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In vitro calcification of orthopaedic implant materials

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The formation of an apatite-like layer is achieved by immersing Ti6Al4V and TiAl2.5Fe substrata in Hank's Balanced Salt Solution (HBSS). The layer was characterized by several techniques, namely X-ray microanalysis, X-ray diffraction and X-ray photoelectron spectroscopy. 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 calcium and phosphate 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 on both 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.

1. 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, calcium phosphates have been coated on to their surfaces. The natural precipitation of a calcium phosphate layer seems to be a simple and low cost method of creating 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 1 μm after 2 weeks of differential immersion [1]. Hanawa and co-workers [3–5] 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 commercially pure titanium [6] and in the passive films of Ti6Al4V 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 techniques, namely X-ray microanalysis (XRMA), X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS). The Ca and P concentrations were determined by atomic absorption spectroscopy and spectrophotometry, respectively.

2. Materials and methods

2.1. Surface preparation

Ti6Al4V and TiAl2.5Fe (Deutsch Titan) samples, 9.15 mm and 5 mm 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 min, followed by a 20 min double rinse with distilled water [8].

2.2. Immersion method

After surface polishing and cleaning, the Ti6Al4V and TiAl2.5Fe 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 h. The pH was recorded as a function of time. The remaining solutions were then stored in 1 ml Eppendorf tubes at room temperature. Ca and P concentrations were later determined by means of atomic absorption spectrometry (Varian SpectAA 300) and spectroscopy (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).

2.3. Rat bone marrow culture

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

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

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plates (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 then performed. The specimens were gold sputter coated, observed by SEM and analysed with XRMA at accelerating voltages of 15 kV and 20 kV, respectively.

3. Results and discussion

3.1. Immersed surfaces

Fig. 1 shows the curves obtained when monitoring 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 a similar pattern consisting of a rapid increase in the pH from the initial 7.5 to 8.5 in the first 2 days; after day 7 both alloys exhibit a decrease in the pH, while the pH of 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.

A reduction in the pH was also detected by 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 HBBS 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 the Ca and the P curves were similar to those for the 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 of the samples 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 the 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 Ti6Al4V is less rough and that the layer formed on TiAl2.5Fe 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 to P ratio of 1.4 in the calcium phosphate precipitate and 1.6 in the "globules". Due to the higher number of "globules" formed on TiAl2.5Fe, it seems that this material more easily allows the formation of a more stable calcium phosphate.

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, $E_{b,C1s} = 285.0$ eV, as reference.

Fig. 4 shows XPS spectra acquired from non-immersed and immersed TiAl2.5Fe samples. Ti4Al4V exhibited similar spectra with respect to the presence of Ca and P after immersion. All samples exhibited a well defined C 1s peak, which is a common contaminant. The presence of the alloy elements can be noticed. Vanadium was never detected on the Ti4Al4V 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.

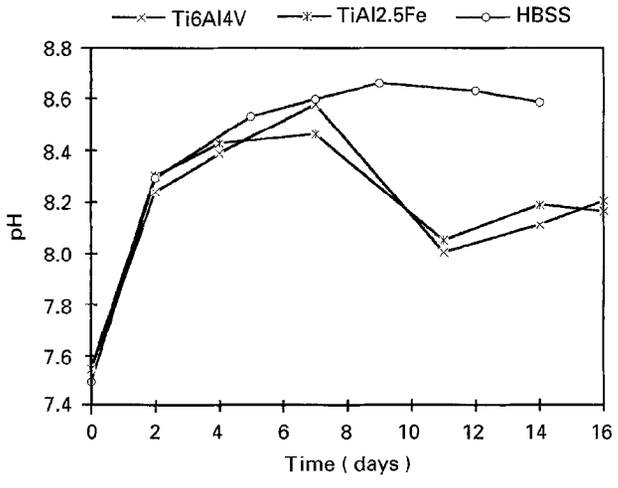

Figure 1 pH changes as a function of time.

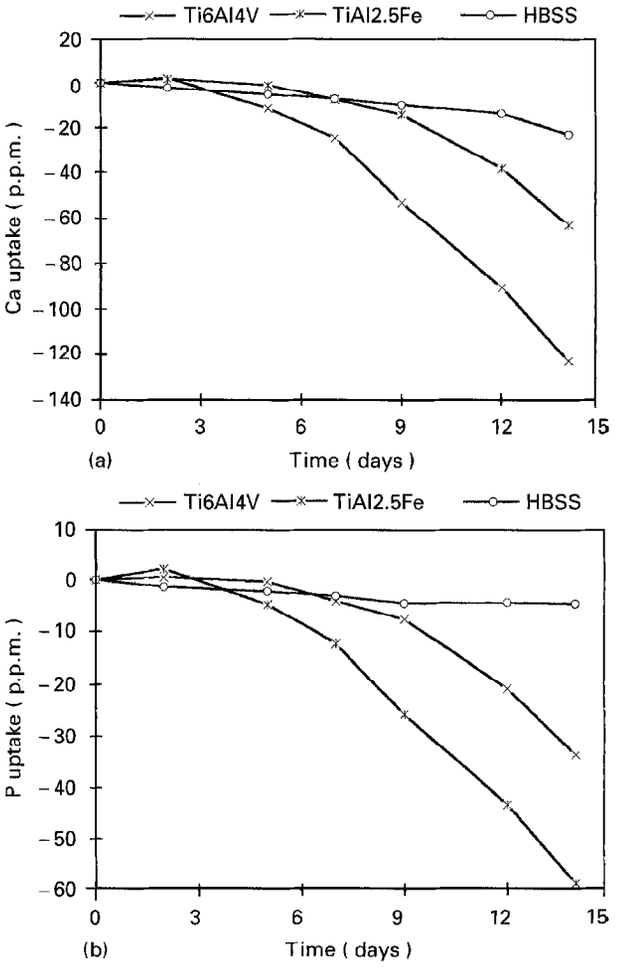

Figure 2 (a) Ca and (b) P uptake after immersion in HBSS.

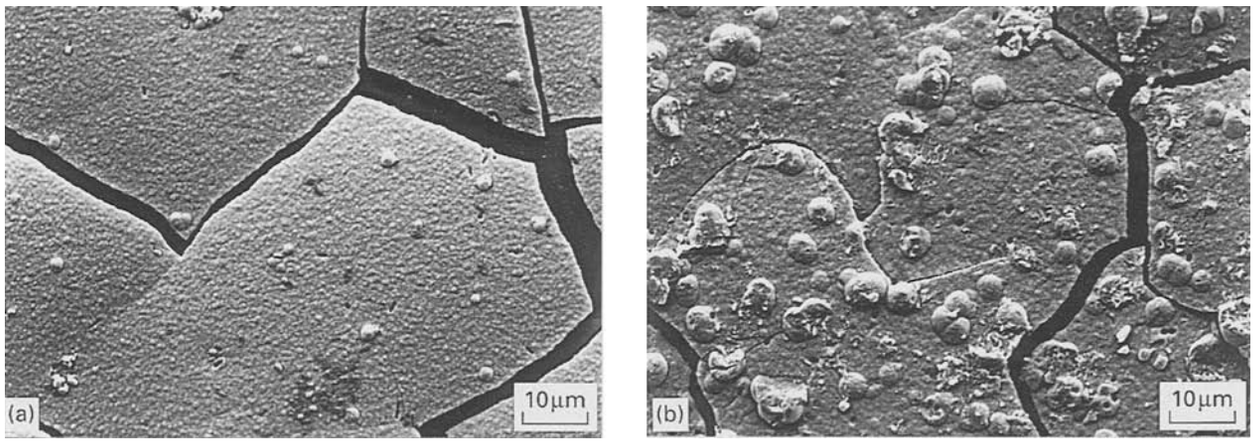

Figure 3 SEM photographs of immersed surfaces of (a) Ti6Al4V and (b) TiAl2.5Fe after 14 days' immersion in HBSS.

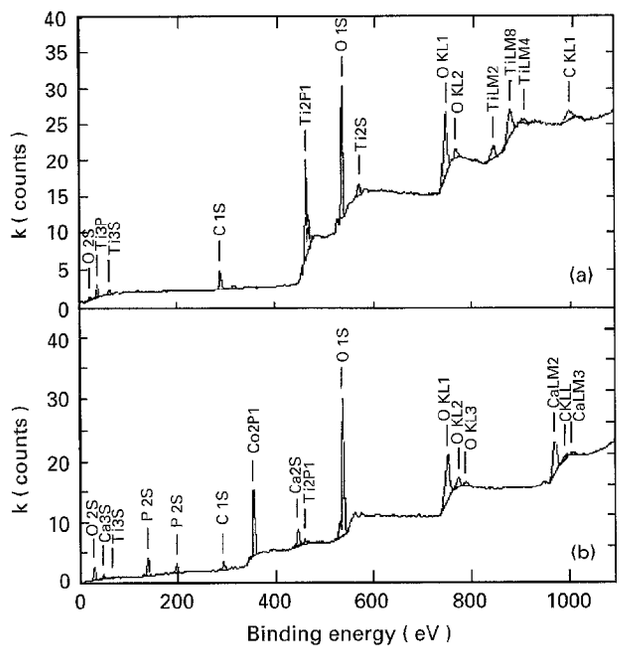

Figure 4 XPS spectra of TiAl2.5Fe (a) before and (b) after 14 days' immersion in HBSS.

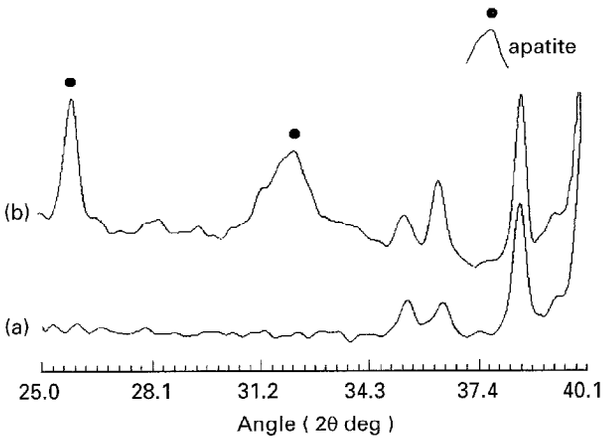

Figure 5 XRD spectra of Ti6Al4V (a) before and (b) after 14 days' immersion in HBSS.

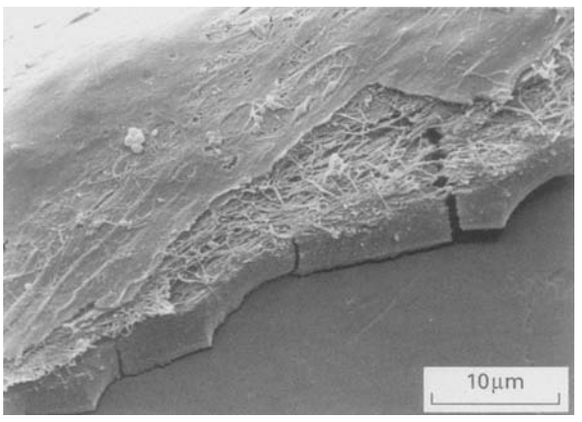

Figure 6 Interface between the metal substratum (A), the apatite-like layer (B) and the cell layer (C).

Fig. 5 shows XRD spectra acquired from the non-immersed and immersed surfaces of Ti6Al4V. On the immersed samples a well-defined [0 0 2] peak and a broader one which is probably constituted by the junction of peaks [2 1 1] and [1 1 2], indicating the amorphous characteristics of the calcium phosphate. These results suggest that the precipitate layer has an amorphous apatite-like structure.

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

Apatite formation on commercial pure titanium 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 a bioliquid. He reported an apatite film grown on Ti6Al4V with a thickness of 7 nm, 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 preferred 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].

3.2. Cell culture

The *in vitro* cell study focused on the cellular responses to the apatite-like layer formed by immersion of the titanium alloys. Fig. 6 shows a photomicrograph of a Ti6Al4V sample previously immersed in HBSS which was inoculated with rat bone marrow. There is 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). 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 only loosely attached [12]. This may be due to differences in the substrata roughness and to the fact that the produced layer is similar to apatite and is capable of causing bone induction. Rougher surfaces may allow the osteoblast cell to have more points of adhesion for the production of extracellular matrix [13, 14]. Light microscopy monitoring of the cell culture as a function of time showed that cell proliferation was attained at an earlier stage with 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]. These were more visible on non-immersed samples due to the poor attachment of the cell layer to the metal substratum.

4. 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. TiAl2.5Fe 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 titanium 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 titanium alloys.

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