion-implantation and corrosion processes. Chapter 3 presents the corrosion results concerning surfaces that were sputter coated with either nitrogen or carbon. Electrochemical characterization of the surfaces was used to provide screening criteria for subsequent biocompatibility studies which are presented in Chapter 4.

Chapter 4 presents the results obtained after an in vitro cell culture which was used to mimic the living tissue/material interface. Scanning electron microscopy
was used to observe the cell layer/substrata interface whose results are shown in section 4.1. Section 4.2 addresses the influence of osteoblast-like cells on the chemical composition of the surface-modified materials. In terms of corrosion and biocompatibility needs this research allowed the determination of the best surface modification technique for the surface treatment of joint-arthroplasty implants.

Development of a new calcium-phosphate coating method was also one of the main objectives of this study and the research involved is presented in Chapter 5. Section 5.1 is focused on the physical-chemical characterization of the coating as well as its suitability as an implant coating. Section 5.2 studies the different calcium-phosphate morphologies obtained for different substrata and the formation of a homogeneous naturally-formed calcium-phosphate coating on titanium alloys. The results obtained herein show the capability of this calcium-phosphate coating to perform as an alternative to plasma-spray coating of orthopaedic implants.
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Chapter 1

Introduction

1 - Introduction
Introduction

A biomaterial is defined as a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body [1]. It is estimated that ca. 2700 different kinds of medical devices [2] exist, made from materials such as polymers, ceramics, metals and composites. Metallic implants are of primary use in orthopaedic surgery as dental implants and orthopaedic prostheses. In the mid-1970s it was estimated that 75 000 total hip arthroplasties and 25 000 total knee arthroplasties were implanted in the USA alone [3], but early 1990s numbers show a drastic increase in these values up to 200 000 of each type of implant [4]. It is a fact that life expectancy is longer and that more prostheses are being implanted in younger patients which creates the need for revision operations within approximately 10 years.

Revision, in arthroplasty, is needed for several reasons: infection, fractures of the stem, loosening of the stem and/or socket. On long term implants, failure may be due to aseptic loosening. On cemented prostheses, the movement between the implant and the cement causes its fragmentation. As a consequence PMMA debris and accelerated wear from other components lead to granuloma, bone resorption and bone cement disintegration. On non-cemented prostheses, aseptic loosening is due to micromovements between the implant and the bone leading to metal debris, accelerated corrosion of the implant and consequent implant loosening. The appearance of metal particles has been observed during revision surgery of orthopaedic implants [5, 6, 7, 8], as well as high concentrations of toxic elements such as Cr (from stainless steel implants), Ti and V (from titanium alloy implants) in the surrounding tissues as a result of wear and dissolution processes [9, 10, 11, 12]. The size of the metal particles is an important factor to take into account when studying the effect of metal debris. The presence
of Co-Cr-Mo and Ti-6Al-4V particles with sizes less than 5-10 μm in cell culture is known to be harmful. Due to the additive effect of particle contact with the cells and metal dissolution, the Co-Cr-Mo alloy presented the most damaging results to cell-culture evolution [13].

It is believed that ion release from implant surfaces may be a significant factor leading to osteolysis surrounding the metal prostheses either by inhibiting the function of osteoblasts or by accelerating the function of osteoclasts. In vitro tests showed that Ti⁴⁺, Al³⁺ and V⁵⁺ ions suppress the Ca deposition in the extracellular matrix (ECM), and at some concentrations [14] they can even be more lethal than Cr⁶⁺, Mo⁶⁺ and Co²⁺ which seem to be the most toxic ions [15]. It was shown that Ni, from stainless steel implants, and Ti, from Ti cp. implants, are released. However, in contrast to Ti, Ni forms complexes with biological ligands and as result permeates the biomembranes, spreads over the body and is eventually excreted by the urine. Ti is a very acidic ion and has very limited capability to link to biological entities which implies that it should stay in the vicinity of the implant. Similar assumptions were made for the Ti-6Al-4V alloy but in this case an added toxicity effect could be inferred due to the presence of Al and V ions [16]. The release of hexavalent Cr was found to become intracellular red blood cell associated in vitro. Cellular uptake of Cr was also documented in red blood cells following corrosion of stainless steel and Co-Cr implants in vivo in patients undergoing total arthroplasty revision and in fibroblasts subjected to products of fretting corrosion of stainless steel and Co-Cr. Corrosion leads to the release of Cr⁶⁺ which can interfere biologically, with harmful consequences [17]. The influence of metallic ions, resulting from the corrosion of stainless steel in mice, caused morphological alterations of the liver and kidneys [18]. The damage caused by corrosion of the implants may not be restricted to the tissues around the implant.

Therefore the need arises to create prostheses that will resist longer, thus avoiding, if possible, a second surgical operation. By modifying the implant
surface through processes such as ion implantation and sputter coating, one may theoretically create a surface with the best characteristics to induce a given tissue response.

THE MATERIALS

Stainless steel and titanium alloys are the metals most widely used in orthopaedic surgery due to their good corrosion and wear resistance.

Stainless steel and titanium alloys owe their good corrosion resistance to the existence of a spontaneously formed oxide film with thicknesses ranging from 1 to 20 nm depending on the conditions under which the film was created.

Immersion [19] and thermal treatment of 316 L stainless steel and Ti-6Al-4V are known to increase the thickness of the passive film. Thermally treated screws of Ti-6Al-4V and 316 L stainless steel were inserted in the cortical bone of rats and showed better bone anchorage than the non-thermally treated controls [20, 21]. An increase in the oxide thickness was also observed in human-retrieved implants which was found to be directly proportional to the implantation time [22]. It appears then that the presence of the oxide layer is not only responsible for the corrosion protection of the materials but also for their biocompatibility.

However, the thicknesses of these films are not constant and homogeneous and have a high degree of nonstoichiometry, variations in crystal structure and a high concentration of defects on the oxide surface. On these sites, corrosion is more likely to occur which may induce adverse cell reactions as a consequence of immune and toxic reactions [8].

Changes in the structure and composition of this film imply different tissue responses as this is the surface in the closest contact with the bone.
Titanium and its alloys show a better induced tissue response when compared to stainless steel. This is probably due to the properties of its oxide film as both materials exhibit close contact of their passive films with the surrounding tissues.

Fig. 1 shows a model of the titanium/tissue interface as proposed by Tengvall et al. [9].

Fig. 1 - Tentative model of the titanium/tissue interface [9]

An inflammatory response always occurs after surgery with release of superoxide and hydrogen peroxide from the inflammatory cells. The occurrence of the Fenton reaction as the precursor of the formation of the hydrated surface layer was suggested by Tengvall et al. [23], Fig.1. This means that the metal ion will react with hydrogen peroxide to form a metal hydroxide according to the following reaction:
\[ M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^- + OH \]

The hydrated surface layer is responsible for the adsorption of proteins, glycoseaminoglycans, inorganic ions and other molecules. It is believed that the presence of hydroxylated Ti on the outermost layers of the oxide is responsible for the better bone apposition and apparent anti-inflammatory action [7]. A similar interface composition was suggested by Healy et al. [19, 24, 25] to interpret the dissolution processes of titanium in a simulated physiological environment.

The presence of this hydroxylated layer appears to be a precursor for the formation of a calcium-phosphate-rich layer on the surface which is responsible for the good biocompatibility exhibited by titanium and its alloys [26].

The incorporation in the passive film of inorganics such as P co-ordinated with O, S or Ca was suggested by P. Tengvall et al. [9] and Kasemo et al. [27]. Such species were observed by Auger electron spectroscopy on human in vivo retrieved prostheses [22]. Similar results were obtained in vitro when Ti cp. and Ti alloys were immersed in a physiological solution [28, 29, 30, 31]. These results suggest that titanium and its alloys are capable of inducing the formation of a calcium-phosphate coating.

The natural precipitation of a calcium-phosphate layer on metals, [29, 26, 32, 33, 27, 34, 35], ceramics [27, 28] bioglasses, [36, 23, 26, 37] and polymers [38] was reported by several groups using different techniques having in common the immersion of substrata in Ca- and P-containing solutions. On metals the layers formed were very thin. Hanawa and coworkers [32] reported the formation of a 7-nm thick layer after 30 days' immersion in Hank’s Balanced Salt Solution and Ducheyne et al. [29] reported the formation of a 1-μm-thick non-homogeneous layer on titanium after 1-2 weeks of immersion in a simulated physiological solution, which is the minimum thickness for the coating to cause bone
induction. Analysis of the composition and structure of this calcium-phosphate layer revealed that it is similar to amorphous hydroxyapatite which in turn is similar to bone. Studies on apatite formation revealed that bone mineral is best imitated when the precipitation involves an aqueous phase [39].

The formation of a naturally precipitated calcium-phosphate coating on titanium implants which is adherent, homogeneous and thick enough to exhibit bioactive properties appears to be an alternative to the production of calcium-phosphate plasma-spray coatings. The formation of such a coating has been one of the research areas of this thesis and is presented in chapter 4.

SURFACE-MODIFICATION TREATMENTS

Ion Implantation

Ion implantation is a surface-modification technique in which an ion beam constituted by ions of one or several elements is introduced in the crystallographic net of the target. The collision mechanisms between the accelerated ions and the atoms of the target are the origin of microstructural substitution and interstitial defects, formation of lacunae and metastable phases. These structural transformations are responsible for the increase in surface hardness and consequent increase in wear resistance [40]. Conversely, they may have a harmful effect as they can act as preferential sites for corrosion to occur. The thickness of the altered layer varies depending mainly on the target structure, beam composition and energy [41].

One of the major advantages of this process, compared to conventional coating techniques like plasma spray and sputter coating, is that it does not produce an
interface between the surface-modified layer and the bulk material, avoiding its later detachment. Because the ion beam penetrates and alters the surface in depth, ion implantation does not change the material’s dimension and therefore may be used as a final treatment.

Precipitates, resulting from nitrogen-ion implantation in iron, were characterized by Rauschenbach et al. [42]. The nature of the precipitates formed during nitrogen-ion implantation varies with the steel carbon concentration and also with the implanted dose. It seems that the carbon concentration and the dose of nitrogen implantation are two competitive factors, whose combination determines the total amount of iron nitrides that will be formed. Results for stainless steel show that the amount and size of nitrides in the as-implanted material increase with the dose, the proportion of nitrides in stainless steel being almost the same as in pure iron at each dose [39, 43, 44].

The ion implantation of N⁺ in Fe results in the formation of crystalline phases in the fluence range of 1x10¹⁶ to 1x10¹⁸ ions/cm². γ-austenite appears in the entire range, but for fluences higher than 4x10¹⁶ ions/cm² other nitride phases appear simultaneously, namely α''-Fe₁₆N₂, ε-Fe₂N₁₋ₓ and α'-martensite [39, 45]. These phase formations, induced by the ion-implantation process, were also observed at ion-implantation temperatures higher than room temperature [46].

The presence of γ-austenite was also reported by Carbucicchio et al. [47] for fluences in the range of 5x10¹⁶ to 1x10¹⁷ ions/cm². Other authors have reported the formation of other nitride phases in steel but all these studies were conducted with ion-implantation doses higher than 2x10¹⁷ ions/cm² [48, 40].

Nitrogen-ion implantation into titanium induces the formation of titanium-nitride and titanium-carbonitride precipitates [49, 39, 50]. According to Hohmuth et al. [49] the diameter of titanium nitrides increases with the implanted fluence and their number increases with implantation fluence below 10¹⁷ ions/cm². However, above this value their number per unit area is fluence-independent and carbonitrides begin to appear. It is known that high-fluence (10¹⁶ to 1.5x10¹⁸
ions/cm²) nitrogen-ion implantation produces TiN crystallites which show preferred orientations. This is due to a collision cascade provoked by the ion-implanted ion resulting in permanent damage defects [46, 51]. A preferential nucleation and/or crystal growth could be sensitive to the direction of these distorted zones. Also high-fluence ion implantation induces large lateral compressive stresses which influence the orientation of the precipitates [47].

Therefore the need arises to create microstructures that will induce materials with high corrosion resistance, high wear resistance and good biocompatibility.

Nitrogen-ion implanted Ti-6Al-4V hip prostheses were tested for their wear resistance. An increase in wear resistance was found on ion-implanted prostheses probably due to an increase in surface hardness [52, 53]. An increase in corrosion resistance of 316 L stainless steel was achieved when the material was ion implanted with B⁺ or P⁺ ions [54].

In vitro and in vivo biocompatibility testing of ion-implanted surfaces showed an intrinsic dependence between the ion-implanted species and the biocompatibility of the surface-treated surfaces.

Howlett et al. [55] investigated the effect of ion implantation with N, P, Mn and Mg ions on silicon single crystals and found no evidence that this surface treatment produced an increase in adherence of human bone-derived cells to silicon substrata. On the other hand, cells were preferentially attached to O ion-implanted surfaces. The ion implantation of Mg onto alumina was studied by Howlett et al. [56] and the results showed that the attachment and spreading of human bone-derived cells was enhanced compared to non-implanted alumina. The cells showed different morphology, being better spread and larger, suggestive of higher metabolic and synthetic activity. Similar results were obtained by Lee et al. [57] when bovine aorta endothelial cells were cultured on polyurethane which was ion implanted with Ne⁺.

In vivo tests performed by Röstlund et al. [58] in rats showed that nitrogen-ion
implantation changes the surface properties, and therefore the biological properties, of pure titanium. They found that up to 6 weeks after implantation, the cell number and structure were the same around ion-implanted and non-implanted implants but, after 6 weeks, differences around the two types of implants began to appear. The numbers of macrophages and giant cells were significantly larger close to the ion-implanted materials. According to the same authors, these results could be due to changes in the physicochemical surface properties of the ion-implanted implants, as all surface-treated and untreated materials had similar surface morphologies. Nevertheless, Johansson et al. [59] studied the influence of nitrogen ion-implanted Ti cp. and Ti-6Al-4V on the cortical bone of rabbits and detected no difference after 3 months' insertion.

Sputter Coatings

Sputter coating is a surface modification technique in which an element can be deposited on a substratum. An electric discharge between an anode (the grounded chamber walls) and a cathode, made up of the material to be deposited, results in the ionization of the surrounding gas. The gas ions are then accelerated by the electric field of the target and their impact results in the sputtering of the target. The vapour thus created condenses on the surfaces of the reactor, especially on the substrata which face the target. The created coating may have different thicknesses and structures depending on the parameters of the process such as pressure and temperature. In the case of nitrogen or carbon sputter coatings, titanium nitrides or titanium carbonitrides are formed on the surface.

It is believed that titanium nitride coatings, in comparison to pure Ti, show a better stability of their oxide layers and that they will therefore possess better biocompatibility properties [60].
Porous Coatings

On total-hip arthroplasties (THA), anchorage of the prosthesis to the bone is crucial for the success of the implant. Nowadays, and due to the improvements achieved both in cement properties and surgical techniques, the aseptic loosening rates of cemented implants have registered a significant drop from 20-25% to 1.7% at a 5 year follow up [61]. Nevertheless, research is still in progress on the production of porous coatings on metal substrata. Clinical results show that these cementless THA are successful on a short timescale [62] because the essential bone responses to implant material occur in a short period of time after implantation. The real advantage of cementless fixation lies in its potential to form a permanent bond with bone [63].

Three main methods are used to apply a porous coating to a metal substratum: sintering, diffusion bonding and plasma spray. In the first two methods pellets and metal fibres, respectively, are applied to the surface and through heat or/and pressure are fused to the substratum. In the plasma-spray method a ceramic powder is heated within the spray nozzle and then projected on to the substratum. The main advantage of the plasma-spray method is that it does not involve the heating of the substratum and therefore leaves its mechanical characteristics intact, whereas in the other methods a reduction of ca. 50% in the substratum mechanical strength was registered. Moreover, clinical results obtained by Bourne et al. [62] on a two year follow-up of cementless bead THA, led to the abandonment of this type of implant due to an increase in thigh pain and the detachment of the metallic-porous coating from the metal substratum [59, 64].

Calcium-phosphate ceramics, because they promote the formation of bone tissue at their surface [65, 58, 66, 67, 68] are the materials of choice for the plasma-spray coating of hip prostheses. Hydroxyapatite (HA), $(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, and tricalcium phosphate (TCP), $\text{Ca}_3(\text{PO}_4)_2$, are the most common calcium
phosphates used in this process, but due to high temperatures involved in the deposition process, phase transformations in the coating may occur [62]. The transition temperature of TCP is around 1200 °C and HA decomposes at 1300 °C. It is known that changes in the calcium-phosphate properties provoke changes in the bone-bonding mechanism and the rate of bone formation [62]. Therefore, fluorapatite (FA) \( \text{Ca}_{10}(\text{PO}_4)_6(\text{F})_2 \) was used as an alternative due to its higher chemical stability during the plasma-spray process. Nevertheless, in vitro studies with FA revealed a detrimental effect in comparison to HA as a result of the damaging effect of fluoride ions on the cells [69].

HA coatings are affected by the composition and purity of the starting powder. They are not identical and may vary among manufacturers in their composition, crystallinity, density, purity and structure [61, 70] thus affecting the coating bioactivity and bioresorbability. It is then difficult to predict the osseointegration of this kind of material in human bone. In vitro studies on the effect of different crystallinities showed that highly crystalline HA have an inhibiting effect on cell proliferation and on alkaline phosphatase. It was proposed that the lower degradability exhibited by the higher crystallinity HA is responsible for this occurrence. It was suggested that the Ca and P released from the amorphous phase may play an important role in the cells' proliferation rate [64, 66] and metabolism [64]. However, a direct contact between the highly crystalline HA and the cells was needed to provoke the inhibition of cell proliferation. This could mean that the roughness of the ceramic has a strong influence on the cytocompatibility of the material. According to de Bruijn et al. [71] when discussing the effect of crystallinity of plasma-sprayed HA coatings on their stability, the coating should be well characterized. A high crystallinity obtained by heat-treatment will result in more stable coatings due to the absence of amorphous phases. Conversely, highly crystalline HA coatings which have been produced without a heat-treatment, will be composed of relatively large areas surrounded by an amorphous phase. Dissolution of this amorphous phase will
release the crystalline domains and thus severe coating breakdown will occur. This event is particularly important in mid- and long-term implantation due to bone resorption [67]. The fixation of the stem depends on bone ingrowth and consequently on bone remodelling. Once bone resorption starts the stem fixation is compromised and failure of the implant might occur. Cell resorption of the HA coating is thought to be one of the factors contributing to the coating degradation. This type of degradation was attributed to the low extracellular pH induced by osteoclasts [66, 67, 72]. In spite of all the calcium phosphates showing a high solubility rate in this range of pH, amorphous HA will be dissolved first due to its higher degradability. Debris emission by the coating is then an unfavourable consequence. It was suggested early on that this type of coating would not present particle migration, due to its osseointegration properties, and in the case of coating detachment, the HA particles would be trapped inside the newly formed bone preventing third-body wear and osteolysis [73]. This has been contradicted by recent clinical studies presented by Bloebaum et al. [64] and Frayssinet et al. [70].

Plasma-spray coatings not only create an interface between the coating and the bone but also have an interface between the coating and the metal bulk. The existence of an interface is associated with a weakening in the mechanical properties and coating delamination may occur. Research on the HA/Ti interface revealed that immersion of coated Ti-6Al-4V specimens in a simulated body fluid degrades the coating bonding by reducing its original strength by 25-33 % [74]. HA microstructure proved to be the major factor affecting its dissolution and consequently bonding degradation. On the other hand, electrochemical experiments performed by Sousa et al. [75] on HA coated Ti-6Al-4V suggest that metal dissolution is probably responsible for this phenomenon. Metal cation hydrolyses in solution, with a consequent decrease in pH; this effect could promote rapid dissolution of the HA at the interface and hence its detachment. Improvement of the bond between the coating and the substratum is therefore
needed. This could be achieved by increasing the surface roughness of the substratum. This would imply an increase in contact area but it would also increase the area of metal dissolution. Reduction of the coating thickness was shown not to be a good alternative. Failure was reported to occur at the HA/bone interface when 50 μm coated Ti-6Al-4V cylinders were implanted in dogs [76].

The production of calcium-phosphate coatings by anodic oxidation of titanium is being investigated as an alternative to plasma-spray. Anodic oxidation of titanium and its alloys is known to increase the materials' corrosion resistance [77]. It allows the production of films with thicknesses higher than the spontaneously-formed oxide film and its technology permits the formation of a homogeneous film on all the implant's surface regardless of the shape. Ishizawa et al. [78, 79, 80] succeeded in the production of an HA-porous film by anodization of titanium in a solution containing β-glycerophosphate and calcium acetate. After a hydrothermal treatment with high-pressure steam, HA crystals with high crystallinity were precipitated covering all the surface. The adhesive strength of these films proved to be twice that achieved with the plasma-spray technique [81] and although the heat treated film was only 1-2 μm thick the bone apposition was equivalent to that obtained using HA ceramics [82]. According to Ducheyne et al. [29] thicknesses of at least 1 μm are needed for the calcium phosphate to cause bone induction. M. Shirkhanzadeh [83] used a potentiostatic technique to deposit HA on Ti-6Al-4V and Cr-Co-Mo using a mixture of Ca(NO₃)₂ and NH₄H₂PO₄ as electrolyte. The coating was steam treated and subsequently heated at 420 °C. A similar electrochemical technique, using a simulated body fluid as electrolyte was used by Ban et al. [84] to deposit calcium phosphates on a pure titanium plate. With these electrochemical methods it is possible to obtain calcium-phosphate coatings with different compositions by varying the chemical composition of the electrolyte. This allows the production of HA coatings according to the requirements of the implant, which is an advantage in the plasma-spray technique.
BONE TISSUE/BIOMATERIAL INTERFACE

When an orthopaedic implant is inserted there is an immediate formation of an interface between the surface of the implant and the surrounding bone. The analysis of the implant/tissue interface from the material-surface point of view was presented previously. The study of the bone response will be analysed herein.

Biocompatibility testing may be achieved by *in vitro* and *in vivo* tests. *In vitro* tests do not exclude the performance of *in vivo* tests but in the case of the biocompatibility study of surface-modified materials they can be regarded as a screening method to assess their biological safety. *In vitro* tests usually precede *in vitro* tests as they can provide faster information about the toxicity of the implant material as well as creating a better-controlled experimental environment which is not possible to achieve when testing biomaterials *in vivo*.

Maniotopoulos *et al.* [85] invented a cell culture method in which an osteoblast-enriched cell population is attained. The addition of dexamethasone, a glucocorticoid, to the cell-feeding medium proved essential to produce this kind of cell culture. Experiments performed without the presence of dexamethasone showed a lower density of osteoblasts. Na-β-glycerophosphate and ascorbic acid were also added to the medium to improve the synthesis of collagen and to promote mineralization.

Davies *et al.* [86, 87] using the same cell culture method as described by Maniotopoulos *et al.* found the existence of 1 μm calcified globules as the first material produced by the cells after which collagen is produced and finally mineralization takes place. The calcified deposits were identified as spheritic foci of calcification and their origin was proved not to be a substratum-mediated process but one which is dependent on the cells expressing their osteoblast phenotype. The observation of these globular accretions was also achieved by
other groups [88, 89]. Other studies [82] on the constitution of the interfacial zone between Ti cp. and the nearest bone cell suggested that the interface cell layer/metal substratum was composed of at least two layers: a proteoglycan-rich layer and a bonding zone between the proteoglycan-rich layer and the metal beneath.

The similarity of this interface to the one observed in vivo suggests that this cell-culture model is valid to perform reliable in vitro studies and infer the in vivo situation.

The formation of a 0.5 μm thick cement-like layer immediately adjacent to the implant was observed on retrieved implants from rats. This cement-like layer, earlier described by Davies et al. [90] was found to be due to the existence of globular accretions. This was observed both on titanium and hydroxyapatite implants [91].

The interface between bone and apatite- and wollastonite- containing glass ceramic showed the presence of calcified globules together with the formation of a mineralized collagen-free area [92]. Linder et al. [93] studied the bone/Ti cp. interface of retrieved implants in rabbits. The Ti cp. surface was separated from the collagen fibres by a gap of 20-50 nm which contained proteoglycans. The collagen was randomly distributed and constituted a layer of ca. 100-500 nm over which ordered collagen was laid. Calcified deposits were present on these 3 layers but their numbers decreased towards the interface.

The cell-culture model presented by Maniotopoulos et al. [85] was used, in this thesis, to assess the biocompatibility of the studied materials as well as to predict the bone/implant interface in vivo.
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Chapter 2

Electrochemical characterization of N⁺-ion implanted biomaterials

2.1 - Electrochemical and surface modifications on N⁺-ion implanted Ti-6Al-4V immersed in HBSS

2.2 - Electrochemical and surface modifications on N⁺-ion implanted Ti-5Al-2.5Fe immersed in HBSS

2.3 - Electrochemical and surface modifications on N⁺-ion implanted 316 L stainless steel immersed in HBSS
Electrochemical and surface modifications on N⁺-ion implanted Ti-6Al-4V immersed in HBSS

The effect of nitrogen-ion implantation on the electrochemical behaviour of Ti-6Al-4V in a simulated physiological solution (HBSS - Hank’s Balanced Salt Solution) was studied by open-circuit potential versus time and cyclic polarization techniques, with the aim of better characterizing the surface prior to biological testing and to choose the most appropriate nitrogen-ion fluence. Three fluences ($10^{15}$, $10^{16}$ and $10^{17}$ ions/cm$^2$) were used. The $10^{16}$ ions/cm$^2$ N⁺ fluence produced the lowest corrosion rate under passive conditions. The highest fluence caused a large increase in the corrosion rate and inhibited the formation of a calcium- and phosphate-rich layer on the electrode surface.

Introduction

The Ti-6Al-4V alloy is commonly used in orthopaedic surgery due to its good biocompatibility and corrosion resistance. However, black debris [1] and high titanium concentrations of non-wear origin [2] have been found in the tissues surrounding titanium implants. It is generally believed that these particles
remain in the tissues without inducing a strong biological reaction. Generally speaking, intolerance to metallic implants is associated with the toxicity of ions released from wear particles and surface oxidation of the implants. It has been postulated [3] that the high amounts of titanium, in the form of TiO₂(aq), found in the tissues around implants, are due to the interaction between titanium (III) and titanium (IV) with biologically-formed oxygen, superoxide and hydrogen peroxide.

Since the corrosion resistance of Ti alloys depends on the presence of an oxide film, it is clear that the first important step in minimizing the release of ionic species is to improve the properties of the surface layers.

Ion implantation has been successfully used in a number of situations to improve wear, fatigue and corrosion of various materials, including titanium alloys. For example, Buchanan et al. [4] have reported a 100-fold decrease in the corrosion rate of Ti-6Al-4V wearing against UHMWPE (ultrahigh molecular weight polyethylene) in isotonic saline when 20% nitrogen was implanted on the surface. Improved wear resistance associated with ion implantation can be the result of [5]: (i) increase in surface hardness; (ii) improvement of the mechanical properties of the oxide (e.g. better adherence to the substrate); or (iii) reduction in friction between contacting surfaces (e.g. solid film lubricant). A decrease in the passive dissolution rate and greater corrosion fatigue endurance in a simulated physiological environment have been found by Yu et al. [6] after implantation of Ti-6Al-4V with 3 x 10^{17} ions/cm².

According to Elder et al. [5] wear improvement requires the formation of a large number of titanium nitrides and possibly carbonitrides. However, it is not clear how such a large number of precipitates affects the corrosion resistance. Furthermore, due to the interaction between the ion beam and the atoms of the target, ion implantation produces microstructural defects in the latter which may be responsible for modifications in the wear and corrosion resistance. Both aspects have been taken into consideration in a project in
which our laboratories have been involved. This paper describes the part of the work related to the corrosion behaviour in the absence of wear.

The effect of several ion-implanted N+ fluences on the corrosion behaviour of Ti-6Al-4V has been studied in HBSS. The research involved the use of electrochemical techniques, namely open-circuit potential versus time and cyclic polarization measurements, aimed at determining the fluence that would induce the best corrosion performance.

X-ray photo-electron spectroscopy (XPS) studies have also been carried out prior to and after immersion in HBSS in order to determine surface modifications induced by the ion implantation process. There was particular interest in studying the accumulation of calcium and phosphate on the surface, in view of previous results reported by one of us [7] and Hanawa [8, 9] obtained with titanium which had not been ion implanted.

Materials and Methods

Ti-6Al-4V samples (Deutsch Titan), 20 mm in diameter and 3 mm thick, were implanted with three different fluences of nitrogen ions, namely: $10^{15}$, $10^{16}$ and $10^{17}$ ions/cm² with a beam energy of 40 keV.

Before the electrochemical experiments the samples were ultrasonically degreased with acetone, thoroughly washed with de-ionized water and dried. The electrical connection was made via a copper electrical wire (diameter 0.5 mm) and by applying a solution of colloidal silver at the region of contact. The electrical junction was protected by polytetrafluoroethylene tape, followed by the application of a non-conductive and water resistant varnish (Lacomit®), as shown in Fig. 1.
Fig. 1. Schematic illustration of the electrical connection to the sample (1) and its protection (2).

The electrochemical cell was made of metilpenthen polymer and had a capacity of 150 mL. All experiments were performed at room temperature (18±2 °C) in HBSS (Gibco), which was de-aerated with a large flux of argon for at least 15 minutes before the tests. The electrolyte composition was the following (g/L): 0.185 CaCl₂; 0.40 KCl; 0.06 KH₂PO₄; 0.10 MgCl₂·6H₂O; 0.10 MgSO₄·7H₂O; 8.00 NaCl; 0.35 NaHCO₃; 0.48 Na₂HPO₄; 1.00 D-glucose. All potentials were measured against the saturated calomel electrode (SCE) and are given on this scale. The counter electrode was a square platinum sheet with an area of 800 mm².

The electrochemical experiments were performed with an EG&G Princeton Applied Research, model 273 A, potentiostat. The potentials were controlled and the data were recorded with the help of software model M352, from EG&G Princeton Applied Research. Several electrochemical parameters were
determined by the least squares method, employing successive iterations of the Stern-Geary equation. Monitoring of the potential as a function of time was carried out for 1 hour before initiating the potential sweep. In cyclic polarization measurements the potential-sweep rate was $10^{-3}$ V/sec starting 0.25 V below the corrosion potential, $E_{\text{corr}}$, and going up to 2 V, followed by a reverse sweep, which finished when the downward curve was well defined.

After corrosion measurements, the samples were washed with de-ionized water and dried in air. The surfaces were observed by scanning electron microscopy (SEM) and analysed by XPS. XPS analysis was performed both on as-implanted samples and after performing polarization curves. A VG Scientific ESCALAB, using Mg Kα as source, was used to obtain the spectra.

Results and Discussion

Electrochemical studies

Fig. 2 gives curves obtained in the open-circuit measurements. A non-implanted surface was used as reference. The increase in nitrogen fluence displaces the curves towards more noble potentials. The figure shows that an increase of ca 350 mV occurs for the highest fluence. Ion implantation of $10^{15}$ ions/cm² has no significant effect on the potential reached at the end of the experiments. The rate of potential change in the initial stages is less significant for the $10^{16}$ ions/cm² and $10^{17}$ ions/cm² doses than for the other surfaces. This may be due to the existence of more stable films, formed as a consequence of the ion-implantation process.
Fig. 2. Open-circuit potential versus time measurements for Ti-6Al-4V not-implanted and ion-implanted with three fluences of N\(^+\) at 40 keV: — not-implanted; — 10\(^{15}\) ions/cm\(^2\); — 10\(^{16}\) ions/cm\(^2\); — 10\(^{17}\) ions/cm\(^2\)

Fig. 3 gives the cyclic polarization curves. For all the curves the reverse branch exhibits lower current densities than the forward branch, which can be attributed to thickening of the oxide during the forward scan. The passive current density decreases with increasing N\(^+\) fluence.

Several electrochemical parameters taken from curves like those in Fig. 3 are given in Table 1. The values obtained are the average of at least two results. The potential for which I=0, E(I=0), is more active than \(i_{corr}\), due to the cathodic polarization of the working electrode prior to the anodic potential sweep.

Analysis of the values given in this table shows that a fluence of 10\(^{15}\) ions/cm\(^2\) N\(^+\) does not significantly modify the corrosion rate, \(i_{corr}\). For 10\(^{16}\) ions/cm\(^2\) N\(^+\), \(i_{corr}\) is ca. 1/3 of the value measured for the non-implanted surfaces. The highest fluence, 10\(^{17}\) ions/cm\(^2\), shows the highest
$E_{\text{corr}}, E(I=0)$ and $i_{\text{corr}}$. Of particular significance is the large increase (nearly 10 times) in $i_{\text{corr}}$.

![Graph showing cyclic polarization curves for Ti-6Al-4V ion-implanted with three fluences of N⁺ at 40 keV: − $10^{15}$ ions/cm²; − $10^{16}$ ions/cm²; − $10^{17}$ ions/cm².]

Fig. 3. Cyclic polarization curves for Ti-6Al-4V ion-implanted with three fluences of N⁺ at 40 keV: $10^{15}$ ions/cm²; $10^{16}$ ions/cm²; $10^{17}$ ions/cm².

Table 1. Summary of the electrochemical data

<table>
<thead>
<tr>
<th>Fluence (ions/cm²)</th>
<th>$E_{\text{corr}}^a$ (mV)</th>
<th>$E(I=0)$ (mV)</th>
<th>$i_{\text{corr}}$ (nA/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-implanted</td>
<td>$-260\pm15$</td>
<td>$-410\pm28$</td>
<td>$4.3\pm0.4$</td>
</tr>
<tr>
<td>$10^{15}$</td>
<td>$-260\pm19$</td>
<td>$-568\pm71$</td>
<td>$4.5\pm1.4$</td>
</tr>
<tr>
<td>$10^{16}$</td>
<td>$-30\pm10$</td>
<td>$-340\pm45$</td>
<td>$1.6\pm0.6$</td>
</tr>
<tr>
<td>$10^{17}$</td>
<td>$+107\pm6$</td>
<td>$-144\pm84$</td>
<td>$36.5\pm16.8$</td>
</tr>
</tbody>
</table>

*a measured in the open-circuit experiments
The formation of $\delta$-TiN$_x$ and TiC$_x$N$_y$ precipitates, induced by the ion-implantation process [10, 11], may explain the above results.

According to Hohmuth et al. [10] the diameter of titanium nitrides increases with the implanted fluence and their number/unit area increases with implantation fluence below $10^{17}$ ions/cm$^2$. However, above this value their number per unit area is fluence-independent and carbonitrides begin to appear. These data could give a plausible explanation for the decrease in corrosion resistance found in our work for the highest fluence ($10^{17}$ ions/cm$^2$). The increase in $i_{corr}$ may be due to a combined effect between an increase in size of $\delta$-TiN$_x$ and the precipitation of TiC$_x$N$_y$. Since large titanium nitrides would tend to detach from the surrounding metallic matrix, the area of attack would be considerably greater than for smaller, coherent precipitates. At the lower fluences the nature and size of the precipitates, and probably also their crystallographic relation with the matrix, are such that they do not decrease the corrosion resistance. Since the improvement found with the intermediate fluence of $10^{16}$ ions/cm$^2$ is probably associated with modifications in the characteristics of the passive oxide film, it was decided to carry out XPS studies.

**Surface Characterization**

Survey XPS spectra were acquired before and after corrosion measurements. Following spectra acquisition, peak identification and quantification were achieved using VG Scientific ESCALAB package software. To take into account some shift caused by charging of the sample surface all spectra were adjusted taking the C 1s peak at 285.0 eV as reference for the adventitious carbon contamination.

All samples exhibited a well-defined C 1s peak, which is usually a common contaminant. Vanadium was never detected, which is in agreement with other
studies [8, 9]. As-implanted samples also showed F as a contamination, which probably comes from the PTFE tape.

Table 2 compares the XPS quantification data for polarized samples. Ca and P were detected on all surfaces after polarization, but the highest concentrations were found for the $10^{16}$ ions/cm$^2$ N$^+$ fluence.

<table>
<thead>
<tr>
<th>Fluence (ions/cm$^2$)</th>
<th>Ti (at.%)</th>
<th>O (at.%)</th>
<th>N (at.%)</th>
<th>Ca (at.%)</th>
<th>P (at.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{15}$</td>
<td>12.7</td>
<td>41.9</td>
<td>0.9</td>
<td>2.7</td>
<td>1.4</td>
</tr>
<tr>
<td>$10^{16}$</td>
<td>13.7</td>
<td>37.8</td>
<td>0.8</td>
<td>3.0</td>
<td>1.7</td>
</tr>
<tr>
<td>$10^{17}$</td>
<td>6.0</td>
<td>27.5</td>
<td>2.9</td>
<td>1.4</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The results obtained by Hanawa [8, 9] showed that calcium phosphate layers are formed on the surface of titanium and its alloys immersed in HBSS. The concentration ratio [Ca]/[P] was 1.67, which is typical of hydroxyapatite. The calculated [Ca]/[P] for the $10^{16}$ ions/cm$^2$ N$^+$ is 1.78, which suggests the existence of an apatite-like layer. Of particular interest is the decrease in the phosphate concentration found on the specimens with the highest N$^+$ fluence (see Table 2), indicating that apatite formation is inhibited. This may affect the biological behaviour of titanium implants, which seem to have a natural tendency to induce calcium phosphate precipitation. Firstly, because studies on the nature of corrosion products formed by the reaction of titanium with solutions containing calcium and phosphate have shown that this material has an ability to induce
the formation of octacalcium phosphate [7] a precursor of the bone mineral, hydroxyapatite. Secondly, because analysis on human retrieved Ti implants have revealed the incorporation of Ca and P in the oxide [12]. However, Johansson et al. [13] did not find a statistically significant difference in bone-metal contact between untreated and ion-implanted Ti-6Al-4V screw-shaped implants inserted in the proximal tibial metaphysis of rabbits. The fluence used was $5.7 \times 10^{17}$ ions/cm². Analysis of their data seems to indicate a tendency to obtain lower averages for the ion-implanted samples.

Conclusions

The following conclusions can be drawn from this work:

1 - Implantation of $10^{16}$ ions/cm² $N^+$ improves the corrosion resistance of Ti-6Al-4V alloy surfaces. This behaviour can probably be explained by the existence of a more stable oxide film formed during the ion implantation process.

2 - A fluence of $10^{15}$ ions/cm² does not bring any improvement.

3 - A fluence of $10^{17}$ ions/cm² $N^+$ is detrimental, probably due to the large size of titanium nitride precipitates and/or appearance of a carbonitride phase.

4 - Corrosion of Ti-6Al-4V alloy in HBSS produces a calcium- and phosphate-rich surface layer, but this process seems to be inhibited when the alloy is implanted with a $N^+$ ion fluence of $10^{17}$ ions/cm².
References

1. K. Merritt and S.A. Brown, Techniques in Orthopaedics, 8, 228-236 (1994)


8. T. Hanawa and M. Ota, Biomater., 12, 767-774 (1991)


Electrochemical and surface modifications on N\textsuperscript{+}-ion implanted Ti-5Al-2.5Fe immersed in HBSS

The electrochemical behaviour of nitrogen ion-implanted Ti-5Al-2.5Fe was studied in HBSS-Hank's balanced salt solution, by open-circuit potential versus time and cyclic polarization techniques. Three fluences (10\textsuperscript{15}, 10\textsuperscript{16} and 10\textsuperscript{17} ions/cm\textsuperscript{2}) were used. All ion-implanted surfaces showed higher corrosion resistance than the non-implanted ones. The corrosion potential of the ion-implanted samples was more noble and their passivation current was lower than the non-implanted ones. X-ray photo-electron spectroscopy showed that the immersion of Ti-5Al-2.5Fe in HBSS produces a Ca- and P-rich surface layer.

Introduction

Titanium and titanium-based alloys have been widely used in restorative surgery as dental and orthopaedic prostheses, pacemakers, heart valves, ear-drum drainage tube coatings, etc. [1, 2]. The success of Ti as a biomaterial is related to its high corrosion resistance, oxide layer structure and mechanical characteristics. The corrosion of Ti and its alloys results in the spontaneous formation of a stable oxide film ca. 4 to 5 nm thick that protects the surface. The
composition of this oxide film is dependent on the surface treatment, temperature and the environment in which it is formed. XPS studies showed that it is composed of a mixture of titanium oxides but the outermost layer is usually TiO₂. The contact surface between the implant and the surrounding tissues is of major importance. It is believed that the characteristics of the titanium oxide layer are responsible for the osseointegration of titanium [1, 3, 4, 5]. According to Tengvall et al. [1] an incorporation of inorganics, such as phosphorus coordinated with oxygen, sulphur or calcium, may be promoted. In fact, the presence of calcium and phosphorus has been detected on the surface of retrieved titanium implants [6]. Similar observations were made on Ti and Ti-6Al-4V when immersed in simulated body fluids [5, 7, 8].

In the ion-implantation process the interaction between the ions of the beam and the ions of the substratum results in the formation of intermetallic phases [9] that may improve the superficial mechanical resistance of the treated material. It is also a surface treatment that does not involve a significant rise in temperature of the substratum or alter the dimension of the implants allowing it to be used as a final treatment. Any element may be ion implanted and there is no interface between the ion-implanted area and the bulk material [10]. All these characteristics may induce an improvement in corrosion and wear resistance of the treated materials, by increasing the surface hardness and altering the characteristics of the passive layer. A significant increase in the wear resistance of Ti-6Al-4V hip prostheses has been achieved by nitrogen-ion implantation [11]. An increase in corrosion resistance has also been found when Ti-6Al-4V is ion implanted with 10¹⁶ ions/cm² of N⁺ [12].

In this work, the effect of several ion-implanted N⁺ fluences on the corrosion behaviour of Ti-5Al-2.5Fe in HBSS, has been studied. Electrochemical techniques, namely open-circuit potential versus time and cyclic polarization were used to determine which fluence would induce the best corrosion performance.
X-ray photo-electron spectroscopy (XPS) studies were also carried out, before and after the electrochemical experiments, in order to determine surface modifications induced by the ion-implantation process.

**Materials and Methods**

Samples of Ti-5Al-2.5Fe (Deutsch Titan) were obtained from a rod of 30 mm diameter. The rod was sliced into 2 mm thick specimens which were cut in quarters and ion implanted with three different fluences of nitrogen ions, namely: $10^{15}$, $10^{16}$ and $10^{17}$ ions/cm$^2$ with a beam energy of 40 keV. Table 1 compares the chemical composition of the Ti-5Al-2.5Fe and the Ti-6Al-4V alloys.

<table>
<thead>
<tr>
<th>Alloy</th>
<th>Al</th>
<th>V</th>
<th>Fe</th>
<th>O</th>
<th>H</th>
<th>N</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti-5Al-2.5Fe</td>
<td>3.0 - 5.0</td>
<td>—</td>
<td>2.0 - 3.0</td>
<td>0.2</td>
<td>0.015</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>Ti-6Al-4V</td>
<td>5.5 - 6.5</td>
<td>3.5 - 4.5</td>
<td>max. 0.25</td>
<td>0.2</td>
<td>0.013</td>
<td>0.07</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Table 1 - Chemical composition of the Ti-5Al-2.5Fe and Ti-6Al-4V alloys
(in % by weight)

Before the electrochemical experiments the samples were ultrasonically degreased with acetone, thoroughly washed with de-ionized water and dried.
The electrical connection was made via a copper electrical wire (diameter 0.5 mm) and by applying a solution of colloidal silver at the region of contact. The electrical junction was protected by polytetrafluorethylene tape, followed by the application of a non-conductive and water resistant varnish (Lacomit®), as described before [12].

The electrochemical cell was made of metilpenthen polymer and had a capacity of 150 mL. All experiments were performed at room temperature (18±2 °C) in HBSS (Gibco), which was de-aerated with a large flux of argon for at least 15 minutes before the tests. The electrolyte composition was the following (g/L): 0.185 CaCl₂, 0.4 KCl, 0.06 KH₂PO₄, 0.1 MgCl₂.6H₂O, 0.1 MgSO₄.7H₂O, 8.00 NaCl, 0.35 NaHCO₃, 0.48 Na₂HPO₄ and 1.00 D-glucose. All potentials were measured against the saturated calomel electrode (SCE) and are given on this scale. The counter electrode was a square platinum sheet of area 800 mm².

The electrochemical experiments were performed with an EG&G Princeton Applied Research, model 273 A, potentiostat. The potentials were controlled and the data were recorded with the help of software model M352, from EG&G Princeton Applied Research. Several electrochemical parameters were determined by the least squares method, employing successive iterations of the Stern-Geary equation. Monitoring of the potential as a function of time was carried out for 1 hour before initiating the potential sweep. In cyclic polarization measurements the potential-sweep rate was 10⁻³ V/sec starting 0.25 V below the corrosion potential, \( E_{\text{corr}} \).

After corrosion measurements, the samples were washed with de-ionized water and dried in air. The surfaces were observed by scanning electron microscopy (SEM) and analysed by XPS. XPS analysis was performed both on as-implanted samples and after performing polarization curves. A VG Scientific ESCALAB, using Mg Kα as source, was used to obtain the spectra.
Results and Discussion

Electrochemical studies

Fig. 1 shows curves obtained in the open-circuit potential versus time measurements. All nitrogen ion-implanted samples show an increase in the final potential ($E_{corr}$) compared to non-implanted ones. Ion-implanted surfaces with $10^{16}$ ions/cm$^2$ exhibit the highest $E_{corr}$. An increase of ca. 130 mV in potential was recorded for this fluence. For the lowest and highest fluences the potential change was not as significant. Increases in potential of ca. 90 mV for the $10^{15}$ ions/cm$^2$ fluence and 70 mV for the $10^{17}$ ions/cm$^2$ fluence were detected. These results may be explained by the formation of more stable films, as a consequence of the ion-implantation process.

Fig. 1. Open-circuit potential versus time measurements for Ti-5Al-2.5Fe not implanted and ion implanted with three fluences of N$^+$ at 40 keV: 
++ not-implanted; — $10^{15}$ ions/cm$^2$; •$10^{16}$ ions/cm$^2$; ×$10^{17}$ ions/cm$^2$
Fig. 2 shows curves obtained in the cyclic polarization measurements. All curves evolve similarly, with the reverse branch showing lower current densities than the forward branch due to the thickening of the oxide during the forward scan. Table 2 gives the electrochemical parameters taken from curves similar to those presented in figure 2.

![Graph](image)

Fig. 2. Cyclic polarization curves for Ti-5Al-2.5Fe ion implanted with three fluences of N\(^+\) at 40 keV:
- \(10^{15}\) ions/cm\(^2\);
- \(10^{16}\) ions/cm\(^2\);
- \(10^{17}\) ions/cm\(^2\)

As the corrosion current densities exhibited by all the materials were very low, comparison between the corrosion resistance was made using other essential parameters in these curves, namely: the passivation potential (\(E_{\text{pass}}\)) and the passivation current density (\(i_{\text{pass}}\)). \(E_{\text{pass}}\) is here defined as the potential after which no significant changes in the current (\(i_{\text{pass}}\)) are registered, as shown in Fig. 3. The protective characteristics of a passive film are revealed by its \(i_{\text{pass}}\). The lower the value the higher its protective properties.
Fig. 3 - Schematic representation of a cyclic polarization curve.

Analysis of the values given in Table 2 shows that ion-implantation modifies the electrochemical behaviour of the Ti-5Al-2.5Fe alloy. Ion-implanted Ti-5Al-2.5Fe is more corrosion resistant than the non-implanted material as shown by the lower \( i_{\text{pass}} \) exhibited by the ion-implanted samples. A significant change in the \( i_{\text{pass}} \) values as a function of the ion-implantation fluence was not observed.

<table>
<thead>
<tr>
<th>Fluence (ions/cm(^2))</th>
<th>( E_{\text{corr}}^a ) (V)</th>
<th>( E_{\text{pass}}^* ) (V)</th>
<th>( i_{\text{pass}}^* ) (A/cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-implanted</td>
<td>-0.123</td>
<td>1.7</td>
<td>4.1x10(^{-5})</td>
</tr>
<tr>
<td>( 10^{15} )</td>
<td>-0.028</td>
<td>0.81</td>
<td>6.0x10(^{-6})</td>
</tr>
<tr>
<td>( 10^{16} )</td>
<td>+0.01</td>
<td>1.18</td>
<td>7.7x10(^{-6})</td>
</tr>
<tr>
<td>( 10^{17} )</td>
<td>-0.059</td>
<td>1.5</td>
<td>6.5x10(^{-6})</td>
</tr>
</tbody>
</table>

\(^a\) measured in the open-circuit experiments

\(^*\)see text for definition
It is known that N\textsuperscript{+}-ion implantation on titanium induces the formation of titanium nitride and carbonitride precipitates [13, 14]. Their size and number are directly proportional to the N\textsuperscript{+}-ion implantation fluences up to 10\textsuperscript{17} ions/cm\textsuperscript{2} [13]. Earlier studies performed by our group on N\textsuperscript{+}-ion implanted Ti-6Al-4V [12] showed that an ion-implantation dose of 10\textsuperscript{16} ions/cm\textsuperscript{2} had a beneficial effect on the corrosion resistance of the material whereas a dose of 10\textsuperscript{17} ions/cm\textsuperscript{2} was detrimental. This detrimental effect was attributed to the existence of larger titanium-nitride precipitates and/or the appearance of carbonitrides. The present results may be attributed to the production of smaller and/or fewer nitride precipitates due to the N\textsuperscript{+}-ion implantation on Ti-5Al-2.5Fe at each dose. The coherence between the precipitates and the matrix may be good enough to avoid a detachment from the matrix and allow the formation of more protective films.

The presence of iron in the alloy may be responsible for the formation of these smaller titanium-nitride precipitates as this element, when ion-implanted with N\textsuperscript{+}, forms iron nitrides [15, 16, 17, 18] leaving fewer N\textsuperscript{+} ions available to form titanium-nitride precipitates.

**Surface Characterization**

Survey XPS spectra were acquired before and after corrosion measurements. Following spectra acquisition, peak identification and quantification were achieved using VG Scientific ESCALAB package software. To take into account some shift caused by charging of the sample surface all spectra were adjusted taking the C1s peak at 285.0 eV as reference for the adventitious carbon contamination.

All samples exhibited a well-defined C1s peak, which is usually a common contaminant. F was also detected on the ion-implanted samples before and after the electrochemical experiments. This is probably due to contamination during
the ion-implantation process as the concentration of this element decreased after the electrochemical experiments. Contamination with Ca was found in all as-implanted samples together with Si and Na.

Table 3 compares the XPS quantification data for samples after cyclic polarization. No iron was found which is in agreement with other studies [19, 20]. Ca and P were detected on all surfaces. The obtained [Ca]/[P] concentration ratios were either very low, 0.5 for the $10^{15}$ ions/cm$^2$ fluence and 0.4 for the $10^{16}$ ions/cm$^2$, or very high, 3.9 for the highest fluence. This suggests that if a calcium-phosphate compound is formed it is not likely to be apatite for which the [Ca]/[P] ratio is 1.67.

Recent *in vitro* work performed with titanium and Ti-6Al-4V indicates that these materials are capable of inducing the precipitation of calcium phosphates on their surfaces [5, 7, 8, 12, 21]. This feature may enhance the biocompatibility of these alloys.

Table 3 - XPS quantification data for the Ti-5A-2.5Fe ion-implanted alloy with $10^{15}$, $10^{16}$ and $10^{17}$ ions/cm$^2$ N$^+$ at 40 keV

<table>
<thead>
<tr>
<th>Fluence (ions/cm$^2$)</th>
<th>Ti (at%)</th>
<th>N (at%)</th>
<th>O (at%)</th>
<th>Ca (at%)</th>
<th>P (at%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{15}$</td>
<td>5.6</td>
<td>0.7</td>
<td>46.9</td>
<td>1.8</td>
<td>3.6</td>
</tr>
<tr>
<td>$10^{16}$</td>
<td>7.2</td>
<td>1.5</td>
<td>35.3</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>$10^{17}$</td>
<td>6.9</td>
<td>2.5</td>
<td>33.9</td>
<td>2.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Surface analysis of *in vivo* retrieved prostheses [1, 3, 6] showed the presence of Ca and P. Studies of the bone/titanium interface performed by Orr et al. [22] showed that the first layer to be deposited on the metal surface, as a result of cell processes, is a calcium-phosphate one. The presence of Ca and P on the surface may induce bone formation after *in vivo* insertion. Clinical results show that calcium phosphates promote the formation of bone tissue at their surface [23].

Conclusions

1 - Nitrogen-ion implantation of Ti-5Al-2.5Fe produces surfaces with lower $i_{\text{pass}}$ and higher $E_{\text{corr}}$ than the non-implanted ones.

2 - Ion implantation with $10^{16}$ ions/cm$^2$ produces surfaces with the noblest corrosion potential. All fluences produce surfaces with similar $i_{\text{pass}}$ indicating that the formed films have similar protective capacities.

3 - The corrosion of Ti-5Al-2.5Fe in HBSS produces a calcium- and phosphate-rich surface layer.

References


Electrochemical and surface modifications on N\textsuperscript{+}-ion implanted 316 L stainless steel

The effect of nitrogen-ion implantation on the electrochemical behaviour of 316 L stainless steel in a simulated physiological solution (HBSS-Hank’s Balanced Salt Solution) was studied by open-circuit potential versus time and cyclic polarization techniques, with the aim of characterizing the surfaces and choosing the best nitrogen-ion fluence. Three fluences (10\textsuperscript{15}, 10\textsuperscript{16} and 10\textsuperscript{17} ions/cm\textsuperscript{2}) were used. The 10\textsuperscript{16} ions/cm\textsuperscript{2} N\textsuperscript{+} fluence improves the corrosion resistance of the 316 L stainless steel.

Introduction

Metals such as stainless steel, titanium alloys and cobalt/chromium alloys are the materials most widely used as orthopaedic prostheses. Stainless steel has good mechanical properties, but has the lowest corrosion resistance among the most common metallic biomaterials [1-3].

The \textit{in vitro} corrosion of stainless steel in physiological solutions shows that
toxic ions such as Cr, Ni and Mo are present both in the solutions and in the corrosion products. Puleo et al. [4] performed a series of cytotoxicity tests on rat bone marrow stromal cells. The ranking of the ions with respect to their toxicity effects was: \( \text{Cr}^{6+} \succ \text{Mo}^{6+} \succ \text{Fe}^{3+} \succ \text{Co}^{2+} \succ \text{Ni}^{2+} \). The presence of these ions \textit{in vivo} may result in the appearance of local inflammatory reactions and ultimately in the loosening of the implant [5-7].

Studies performed on mice by Pereira et al. [8] indicate that metallic ions, resulting from the corrosion of stainless steel, accumulate in the liver and kidneys and are responsible for morphological changes in these organs. It is not possible to extrapolate this model to the human body but these results may indicate that the extent of the damage provoked by the corrosion of stainless steel implants may not occur immediately after implantation in the human body.

Wear of total joint replacements is responsible for the accumulation of debris around the implant, resulting in the long-term failure of these prostheses. The mechanism of aseptic loosening is not fully understood but it has been found that along with the metal debris, macrophages and T-lymphocytes are present around the implant. This indicates the close relationship between the cells and wear particles. It is therefore probable that improving the wear properties of the joint prostheses will improve their longevity [9, 10].

Ion implantation is a surface-treatment technique that modifies the materials' surface characteristics. Microstructural defects due to the interaction between the ions of the ion beam and the atoms of the target are produced in the latter. These defects may be responsible for modifications in wear and corrosion resistance and biocompatibility. High-fluence nitrogen ion implantation improves the tribological properties of titanium alloys [10]. It is known that iron nitride precipitates are formed as a result of the nitrogen ion-implantation process [11] and their presence is assumed to be responsible for the enhancement in wear resistance found on nitrogen ion-implanted iron [12].
Howlett et al. have studied the influence of several ion-implanted ions on silicon wafers [13] and Mg onto alumina [14]. In the first case, they found no evidence that ion-beam implantation with nitrogen, phosphorus, manganese or magnesium ions produces an increase in adhesion of human bone-derived cells to silicon substrata. Nevertheless, they found an increase in the cells’ attachment to surfaces which were ion implanted with oxygen. In the second case, they detected an increase in both attachment and spreading of human bone-derived cells on Mg ion-implanted alumina compared to the non-implanted surfaces. In vivo studies performed by Johansson et al. [9] on rabbit cortical bone showed no major differences between nitrogen ion-implanted titanium and Ti-6Al-4V and the non-implanted materials after three months insertion. Röstiund et al. [15] found no significant differences between nitrogen-ion-implanted and non-implanted titanium after 1 week of implantation into the abdominal wall of rats. It was found that nitrogen-ion implantation changed the biological properties of pure titanium only six weeks after the surgery.

The effect of several ion-implanted N+ fluences on the corrosion behaviour of 316 L stainless steel in HBSS has been studied in this work. Electrochemical techniques, namely open-circuit potential versus time and cyclic polarization, were used to determine which fluence would induce the best corrosion performance.

X-ray photo-electron spectroscopy (XPS) studies were also carried out, before and after the electrochemical experiments, in order to determine surface modifications induced by the ion-implantation process.

Materials and Methods

316 L stainless steel samples (Aubert & Duval - Z2CND17-12), 20 mm in diameter and 3 mm thick, were implanted with three different fluences of N+
ions, namely: $10^{15}$, $10^{16}$ and $10^{17}$ ion/cm$^2$, with a beam energy of 40 keV.

Before the electrochemical experiments the samples were ultrasonically degreased with acetone, thoroughly washed with de-ionized water and dried. The electrical connection was made via a copper electrical wire (diameter 0.5 mm) and by applying colloidal silver at the region of contact. The electrical junction was protected by polytetrafluorethylene tape, followed by the application of a non-conductive and water-resistant varnish (Lacomit®), as described in a previous work [16].

The electrochemical cell was made of metilpenthen polymer and had a capacity of 150 mL. All experiments were performed at room temperature (18±2 °C) in Hank’s Balanced Salt Solution (Gibco), which was de-aerated with argon for at least 15 minutes before the tests. The electrolyte composition was the following (g/L): 0.185 CaCl$_2$, 0.4 KCl, 0.06 KH$_2$PO$_4$, 0.1 MgCl$_2$.6H$_2$O, 0.1 MgSO$_4$.7H$_2$O, 8.00 NaCl, 0.35 NaHCO$_3$, 0.48 Na$_2$HPO$_4$ and 1.00 D-glucose. All potentials were measured against the saturated calomel electrode (SCE) and are given on this scale. The counter electrode was a square platinum sheet of area 800 mm$^2$.

The electrochemical experiments were performed with an EG&G Princeton Applied Research, model 273 A, potentiostat. The potentials were controlled and the data were recorded with the help of software model M352, from EG&G Princeton Applied Research. Several electrochemical parameters were determined using the least squares method, employing successive iterations of the Stearn-Geary equation. Monitoring of the potential as a function of time was carried out for one hour before initiating the potential sweep. In cyclic polarization measurements the potential sweep rate was $10^{-3}$ V/sec starting 0.25 V below the corrosion potential, $E_{corr}$. The reverse sweep finished when the downward curve was well defined.

After the corrosion measurements, the samples were washed with de-ionized water and dried in air. The surfaces were observed by scanning electron
microscopy (SEM) and analysed by XPS. XPS analysis was performed both on as-implanted samples and after corrosion measurements. A VG Scientific ESCALAB, using Mg Kα as source, was used to obtain the spectra.

Results and Discussion

Electrochemical studies

Fig. 1 gives curves obtained in the open-circuit measurements. A non-implanted surface was used as reference. The figure shows that ion implantation with $10^{15}$ ions/cm² has no significant influence on the final potential. Fluences of $10^{16}$ and $10^{17}$ ions/cm² displace the curves towards more noble potentials. A dose of $10^{16}$ ions/cm² produces the highest shift in potential. This may be due to the existence of more noble surface layers, formed during the ion-implantation process.

Fig. 2 shows the cyclic polarization curves. All materials, including the non-implanted stainless steel, show very low corrosion current densities in the range of nA/cm². Fig. 3 shows a schematic representation of a polarization curve where some parameters are signalled, namely: the passivation potential, $E_{\text{pass}}$, the passivation current density, $i_{\text{pass}}$ and the pitting potential, $E_p$. 
Fig. 1 - Open-circuit potential versus time curves for 316 L stainless steel not implanted and ion-implanted with three fluences of $N^+$ at 40 keV:
- o - not-implanted; — $10^{15}$ ions/cm$^2$; • $10^{16}$ ions/cm$^2$; × $10^{17}$ ions/cm$^2$

Fig. 2 - Cyclic polarization curves for 316 L stainless steel not implanted and ion-implanted with three fluences of $N^+$ at 40 keV:
— $10^{15}$ ions/cm$^2$; • $10^{16}$ ions/cm$^2$; × $10^{17}$ ions/cm$^2$
Fig. 3 - Schematic representation of a polarization curve

Observing the curves presented in Fig. 2, one notices that the lowest fluence gives a first anodic current density maximum ($E_{\text{pass}}$) at -0.28 V, with a passive step up to 0 V. Between 0 V and 0.5 V, a rise in current was detected which was probably due to localized corrosion. Above this point, transients in current were detected. This indicates the initiation and recovery of pits formed on the surface. Above 0.75 V ($E_p$) a sharp rise in current was observed as a result of pitting attack. The currents detected on the reverse scan were higher than those on the forward scan, indicating corrosion of the surface. SEM observations, after the electrochemical experiments, showed the presence of pits on the surface. Similar electrochemical behaviour was revealed by the ion-implanted materials with the highest dose. In this case $E_{\text{pass}}$ was not evident and the transition potential to the passive state could not be determined. The $E_p$ was lower than in the samples implanted with $10^{15}$ ions/cm$^2$. SEM observations of ion-implanted materials with $10^{16}$ ions/cm$^2$ showed the presence of no pits. The cyclic polarization curves showed that the recorded currents on the reverse scan were lower than those on the forward scan. The highest potential reached before a sharp rise in current was detected at 0.96 V. This potential was considered as the "pitting potential" to allow a comparison with the other materials. At this potential, the
$10^{16}$ ions/cm$^2$ sample exhibited the lowest current density of the three ion-implanted samples.

The comparison between the corrosion resistance of these materials can be made by comparing essential potentials in these curves [17], namely the passivation potential and the pitting potential as schematically shown in Fig. 3.

The protective characteristics of a passive film are shown by the value of $i_{\text{pass}}$. The lower this value the higher are its protective properties. Comparing the values of $E_p$ between these materials gives information on their resistance to localized attack. A material's resistance to localized attack is directly proportional to the value of $E_p$.

The values, taken from curves similar to those in Fig. 2, are given in Table 1. The corrosion potential, $E_{\text{corr}}$, and the passivation current, $i_{\text{pass}}$, are also given and the values obtained are the average of two results.

<table>
<thead>
<tr>
<th>Fluence (ions/cm$^2$)</th>
<th>$E_{\text{corr}}$ $^a$ (V)</th>
<th>$E_{\text{pass}}$ (V)</th>
<th>$E_p$ (V)</th>
<th>$i_{\text{pass}}$ (A/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-implanted</td>
<td>-0.17</td>
<td>-</td>
<td>0.85</td>
<td>2.8x10^{-6}</td>
</tr>
<tr>
<td>$10^{15}$</td>
<td>-0.15</td>
<td>-0.29</td>
<td>0.75</td>
<td>4x10^{-6}</td>
</tr>
<tr>
<td>$10^{16}$</td>
<td>-0.028</td>
<td>-0.25</td>
<td>0.96</td>
<td>2x10^{-6}</td>
</tr>
<tr>
<td>$10^{17}$</td>
<td>-0.065</td>
<td>-</td>
<td>0.38</td>
<td>4x10^{-7}</td>
</tr>
</tbody>
</table>

$^a$ measured in the open-circuit experiments

Analysis of the values given in Table 1 shows that $N^+$-ion implantation does not change the corrosion resistance significantly. The $E_p$ were similar on all studied materials except for the highest fluence where a decrease in $E_p$ of ca. 0.50 V was registered. On the other hand, the highest fluence exhibited the lowest
$i_{\text{pass}}$. According to Rauschenbach et al. [18] implantation of $N^+$ in Fe results in crystalline phases in the range $1 \times 10^{16}$ to $1 \times 10^{18}$ ions/cm$^2$. $\gamma$-austenite appears in the entire range, but from fluences higher than $4 \times 10^{16}$ ions/cm$^2$ other nitride phases appear simultaneously, namely $\alpha''$-Fe$_{16}$N$_2$, $\varepsilon$-Fe$_2$N$_{1-x}$ and $\alpha'$-martensite [18, 19]. The presence of $\gamma$-austenite was also reported by Carbucicchio et al. [20] for fluences in the range of $5 \times 10^{16}$ to $1 \times 10^{17}$ ions/cm$^2$. Other authors have reported the formation of other nitride phases in steel but all these studies were conducted with ion-implantation doses higher than $2 \times 10^{17}$ ions/cm$^2$ [21, 22].

The nature of the precipitates formed during nitrogen-ion implantation varies with the carbon concentration and also with the implanted dose. It seems that the carbon concentration and the dose of nitrogen implantation are two competitive factors, whose combination determines the total amount of iron nitrides that will be formed [22]. Results for stainless steel show that the amount and size of nitrides in the as-implanted material increase with the dose, the proportion of nitrides in stainless steel being almost the same as in pure iron at each dose [12, 18, 22].

The results obtain herein may be explained by the formation of iron nitrides as a consequence of the ion-implantation process. A joint effect between the formation of $\gamma$-austenite, $\alpha''$-Fe$_{16}$N$_2$, $\varepsilon$-Fe$_2$N$_{1-x}$ and $\alpha'$-martensite and an increase in size of the iron nitrides may explain the low $E_p$ exhibited by the $10^{17}$ ions/cm$^2$ samples. Larger precipitates will tend to detach from the metallic matrix increasing the area of attack. For the $10^{15}$ ions/cm$^2$ the $\gamma$-austenite formed may be coherent with the metallic matrix in such a way that no significant changes in the electrochemical parameters have been registered. Ion implantation with $10^{16}$ ions/cm$^2$ produces a rise towards more noble potentials of the $E_p$ and a slight reduction of the $i_{\text{pass}}$ compared to that of the non-implanted material. Changes in the oxide film composition may explain both these results and the fact that no pits were seen after the cyclic polarization experiments.
Surface Characterization

Survey spectra were acquired before and after corrosion measurements. Following spectra acquisition, peak identification and quantification were achieved using VG Scientific ESCALAB package software. All spectra were calibrated using C1s binding energy, Eb, C1s = 285.0 eV, as reference. All samples exhibited a well defined C1s peak, which is usually a common contaminant.

Table 2 compares the XPS quantification data for samples which were submitted to corrosion testing. Calcium and phosphorus were detected on all surfaces after corrosion testing but the highest concentrations were found for the $10^{16}$ ions/cm$^2$ fluence. On all surfaces a higher concentration of P than Ca was detected.

<table>
<thead>
<tr>
<th>Fluence (ions/cm$^2$)</th>
<th>Fe (at%)</th>
<th>Cr (at%)</th>
<th>Ni (at%)</th>
<th>N (at%)</th>
<th>O (at%)</th>
<th>Ca (at%)</th>
<th>P (at%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{15}$</td>
<td>1.41</td>
<td>3.18</td>
<td>—</td>
<td>2.75</td>
<td>37.19</td>
<td>1.34</td>
<td>1.67</td>
</tr>
<tr>
<td>$10^{16}$</td>
<td>2.67</td>
<td>0.2</td>
<td>0.2</td>
<td>1.6</td>
<td>42.7</td>
<td>3.16</td>
<td>8.62</td>
</tr>
<tr>
<td>$10^{17}$</td>
<td>1.08</td>
<td>3.38</td>
<td>—</td>
<td>0.98</td>
<td>41.3</td>
<td>0.75</td>
<td>3.41</td>
</tr>
</tbody>
</table>

Electrochemical experiments performed on 316 L stainless steel by Sousa et al. [23] in calcium phosphate and protein solutions showed that there was an inhibition of corrosion caused by the presence of calcium and phosphate ions. It was determined that these ions influence the kinetics of film growth and its thickness. On solutions where Ca and P were present a rise of the $E_p$ was noticed. The increase in thickness is consistent with increased breakdown potentials,
since thicker films should require higher electrode potentials. The rupture of the passive films depends on a series of factors, namely their composition, structure and thickness. It was also determined that calcium phosphate acts as an inhibitor of pit propagation.

It seems that the material surface structure resulting from nitrogen-ion implantation with $10^{16}$ ions/cm² allows the formation of more protective films whose composition, formed during polarization, is richer in Ca and P. The incorporation of these ions on the film formed during the anodic polarization may explain the high potentials attained without film breakdown. The presence of these ions in the surface film may interfere with the *in vivo* behaviour of the stainless-steel implants as it is known that calcium phosphates enhance the bone formation at their surface [24].

**Conclusions**

Nitrogen-ion implantation with $10^{16}$ ions/cm² slightly improves the corrosion resistance of 316 L stainless steel. The formation of more stable films as a consequence of the ion-implantation process may explain this behaviour.

Fluences of $10^{15}$ ions/cm² do not bring any improvement and $10^{17}$ ions/cm² reduces the $i_{pass}$.

Corrosion of 316 L stainless steel in HBSS produces a calcium- and phosphate-rich surface layer.
References


Chapter 3

Electrochemical characterization of sputter-coated 316 L stainless steel

3 - Electrochemical impedance spectroscopy of nitrogen and carbon sputter-coated 316 L stainless steel
Electrochemical impedance spectroscopy of nitrogen and carbon sputter-coated 316 L stainless steel

The effect of nitrogen and carbon sputter coatings on the electrochemical behaviour of 316 L stainless steel in a simulated physiological solution (Hank’s Balanced Salt Solution) was studied by electrochemical impedance spectroscopy with the aim of characterizing the surfaces and choosing the best coating. Coatings with different thicknesses and structures were tested. The results showed that thicker sputter coatings produce surfaces with higher charge transfer resistance and lower interface capacities. This indicates that these materials probably owe their high corrosion resistance to the formation of more protective films.

Introduction

Orthopaedic alloys such as stainless steel and titanium alloys owe their good corrosion resistance to the spontaneous formation of a passivating film on their surfaces. The structure and composition of these films are crucial for the successful integration of an implant in the tissues as they constitute the surface where cells will be attached. Controlling the surface characteristics implies controlling the biological response [1]. Gomi et al. [2] showed that the roughness
of a polystyrene surface influences the amount and spatial distribution of bone formed from a bone marrow cell culture brought into contact with it. Similar studies performed on titanium with different surface roughnesses showed that not only roughness but also the surface topography are important in the biological performance [3, 4]. Cell cultures performed on different substrata also exhibit different levels of cell response. Titanium and titanium alloys seem to be better substrata for osteoblast proliferation than stainless steel or Co-Cr alloys [5, 6] and bioactive glass showed the best results when compared to hydroxyapatite, stainless steel and Ti-6Al-4V [7]. Modifying a biomaterial surface by means of thermal treatment, ion implantation, sputter coating, plasma-spraying or any other surface treatment may enhance or decrease its biocompatibility. An increase in bone growth was detected when heat-treated titanium screws were implanted in rats [8]. Cell culture tests performed on plasma-sprayed hydroxyapatite with different crystallinities showed that the degree of crystallinity has a direct effect on the bone formation rate and bonding strength between the hydroxyapatite and the bone tissue [9]. In vivo tests performed by Röstlund et al. [10] in rats showed that nitrogen ion implantation changes the surface properties, and therefore the biological properties, of pure titanium. Surface modification thus seems to be the key to the control of biological responses [1].

Sputter coating is a surface modification technique in which an element can be deposited on a substratum. An electric discharge between an anode (the grounded chamber walls) and a cathode, made up of the material to be deposited, results in the ionization of the surrounding gas. The gas ions are then accelerated by the electric field of the target and their impact results in the sputtering of the target. The vapour thus created condenses on the surfaces of the reactor, especially on the substrata which face the target.

The work described in this paper is part of a project, in which our laboratories have been involved, where corrosion, mechanical characterization and
biological evaluation are being studied for several surface modification techniques. This research focused on the corrosion characterization of 316 L stainless steel surfaces modified by nitrogen and carbon sputter coating. The medium used was Hank's balanced salt solution (HBSS). Electrochemical-impedance spectroscopy was used to characterize the surfaces. This technique allowed the determination of several electrochemical parameters such as the charge transfer resistance ($R_{te}$) and the interface capacity ($C_i$). The detection of pitting was achieved by acquiring impedance spectra at d.c. potentials higher than the pitting potential. It was found that thicker sputtered coatings tend to produce surfaces with higher corrosion resistance.

Theoretical Synthesis

The electrochemical cell, because it presents an impedance to a small sinusoidal excitation, may be represented by an equivalent electrical circuit [11]. A typical circuit of a corroding electrode may be electrically described as a parallel RC circuit where the resistance mimics the faradaic process and the capacitor the double-layer capacitance. The effect of the solution resistance is taken into account by adding a further resistor in series. It is possible to interpret and calculate the corrosion parameters by using this analogy.

The most common way to represent the impedance of an electrode is by plotting the imaginary part as a function of the real part. This is known as the Nyquist representation. The solution resistance, $R_\Omega$, is calculated by the interception of the curve with the x-axis at high frequencies. In the Nyquist plot the RC circuit gives a response represented by a semi-circle whose radius is the charge transfer resistance, $R_{te}$. The interface capacitance, $C_i$, is calculated by the expression:
\[ C_i = \frac{1}{\omega_{\text{max}} R_{\text{tc}}} \]

where \( \omega_{\text{max}} \) is the frequency at which the imaginary part is maximum.

For highly passive orthopaedic alloys other data plots provide more experimental convenience and reliability of data [12]. The Bode representation consists of a plot of the log (frequency) versus log |Z|, where |Z| is the modulus of the impedance vector Z. On this representation, it is usual to plot simultaneously the variation of the phase angle. This allows the detection of maxima in electrochemical processes that exhibit several time constants. Other advantages of the Bode plot are less sensitivity to electrochemical noise, especially at low frequencies, and simplification of the analysis of Nyquist plots, for which data are difficult to interpret. \( R_{\Omega}, R_{\text{tc}} \) and \( C_i \) can be obtained from the Bode plot. The first two parameters may be found, respectively, by fitting the high- and low-frequency values to a straight line and intersecting it with the y-axis. \( C_i \) is calculated by use of the equation: \( |Z|_{\omega=1} = 1/C_i \).

The software used to acquire and analyse the data allows the representation of the data by both Nyquist and Bode plots. The calculation of \( R_{\Omega} \) and \( R_{\text{tc}} \) is achieved by fitting a semi-circle to the data plotted in the Nyquist representation.

**Materials and Methods**

Samples of 316 L stainless steel (Aubert & Duval Z2CND17-12), 20 mm in diameter and 3 mm thick, were cut in half and then nitrogen- and carbon-sputter coated.

Before the electrochemical experiments the samples were ultrasonically degreased with acetone, thoroughly washed with de-ionized water and dried. The electrical connection was made via a copper electrical wire (diameter 0.5
mm) and by applying a solution of colloidal silver at the region of contact. The electrical junction was protected by polytetrafluorethylene tape, followed by the application of a non-conductive and water resistant varnish (Lacomit®), as indicated in a previous paper [13].

The electrochemical cell was made of metilpenthen polymer and had a capacity of 150 mL. All experiments were performed at room temperature (18±2 °C) in HBSS (Gibco), which was de-aerated with a large flux of argon for at least 15 minutes before the tests. The electrolyte composition was the following (g/L): 0.185 CaCl2; 0.40 KCl; 0.06 KH2PO4; 0.10 MgCl2.6H2O; 0.10 MgSO4.7H2O; 8.00 NaCl; 0.35 NaHCO3; 0.48 Na2HPO4; 1.00 D-glucose. All potentials were measured against the saturated calomel electrode (SCE) and are given on this scale. The counter electrode was a square platinum sheet with an area of 800 mm².

Monitoring of the potential as a function of time was carried out 1 hour before starting the electrochemical impedance measurements. The impedance measurements were performed at the following d.c. potentials: corrosion potential measured 1 hour after immersion, 100 mV, 500 mV, 700 mV and 1000 mV. Some samples were also subjected to 1500 and 2000 mV. These potentials were chosen in order to investigate the material response as a function of the d.c. polarization. It has been assumed that the electrode reaches its resting potential 1 hour after immersion.

The amplitude of the applied sinusoidal wave was 10 mV and all the experiments were performed by scanning from higher to lower frequencies, beginning at 10 kHz and ending at 10 mHz. A high impedance voltmeter was used to measure the potential as a function of time. A frequency response analyser (Solartron 1250) and a potentiostat (Thompson and Ass.) were used to perform the impedance tests. The monitoring of the experiments, data acquisition and treatment were made by IMPFRA - Impedance Software [14].

After the corrosion tests some surfaces were observed with a scanning electron microscope.
Results and Discussion

Table 1 shows the results obtained from the a.c. impedance measurements. N and C, in this table, indicate that the samples were sputter-coated with nitrogen and carbon, respectively. N2 samples have thicker sputter coatings than N1 samples and are similar, in thickness, to C3 and C4 samples. The thickness ranking of the carbon-sputter coatings are as follows: C1 > C2 > C3 = C4. C3 and C4 surfaces have the same characteristics except for the later vacuum impregnation (after sputter coating) with POLYTROL of the C4 samples [15]. POLYTROL is a polymeric solution. Each measurement is the average of two tests.

Table 1 - Summary of the electrochemical impedance parameters for d.c. potentials after 1 hour immersion

<table>
<thead>
<tr>
<th>Sample</th>
<th>E (mV)</th>
<th>RΩ (Ω.cm²)</th>
<th>Rtie (Ω.cm²)</th>
<th>Ci (F.cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>-40</td>
<td>11</td>
<td>118350</td>
<td>1.1E-4</td>
</tr>
<tr>
<td>N2</td>
<td>-5</td>
<td>13</td>
<td>146330</td>
<td>9.2E-5</td>
</tr>
<tr>
<td>C1</td>
<td>2</td>
<td>11</td>
<td>130102</td>
<td>1.0E-4</td>
</tr>
<tr>
<td>C2</td>
<td>0</td>
<td>10</td>
<td>117942</td>
<td>1.1E-4</td>
</tr>
<tr>
<td>C3</td>
<td>-15</td>
<td>10</td>
<td>118103</td>
<td>1.6E-4</td>
</tr>
<tr>
<td>C4</td>
<td>17</td>
<td>10</td>
<td>133387</td>
<td>1.0E-4</td>
</tr>
</tbody>
</table>

Except for RΩ, the values shown in this table were calculated from the Bode representation. The value of RΩ obtained is 10 ± 2 Ω.cm². In the Nyquist plot, data could not be fitted to a semi-circle when applying the d.c. potential measured after 1 hour of immersion. Comparing the Rtie values on Table 1, one observes that the higher value is attained for the N2 coating. Carbon sputter coatings C1 and C4 show the highest Rtie values for this type of coating. This indicates that these surfaces are more corrosion resistant, probably due to the formation of thicker protective films. In fact, the Ci values obtained for these
surfaces are lower, which indicates that thicker films were formed. This can only be inferred assuming that there is no contribution from the double layer [16].

Fig. 1 shows experimental Bode plots obtained for a N2 sample at different applied d.c. potentials. Up to 1000 mV all curves evolve identically, showing a first plateau at high frequencies followed by an increase in slope until a second plateau at low frequencies is reached.

![Bode plots](image)

**Fig. 1 - Bode plots obtained for a N2 sample at d.c. potentials of:**
- $-6$ mV
- $100$ mV
- $500$ mV
- $1000$ mV
- $1500$ mV and $2000$ mV

This electrochemical response can be modelled by an equivalent electrical circuit similar to that previously described. The slope of the straight line between the plateaux is $-0.91 \pm 0.1$ which indicates that the response is almost purely capacitive at these frequencies. A pure capacitive response shows a slope of $-1$. The electric equivalent circuit previously mentioned may also be used to model these corroding surfaces. Bundy *et al.* [12] performed a.c. impedance tests on 316L stainless steel, Ti-6Al-4V and Co-Cr-Mo alloys. The results showed that a similar electric equivalent circuit could fit the data obtained. A.c. tests performed on passivated titanium by Sousa *et al.* [17] also revealed a characteristic RC
behaviour. For applied d.c. potentials $\geq 1000$ mV the values of $R_{tc}$ decrease and $C_i$ increases with increasing applied potential, indicating a decrease in corrosion resistance and a less thick, and therefore less protective, passivating film. This behaviour was observed for all the samples tested. The electric equivalent circuit that may represent the corrosion of a N2 surface at d.c.$=2$V is a series of two resistors: $10 \ \Omega$ ($R_{\Omega}$) $+$ $8 \ \Omega$ ($R_{tc}$). The curve is almost parallel to the x-axis which indicates an almost pure resistive interface.

Fig. 2 shows a Nyquist plot obtained for a C1 sample. It is clear that with increasing applied d.c. potential the data are more clearly defined by a semi-circle whose radius is decreasing. This indicates a reduction in corrosion resistance with increasing applied potential.

![Nyquist plot](image)

Fig. 2 - Nyquist plot obtained for a C1 sample at d.c. potentials of:

--- $\bullet$ --- 2 mV, ---$+$--- 100 mV, $\circ$ --- 500 mV, $\bullet$ --- 700 mV and $-$ 1000 mV

This behaviour may be explained by assuming that the surfaces after 1 hour immersion are in the passive state. The applied d.c. potentials, up to 1V, fall in the passive region as the slopes obtained on the Bode plots are similar to pure
capacitor responses. As the applied potential increases from 100 mV to 700 mV, \( R_{tc} \), \( C_i \) and the value of the slopes do not change significantly. This indicates that the materials are still in the passive state. The application of d.c. potentials higher or equal to 1000 mV drives the surfaces to a transpassive region, causing a decrease in the corrosion resistance. In fact, cyclic polarization measurements performed on C3 and C4 samples showed that their pitting potential was *ca.* 800 mV.

Comparing the \( R_{tc} \) values in Table 1 one observes that the surfaces with thicker sputter coatings exhibit higher corrosion resistance. POLYTROL impregnation also seems to be an effective treatment as it raises the \( R_{tc} \) value up to the typical \( R_{tc} \) obtained with thicker coatings.

**Conclusions**

1. Thicker coatings seem to produce materials with higher corrosion resistance. POLYTROL impregnation after carbon sputter increases the corrosion resistance.

2. The high \( R_{tc} \) attained by the N2 surfaces is accompanied by the lowest \( C_i \). The surface film produced is then the most protective.

3. Applied d.c. potentials \( \geq 1 \) V decrease \( R_{tc} \) significantly. Pitting potential is between 700 and 1000 mV. The impedance response obtained by applying 1000 mV showed that at this potential the material is in the transpassive region, therefore the pitting potential is lower than 1 V.

4. The corrosion interface of nitrogen- and carbon-sputter coated 316 L stainless steel, up to 1 V, may be represented by a three-element equivalent electric circuit. The comparison between the experimental Bode curves and a curve obtained from a response from a built-up three element electric circuit, as described before, show their similarity.
References


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Chapter 4

Biocompatibility testing of surface-modified biomaterials

4.1 - *In vitro* testing of surface-modified biomaterials

4.2 - XPS characterization of surface films formed on surface-modified implant materials after cell culture
In vitro testing of surface-modified biomaterials

The influence of surface modification treatments such as ion implantation and sputter coating on an in vitro rat bone-marrow cell culture was studied by scanning electron microscopy and X-ray microanalysis. 316 L stainless steel, Ti-6Al-4V and Ti-5Al-2.5Fe were nitrogen ion-implanted with three fluences: $10^{15}$, $10^{16}$ and $10^{17}$ ion/cm$^2$ with an energy beam of 40 keV. Both nitrogen- and carbon-sputter-coated 316 L stainless steel samples were also studied. Polished 316 L stainless steel, Ti-6Al-4V, Ti-5Al-2.5Fe and Thermanox™ were also studied in order to give comparative information. The materials were inoculated with a droplet of cell suspension and were maintained for 3 weeks. A mineralized extracellular matrix was formed on all materials except on nitrogen sputter-coated 316 L stainless steel. The morphology of the cell cultures obtained on nitrogen-ion implanted materials was similar to those obtained on the untreated materials and Thermanox™. The observation of the interface between the cell layer and the substrata showed the presence of Ca- and P- rich globular deposits associated with collagen fibres. A higher density of these globular deposits was observed on the ion-implanted materials.

Introduction

Biological evaluation is crucial when testing materials for human use. Testing the biocompatibility of a specific material implies its rejection if it shows toxic
effects on cells. In vitro and in vivo tests can assess the biocompatibility of a material. Although in vitro tests do not avoid in vivo testing [1, 2, 3] they are generally accepted as a first-order method to test for toxicity of a material. Evaluation of several in vitro parameters such as cell death, cell adhesion, cell morphology, cell proliferation and biosynthetic activity may give an indication of the toxicity of the material [1]. Cell culture tests may also provide important information when studying the biomaterial/living tissue interface since they are studied in a more controlled environment than in an in vivo situation [4]. On the cell culture of osteoblasts it was observed that their behaviour mimics that of their counterparts in vivo [5]. This implies that the extracellular matrix formed is similar to that produced in vivo. It was suggested by Davies et al. [5] that these methods are more suitable for the investigation of interfacial reaction with modified material surfaces.

It is known that surface modification of biomaterials may promote desirable reactions, such as an increase in cell adhesion, or prevent undesirable effects such as the triggering of blood coagulation [1]. Controlling the surface characteristics implies controlling the biological response [6]. An increase in biocompatibility was detected when human venous endothelial cells were cultured on polycarbonate urethanes which were modified by introducing hydroxyl groups into the polymer [7]. Howlett et al. [8] investigated the effect of ion implantation with N, P, Mn and Mg ions on silicon single crystals and found no evidence that this surface treatment produced an increase in adherence of human bone-derived cells to silicon substrata. On the other hand, cells were preferentially attached to O ion-implanted surfaces. The ion implantation of Mg onto alumina was studied by Howlett et al. [9] and the results showed that the attachment and spreading of human bone-derived cells was enhanced compared to non-implanted alumina. The cells showed different morphology, being better spread and more voluminous, suggestive of higher metabolic and synthetic activity.
Similar results were obtained by Lee et al. [10] when bovine aorta endothelial cells were cultured on polyurethane which was ion implanted with Ne⁺.

In vivo tests performed by Röstlund et al. [11] in rats showed that nitrogen-ion implantation changes the surface properties, and therefore the biological properties, of pure titanium. They found that up to 6 weeks after implantation, the cell number and structure were the same around ion-implanted and non-implanted implants but, after 6 weeks, differences around the two types of implants began to appear. The numbers of macrophages and giant cells were significantly larger close to the ion-implanted materials. According to the same authors, these results could be due to changes in the physicochemical surface properties of the ion-implanted implants, as all surface-treated and untreated materials had similar surface morphologies. Nevertheless, Johansson et al. [12] studied the influence of nitrogen ion-implanted Ti cp. and Ti-6Al-4V on the cortical bone of rabbits and detected no difference after 3 months' insertion.

The work here described is part of a study in which titanium alloys and stainless steel were surface modified by ion-implantation and sputter-coating techniques in order to improve their performance as implant materials. The surface-modified materials were previously characterized for their corrosion behaviour and this research is aimed at evaluating their biocompatibility. The surface-modified samples that produced better in vitro corrosion resistance were inoculated with rat bone marrow cells in order to study the surface/cell culture interface and to evaluate their biocompatibility. The osteoblast-cell culture was chosen because the materials in this study are being developed with the objective of bone implantation. Scanning electron microscopy (SEM) was used to observe the surfaces of the materials and the tissue immediately adjacent. X-ray microanalysis (XRMA) was used whenever appropriate.
Materials and Methods

Cell substrata

Surface-modified materials

316 L stainless steel (Aubert and Duval), Ti-6Al-4V and Ti-5Al-2.5Fe samples (Deutsch Titan) were cut from rods of 20 mm, 15 mm and 30 mm in diameter, respectively, and were ion implanted with three different fluences of nitrogen ions, namely: 10^{15}, 10^{16} and 10^{17} ions/cm² with a beam energy of 40 keV.

Samples of 316 L stainless steel (Aubert and Duval), were sliced from a rod of 20 mm in diameter and then nitrogen- or carbon- sputter coated (samples N2 and C1 from chapter 2).

All samples were ultrasonically cleaned in 90% ethanol for 20 min., followed by a 20-min. double rinse with distilled water before steam sterilization [13].

Untreated materials

Samples of 316 L stainless steel, Ti-6Al-4V and Ti-5Al-2.5Fe were cut, ground on SiC paper and polished down to 1μm in diamond paste. The surface degreasing and cleaning was performed using the same protocol as for the surface-modified materials.

Thermanox™ coverslips (polyethyleneterephthalate), of 13 mm diameter, were used as a control substratum.

Cell Culture

The samples were steam sterilized before cell culture for 20 minutes. A primary droplet rat bone marrow culture (RBMC) was performed according to the method described by Maniotopoulos et al. [14] which induces an osteoblast-enriched cell population.
Young adult male Wistar rats, of about 100-120 g, were sacrificed. The epiphyses were removed and the bone marrow was flushed out from each dyaphise with α-minimum essential medium (Gibco), 15% foetal calf serum (Gibco) and 10% antibiotics (amphotericin B (Sigma), penicillin G (Sigma) and gentamycin (Gibco)). The cells were then grown on this medium to which 1% 1M Na-β-glycerophosphate (Sigma), 1% 5 mg.mL⁻¹ ascorbic acid (Sigma) and 1% 10⁻⁸ M dexamethasone (Sigma) were added. A droplet culture of the bone marrow cell suspension was placed on every sample. The cell culture was performed either in 6- or 24-well tissue culture plates (Costar) depending on the sample's dimension and kept in a humidified atmosphere of 95% air - 5% CO₂ at 37 °C. The cultures were observed, every 48 hours, with a light microscope. The cultures were maintained for 19 days and then prepared for observation with SEM.

The specimens were then fixed overnight, at room temperature, in 1.5% glutaraldehyde in the same buffer. Dehydration in a graded series of ethanol and critical point drying (Balzers CPD 030) from carbon dioxide followed. The specimens were gold sputter coated, observed by SEM and analysed with XRMA at accelerating voltages of 15 kV and 20 kV, respectively.

**Results and Discussion**

*Thermanox™ culture*

Figs. 1a and 1b show the surface of the cell layer on Thermanox™. Fig. 1a presents the morphology of the osteoblasts cultured on Thermanox™. A compact cell layer was formed on top of the materials. Cells exhibit a morphology typical of osteoblasts [15, 16]: a polygonal shape with dorsal ruffles and cell processes. This indicates that the cells have a normal level of activity. Round-shaped cells can also be observed which usually indicates bad condition. It can
Fig. 1 - SEM photomicrographs of osteoblasts cultured on Thermanox™. (a) surface of the cell layer where osteoblasts (arrow) and collagen fibres can be identified. (b) higher magnification photomicrograph showing the interface between the cell layer and the substratum where globular deposits (arrow) can be easily identified.
also be seen that the extracellular matrix (ECM) is mainly composed of collagen fibres in which small globules are visible. XRMA of these globules show that they are rich in Ca and P. Fig. 1b shows a higher magnification of the cell layer where a crack due to critical-point drying allowed the observation of the interface between the cell tissue and the Thermax™. On the surface of the substratum, globular deposits can be seen. In order to expose the interface fully, the cell layer was mechanically detached. A net of collagen fibres enveloping the globules and flat cells could be observed on the cell layer that was adjacent to the metal surface. On the Thermax™, remains of attached ECM with globules could be observed as well as negative imprints from the globules that were detached with the cell layer.

Untreated materials

Figs. 2 to 5 represent SEM photomicrographs of several aspects of the RBMC on 316 L stainless steel, Ti-6Al-4V and Ti-5Al-2.5Fe. The cell cultures performed on these metals showed a similar morphology to each other, and also to those observed for the cultures performed on Thermax™. The upper layer of the cell culture showed the presence of polygonal and round cells with extensive cell processes. The ECM seems to be composed mainly of collagen fibres. The detachment of the cell layer from the substratum showed the presence of the Ca- and P-rich globules trapped in a complex net of collagen. Flattened cells were also visible. The structure of the RBMC on Ti-5Al-2.5Fe showed, in certain areas, clusters of polygonal cells, with a pronounced cell body and intense filapodia well attached to the ECM. From their morphology they appear to be osteocytes. The exposure of the metal/cell layer interface showed the presence of a high density of calcified globules.
Fig. 2 - SEM photomicrograph of the cell layer surface on 316 L stainless steel after 19 days of culture showing polygonal cells (arrow), round cells (arrow head) and collagen fibres.
Fig. 3 - SEM photomicrograph of Ti-6Al-4V after 19 days of cell culture. The detachment of the cell layer shows the existence of globular deposits (arrow) where collagen fibres are incorporated (arrow head).
Fig. 4 - SEM photomicrograph of a RBMC cell layer surface on Ti-5Al-2.5Fe showing the presence of osteocytes (arrow).
Fig. 5 - SEM photomicrograph of a RBMC on Ti-5Al-2.5Fe showing the cell layer surface. The ECM is mainly composed of collagen fibres (arrow head). Note the lower density of globular deposits (arrow) trapped in the collagen fibres when compared with the density observed at the interface with the substratum.
Ion-implanted materials

Figs. 6 to 10 show various aspects of the histology of the RBMC culture performed on the ion-implanted metals. All the cell cultures showed the same surface morphology as those obtained for the untreated materials: osteoblast-like cells with dorsal ruffles and filapodia in an extracellular matrix mainly composed of collagen where globular material is trapped. The detachment of the cell layer allowed the observation of the interface between the cell layer and the material’s surface. The layer adjacent to the substratum was composed of an intricate net of collagen fibres containing trapped calcified globules. Osteoblast-like cells were also observed. They displayed a flat shape and cell processes indicating that they were well attached to the substratum. Comparing the ion-implanted material’s surface after the cell layer detachment with equivalent surfaces of the untreated materials, it is possible to observe that the metal surface in the former case is covered with a continuous layer of globules where calcified fibres of collagen can be seen.

Sputter-coated 316 L stainless steel

The cell layer grew heterogeneously on these materials. The nitrogen sputter coating induced worse in vitro cell response. Fig. 11 shows a nitrogen sputter-coated sample after 19 days of cell culture. Most of the surface was only covered by widely dispersed polygonal cells. Contacts between cells could easily be identified but no ECM was produced. Nevertheless, small clusters of cells with a morphology similar to that obtained on Thermanox™ could be observed. Fig. 12 shows a photomicrograph of a surface cell layer formed on a carbon sputter-coated stainless steel. Fig. 13 presents a photomicrograph of the same material in which the cell layer has been mechanically detached to expose the substratum surface. As for the nitrogen sputter-coated samples, not all the surface was completely covered with a cell layer. But, in this case, where the cell layer was present its aspect seemed to be smoother than the cell layer surfaces previously
Fig. 6 - SEM photomicrograph of a cracked cell layer on a N⁺- ion implanted with $10^{15}$ ions/cm² 316 L stainless steel. Collagen fibres (arrow head) and globular deposits can be identified in the ECM (arrow) and on the substratum (*).
Fig. 7 - SEM photomicrograph from a cell layer that has been detached from a N⁺ ion implanted with 10¹⁶ ions/cm² 316 L stainless steel sample. Many globular deposits (arrow) surrounded by collagen fibres can be observed as well as flattened cells (arrow head). Note the high density of globules.
Fig. 8 - SEM photomicrograph from a cell layer that has been detached from a N⁺ ion implanted with $10^{17}$ ions/cm² Ti-6Al-4V sample showing many globular deposits (arrow) trapped in a net of collagen fibres.
Fig. 9 - SEM photomicrograph from a N⁺- ion implanted with 10¹⁷ ions/cm² Ti-6Al-4V sample showing the metal substratum after the removal of the cell layer. Osteoblast-like cells (arrow) and globular deposits (arrow head) can be identified.
Fig. 10 - SEM photomicrograph at a higher magnification of a N⁺- ion implanted with 10¹⁷ ions/cm² Ti-6Al-4V sample showing the substratum after the removal of the cell layer. A layer of globular deposits (arrow) covers all the surface. Calcified collagen fibres (arrow head) can also been seen.
Fig. 11 - SEM photomicrograph of a nitrogen-sputter coated sample after 19 days of RBMC. No ECM was formed. Spreaded polygonal shaped cells (arrow) with cells process (arrow head) can be identified.
Fig. 12 - SEM photomicrograph of a carbon-sputter coated sample after 19 days of RBMC. Note the smoother appearance of the cell layer surface.
Fig. 13 - SEM photomicrograph of a nitrogen-sputter coated sample after the removal of the cell layer. Clusters of globular deposits (arrow) and osteoblast-like cells (arrow head) were observed.
described, as no individual cells are protruding. At higher magnifications it was possible to identify fibres of collagen as the main constituent of the ECM. Cracks induced by the critical-point drying procedure allowed the observation of the inner layers. It was possible, in some areas, to observe the presence of calcified globules associated with calcified collagen fibres. Osteoblast-like cells that initially colonized the surface were also observed.

In this study, a comparison between the cell cultures performed on the above-mentioned materials was achieved. Both the cell layer surface and the substratum/cell layer interface were observed. The influence of the substratum will be preferentially seen in the cell layer immediately adjacent to the material. It was previously demonstrated by other groups that bone-like tissue can be grown, in culture, on Ti cp. [5, 16, 17], Ti-6Al-4V [5, 16], calcium phosphates [18] and polymers [2, 5, 19, 20, 21, 22]. Our results clearly show that a bone-like tissue can also be grown, in culture, on 316 L stainless steel, Ti-5Al-2.5Fe and on the nitrogen ion-implanted metals previously mentioned.

Based on the light microscopy and SEM observations, it seems that the culture evolves in three stages, in the same way as the model proposed by Davies et al. [5]: cell colonization and multilayering, initial multicellular mineralized matrix production and bone formation on the substrata. After cell inoculation the cells start to spread on the substrata and to multilayer. According to the studies performed by Maniakopoulos et al. [14] the multilayering of these cultures, prior to confluence, is an indication that bone nodule formation will occur. By observing the surface of the substrata and the tissue immediately adjacent, either by mechanically detaching the cell layer or by observing the cracks induced by the critical-point drying process, it was possible to detect the presence of Ca- and P-rich globular deposits which seem to be formed first followed by the production of collagen fibres. These deposits were identified by Davies et al. [17] as spheritic
foci of calcification and their origin was proved not to be a substratum-mediated process but one which is dependent on the cells expressing their osteoblast phenotype. The observation of these globular accretions was also achieved by other groups [5, 21, 23]. Further studies [17] on the constitution of the interfacial zone between Ti cp. and the nearest bone cell suggested that the interface cell layer/metal substratum was composed of at least two layers: a proteoglycan-rich layer and a bonding zone between the proteoglycan-rich layer and the metal beneath.

The extracellular matrix observed on the samples appears to be mainly constituted by collagen. Osteoblast-cell cultures obtained from rat neonatal parietal bones were grown by Puleo et al. [16] on stainless steel, Ti-6Al-4V, Co-Cr alloy, HA and several polymers. They showed that 50-55% of the protein synthesized by these cultures was collagen.

The fact that the density of these globular deposits is higher on ion-implanted materials suggests that this surface modification provokes changes to the surface chemistry which are beneficial as they are a precursor of bone formation. SEM observations of retrieved implants showed that the interface formed between the implant and the bone consists of a cement-like matrix which originated from globular deposits [5, 24]. The sequence of events occurring at the implant surface in vivo is similar to that described in vitro on both Ti and HA.

Conclusions

1 - A bone-like tissue grew, in culture, on all studied materials except on the nitrogen sputter-coated samples.

2 - The SEM observation of the cell layer surface on all the materials, except on the sputter-coated samples, showed that the morphology was similar, consisting of osteoblast-like cells and an ECM made up mainly of collagen where Ca- and P-rich globules were trapped.
3 - The study of the interface substrata/cell layer showed the presence of Ca- and P-rich globular deposits in higher density than on the cell layer surface.

4 - Enhanced production of globules is seen on nitrogen ion-implanted metals, which indicates that bone is more likely to be formed on these surfaces.

5 - 316 L stainless steel sputter coated with either nitrogen or carbon does not seem a suitable material for biomedical use. Cells grew heterogenously on such surfaces and on some areas they were not even able to form an ECM.

References


XPS characterization of surface films formed on surface-modified implant materials after cell culture

Nitrogen ion-implanted Ti-6Al-4V, Ti-5Al-2.5Fe and 316 L stainless steel and nitrogen or carbon sputter-coated samples were inoculated with rat bone marrow. The interface between the cell layer and the substrata was studied by X-ray photo-electron spectrometry and observed by scanning electron microscopy. Ca and P were detected on all materials after in vitro cell culture. Titanium appears to be present mainly in the form of TiO₂.

Introduction

The surface characteristics of biomaterials play an important role in the tissue response. The objective of modifying the surface characteristics of a biomaterial by means of a surface treatment, such as ion implantation and sputter coating, is to improve the corrosion and wear resistance as well as its biocompatibility. Ion implantation has been successfully used to promote wear resistance [1], corrosion resistance [2] and biocompatibility [3].

The study of the interface between the living tissue and the biomaterial is of crucial importance in understanding the mechanisms of cell bonding. Parameters such as surface roughness, oxide composition and thickness and the existence of
contaminants on the surface are known to affect the biological response both in vivo and in vitro [4, 5, 6, 7], thereby determining the success of cell attachment.

An osteoblast-cell culture was used to mimic the bone/biomaterial interface. This interface has been investigated, in vitro, by Davies et al. [8] and de Bruijn et al. [9] on titanium and calcium phosphates. They reported that the interface produced consists of a layer of Ca- and P-rich globular deposits adjacent to the implant surface over which collagen fibres are deposited. A similar interface was also observed to have been created in vivo [10].

The work here described is part of a study in which titanium alloys and stainless steel were surface modified by ion-implantation and sputter-coating techniques in order to improve their performance as implant materials. The surface-modified materials were previously characterized according to their corrosion behaviour and the surfaces that produced better in vitro corrosion resistance were tested for biocompatibility. The objective of this study is the surface characterization of the nitrogen ion-implanted and sputter-coated materials after rat bone marrow cell culture. Scanning electron microscopy (SEM) was used to observe the surfaces of the materials and the tissue immediately adjacent. The chemical composition of the substrata was determined by X-ray photo-electron spectrometry (XPS).

Materials and methods

Materials

Ti-6Al-4V and Ti-5Al-2.5Fe samples (Deutsch Titan) were cut from rods of 15 and 30 mm in diameter, respectively, and were ion implanted with two different fluences of nitrogen ions, namely: $10^{15}$ and $10^{16}$ ions/cm$^2$ with a beam energy of 40 keV. 316 L stainless steel (Aubert & Duval) samples were obtained from a rod of 20 mm in diameter and nitrogen ion-implanted with $10^{16}$ and $10^{17}$ ions/cm$^2$
with the same energy.

Samples of 316 L stainless steel (Aubert & Duval) were sliced from a rod of 20 mm in diameter and then nitrogen- or carbon- sputter coated (N2 and C1 samples from chapter 2).

All samples were ultrasonically cleaned in 90% ethanol for 20 min., followed by a 20-min. double rinse with distilled water before steam sterilization [11].

**Cell Culture**

The samples were steam sterilized before cell culture for 20 minutes. A primary droplet rat bone marrow culture (RBMC) was performed according to the method described by Maniotopoulos *et al.* [12] which induces an osteoblast-enriched cell population.

Young adult male Wistar rats, of about 100-120 g, were sacrificed. The epiphyses were removed and the bone marrow was flushed out from each dyaphisise with α-minimum essential medium (Gibco), 15% foetal calf serum (Gibco) and 10% antibiotics (amphotericin B (Sigma), penicillin G (Sigma) and gentamycin (Gibco)). The cells were then grown on this medium to which 1% 1M Na-β-glycerophosphate (Sigma), 1% 5 mg.mL⁻¹ ascorbic acid (Sigma) and 1% 10⁻⁸ M dexamethasone (Sigma) were added. A droplet culture of the bone marrow cell suspension was placed on every sample. The cell culture was performed either in 6- or 24-well tissue culture plates (Costar) depending on the sample dimension and kept in a humidified atmosphere of 95% air - 5% CO₂ at 37 °C. The cultures were observed with a light microscope every 48 hours. The cultures were maintained for 19 days and then the samples were prepared for XPS analysis.

Samples were also prepared for scanning electron microscopy (SEM). Briefly, the specimens were fixed in 1.5% glutaraldehyde followed by dehydration in a graded series of ethanol and critical point drying (Balzers CPD 030) from carbon dioxide. The specimens were gold sputter coated and observed by SEM with an accelerating voltage of 15 kV.
X-ray photo-electron spectrometry

After the 19 days of cell culture, the samples were thoroughly washed in Physiological Body Solution (PBS) and the cell layer was scraped with a rubber policeman. The samples were then washed twice with PBS and rinsed in distilled water. A VG Scientific ESCALAB, using Mg Kα as source, was used to obtain the spectra. Survey XPS spectra from 0 to 1100 eV were acquired after cell culture on all surfaces. Survey spectra were also acquired on surface-modified materials which were not subjected to cell culture in order to provide comparison data. Following spectra acquisition, peak identification and quantification were achieved using VG Scientific ESCALAB package software. High-resolution spectra for the Ca 2p, P 2p, O 1s, Ti 2p and C 1s were also obtained and computer curve-fitted employing a Gaussian model, using the same package software, to obtain the best binding energy (BE) values. To take into account some shift caused by charging of the sample surface, all spectra were adjusted taking the C 1s peak at 285.0 eV as reference for the adventitious carbon contamination.

Results and Discussion

Wide scans were performed on nitrogen ion-implanted and sputter-coated samples before and after RBMC. C and O, as well as the alloy elements, were detected in all samples before cell culture. On the titanium alloys V was not observed as this element does not contribute to the formation of the surface film [13, 14]. Si, probably having its origin in the sample preparation process, was a common contaminant on all ion-implanted samples before cell culture. F was detected on the ion-implanted titanium alloys before RBMC. After cell culture, Al, Si and F signals were no longer detected. An exception was noticed for the Ti-6Al-4V ion-implanted samples with 10^{16} ions/cm^2 where F was detected after
RBMC but the peak height was reduced by ca. 75 % which implies a reduction in its concentration. Ca was detected as a contaminant on ion-implanted Ti-5Al-2.5Fe samples. After cell culture, Ca and P were easily detected on all materials investigated. N, also present on all samples before RBMC, showed an enhanced signal after cell culture, indicating that part of the N is of biological origin. A similar result may be deduced from the width analysis of the C 1s peak. The peak broadening, after cell culture, suggests that there are contributions from more chemical states than is the case for the usual C contamination. Ti concentration on the titanium alloys and Fe concentration on the stainless steels were reduced after cell culture. Ni and Cr, detected on the ion-implanted 316 LL stainless steel before cell culture, were unseen after RBMC. Ni and Cr peaks were easily observed on all sputter-coated materials before and after cell culture.

Other groups [13, 15, 16, 17] have studied the surface composition of Ti cp., Ti-6Al-4V by XPS. Ti, O and C were always detected. Ca and traces of S, Cl, P, Si, Na, Cu, Zn, Sn and Pb were also found on Ti cp. samples before sterilization. Surface analysis of in vivo retrieved titanium [18, 19] and stainless steel samples [18] showed that Ca and P were always present. Similar results were obtained in this work by studying the surfaces after cell culture. Ca and P may have their origin from the cell-culture medium, as it is known that these elements are present on the surface after immersion of Ti-6Al-4V, Ti-5Al-2.5Fe and 316 L stainless steel in SBF or HBSS [13, 14, 15, 20]. Another probable origin of the Ca and P is related to the extra-cellular matrix produced by the osteoblast-like cells. SEM observations of the interface substrata/cell layer [21] showed that Ca- and P-rich globular deposits are produced by these cells as soon as they colonize the material. Even after mechanically removing the cell layer, some of these globular accretions were still attached to the surface. While preparing the surfaces for XPS spectra acquisition the cell layer was scraped with a rubber policeman but the adhesion of the cell layer to the material surface, especially on ion-implanted titanium alloys, was very strong, leaving some cell-layer leftovers attached to the surfaces. Care
was taken to choose the cleanest areas of the samples for spectra acquisition.

The reduction in concentration of most elements and the disappearance of the contaminants after RBMC may be a consequence of surface oxide thickening due to both cell contact and interaction with the cell-feeding media. By comparing the morphologies of the different cell layers produced on the surface-modified materials, one notices that the cell response to the sputter-coated materials was worse compared to that of ion-implanted ones. The cell layer grew heterogeneously on these surfaces and on nitrogen-sputter coated samples an extra-cellular matrix was not produced even after 19 days of culture. The detection of alloy elements such as Cr and Ni after cell culture may explain these results since the toxicity of these elements is well known [22, 23].

High-resolution spectra were acquired, on nitrogen ion-implanted Ti-6Al-4V, for C 1s, O 1s, Ti 2p, P 2p and Ca 2p. This provided an indication of the chemical composition of the calcium phosphate and the titanium oxide formed on the surfaces. The high-resolution spectra were very similar for both fluences. The binding energies obtained differed by max. \( \pm 0.2 \) eV. Figs. 1 to 5 show the high-resolution spectra of C 1s, O 1s, Ti 2p, P 2p and Ca 2p.

C 1s spectra - The spectra were dominated by a shoulder at high energies and it was necessary to employ two peaks to achieve the best fit. The main peak had a binding energy of 288.2 \( \pm 0.1 \) eV which indicates that the carbon is bonded to O or hydroxyl groups [15]. The Full Width Half Maximum (FWHM), of the order of 2.7 eV for the lower binding-energy peak and 2.5 eV for the higher binding-energy peak, suggests that there are overlapping peaks. Therefore carbon may be present as a mixture of compounds, probably as sodium or calcium carbonates since C 1s shows standard binding energies for these compounds of 289.4 eV and 289.8 eV, respectively [24]. Nevertheless, the main source of contamination is probably due to organic molecules having an origin either in the cell culture or simply in the exposure to air.
Fig. 1 - XPS C 1s high-resolution spectrum from a N⁺ -ion implanted Ti-6Al-4V with 10¹⁵ ions/cm² sample.

O 1s spectra - The main peak has a BE of 534.6 ± 0.0 eV and a FWHM of 2.73 ± 0.0 eV which indicates, as for the C 1s peak, the overlapping of more than one peak. A shoulder at lower BE (531.2 eV) was also detected. Armstrong et al. [25] determined the standard O 1s for TiO₂ = 533.3 eV which is similar to the BE of the main peak. Binding energy differences between two major transitions can also indicate the chemical composition of a metal oxide. By calculating the BE difference between the O 1s and the Ti 2p 3/2, ΔBE = O (1s) - Ti (2p 3/2) = 75.1 eV which, according to the data obtained by the same authors, corresponds to TiO₂. On the other hand, high-resolution spectra obtained by other groups [14, 15, 16, 26] suggest that the BE for the oxide appears in the low-energy range (ca. 531 eV) followed by other components at higher BE, namely hydroxide/hydroxyl groups, phosphate or chemisorbed water. Taking this into account, it is possible that titanium oxide contributes mainly to the O 1s peak at BE 531.2 eV, and that the main peak at 534.6 eV can be mostly attributed to the presence of phosphates and
adsorbed water. Recalculating $\Delta$BE one obtains the value of 71.7 eV which is closer to the values obtained by Armstrong et al. [23] for TiO$_2$. The analysis of the Ti 2p spectra also suggests that the titanium is mostly present in the form of TiO$_2$.

![Graph showing XPS O 1s high-resolution spectrum from a N+ -ion implanted Ti-6Al-4V with 10$^{15}$ ions/cm$^2$ sample.](image_url)

Fig. 2 - XPS O 1s high-resolution spectrum from a N$^+$ -ion implanted Ti-6Al-4V with 10$^{15}$ ions/cm$^2$ sample.

Ti 2p spectra - The Ti 2p peak was a doublet peak, Ti 2p3 at 459.5 ±0.1 eV and Ti 2p1 at 465.0 ±0.2 eV. This indicates that the titanium is present mainly in the oxide state [22] and by taking into account the BE of the peaks [16, 24] and the separation between them [24] it is probable that the oxide present is TiO$_2$ which is in accordance with results from the O 1s spectra. No contributions were detected between the main peak and 454.0 eV which is the BE value for Ti metal. This suggests first, that the surface film is too thick to allow photo-electrons from the bulk metal to reach the surface and second, that there are no sub-oxides, like TiO and Ti$_2$O$_3$, present [15, 23].
Fig. 3 - XPS Ti 2p high-resolution spectrum from a N⁺-ion implanted Ti-6Al-4V with $10^{15}$ ions/cm² sample.

P 2p spectra - This was a single peak with a BE of $136.0 \pm 0.1$ eV and a FWHM of $2.39$ eV indicating a probable overlapping peak. According to the literature [22, 13, 14, 24, 27, 28] there is no clear indication as to the nature of the chemical compound in which this element is present. A probable mixed Ca and P compound may be responsible for its high BE.

Ca 2p spectra - This was a doublet peak with Ca2p 1/2 = $353.8 \pm 0.1$ eV and Ca 2p3/2 = $350.2 \pm 0.1$ eV. The FWHM of Ca 2p3/2 is ca. 2.1 which is relatively large for Ca which has a value of 1.68 eV [22]. This suggests that the peak overlaps with other contributions. As for the P 2p, Ca 2p 3/2 has a BE which enables the identification of the Ca compound. Comparison of the BEs of P 2p and Ca 2p 3/2 suggests that a Ca- and P- compound may have been formed, probably a calcium-hydrogen phosphate.
Fig. 4 - XPS P 2p high-resolution spectrum from a N⁺-ion implanted Ti-6Al-4V with 10¹⁵ ions/cm² sample.

Fig. 5 - XPS Ca 2p high-resolution spectrum from a N⁺-ion implanted Ti-6Al-4V with 10¹⁵ ions/cm² sample.
It is possible that the features obtained in the XPS spectra are mainly a consequence of the Ca- and P-rich globular deposits formed by the osteoblast-like cells. As mentioned before, it is also possible that a calcium phosphate may have been formed as a result of the immersion of the samples in the cell-feeding medium which is composed partly of compounds of Ca and P. A third possibility is the joint effect of both osteoblast-like cells and medium.

Conclusions

1 - In vitro cell culture seems to be a fairly reliable method to test biomaterials in vitro because the interface formed is both morphologically and chemically similar to those observed in vivo.

2 - The bad cell culture results exhibited by the sputter-coated 316 L stainless steel seem to result from the existence of Cr and Ni ions on the metal surface.

3 - SEM observations of the interface between the cell layer and the substrata together with the XPS results suggest that the surface of the nitrogen ion-implanted Ti-6Al-4V is mainly composed of TiO$_2$ and a biologically produced calcium-phosphate compound.

References


Chapter 5

Development and characterization of a calcium-phosphate coating

5.1 - *In vitro* calcification of orthopaedic implant materials

5.2 - The influence of substrate material and surface finishing on the morphology of the calcium-phosphate coating
In vitro calcification of orthopaedic implant materials

The formation of an apatite-like layer is achieved by immersing Ti-6Al-4V and Ti-5Al-2.5Fe substrata in Hank's Balanced Salt Solution (HBSS). The layer was characterised by surface analysis techniques, namely X-ray microanalysis (XRMA), X-ray diffraction (XRD) and X-ray photoelectron spectrometry (XPS). The results suggest that the layer produced by immersion in HBSS is in the form of an amorphous apatite. The pH and the concentrations of Ca and P were monitored as a function of time. In vitro tests with rat bone marrow were performed in order to mimic the bone/biomaterial interface. They were performed both on immersed and non-immersed samples. The in vitro bone marrow results suggest that the apatite-like layer formed may improve the bone bonding characteristics of the studied titanium alloys.

Introduction

The surface properties of orthopaedic implants are of major importance in the initial stages of contact with surrounding bone tissue. In order to improve the bioactivity of titanium alloys and other metals, their surfaces have been coated with calcium phosphates. The natural precipitation of a
calcium-phosphate layer seems to be a simple and a low cost method to create a biologically equivalent apatite.

The formation of such films has been reported by several groups [1, 2]. The thickest layer formed on titanium and its alloys, after immersion in a simulated physiological solution, was 1μm after 2 weeks of differential immersion [1]. Hanawa and co-workers [3, 4, 5] also reported the formation of very thin calcium phosphate films after 30 days of immersion.

Calcium phosphates have also been detected in the corrosion products of titanium c.p. [6] and in the passive films of Ti-6Al-4V after electrochemical tests in a simulated physiological solution [7]. Therefore, titanium and its alloys seem to be capable of inducing the formation of a calcium phosphate coating.

The formation of an apatite-like layer is achieved by immersing titanium alloys in Hank's Balanced Salt Solution (HBSS). The precipitate layer formed on the surface of the metals was studied by several surface analysis techniques, namely, XRMA, XRD and XPS. The Ca and P concentrations were determined by atomic absorption spectrometry and spectrophotometry, respectively.

Materials and Methods

Surface Preparation

Ti-6Al-4V and Ti-5Al-2.5Fe (Deutsch Titan) samples, 9.15 mm and 5 mm in diameter respectively and 1.5 mm thick, were ground using SiC papers and polished with diamond paste. All samples were ultrasonically cleaned in
90% ethanol for 20 minutes followed by a 20 minutes double rinse with distilled water [8].

**Immersion Method**

After surface polishing and cleaning Ti-6Al-4V and Ti-5Al-2.5Fe samples were immersed in HBSS at 37 °C for 14 days in single polyethylene containers. To allow a constant supply of solution this was changed every 48 hours. The pH was recorded as a function of time. The remaining solutions were then stored in 1 mL Eppendorf™ at room temperature. Ca and P concentrations were later determined by means of Atomic Absorption Spectrometry (Varian SpectAA 300) and spectrophotometry (Vitalab 21, Vitalab Scientific), respectively. All the results are the average of at least three measurements.

The surfaces, before and after immersion, were analysed by XRD (Philips Thin-film XRD), XRMA (Voyager XRMA system, NORAN Instruments) and XPS (VG Scientific ESCALAB). All surfaces were observed by scanning electron microscopy (SEM) (Philips SEM 525M).

**Rat Bone Marrow Culture**

The samples were steam sterilised before cell culture for 20 minutes. A droplet rat bone marrow culture was performed on immersed and non-immersed samples, according to the method described by Maniatisopoulos et al. [9] which induces an osteoblast differentiated population.

Thermanox™ coverslips (polyethyleneterephthalate), of 13 mm diameter, were used as a control substratum. Cell culture was performed in 24-well tissue culture plate (Costar).

The specimens were then fixed overnight, at room temperature, in 1.5% glutaraldehyde in the same buffer. Dehydration in a graded series of ethanol and critical point drying (Balzers CPD 030) from carbon dioxide were
followed. The specimens were gold-sputter coated, observed by SEM and analysed with XRMA at accelerating voltages of 15 kV and 20 kV, respectively.

Results and Discussion

Immersed surfaces

Fig. 1 gives curves obtained in the monitoring of the pH as a function of time. A container with only HBSS was used as reference. From day 0 until day 7 all curves show similar laws consisting of a rapid increase in the pH, from initial 7.5 to 8.5, in the first two days; after day 7 both alloys exhibit a decrease in the pH while the HBSS is maintained at an approximate value of 8.6. This effect may be due to the precipitation of a solid phase from the solution.

Fig. 1 - pH changes as a function of time
A reduction in the pH was also detected by J. Arends et al. [10] when hydroxyapatite is precipitated from an aqueous solution. The precipitation of calcium phosphates from a simulated body fluid onto silica gel has been shown to be possible for pH values higher than 7.2 [2].

Fig. 2 shows the Ca and P concentrations in the remaining HBSS as a function of time. In the solutions in contact with the alloys there was a monotonic decrease in the concentrations of Ca and P, in contrast to the HBSS control. Until day 5 both Ca and P curves were similar to those for HBSS. In the solutions which were in contact with the metal alloys the concentrations of Ca and P started to decrease between day 5 and 7. This was attributed to the growth of precipitate nuclei on the surfaces from the HBSS solution. Similar behaviour was also found by Li [2] after immersion of silica-gel and gel-derived titania in a Simulated Body Fluid. These findings together with the drop in the pH value suggest the formation, from the HBSS solution, of a Ca- and P-rich precipitate with an induction time of about 6 days. The time to nucleation seems to be similar for both the titanium alloys.

SEM observations showed that all samples were completely covered with a brittle layer (Fig. 3). Comparing the morphology between the layers formed on both alloys, one notices that the one formed on Ti-6Al-4V is less rough and that the layer formed on Ti-5Al-2.5Fe shows a higher density of “globules”. Xrma performed on the precipitate layer showed the presence of Ca and P. Semi-quantitative analysis revealed an average Ca/P ratio of 1.4 in the calcium phosphate precipitate and a Ca/P of 1.6 in the “globules”. Due to the higher number of “globules” formed on Ti-5Al-2.5Fe it seems that this material more easily allows the formation of a more stable calcium phosphate.
Fig. 2 - Ca (a) and P (b) uptake after immersion into HBSS
Fig. 3 - SEM photographs of immersed surfaces of Ti-6Al-4V (a) and Ti-5Al-2.5Fe (b) after 14 immersion.

Survey XPS spectra were acquired before and after immersion in HBSS. Following spectra acquisition, peak identification and quantification were achieved using VG Scientific ESCALAB package software. All spectra were calibrated using C 1s binding energy, $E_b$, $E_b, C_{1s} = 285.0$ eV, as reference.

Fig. 4 shows XPS spectra acquired on non-immersed and immersed Ti-5Al-2.5Fe samples. Ti-6Al-4V exhibited similar spectra concerning the presence of Ca and P after immersion.
Fig. 4 - XPS spectra of Ti-5Al-2.5Fe before (a) and after 14 days (b) immersion in HBSS

All samples exhibited a well defined C 1s peak, which is usually a common contaminant. The presence of the alloy elements can be noticed. Vanadium was never detected on the Ti-6Al-4V surfaces, which is in agreement with other studies [3, 4]. The presence of Ca and P was only detected on the immersed samples of both titanium alloys.

Fig. 5 shows XRD spectra acquired on non-immersed (a) and immersed surfaces (b). On the immersed samples one can observe the appearance of a well defined [002] peak and a broader one which is probably constituted by the junction of peaks [211] and [112] indicating the amorphous characteristics of the calcium phosphate. These results suggest that the precipitate layer has an amorphous apatite-like structure.
Fig. 5 - XRD spectra of Ti-6Al-4V before (a) and after 14 days (b) immersion in HBSS

The calculated thickness of this calcium phosphate precipitate layer is ca. 5 μm, Fig. 6. Some samples showed thicker precipitate films.

Apatite formation on commercial pure Ti was also found by Ducheyne et al. [1] after 2 weeks immersion time. The reported thickness of the formed layer was 1 μm. Hanawa [3] also reported that apatite is naturally formed on titanium when titanium is immersed in a solution whose pH is similar to that of the bioliquid. They reported a thickness of 7 nm of the apatite film grown on Ti-6Al-4V which makes it impossible for this layer to exhibit any properties of calcium phosphate in this environment. Thicknesses of least 1 μm are needed for the calcium phosphate to show its properties and to cause bone induction.

The above results seem to indicate that a calcium phosphate with an apatite-like structure is naturally formed on the surfaces of the titanium alloys. The thickness of this layer makes it a more suitable surface for bone induction.
A possible explanation of these results is the presence of hydroxyl groups on the passive films of the titanium alloys [11]. It was proposed that apatite induction could take place on a negatively charged surface with sufficient hydroxyl groups [2].

Cell culture

The in vitro cell study focused on the cell responses to the apatite-like layer formed by immersion of the titanium alloys. Fig. 6 shows a photomicrograph of a Ti-6Al-4V sample previously immersed in HBSS which was inoculated with rat bone marrow. The figure shows an interface between three clearly distinguished zones: a first zone, A, which is the metal substratum, a second zone, B, the apatite-like coating and finally the cell layer, C.

Fig. 6 - Interface between the metal substratum (a), the apatite-like layer (b) and the cell layer (c)
After 21 days of culture, a mineralized extracellular matrix is clearly visible beneath the layer of surface cells. After critical point drying and gold sputtering the cell layer was still attached to the apatite. The cell layer was never seen detached from the apatite-like layer in the immersed samples. This indicates that the cell layer is firmly attached to the apatite. It was observed that on non-immersed samples the cell layer was easily detached during the critical point drying procedure indicating that they were loosely attached [12]. This may be due to differences in substrata roughness and to the fact that the produced layer is similar to apatite and is able to cause bone induction. Rougher surfaces may allow the osteoblastic cell to have more points of adhesion and to produce an extracellular matrix [13, 14]. Light microscope monitoring of the cell culture as a function of time showed that cell proliferation was attained at an earlier stage for the immersed samples.

It was also possible to identify, in areas where the cell layer was removed, the presence of afibrillar accretions [12, 15, 16]. This was more visible on non-immersed samples due to the poor attachment of the cell layer to the metal substratum.

Conclusions

XRMA together with XPS and XRD suggest that the calcium phosphate layer produced by immersion in HBSS is mainly in the form of an amorphous apatite-like layer. Ti-5Al-2.5Fe alloy seems to allow formation of a more stable calcium phosphate more easily, as evidenced by the higher density of calcium phosphate rich globules.

The results indicate that the Ti surface acts as an inducer of the apatite from the HBSS solution.
The in vitro bone marrow results suggest that the apatite-like layer formed may improve the bone bonding characteristics of the studied Ti alloys.

References


4. T. Hanawa and M. Ota, Biomaterials 12, 767-774 (1991)


The influence of substrate material and surface finishing on the morphology of the calcium-phosphate coating

The formation of an apatite-like layer was achieved by immersing Ti-6Al-4V, Ti-Al-2.5Fe and 316 L stainless steel substrates in Hank’s balanced salt solution (HBSS). The layer was characterized by surface analysis techniques, namely X-ray microanalysis and X-ray diffraction, and the morphology was observed by scanning electron microscopy and atomic force microscopy. The concentrations of Ca and P were monitored as a function of time.

The morphology of the precipitate layer seems to be dependent both on type of metal substrate and its surface finish. Polished Ti-6Al-4V and Ti-Al-2.5Fe surfaces exhibit a plate precipitate morphology whereas rougher surfaces show scattered crystal-like precipitation.

The results suggest that the layer produced by immersion of polished titanium alloys in HBSS is constituted by an amorphous apatite.

Introduction

The clinical use of hydroxyapatite as a load-bearing biomaterial is very limited due to its brittleness; on the other hand, the biocompatibility of metals is poor compared to that of hydroxyapatite [1, 2]. As a consequence, calcium
phosphates, particularly hydroxyapatite, have been extensively used as a coating onto metals to increase their biocompatibility. The mechanical strength of the metallic substrate is combined with the bioactive character of the hydroxyapatite [3]. The natural precipitation of a calcium phosphate layer seems to be a simple, low cost method to create a biologically equivalent apatite capable of enhancing the bioactivity of metal alloys.

Previous studies, performed by our group [4] on titanium alloys immersed in HBSS, showed the formation of an amorphous apatite layer which may improve the bone-bonding characteristics of these alloys.

The formation of a natural precipitated hydroxyapatite layer on several materials was reported by several groups [5-8]. Hanawa and co-workers [9-11] also reported the formation of very thin calcium-phosphate films after 30 days of immersion.

The formation of rich calcium-phosphate deposits was achieved by immersing titanium alloys and stainless steel in HBSS. The precipitate layers formed were studied by X-ray microanalysis (XRMA) and X-ray diffraction (XRD) and the morphology was observed by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The Ca and P concentrations were determined by atomic absorption spectrometry and spectrophotometry, respectively.

Materials and Methods

Ti-6Al-4V and Ti-Al-2.5Fe samples, 9.15 mm and 5 mm in diameter respectively and 1.5 mm thick, were used. They were ground flat in SiC papers, 1200, 4000 grit and diamond polished down to 1 µm. 316L stainless steel
samples, ca. 80 mm², were also ground in SiC papers, 1200 and 4000 grit. All samples were ultrasonically cleaned in 90% ethanol for 20 minutes followed by a 20-minute double rinse with distilled water and dried under a flow of hot air. The surface roughnesses were measured with a laser profilometer (Perkin Elmer). Table 1 shows the results of the following roughness parameters: $R_a$ - arithmetic mean of the roughness height, $R_z$ - mean peak-to-valley height and $R_{\text{max}}$ - maximum roughness depth.

<table>
<thead>
<tr>
<th>Surface finish</th>
<th>$R_a$ (µm)</th>
<th>$R_z$ (µm)</th>
<th>$R_{\text{max}}$ (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti-6Al-4V 1200 grit</td>
<td>0.47±0.01</td>
<td>3.74±0.04</td>
<td>5.13±0.08</td>
</tr>
<tr>
<td>Ti-6Al-4V 4000 grit</td>
<td>0.24±0.03</td>
<td>1.91±0.31</td>
<td>2.46±0.54</td>
</tr>
<tr>
<td>Ti-6Al-4V 1 µm</td>
<td>0.03±0.00</td>
<td>0.35±0.05</td>
<td>0.48±0.03</td>
</tr>
<tr>
<td>Ti-Al-2.5Fe 1200 grit</td>
<td>0.42±0.03</td>
<td>2.97±0.35</td>
<td>3.47±0.48</td>
</tr>
<tr>
<td>Ti-Al-2.5Fe 4000 grit</td>
<td>0.23±0.01</td>
<td>1.97±0.18</td>
<td>2.46±0.34</td>
</tr>
<tr>
<td>Ti-Al-2.5Fe 1 µm</td>
<td>0.04±0.01</td>
<td>0.28±0.11</td>
<td>0.36±0.19</td>
</tr>
<tr>
<td>316 L 1200 grit</td>
<td>0.3±0.06</td>
<td>2.32±0.47</td>
<td>2.96±0.03</td>
</tr>
<tr>
<td>316 L 4000 grit</td>
<td>0.04±0.01</td>
<td>0.35±0.1</td>
<td>0.46±0.1</td>
</tr>
</tbody>
</table>

After surface polishing and cleaning, all samples were immersed in HBSS at 37 °C for 14 days in separate polyethylene containers. To allow a constant supply of solution this was changed every 48 hours. Empty polyethylene containers were used as reference. A sample of each retrieved solution was stored in 2 mL Eppendorf™ at 4 °C. Ca and P concentrations in these solutions were later determined by means of atomic absorption spectrometry (Varian SpectAA 300)
and spectrophotometry (Vitalab 21, Vitalab Scientific), respectively. All the results are the average of at least three measurements.

All surfaces were observed, before and after immersion, by scanning electron microscopy (Philips SEM 525M) and analysed by XRMA (Voyager XRMA system, NORAN Instruments). XRD (Philips Thin-film XRD) was used to determine the structure of the precipitate layer, and AFM was used to observe its morphology on polished titanium alloys.

Results and Discussion

Fig. 1 shows the Ca concentrations as a function of time. In the solutions that were in contact with the alloys, a monotonic decrease of the concentrations of Ca was detected. The same phenomenon was also observed for the reference HBSS. Until day 5 all curves have similar forms but after day 5 a higher decrease for the Ti-6Al-4V 1 μm samples is apparent which reaches 123±1.5 ppm. For both Ti-6Al-4V 4000 and Ti-Al-2.5Fe 1 μm samples the Ca concentration decreased more rapidly after day 7 attaining similar final Ca uptake concentrations, 61±2.3 and 63±1.5 ppm, respectively. All other surfaces exhibited Ca uptake values between 5 and 20 ppm.
Fig. 1 - Ca concentration as a function of time

Fig. 2 shows the P concentration as a function of time. The P uptake curves, like the Ca determinations, also showed a decrease as a function of time. The Ti-6Al-4V 4000 and 1 μm and the Ti-Al-2.5Fe 1 μm showed the highest P uptake; 29±2.1, 34±1.5 and 58.5±2.2 ppm, respectively. These findings suggest that a Ca and P rich precipitate is formed on all the surfaces from the HBSS. In fact, it was possible to see a white film deposited on the polyethylene that contacted with the HBSS. Ti-Al-2.5Fe 4000 and 1200 showed the lowest Ca and P uptake. The decrease in both Ca and P was attributed to the growth of precipitate nuclei on the surfaces from the HBSS solution. Similar behaviour was found by Li et al. [6] after immersion of silica-gel and gel-derived titania in a Simulated Body Fluid; Radin et al. [12] also reported a decrease in Ca and P concentration in Simulated Physiological Solution after the immersion of ceramic particles.
Fig. 2 - P concentration as a function of time

Fig. 3 shows SEM photomicrographs of the metal surfaces after immersion in HBSS. Comparing the photographs on Fig. 3 it can be seen that the precipitate layer has a plate morphology on which "globules" and/or crystals grow. XRM revealed a higher quantity of Ca and P on these particles than in the plate precipitate. It was possible to observe that the plates fractured on some of the surfaces, namely Ti-6Al-4V 1200 and 1 µm, Ti-Al-2.5Fe 1 µm and stainless steel 1200. The orientation of the fractures does not seem to depend on the orientation of the grinding flaws as it is possible to observe a random cracking of the plates. The precipitate formed on Ti-6Al-4V 4000 shows a continuous texture at the same magnification as the other observations. It was only possible to detect fractures on these surfaces, on the Ca and P rich layer, at magnifications higher than 2400 x.
Fig. 3 - SEM photomicrographs of the metal surfaces after immersion in HBSS: A - Ti-6Al-4V 1200; B - Ti-6Al-4V 4000; C - Ti-6Al-4V 1 μm; D - Ti-Al-2.5Fe 1 μm; E - Ti-Al-2.5Fe 4000; F - stainless steel 1200
Li et al. [6] performed a series of experiments in which silica-gel was immersed in SBF. They suggest that the regulation of apatite growth is related to the Ca/P molar ratio of the fluids. Fugishiro et al. [1] obtained different HA morphologies by immersing Fe and Ti in Ca(edta)$^{2-}$—NaH$_2$PO$_4$ solution. Various concentrations of Ca(edta)$^{2-}$ had a direct effect on the morphology of the hydroxyapatite film.

The SEM observations suggest that the morphology of the precipitate layer seems to be dependent both on material and surface finishing as the immersion fluid was the same in all experiments.

Fig. 4 shows an AFM photomicrograph from a Ti-Al-2.5Fe 1 μm sample. It is apparent that the calcium phosphate-rich coating is constituted by the agglomeration of spherical particles. Similar results were obtained for the Ti-6Al-4V 1 μm surfaces. It seems that the formation of the coating starts with heterogeneous precipitation of nuclei which gather with time until all the surface is covered.

Fig. 4 - AFM photomicrograph of a Ti-Al-2.5Fe 1 μm sample after immersion in HBSS. Increasing magnification from field 0 to 3. Scanning length from field 3 - 1.5 μm
It was noticeable that the Ti-Al-2.5Fe alloy surfaces 4000 and 1200 did not exhibit plate precipitates. It was only possible to observe small scattered deposits which had a similar morphology to crystals. XRMA acquisition on the flat-ground surfaces showed the presence of no Ca or P. The same acquisition on the crystals showed the presence of the alloy elements, Ca and P, associated with Si. Si seems to act as a nucleus for the precipitation and growth of the crystals. This impurity is probably due to the SiC emery paper used during the surface preparation. Either the degreasing and cleaning of the surface was not sufficient, on these surfaces, to remove the SiC or some SiC particles might be anchored in the alloy's surface as Ti-Al-2.5Fe is a softer material than the other alloys.

Fig. 5 exhibits XRMA spectra acquired in a Ti-6Al-4V 4000 sample before and after immersion in HBSS. One can observe the presence of the alloy elements as well as very well defined Ca and P peaks on the after-immersion spectra. The calculated Ca/P ratio is 1.56±0.03 which indicates that the precipitate probably consists mainly of tricalcium phosphate.

Fig. 5 - XRMA spectra acquired on a Ti-6Al-4V 4000 sample before (A) and after immersion (B) in HBSS
Fig. 6 shows XRD spectra acquired on non-immersed (A) and immersed (B) Ti-6Al-4V 1 μm surfaces. On the immersed samples one can observe the appearance of a well defined [002] peak and a broader peak which seems to be constituted by the junction of peaks [211] and [112] indicating the amorphous characteristics of the calcium phosphate. These results suggest that the precipitate layer has an amorphous apatite-like structure. Similar results were obtained for the Ti-Al-2.5Fe 1 μm samples.

Fig. 6 - XRD spectra acquired on a non-immersed (A) and immersed (B) Ti-6Al-4V 1 μm surface

The thickness of this layer was previously determined by SEM observations and is ca. 5 μm. Li et al. [6] monitored the development of hydroxyapatite deposits on gel-derived titania, as a function of time, after immersion in Simulated Body Fluid. In the initial stages they detected scattered precipitates all over the surface which increased in number and size until, eventually, all the surface was covered by a 10 μm coating. Ducheyne et al. [5] reported the formation of small deposits on titanium discs after 1-day exposure to a
Simulated Physiological Solution. Two weeks of differential immersion were needed to produce an apatite layer with a thickness of 1 \( \mu \text{m} \).

Hanawa et al. [9] also reported that apatite is naturally formed on titanium when titanium is immersed in a solution whose pH is similar to that of the bioliquid. They reported a thickness of 7 nm of the apatite film grown on Ti-6Al-4V which makes it impossible for this layer to exhibit any properties of calcium phosphate in this environment.

Our results seem to indicate that a calcium phosphate with an apatite-like structure is naturally formed on the surfaces of polished titanium alloys. The thickness of this layer makes it a suitable surface for bone induction. Thicknesses of at least 1 \( \mu \text{m} \) are needed for the calcium phosphate to show its properties and cause bone induction [5].

Conclusions

The morphology of calcium-phosphate precipitates depends on the metal substrate and its surface characteristics.

It is possible to produce a naturally formed calcium phosphate coating by immersing metals such as titanium alloys and stainless steel in HBSS.

Ti-6Al-4V 4000 seems to be the surface that is most favourable to produce a continuous and more adherent apatite-like coating capable of bone induction.

References


Chapter 6

General discussion
General discussion

In view of the results obtained in the previous chapters, a relation between the surface modification treatments (ion implantation, sputter coating and calcium-phosphate coating) and the cell culture results will be made.

Surface-modification techniques such as N⁺- ion implantation and nitrogen- or carbon- sputter coating change the electrochemical characteristics of the bulk material. The results obtained in this thesis show that N⁺- ion implantation with $10^{16}$ ions/cm² increases the corrosion resistance of the Ti-6Al-4V and Ti-Al-2.5Fe alloys. The $10^{15}$ ions/cm² does not bring any significant improvement and $10^{17}$ ions/cm² is detrimental when implanted in Ti-6Al-4V. After the corrosion tests, all the ion-implanted surfaces also showed the presence of Ca and P. Electrochemical impedance spectroscopy tests performed on both nitrogen- and carbon- sputter-coated 316 L stainless steel demonstrated that an increase in thickness of coating was accompanied by an increase in corrosion resistance.

Nitrogen was the common element used in ion implantation and sputter coating to provoke physico-chemical changes in the materials. As a consequence similar cell culture results would be expected. The cell-culture results obtained showed two opposite behaviours. A good result from the ion-implanted samples, where a cell layer was grown after 19 days of culture, contrasted with a bad cell-culture result from the nitrogen- sputter coated samples, where only a few osteoblasts had attached to the substratum and no extracellular matrix or cell layer was produced.

The cell suspension used on all the samples was the same, so in spite of the presence of nitrogen on all the surfaces their physico-chemical characteristics
appear not to be the same. Similar results might nevertheless have been expected for the stainless steel samples, either ion-implanted or nitrogen sputter coated. XPS analysis of the surfaces after cell culture provided the answer. The presence of Cr and Ni ions, known for their toxicity, was detected on the sputter-coated samples but they were absent on ion-implanted stainless-steel surfaces.

All ion-implanted materials showed similar in vitro results obtained with rat bone marrow cells. According to SEM observations an increase in Ca- and P-rich globules was detected on ion-implanted surfaces compared with untreated materials. The production of these globular accretions is an indication that the osteoblasts are expressing their phenotype and they are also the first stage leading to bone formation. Therefore, N⁺⁺ ion implantation enhances the biocompatibility of the titanium alloys and 316 L stainless steel whilst nitrogen-sputter coating is detrimental.

The calcium-phosphate coating method described in this thesis shows that the formation of a Ca- and P-rich layer is directly related to the type of metal substratum and roughness.

The formation of a homogeneous and adherent calcium-phosphate layer was only achieved when Ti-6Al-4V surfaces were mechanically ground down to a roughness mean height of 0.24 ±0.03 μm. On all other surfaces the calcium-phosphate coating either did not adhere well to the substratum or only scattered calcium-phosphate crystals were formed. Surface analysis results on the coating showed that its composition is apatite-like. As mentioned in Chapter 1, it is known that calcium phosphates enhance bone formation at their surfaces and that coating thicknesses of at least 1 μm are needed for the calcium phosphate to cause bone induction. SEM observations of the apatite-like coating showed that its thickness is ca. 5 μm. In fact, rat bone marrow culture performed on samples that were coated by this immersion method showed that cell proliferation was achieved at an earlier stage when compared to non-immersed surfaces.
The results obtained in this thesis show that surface modification of biomaterials by means of N⁺ ion implantation, sputter coating or immersion in a simulated body fluid have a direct influence on the material's biological behaviour.
Conclusions

In summary the following conclusions can be drawn from this thesis:

Surface modified materials

N⁺- ion implanted Ti-6Al-4V

1 - Implantation of 10¹⁶ ions/cm² N⁺ improves the corrosion resistance of Ti-6Al-4V alloy surfaces. This behaviour can probably be explained by the existence of a more stable oxide film formed during the ion implantation process.

2 - A fluence of 10¹⁵ ions/cm² does not bring any improvement.

3 - A fluence of 10¹⁷ ions/cm² N⁺ is detrimental, probably due to the large size of titanium nitride precipitates and/or appearance of a carbonitride phase.

4 - Corrosion of Ti-6Al-4V alloy in HBSS produces a calcium- and phosphate-rich surface layer, but this process seems to be inhibited when the alloy is implanted with a N⁺-ion fluence of 10¹⁷ ions/cm².

N⁺- ion implanted Ti-5Al-2.5Fe

5 - Nitrogen-ion implantation of Ti-5Al-2.5Fe produces surfaces with lower passivation current density and higher corrosion potential than the non-implanted ones.

6 - Ion implantation with 10¹⁶ ions/cm² produces surfaces with the noblest corrosion potential. All fluences produce surfaces with similar ipass indicating that the formed films have similar protective capacities.
7 - The corrosion of Ti-5Al-2.5Fe in HBSS produces a calcium- and phosphate-rich surface layer.

*N+ ion implanted 316 L stainless steel*

8 - Nitrogen-ion implantation with $10^{16}$ ions/cm² improves the corrosion resistance of 316 L stainless steel. The formation of more stable films as a consequence of the ion-implantation process may explain this behaviour.

9 - Fluences of $10^{15}$ ions/cm² do not bring any improvement and fluences of $10^{17}$ ions/cm² reduce the $i_{pass}$.

10 - Corrosion of 316 L stainless steel in HBSS produces a calcium- and phosphate-rich surface layer.

*Nitrogen- and carbon- sputter coated 316 L stainless steel*

11 - Thicker sputter coatings seem to produce materials with higher corrosion resistance. POLYTROL impregnation after carbon sputter increases the corrosion resistance.

12 - The high charge transfer resistance ($R_{tc}$) attained by the N2 surfaces is accompanied by the lowest interface capacitance. The surface film produced is then the most protective.

13 - Applied d.c. potentials ≥ 1 V decrease $R_{tc}$ significantly. Pitting potential is between 700 and 1000 mV. The impedance response obtained by applying 1000 mV showed that at this potential the material is in the transpassive region, therefore the pitting potential is lower than 1 V.

14 - The corrosion interface of nitrogen- and carbon-sputter coated 316 L stainless steel, up to 1 V, may be represented by a three-element equivalent electric circuit. The comparison between the experimental Bode curves and a curve obtained from a response from a built-up three element electric circuit, as described before, show their similarity.
**In vitro testing and surface analysis**

15 - A bone-like tissue grew, in culture, on all studied materials except on the nitrogen sputter-coated samples.

16 - The SEM observation of the cell layer surface on all the materials, except on the sputter-coated samples, showed that the morphology was similar, consisting of osteoblast-like cells and an extracellular matrix made up mainly of collagen where Ca- and P-rich globules were trapped.

17 - The study of the interface substrata/cell layer showed the presence of Ca- and P-rich globular deposits in higher density than on the cell layer surface.

18 - Enhanced production of globules is seen on nitrogen ion-implanted metals, which indicates that bone is more likely to be formed on these surfaces.

19 - 316 L stainless steel sputter coated with either nitrogen or carbon does not seem a suitable material for biomedical use. Cells grew heterogeneously on such surfaces and on some areas they were not even able to form an extracellular matrix.

20 - *In vitro* cell culture seems to be a fairly reliable method to test biomaterials *in vitro* because the interface formed is both morphologically and chemically similar to those observed *in vivo*.

21 - The bad cell culture results exhibited by the sputter-coated 316 L stainless steel seem to result from the existence of Cr and Ni ions on the metal surface.

22 - SEM observations of the interface between the cell layer and the substrata together with the XPS results suggest that the surface of the nitrogen ion-implanted Ti-6Al-4V is mainly composed of TiO$_2$ and a biologically produced calcium-phosphate compound.
Calcium-phosphate coating

23 - XRMA together with XPS and XRD suggest that the calcium phosphate layer produced by immersion in HBSS is mainly in the form of an amorphous apatite-like layer.

24 - Ti-5Al-2.5Fe alloy seems to allow formation of a more stable calcium phosphate more easily, as evidenced by the higher density of calcium phosphate-rich globules.

25 - The results indicate that the Ti surface acts as an inducer of the apatite from the HBSS solution.

26 - The in vitro bone marrow results suggest that the apatite-like layer formed may improve the bone bonding characteristics of the studied Ti alloys.

27 - The morphology of calcium phosphate precipitates depends on the metal substrate and its surface characteristics.

28 - It is possible to produce a naturally formed calcium phosphate coating by immersing metals such as titanium alloys and stainless steel in HBSS.

29 - Ti-6Al-4V 4000 seems to be the surface that is most favourable to produce a continuous and more adherent apatite-like coating capable of bone induction.