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Recubrimientos biocompatibles obtenidos por Proyección Térmica y estudio *in vitro* de la función osteoblástica

Memoria presentada para optar al grado de Doctor en Ciencias Químicas por Mireia Gaona Latorre, bajo la dirección del Profesor Josep Maria Guilemany Casadamon y el Profesor Javier Fernández González

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CAPÍTULO 7:

Evaluación y comparación de la respuesta biológica de recubrimientos de hidroxiapatita por proyección térmica de alta velocidad

7.1 Introducción

En los capítulos 5 y 6 se optimizaron las condiciones de proyección para la obtención de recubrimientos de HA mediante HVOF, se mejoró la adherencia de estos recubrimientos tras inmersión en SBF y se realizó su caracterización en cuanto a sus propiedades físico-químicas. Para completar su caracterización, en este capítulo se han estudiado algunos indicadores de la respuesta celular de dichos recubrimientos, lo cual es indispensable en el análisis integral de un biomaterial.

El efecto de la hidroxiapatita sobre la respuesta en un tejido adyacente aún no ha sido plenamente descrito. A pesar que varios autores han demostrado que la

respuesta celular depende de las características físico-químicas del sustrato, de la composición química [1,2], la cristalinidad [3,4], y la rugosidad [5], las características superficiales necesarias para una óptima osteointegración no son totalmente comprendidas.

Los estudios *in vitro* son el primer paso para la comprensión de la interacción material-célula dado que estos sistemas no presentan la complejidad de los sistemas *in vivo*. Los cultivos celulares son un procedimiento para el estudio de células vivas en un medio artificial que permite reproducir, de forma bastante fiable, las condiciones biológicas que las células tienen en su lugar de origen. Como los recubrimientos desarrollados en esta Tesis Doctoral tienen como finalidad ser utilizados en aplicaciones de reparación y regeneración ósea, el cultivo celular idóneo sería el de células obtenidas del órgano de interés, en este caso de hueso. Los estudios *in vitro* se han realizado utilizando células óseas, concretamente osteoblástos ya que se trata de células formadoras de la matriz ósea, y se encuentran constantemente en el frente de avance del hueso que crece o se desarrolla.

La introducción del cultivo de osteoblastos humanos ha permitido la obtención modelo experimental más fidedigno evitando las posibles conclusiones erróneas al utilizar otro tipo de cultivos celulares que, como consecuencia, no permitiera una extrapolación de sus resultados a que sucede “in vivo”.

Cabe mencionar que la biocompatibilidad de los recubrimientos de HA obtenidos por proyección térmica de HVOF apenas ha sido estudiada por lo que los resultados mostrados en esta tesis pretenden ser un referente en estos estudios .

7.2 Objetivos

La finalidad de este capitulo fue analizar la respuesta celular sobre 3 recubrimientos de HA obtenidos por HVOF. Los recubrimientos seleccionados para el estudio fueron:

- Recubrimientos obtenidos con las condiciones optimizadas (82% HA cristalina)
- Recubrimientos totalmente cristalinos conseguidos tras un tratamiento térmico (700ºC, 1 hora)
- Recubrimientos con cristalinidad gradual (interfaz substrato-recubrimiento 100% cristalina, superficie del recubrimiento 82% HA cristalina)

Para dicho motivo se realizaron cultivos de células osteoblásticas sobre los diferentes recubrimientos, siendo los objetivos de este capítulo:

1. Determinar la viabilidad y proliferación de osteoblastos humanos sobre los diferentes recubrimientos de HA.

2. Determinar el comportamiento de diferenciación de osteoblastos humanos sobre los diferentes recubrimientos.

7.3 Resultados

En el artículo *"Testing the biocompatibility of HVOF-sprayed hydroxyapatite coatings using human osteoblasts cultured in vitro"* que se adjunta con este capítulo se muestran los resultados obtenidos de los ensayos *in vitro* realizados con cultivos de osteoblastos sobre los tres recubrimientos de HA seleccionados.

Testing the biocompatibility of HVOF-sprayed hydroxyapatite coatings using human osteoblasts cultured in vitro.

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Resumen:

En este trabajo se estudió la respuesta celular de 3 recubrimientos de HA y su adherencia a un substrato de Ti6Al4V tras un ensayo de inmersión en SBF. Los recubrimientos presentaban una rugosidad similar, pero diferente composición química por lo que las diferencias en los resultados vendrán dados por esta última variable.

Se sembraron celulas osteoblásticas sobre los recubrimientos y se cultivaron durante 1, 3, 7 y 14 días para estudiar su morfología, viabilidad, proliferación y diferenciación. El hecho más significativo fue que las células estaban más diferenciadas en recubrimientos parcialmente amorfos que totalmente cristalinos, pero a su vez estos recubrimientos eran los que fallaban por adherencia tras inmersión en fluidos corporales simulados (SBF).

Por este motivo los recubrimietnos de cristalinidad gradual se presentan como alternativa ya que presentan una buena adherencia al substrato por la alta cristalinidad de la interfaz que evita que se disuelva en SBF y exista un fallo adhesivo y un buen comportamiento *in vitro* facilitado por la capa parcialmente amorfa que le permite la deposición de una capa de apatita y aumenta los niveles de diferenciación de los osteoblastos.

Testing the biocompatibility of HVOF-sprayed hydroxyapatite coatings using human osteoblasts cultured *in vitro*

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ABSTRACT

Here we study the influence of crystallization on the properties of high velocity oxy-fuel sprayed hydroxyapatite (HA) coatings following heat treatment. We examined three types of coating: as-sprayed HA, heat treated and graded crystalline HA. The HA coatings were characterized using scanning electron microscopy and X-ray diffraction, while the bonding strength of the coatings to the substrates was determined using the standard test ASTM F1147-99. The crystallization of the coating after heat treatment increased the bonding strength of the coatings after immersion in simulated body fluid (SBF), measured at various points in time. The biocompatibility of the coatings on titanium alloy substrates was studied by means of human osteoblastic cultures. Cellular behavior was studied in terms of morphology, viability, proliferation, and differentiation of osteoblasts. Viability and proliferation rates were analyzed quantitatively using colorimetric tests; cell attachment and morphology were analyzed qualitatively using environmental scanning electron microscopy. Our results show that osteoblast viability and proliferation were not sensitive to the chemical or phase features of the HA coatings. However, osteoblastic differentiation was higher in the presence of amorphous calcium phosphate (ACP). Thus, graded crystalline HA coatings were produced here in order to achieve a balance between the behavior of the as-sprayed coatings *in vitro* and the positive effects obtained in terms of the adhesive strength of the crystalline coatings.

Keywords: Thermal spray, high velocity oxy-fuel (HVOF), hydroxyapatite coating (HA), bond strength, *in vitro* testing, osteoblast culture.

1. INTRODUCTION

Improving the quality and performance of orthopedic implants requires the development of coatings that present better properties and bio-performance characteristics than existing devices. Several techniques are available for the ceramic coating of a metal substrate, including ion-beam sputtering, electrophoretic deposition, RF-magnetron sputtering, laser ablation, thermal spraying technologies and biomimetic coatings [1-5]. Thermal spraying, or more particularly Atmospheric Plasma Spraying (APS), has become the main technique for applying hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) coatings onto metal implants in order to improve fixation and bone growth. The main advantages of plasma spraying are the rapid rates of deposition that can be achieved at relatively low cost. However, due to the extremely high temperature of the plasma flame and the rapid cooling rate, the phase and structure of the HA coatings differ considerably from those of the feedstock [6, 7].

In recent years, the use of high velocity oxy-fuel spraying (HVOF) has allowed the production of HA with a similar set of advantages to those offered by APS, but which additionally offer a low content of amorphous calcium phosphate (ACP) thanks to the high flame velocity and moderate temperatures required [8, 9]. The high particle velocities achieved in HVOF are believed to enhance the adhesion strength of the HA coating on the metallic substrate, while the moderate flame temperature is particularly suited to the deposition of materials with low melting temperatures or low phase transformation points. However, few studies analyzing the production of HA coatings with HVOF technologies have been reported.

Similarly, the effects of the crystallization of the amorphous phase on the coating properties of HVOF-sprayed calcium phosphate coatings are poorly understood. Previous studies have reported that such coatings are 100% HA crystalline following appropriate heat treatment [9]. Furthermore, they are stable in simulated body fluid (SBF), since crystalline HA is more stable than other calcium phosphate phases in physiological conditions, due to its lower solubility and slower resorption kinetics. The dissolution rate of different CP phases in the SBF has been reported to occur in the following order: Amorphous Calcium Phosphate (ACP) > > Tetracalcium Phosphate (TTCP) > α Tricalcium Phosphate (α-TCP) > β Tricalcium Phosphate (β-TCP) >> hydroxyapatite (HA). The relative insolubility of hydroxyapatite compared to that of

other calcium phosphate phases is expected, since it is the only stable calcium phosphate compound at pH above 4.2. [10]

Although several studies have examined the cellular behavior of HA plasma spray coatings *in vitro* only a few report their findings for HVOF coatings [11]. In this study, HA coatings were obtained by HVOF spraying and biological studies were then performed on them. Human osteoblast cells were cultured on three different HVOF-sprayed hydroxyapatite coatings. Cell proliferation, attachment and morphology were analyzed using the BrdU test, the MTT test and environmental scanning electron microscopy (ESEM) respectively. Moreover, osteoblastic differentiation was studied by means of alkaline phosphatase (ALP) activity.

2. MATERIALS AND METHODS

Preparation of HA coatings

HA powder CAPTAL 30 (Plasma Biotal, UK) was deposited onto previously grit blasted Ti6Al4V substrates with a Sulzer Metco DJH 2600 HVOF system. The spraying parameters employed are reported elsewhere [9]. Three different HA HVOF-sprayed coatings were examined: an as-sprayed coating, a heat treated coating and a combination of these two coatings which comprised a heat treated coating as the bond coat with an HA as-sprayed coating on the surface.

It has been reported that the amorphous-crystalline phase transformation of HA takes place between 500ºC and 700ºC [12, 13]. Therefore, the as-sprayed coatings were heat treated at 700°C for an hour in air atmosphere followed by a slow cooling back to room temperature within the furnace. Some coatings were re-sprayed on the substrates after the heat treatment. Then, HA as-sprayed coatings were deposited onto the thermally treated coatings.

Before conducting the cell culture experiments, all the coated samples were sterilized by means of gamma radiation (25kGy) to ensure conditions were sterile.

Structure and phase characterization

Cross-sections of the coatings were examined using scanning electron microscopy (SEM). The samples were cut with a diamond saw, vacuum impregnated with a low viscosity epoxy resin (Caldo Fix, Struers, USA) and polished using standard metallographic procedures. Ten images of the cross-sections of each coating were analyzed via image analysis to determine the porosity levels. Finally, the samples were etched with an acidic solution (2% HNO_3) that preferably dissolves the amorphous calcium phosphate phase.

Quantitative phase composition analyses of the crystal structure of the crystalline phases for each coating were carried out using the Rietveld method. The method uses a least squares approach to refine a theoretical line profile until this matches the measured profile [14] and this then enables us to obtain structural parameters directly from X-ray powder diffraction patterns. Coatings were scraped off the substrate and mixed with ZnO as standard. Then the mixtures were analyzed on a Siemens $\theta/2\theta$ D-500 diffractometer in Bragg-Brentano geometry. Cu Kα radiation was employed (λ= 1.5418 A) with a graphite monochromator and data were collected from 8 to 80º (2θ) with a step size of 0.02º and a counting time of 24 s.

The average roughness (Ra) was measured using a Surftest 301 roughness tester (Mitutoyo, Tokyo, Japan), where Ra is the average deviation of the profile from a mean line.

Bond strength

The coated samples were immersed in SBF, prepared in line with Kokubo [15], for periods of between 1 and 28 days. The samples were rinsed with distilled water and dried in air after immersion. The bond strength was then measured using the tensile test ASTM F1147-99 designed especially for calcium phosphate coatings [16].

Cell culture

Human osteoblast cultures were obtained from three different trabecular bone of total knee replacement surgery patients with the patients' previous written informed consent**.** The study was conducted in accordance with the Declaration of Helsinki of 1975, revised in 1983, and approved by our local Ethics Committee (Hospital del Mar-IMIM). Samples were removed from the surgically excised bone in the operating room and immediately stored in sterile plastic bottles with Dulbecco's Modified Eagle's Medium (DMEM).

Primary osteoblast cultures were prepared according to a modification developed in our laboratory [17] of a previously published procedure [18]. The bone was first cleared of soft tissue and then washed several times with phosphate buffer (PBS). Clean bone fragments were cut into explants of 1-2 mm that were then placed in culture plates. The explants were kept in DMEM containing 10% fetal calf serum (FCS) at 37ºC for one week. After the first week, cells were obtained from the surroundings of the explants and the serum was subsequently changed every three or four days. The cells were cultured in DMEM media (4.5 g/l of glucose) supplemented with pyruvate (1 mM), glutamine (2 mM), penicillin (56 U/ml), streptomycin (56 µg/ml), fungizone (1.5 µg/ml) and 10% of FCS. The cultures were stored at 37ºC in a damp air atmosphere containing 5% CO_2. We obtained full plate coverage at four to five weeks, at which point the cells were treated with 0.25% trypsin/EDTA (Biological industries), subcultured and frozen with liquid nitrogen.

The coated samples were then placed on a 24-well polystyrene culture plate (Nunk A/S) and immersed in Hank's balanced salt solution (HBSS) (Sigma Aldrich) overnight at room temperature. After removing Hank's solution, coated samples were seeded with 12000 cells for cell morphology, viability and proliferation and 200000 cells for differentiation studies. After 24 hours of cell culture, the coated samples were removed from the original well in order to ensure that the results obtained were provided solely by the cells on the coating.

Cell morphology and attachment

Cell morphology and attachment onto the coatings were observed by means of Environmental Scanning Electron Microscopy (ESEM) (Electroscan 2020, Boston, MA). The use of ESEM enables us to observe the biological specimens that have not been coated with a conductive material such as gold or carbon. Cells were fixed with 2% glutaraldehyde in 0.1M sodium cacodylate buffer for 2 hours at 4ºC prior to ESEM observation.

Cell viability and proliferation assays

Cell viability was evaluated using an MTT assay kit (Roche Diagnostics GmbH) at 3, 7 and 14 days of culture. This experiment is based on the cleavage of the yellow tetrazolium salt (MTT) to purple formazan crystals by metabolically active cells. The resulting colored solution was quantified using a spectrophotometer (550 nm). Cell proliferation was determined at 3, 7 and 14 days of culture using a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis (Roche Diagnostics GmbH). Before the proliferation assay, cells were synchronized by incubation in serum-free medium with 0.1% BSA for 24 hours. Then, serum-containing medium was added 24h before BrdU. The absorbance was measured at 450 nm in a spectrophotometer.

Cells cultured on 24-well polystyrene plate were used as a control (CPS; culture polystyrene) in both experiments. As approximately a quarter of the original cells were deposited on the material after 24 hours of seeding, 3000 cells were then seeded in the positive control (CPS) to balance out cell numbers with regard to the seeded materials. Samples of each coating studied with no seeded cells were used as negative controls.

Alkaline phosphatase activity

Osteoblastic differentiation levels were evaluated with the expression of alkaline phosphatase (ALP) activity [19]. HA coated samples were placed individually in 24-well plates and cultured for 7 and 14 days in DMEM supplemented with 10% FBS, 100 mg/ml of ascorbic acid, 10^{-8}M of vitamin K and D. The cell layers were washed with PBS and 1% Triton X-100 (Serva) in 1 M tris-HCl (pH=10). Then, the ALP

activity in the osteoblast lysates was determined using a commercially available kit (Sigma). ALP activity was determined by the concentration of p-nitrophenol liberated as a result of the conversion from p-nitrophenyl phosphate. The p-nitrophenol was measured at 450 nm by means of a spectrophotometer.

Statistical analysis

Data from every assay were expressed as mean ± standard deviation (SD) and statistical differences were determined by U-Mann Whitney test. Data from each independent experiment were normalized to one of the materials within the experiment. Each experiment was performed in triplicate and repeated at least three times. P-values less than 0.05 were considered significant

3. RESULTS

Microstructure characterization

Figure 1 shows high magnification SEM micrographs of the cross-sections of etched HA coatings. The chemical etching preferentially removed amorphous material, revealing the lamellar coating structure of the as-sprayed coating, but there was no similar removal on the heat treated coatings. Figure 1c shows the microstructure of the graded crystalline coating, where as-sprayed coating was deposited onto a previously heat treated coating. Here, the coating in contact with the substrate was 100% crystalline and no removal of ACP was observed.

A total of 15 thickness, roughness and porosity measurements were recorded for each coating. The thickness of the as-sprayed coatings was 68.6 ± 6.0 μm and its surface roughness was 4.2 ± 0.4 μm. After heat treatment, the cross-sectional thickness was found to be 67.6 ± 5.4 μm and the coating roughness was 4.1 ± 0.7 μm. However a change in porosity can be deduced from the SEM images between as-sprayed and crystallized coatings. The porosity of the as-sprayed coatings was 13 ± 2 %, whereas for the annealed coating was 6 ± 1 %

Phase analysis using XRD

The XRD pattern of the as-sprayed HA and the post treated coatings are shown in Fig 2. The only crystalline phase present in the coatings was HA (Powder Diffraction File 9-432 JCPDS). No other crystalline calcium phosphate phases such as tricalcium phosphate ($Ca_3(PO_4)_2$,α- or β-TCP), tetracalcium phosphate ($Ca_4P_2O_9$, TTCP) or CaO were found in the as-sprayed coating as a result of the thermal decomposition of HAp during coating deposition. Experimental and calculated XRD patterns presented a very good Rietveld fit (chi^2 = 2.79). The content of crystalline HA was 82% with 18% ACP in the as-sprayed coating, whereas the heat treated coating was 100% crystalline. This result is in line with the chemical stability of etching with HNO_3 (2%).

The HA cell parameters refined by the Rietveld analysis are summarized in Table 1. The c-axis dimension of the HA powder was lower than the corresponding value for the coatings. Some studies have proposed a hexagonal crystal structure for the oxyapatite with lattice parameters similar to those of hydroxyapatite but with a larger c-axis, on the basis of high-resolution electron microscopy following the on-beam decomposition of hydroxyapatite [20].

HA and oxyapatite (OHA) have a hexagonal crystal structure, but the larger c-axis dimension of oxyapatite is shown as a shift in the (00l) plane diffraction to smaller angles according to Bragg's Law. The (004) plane diffraction position using Cu Kα radiation for different compounds is shown in Table 2. β-TCP has a rhombohedral crystal structure and the plane (004) diffracts at 2θ= 52.9º, whereas the plane of titanium diffracts at 53º. It would appear that β-TCP is not present in the coating as the main diffraction peaks are not observed. The diffraction peaks for (002) and (004) planes for copper radiation ($K\alpha_1$, 1.544 A; Kα 2 , 1.540 A) are shown in Table 2.

Bond strength

The bond strength data were acquired following the ASTM F1147-99, where each value is the average of three readings. The results are summarised in Figure 3. The tensile adhesion values were 37.5 ± 4.8 MPa,

44.2 ± 8.3 MPa and 46.7 ± 4.0 MPa for the as-sprayed, the heat-treated and the graded crystalline respectively. An adhesive-cohesive failure was found for all the coatings before immersion in SBF. However, the bond strength values of the as-sprayed coatings decreased dramatically with immersion time and a change in the failure of the coating was observed, so that after immersion a completely adhesive failure was recorded. The bond strength values of the graded crystalline coatings also decreased with immersion time but not as noticeably as the values recorded for the as-sprayed coatings during the first few days of immersion. The image analysis revealed that adhesive failure occurred between the crystalline and the top as-sprayed coating, so the underneath crystalline coating was not degraded by the SBF. Additionally, no significant deterioration of bond strength was observed after 28 days of immersion for the heat treated coating and the adhesion values ranged from 36.9 MPa to 47.0 MPa.

Cell morphology

High-resolution morphological techniques, i.e. ESEM imaging, allowed us to observe the attachment and growth of cells onto materials after varying times of culture. The typical morphology of a cell attached to the as-sprayed coating surface is shown in Fig. 4. Figures 5, 6 and 7 show the time evolution of the osteoblast cultures for the as-sprayed, the heat treated, and the graded coatings respectively. These images are representative of a series of repeated experiments, demonstrating that the adhesion characteristics were consistent and reproducible. After three days of incubation, the cells were attached to and proliferated on all the coating surfaces. The population of osteoblasts increased over time and the cells exhibited a flattened and well-spread shape on all coatings. No apparent differences in cell morphology on any of the surfaces were found. ESEM observations after 14 days of culture showed a confluent cell layer for the three coatings. Moreover, Ca-P globules appeared on the as-sprayed surfaces suggesting that extracellular matrix mineralization was starting. The globules did not appear on the heat treated surface.

Cell viability assay

The MTT tests were used to assess the cytotoxicity of the HA coatings. This test facilitates the quantitative measurement of cell viability. The MTT was assayed at 3, 7 and 14 days of osteoblast cultures (Figure 8). The materials were not cytotoxic to osteoblasts and the cell cultures were prepared in optimal conditions

since cell viability increased in the case of all three coatings and a better cell viability even than CPS (higher absorbance) was recorded. The increase in cell viability with time indicates that the cells were growing on the materials, with higher colorimetric detection representing more living cells. No statistically significant differences were recorded between the different materials, or with the CPS control, as regards the number of cells that attached after a given number of days of incubation.

Cell proliferation assay

Cell proliferation was expressed as the absorbance after 3, 7 and 14 days of culture for the three coatings and the controls (Figure 9). DNA content increased from day 3 to day 14 on each coating and CPS studied. There were no significant differences in DNA content between the coated substrates and CPS. Both assays (MTT and BrdU) showed complementary results which are consistent with cellular adhesion on the material. Thus, as the BrdU value increased, the MTT also increased.

Cell differentiation

Differentiated osteoblasts express ALP activity. Therefore, it is important to measure ALP activity so as to ensure that the results obtained in the different assays correspond to osteoblast cultures. Fig. 10 shows the ALP activity of cells cultured on HA coatings and CPS plate after 7 and 14 days of cell culture. The results from three different line osteoblast cultures showed similar features in terms of ALP activity. The ALP activity of the cells cultured on HA coatings increased with time. After 14 days of culture the ALP activity was significantly higher on the as-sprayed HA coatings compared to activity levels on the heat treated coatings and CPS. Interestingly the CPS was the material with the lowest ALP activity.

4. DISCUSSION

The HVOF as-sprayed HA coatings exhibited both high crystallinity levels (82%) and low degradation in the absence of other bioactive calcium phosphates, such as tricalcium phosphate (TCP), tetracalcium phosphate (TTCP) or the non biocompatible CaO. The high level of crystallinity and the correspondingly

low degradation of the HA coatings in SBF were probably the result of the low temperatures of the HVOF flame and a possible plastic deformation of the HA particles at a temperature below melting point.

In general, HVOF-sprayed HA coatings exhibited good bond strength. After immersion, the failure was found to be completely adhesive due to the concentration of ACP at the interface and its preferential dissolution in SBF. During the thermal spray process, the cooling rate of the first lamella was controlled by rapid heat dissipation to the metallic substrate and the dehydroxylated region near the substrate solidified to form amorphous calcium phosphate. Thus, the as-sprayed coating is more amorphous at the interface substrate/coating than on the surface. However, 100% crystalline coatings were obtained after an optimal heat treatment and as crystalline HA is very stable in SBF, no dissolution occurred. The heat treated coatings maintained the same value of adhesion to the substrate after the immersion test. A heat treatment at 700ºC for 1 hour succeeded in effectively converting the amorphous calcium phosphate into crystalline phases. The HA structure with the deficient hydroxyl group showed a greater tendency to be converted after spraying into the amorphous phase. This amorphous phase is thermodynamically metastable and an appropriate thermal treatment could induce crystallization. In addition, atmospheric moisture may react with amorphous oxyapatite so that OH groups might recover and promote the reconstitution of the amorphous into the crystalline phase [21]. So, the annealing treatment was therefore useful at 700°C and contributed significantly to an improvement in the bond strength of the HA coatings.

Taking this phenomenon into account, the graded crystalline coatings were obtained in order to achieve a similar adhesion to the substrate after immersion in SBF to that achieved for the annealing coatings, while maintaining a low amorphicity on the surface that might induce the deposition of apatite on the top of the coating. The bond strength values decreased with immersion time as observed in the as-sprayed coating, but the main difference was that after the pull test in the graded crystalline coatings the crystalline HA coating remained fixed to the substrate. If this top coat is degraded in the human body, then this annealed coating might reduce or even prevent altogether the release of metal ions from the Ti-6Al-4V substrate to the surrounding living tissue and attached bone cells.

In vitro techniques are useful for providing an understanding of cell interaction with biomaterials and they constitute an initial approach for the development of suitable biomaterials for *in vivo* studies. Okumura *et al.* [22] reported that the critical event for bone bonding is cell adhesion onto the HA surface followed by osteoblastic differentiation. The ESEM technique was used to observe the evolution of cell adhesion on the HA coatings. As shown in Fig. 5, there did not appear to be any major morphological differences between the cells cultured on the different surfaces. The osteoblastic cells adhered on rough surfaces adapted to the irregularities with filopodia extensions (Fig 4), proliferated and formed confluent cultures by day 14. Based on the ESEM images, HA as-sprayed surfaces have been shown to induce the deposition of calcium phosphate mineral nuclei on their surface after 3 days. The capacity of the as-sprayed coating to mineralize has been assessed by means of an immersion test using a simulated body fluid solution in a previous study conducted by the authors [9]. Apatite or calcium phosphate mineral deposition can be expected therefore to contribute to improved bone bonding *in vivo* and help fill in any pore volumes.

In this study cell lines from three patients were employed. Cell adhesion, viability and proliferation results were not influenced by the source of the cells. MTT and BrdU assays (Figs. 8 and 9) showed complementary results, which are consistent with cellular adhesion on the materials (Figs. 5, 6 and 7). The cells seeded on the coated samples proliferated at the same rate as on the CPS throughout all the culture period, which suggests that there was no preferential cell growth in the control CPS. Thus, the coatings studied can be considered as being as good as CPS for cell culture in terms of their cell proliferation and viability. Moreover, the proliferation levels of osteoblastic cells on the three hydroxyapatite coatings were statistically identical at 3, 7 and 14 days of culture.

The number of living cells on the coatings and the CPS (MTT test) did not differ significantly (Fig. 8). Cell viability and proliferation data are coherent with the ESEM micrographs and did not depend on the crystallinity of the coatings. The main difference between the coatings was their phase composition, but their surface roughness was exactly the same, indicating that the crystallinity of the coating does not seem to be an important parameter for cell growth. Our studies clearly indicate, therefore, that HA surfaces function as excellent substrates for the proliferation and viability of bone cells.

The ALP activity assay suggests that the cells were differentiated on all the coatings, but there was a significant difference in ALP activity between the coatings and CPS. The alkaline-phosphatase activity of osteoblasts seeded on HA coatings was significantly higher than that of the controls (Fig. 10). However, ALP was very sensitive and displayed a high standard deviation. Indeed, all cultures displayed similar behavior, with different ranges of values. At 14 days, the ALP levels in all coatings were significantly higher than they were at 7 days ($p < 0.05$), though not in the case of the CPS. When comparing all coatings, the heat treated coating displayed the lowest ALP activity level at 14 days of culture, but no significant differences were observed in the first week. As the BrdU and MTT tests recorded similar results for the different coatings, a similar quantity of cells was deposited onto each coating, but the higher ALP activity values in as-sprayed surfaces were not influenced by the larger number of living cells. However, the ALP function of the osteoblasts was higher when ACP was present in the coating. A number of *in vitro* studies show that osteogenic differentiation is higher in cultures on amorphous calcium phosphates substrates compared to crystalline substrates [23]. In contrast, other *in vitro* and in vivo studies found that the dissolution of the amorphous phase inhibited the osteogenic differentiation and bone formation [24, 25]. The first behaviour is in agreement with this study, which shows that the release of Ca^{2+} and HPO_4^{2-} ions in the vicinity of the cells will therefore stimulate osteoblastic ALP activity. Moreover, significant mineralized matrix formation was observed by means of ESEM on all as-sprayed surfaces after 3 days, so these results agree with the ALP activity result.

High crystalline HA coatings are therefore valuable for the long-term survival of coatings in application and mechanical performances in terms of their resorbability since the amorphous phase has much greater resorbability in human fluids than crystalline hydroxyapatite, although it shows an inhibiting effect on cell proliferation as measured by ALP activity. By contrast, the presence of amorphous calcium phosphate (ACP) enhances the solubility of the coating in SBF, which might jeopardize the adhesion of the coating, but should nevertheless accelerate the fixation of the implant. The graded crystalline coatings obtained in this study presented a crystalline HA layer next to the substrate that ensured good mechanical properties in human fluids while an as-sprayed surface ensured osteoblastic differentiation.

5. CONCLUSIONS

- The HVOF-sprayed HA coating presented a higher crystallinity than that obtained with plasma spraying.
- The rapid solidification of crystalline HA as an amorphous phase in the HVOF-sprayed coating is reversible. 100% HA crystalline coatings were obtained after a one-hour heat treatment at 700ºC .
- The HVOF-sprayed HA coating exhibited high bond strength. Bond strength decreased in the case of as-sprayed coatings after immersion in SBF, but it remained constant for the heat treated coatings. This phenomenon is related to amorphous phase dissolution at the interface substrate-coating in as-sprayed coatings.
- Osteoblast viability and proliferation is not sensitive to the chemical and phase features of the hydroxyapatite coatings. A confluent monolayer of osteoblasts was observed on each coating after 14 days of incubation. Moreover, *in vitro* studies show that osteoblastic differentiation is higher in cultures on partially amorphous substrates than on HA crystalline substrates.
- The graded crystalline HA coatings were produced in order to achieve a balance between the *in vitro* behaviour of the as-sprayed coatings and the positive effect of crystalline coatings on adhesive strength. Both properties are desirable in biocompatible coatings and research efforts are moving increasingly in this direction.

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Table Captions

Table 1: Cell parameters of the crystalline phases recorded in the coatings.

Table 2: Position of the (002) and (004) diffraction peaks for HA feedstock powder and as-sprayed and post treated HVOF coatings.

Figure Captions

Figure 1: SEM micrographs of the cross-sections of etched HA HVOF coatings (a) as-sprayed, (b) heat treated, (c) combination of both coatings.

Figure 2: XRD patterns of (a) as-sprayed and (b) heat treated coatings.

Figure 3: Evolution in the adhesion to the substrate with immersion time.

Figure 4: Osteoblast morphology on an as-sprayed HA coating alter 1 day of seeding.

Figure 5: As-sprayed HA coating after (a) 1 day, (b) 3 days, (c) 7 days and (d) 14 days of cell culture.

Figure 6: Post heat treatment HA coating after (a) 1 day, (b) 3 days, (c) 7 days, (d) 14 days of cell culture.

Figure 7: Graded crystalline HA coating after (a) 1 day, (b) 3 day (c) 7 days, (d) 14 days of cell culture.

Figure 8: Cell viability (MTT assay) of osteoblasts cultured for 3, 7 and 14 days on the HA coatings and CPS. Results represent mean of three independent experiments. An asterisk in the figures indicates data showing statistically significant differences ($p<0.05$) between the days of cell culture.

Figure 9: Osteoblast proliferation (BrdU test) of osteoblasts cultured for 3, 7 and 14 days on the HA coatings and CPS. Results represent mean of three independent experiments. An asterisk in the figures indicates data showing statistically significant differences ($p<0.05$) between the days of cell culture.

Figures

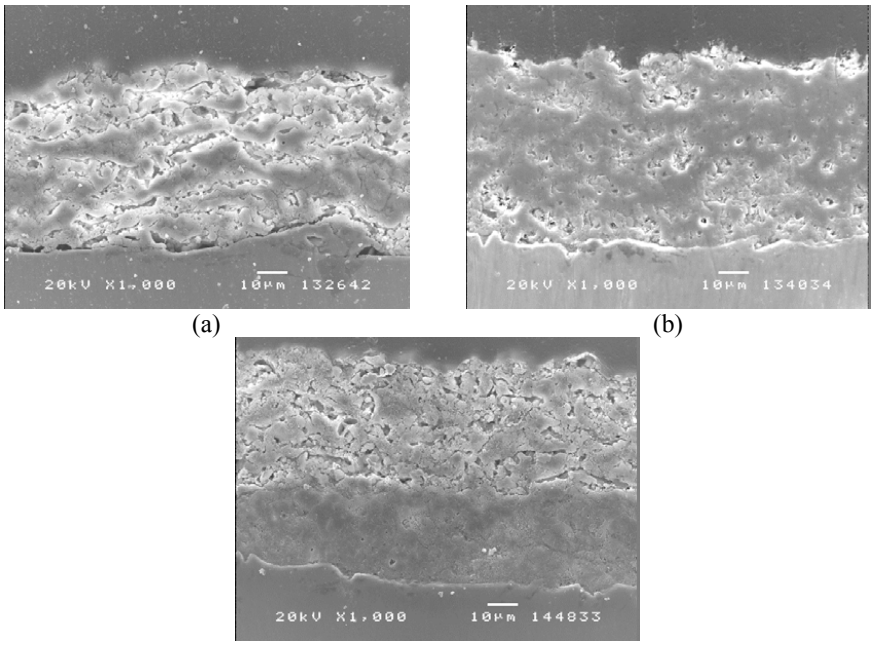


Figure 1: Etched cross-section SEM view of HA HVOF coatings (a) as-sprayed, (b) heat treated, (c) combination of both coatings.

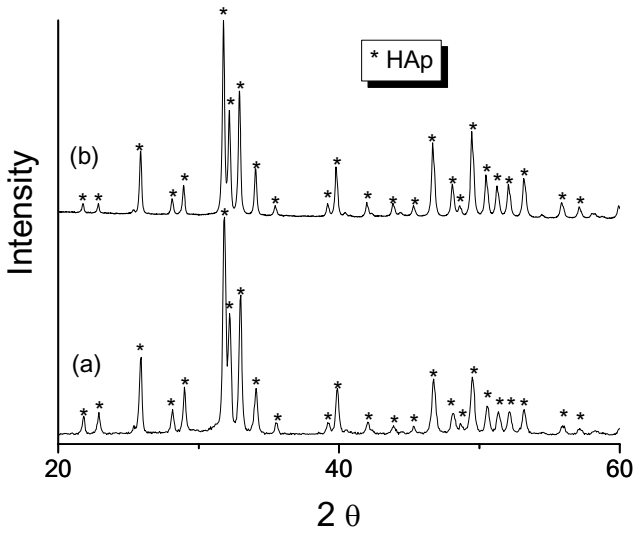


Figure 2: XRD patterns of (a) as-sprayed coating and (b) heat treated coating.

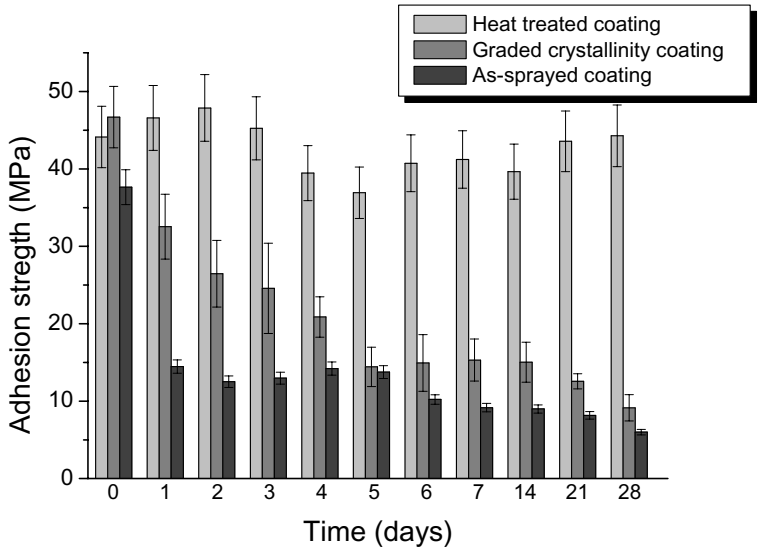


Figure 3: Evolution of the adhesion to the substrate with the immersion time.

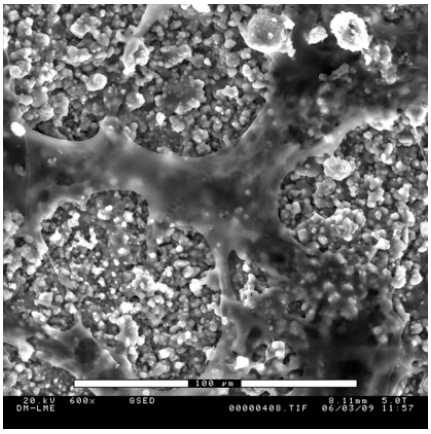


Figure 4: Osteoblast morphology on an as-sprayed HA coating after 1 day of seeding

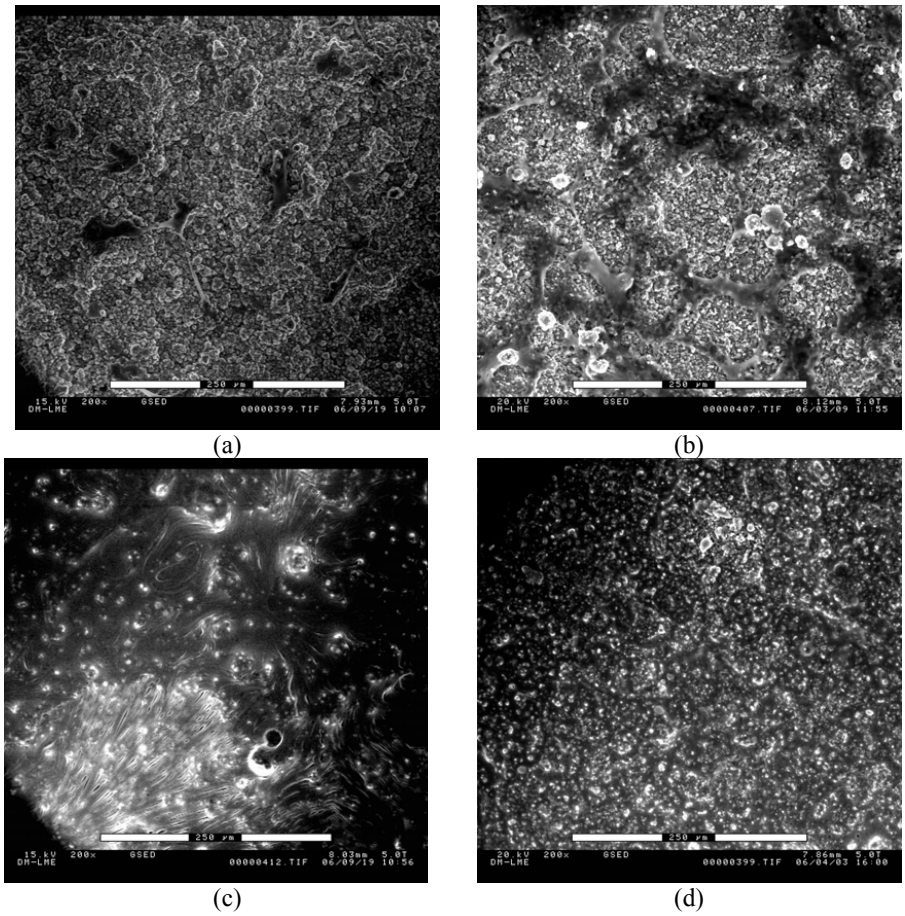


Figure 5: As-sprayed HA coating. (a) 1 day, (b) 3 days, (c) 7 days and (d) 14 days of cell culture.

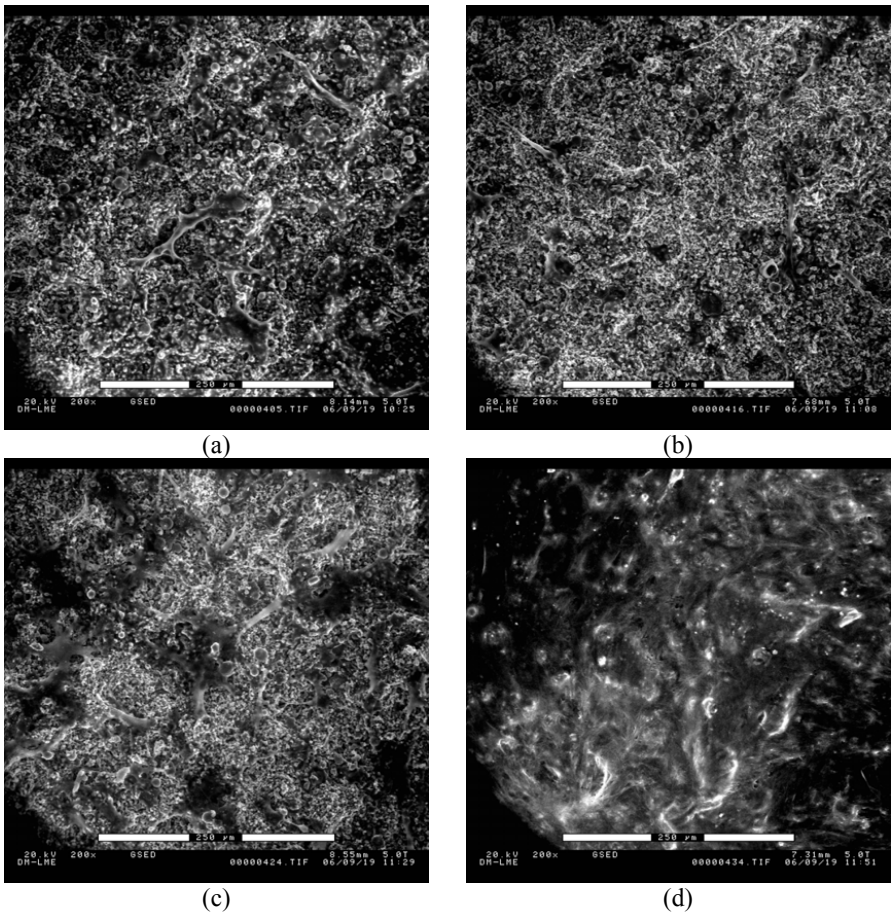


Figure 6: Post heat treatment HA coating. (a) 1 day, (b) 3 days, (c) 7 days, (d) 14 days of cell culture.

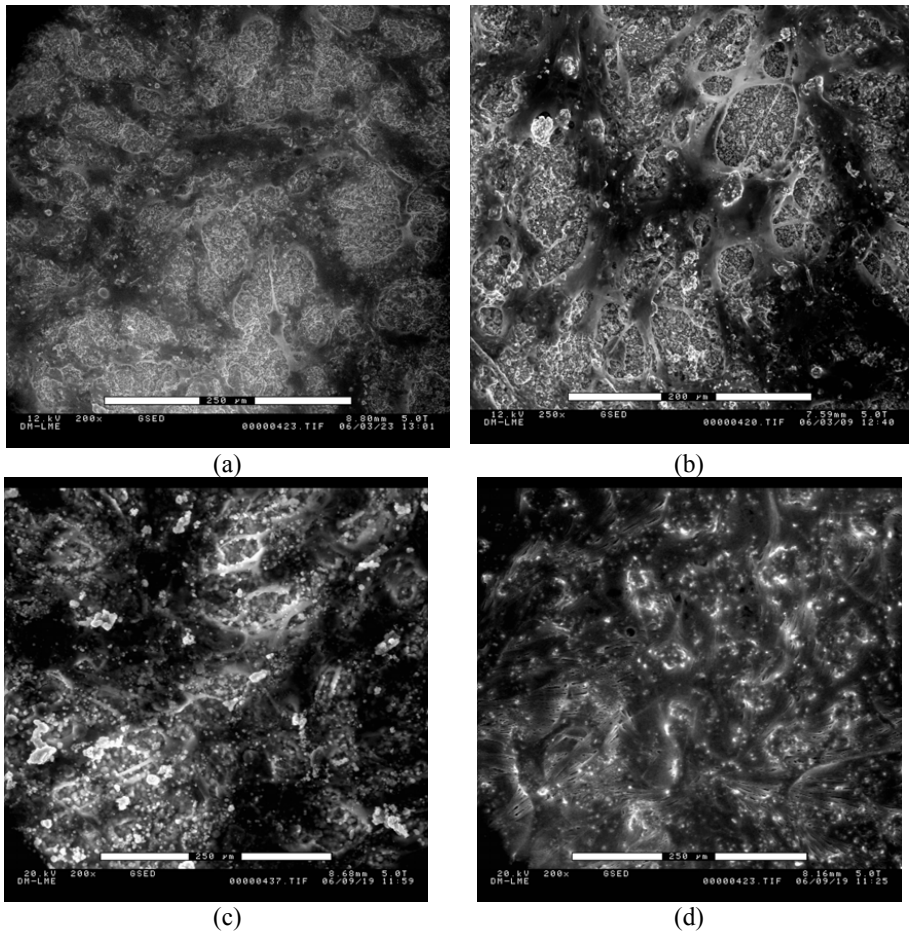


Figure 7: Graded crystalline HA coating. (a) 1 day, (b) 3 day (c) 7 days, (d) 14 days of cell culture.

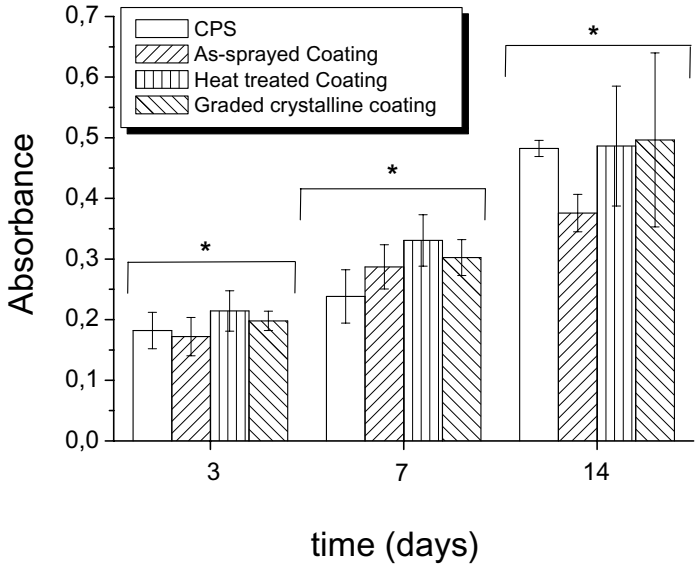


Figure 8: Cell viability (MTT assay) of osteoblasts cultured for 3, 7 and 14 days on the HA coatings and CPS Results represent mean of three independent experiments. An asterisk in the figures emphasizes the data that show differences statistically significant with p<0.05 between the days of cell culture.

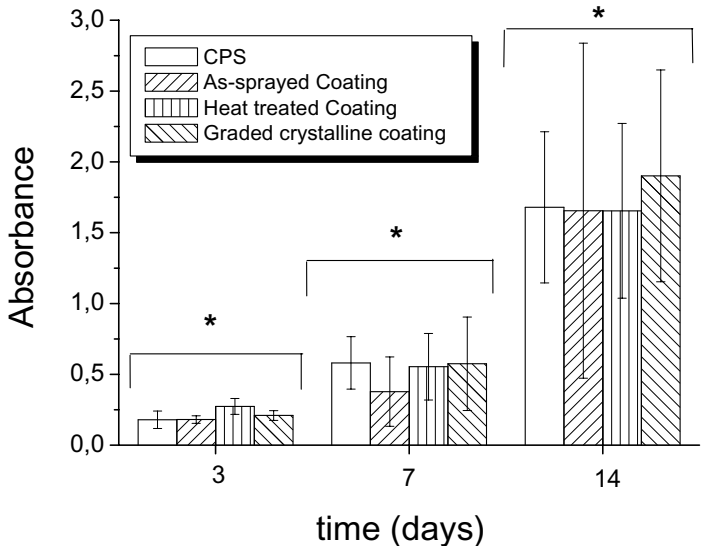


Figure 9: Osteoblast proliferation (BrdU test) of osteoblasts cultured for 3, 7 and 14 days on the HA coatings and CPS. Results represent mean of three independent experiments. An asterisk in the figures emphasizes the data that show differences statistically significant with p<0.05 between the days of cell culture.

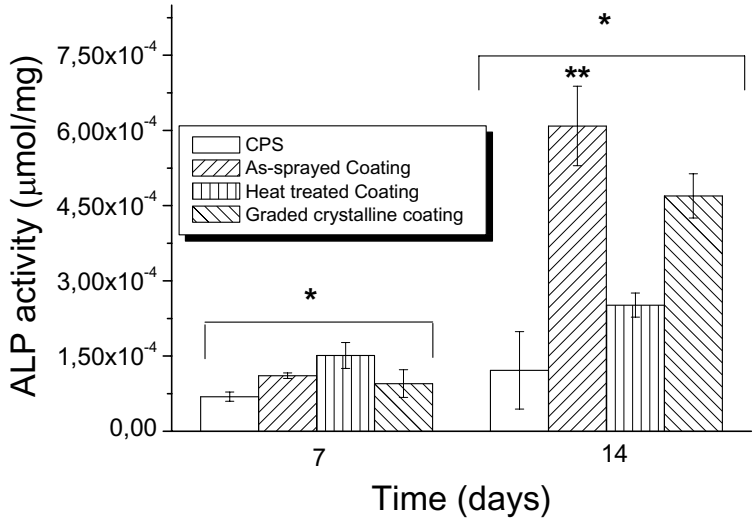


Figure 10: ALP activity of adhered osteoblast on the HA coatings and the control (CPS) after cell culture for 7 and 14 days. Results represent mean of three independent experiments An asterisk (*) emphasizes the data that show differences statistically significant with $p<0.05$ between the days of culture. Two asterisks (**) emphasize the data that show differences statistically significant with $p<0.05$ between the studied coatings.

7.4 Discusión de resultados

7.4.1 Morfología celular.

La observación de cultivos celulares mediante ESEM es poco conocida por lo que existe una carencia de protocolos para la fijación. En esta Tesis doctoral se emplearon dos tipos de fijación diferentes. La primera se realizó utilizando una disolución de paraformaldehído (PF) al 4% en solución tampón fosfato (PBS) durante 5 minutos a temperatura ambiente conservándose en PBS a 4ºC hasta su observación por ESEM y la segunda empleando una disolución de glutaraldehido (GA) al 2% en tampón cacodilato durante 2 horas conservándose en tampón cacodilato a 4ºC hasta su observación por ESEM [6]. Mediante ésta primera, no se pudieron observar células en la superficie de los recubrimientos, por lo que se rechazó su utilización pasando a realizar el resto de las fijaciones con la disolución de GA.

Morfológicamente, las células cultivadas sobre los diferentes recubrimientos no presentaron diferencias significativas. En todas las superficies se observó como las células osteoblásticas se adhirieron adaptándose íntimamente a las irregularidades de los recubrimientos con extensiones citoplasmáticas (figuras 7.1 y 7.2), por lo que los recubrimientos obtenidos presentan una rugosidad óptima para la adherencia de los osteoblastos y el anclaje de las células no depende de las fases presentes en los recubrimientos.

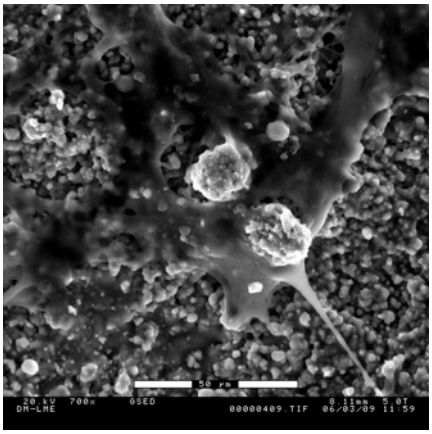


Figura 7-1 Micrografía que muestra la extensión citoplasmáticas de un osteoblasto sobre recubrimiento con 82% de HA cristalina obtenido por HVOF. (ESEM x700)

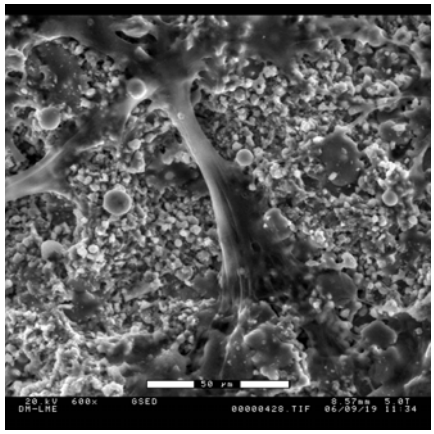
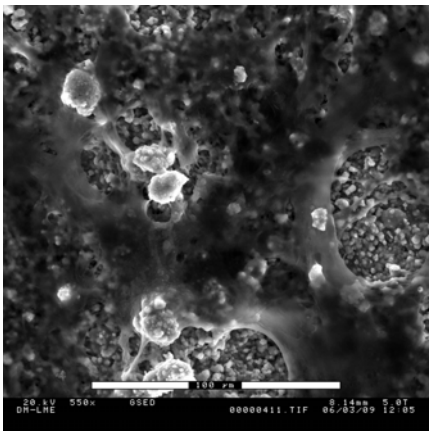
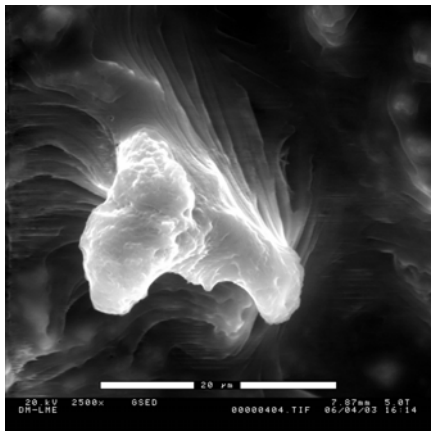


Figura 7-2 Micrografía donde se muestra un osteoblasto con extensiones citoplasmáticas ancladas en la rugosidad superficial de un recubrimiento de HA totalmente cristalino. (ESEM x600)

Tras 3 días de cultivo se observó la presencia de núcleos de fosfato de calcio sobre las superficies parcialmente amorfas (Figura 7-3). Este hecho probablemente también esté relacionado con la capacidad que presentaban estos recubrimientos de depositar apatita cuando están inmersos en una solución SBF como se observó en el Capítulo 6. El conjunto de estos factores podría contribuir a la mejora de la unión del hueso y el recubrimiento *in vivo.*



(a)



(b)

Figura 7-3: Núcleos de fosfato de calcio que se observaron en los cultivos celulares sobre recubrimientos con ACP

A pesar de estos resultados se observó, mediante las imágenes de ESEM, una menor densidad de osteoblastos en los recubrimientos totalmente cristalinos para 3 y 7 días de cultivo comparado con los recubrimientos parcialmente amorfos, pero tras 14 días todos los cultivos habían llegado a la confluencia.

7.4.2 Viabilidad y proliferación celular

Los resultados de viabilidad y proliferación mostraron que la respuesta celular era comparable entre los cultivos de control y los de los diferentes recubrimientos. Este hecho puede indicar que estas dos variables son independientes de las fases presentes en los recubrimientos ya que es la única diferencia entre los recubrimientos ensayados. Un aspecto interesante sería estudiar y evaluar la respuesta celular en recubrimientos de HA con diferentes rugosidades. Para ello se deberían utilizar polvo de diferente granulometría y diferentes condiciones de proyección.

7.4.3 Diferenciación celular y actividad osteoblástica.

En este estudio se observó que las células cultivadas sobre recubrimientos con ACP, presentaban mayor actividad de la ALP y, como se observó en las imágenes de ESEM, una producción de núcleos de fosfato de calcio. Este hecho sugiere que las células se encuentran en un estado diferenciado y, por lo tanto, es de esperar una mineralización de su matriz extracelular. Estos resultados son consistentes con Sugawara et al. [7], que demostró que la actividad enzimática de la ALP es esencial para el proceso de la mineralización de células osteoblásticas observándose la ausencia de formación de hueso en células cultivadas con la presencia del tetramisole, un inhibidor de la actividad de la ALP. La formación de esta matriz extracelular es el paso final en la diferenciación de los osteoblastos, y una etapa esencial en la osteointegración [8] y, a pesar del recelo de extrapolar los resultados *in vitro* al comportamiento *in vivo* del material, las superficies con ACP pueden

conseguir la osteointegración a corto plazo más rápido que los recubrimientos 100% cristalinos.

Por este motivo los recubrimientos de cristalinidad gradual se desarrollaron para que tuvieran una buena adherencia al substrato por la alta cristalinidad de la interfaz que evita que se disuelva en medios fisiológicos y ponga en compromiso el anclaje del recubrimiento y un buen comportamiento *in vitro* facilitado por la capa parcialmente amorfa que le permite la deposición de una capa de apatita cuando están en inmersión y una mayor diferenciación de las células osteoblásticas. Como se observó en el Capítulo 6 tras un ensayo de inmersión en SBF el fallo en la adherencia de este tipo del recubrimientos ocurre en la interfaz del recubrimiento parcialmente amorfo-recubrimiento totalmente cristalino. Si esto ocurriese la capa totalmente cristalina unida al substrato metálico también presenta una buena biocompatibilidad en términos de viabilidad y proliferación aunque una generaría una menor actividad osteoblástica.

7.5 Conclusiones del capítulo

- Todos los recubrimientos estudiados han demostrado una buena respuesta en los ensayos de viabilidad, proliferación y diferenciación celular por lo que las diferencias en las fases presentes en los recubrimientos no afecta a la citocompatibilidad.

- La viabilidad y la proliferación celular no esta influida por las diferentes fases de fosfato de calcio presentes en los recubrimientos.

- La presencia de ACP en los recubrimientos induce una mayor actividad de la ALP y una producción de núcleos de fosfato de calcio. Este hecho puede tener un papel fundamental en la rápida mineralización de la matriz extracelular y, en consecuencia, se puede esperar una osteointegración a corto plazo más rápida.

- Los recubrimientos de cristalinidad gradual permiten una tener una buena adherencia al substrato, que le proporciona la capa cristalina, y un buen comportamiento *in vitro* facilitado por la capa parcialmente amorfa. El fallo de la adherencia en medio fisiológico ocurre entre la capa cristalina y la parcialmente amorfa, por lo que aunque exista un fallo en uso de la adherencia de la capa amorfa, la cristalina también presentó buenos resultados de proliferación y viabilidad celular así como evitar el desprendimiento de iones metálicos del substrato al medio fisiológico.

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