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In vitro cellular response to titanium electrochemically coated with hydroxyapatite compared to titanium with three different levels of surface roughness

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The *in vitro* response of primary human osteoblast-like (HOB) cells to a novel hydroxyapatite (HA) coated titanium substrate, produced by a low temperature electrochemical method, was compared to three different titanium surfaces: as-machined, Al_2O_3-blasted, plasma-sprayed with titanium particles. HOB cells were cultured on different surfaces for 3, 7 and 14 days at 37 °C. The cell morphology was assessed using scanning electron microscopy (SEM). Cell growth and proliferation were assessed by the measurement of total cellular DNA and tritiated thymidine incorporation. Measurement of alkaline phosphatase (ALP) production was used as an indicator of the phenotype of the cultured HOB cells. After three days incubation, the electrochemically coated HA surface produced the highest level of cell proliferation, and the Al_2O_3-blasted surface the lowest. Interestingly, as the incubation time was increased to 7 days all surfaces produced a large drop in tritiated thymidine incorporation apart from the Al_2O_3-blasted surface, which showed a small increase. Cells cultured on all four surfaces showed an increased expression of ALP with increased incubation time, although there was not a statistically significant difference between surfaces at each time point. Typical osteoblast morphology was observed for cells cultured on all samples. The HA coated sample showed evidence of a deposited phase after three days of incubation, which was not observed on any other surface. Cells incubated on the HA

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coated substrate appeared to exhibit the highest number of cell processes attaching to the surface, which was indicative of optimal cell attachment. The crystalline HA coating, produced by a low temperature route, appeared to result in a more bioactive surface on the c.p. Ti substrate than was observed for the other three different Ti surfaces.
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1. Introduction

Several authors [1–8] have studied the influences of the surface topography and/or chemical composition of a biomaterial on cellular and molecular responses. In general, it has been shown that using rougher surfaces and choosing materials with a chemical composition that is comparable to bone mineral can enhance the behavior of osteoblast-like cells *in vitro*. This knowledge has been used to improve the *in vivo* biological response to ''bioinert'' metallic implants, e.g. hip implants. Here, HA coatings or porous Ti-bead coatings have been applied to the surfaces of the implant materials to produce a more favorable chemical composition or surface roughness, respectively. Applying a HA coating to an implant surface can result in the rapid formation of a strong bond between implant and bone, leading to stronger and faster fixation of the implant, compared to non-coated implants. This feature would be particularly important when dealing with dental implants used in two-stage surgeries, such as titanium tooth root implants, where speeding up the rate of osseointegration can result in simplifying the procedure to a one-stage surgery. However, some authors have stated that coated and non-coated titanium exhibit the same tissue responses after long periods of time [9, 10]. When the surface of an implant is not highly bioactive, such as with commercially pure titanium, surface properties such as chemical purity and topography must be optimized in order to improve the level of osseointegration as well as to reduce the necessary time for bone bonding to occur. Smooth surfaces result in poor bony adhesion and are predominantly anchored by soft tissue, while rough surfaces at a level that allows vascular in-growth will encourage bone ingrowth/apposition, and are strongly desired for endosseous implants.

A variety of surface treatments have been used to produce a topography on titanium implants that is more conducive to bone apposition; these treatments include blasting with ceramic beads, etching and plasma-spraying with titanium beads. Several authors have studied the influence of the surface roughness on the *in vivo* bony response [11–15] and *in vitro* cell response [3–7] to such titanium dental implants. Surface properties such as macro- and micro-topography and chemical composition have been shown to affect the short-term cellular responses (cell migration and anchorage) and long term (cell differentiation and matrix expression). For example, Keller *et al*. [8] associated osteoblast adhesion to metallic substrates and differences in cell morphology to changes in surface roughness. Kieswetter *et al*. [6] studied the effect of surface roughness of titanium on the local production of growth factors and cytokines by MG-63 cells. These authors observed that roughness affected cell proliferation and differentiation, and the production of cell matrix by MG-63 cells, as well as the production of cytokines and growth factors. A lower level of cellular proliferation was observed on the rougher surfaces as well as differences in cell morphology when compared to flat surfaces. This result was associated to a more advanced stage of differentiation, which was preceded by a drop in proliferation.

The interactions between cells and biomaterials are favored by the adsorption of proteins to the surface and calcium phosphate coatings have the advantage of facilitating this adsorption [16]. Among the calcium phosphates, HA ($Ca/P = 1.67$) has the largest capacity of adsorbing proteins to its surface [17]. Correspondingly, HA coatings have been a very popular method of improving the osseointegration of titanium implants. The most common and most widely reported method of applying a HA coating is using the plasma-spraying technique, and the performance of these coatings compared to different titanium surfaces have been reported both *in vivo* [9, 10, 14] and *in vitro* [16]. The plasma-spraying method has considerable disadvantages, however, and these are due mainly to the high temperatures used to spray the coatings. Examples of these disadvantages are poor control of crystallinity and phase purity of the HA coating, rapid and uncontrolled dissolution of the HA coating and a lack of a strong substrate-coating interface; these factors have been described in a recent review by Sun *et al*. [18].

In the present study the *in vitro* cellular response of primary human osteoblast cells cultured on the surface of a novel HA-coated titanium surface, produced by a low temperature electrochemical technique, was compared to the cellular response on three different titanium surfaces. The main aim of this study was to assess if this HA coating would behave in a favorable manner to non-coated, but surface modified, titanium substrates.

2. Materials and methods

2.1. Materials preparation

Four different surface finishes were applied to commercially pure (c.p.) titanium sheets: as-machined; surface blasted with Al_2O_3 particles in the size range 250–600 μm; plasma-sprayed with titanium beads in the size range 300–600 μm; electrochemically coated with HA using the method described elsewhere [19]. The titanium sheets with three different levels of roughness (i.e. non-HA coated) were supplied by CONEXAO Sistemas de Proteses Ltd.

Eight samples (10×10 mm) of each surface condition were used for the cell culture study: three samples for proliferation measurement, two for biochemical assess-

ment of alkaline phosphatase and two for evaluation of the cell morphology using SEM. The sheets were cleaned with acetone in an ultra-sonic bath and sterilized under Gamma irradiation with a dose of 27.4 KiloGray prior to cell culture.

2.2. Surface characterization

An indication of the roughness of the four different surface finishes was obtained by carrying out profilometry measurements using an optical laser profilometer (Mahr GmbH). The measured parameters were: R_a, the arithmetic average of the absolute values of all points of the profile; R_q, the root-mean-square of the values of all points and R_z, the average value of the absolute heights of the five highest peaks and the depth of the five deepest valleys. For each surface condition the roughness parameters were measured in five different regions.

Characterization of the surface morphology of the four different substrates was carried out using scanning electron microscopy (JEOL 6300 SEM); surfaces were coated with gold prior to analysis.

2.3. *In vitro* cell culture

Primary human osteoblasts (HOB) were isolated from the femoral head of a patient undergoing total hip replacement surgery, as described in detail elsewhere [20, 21]. HOB cells were seeded (50 μl) on the four different materials and on control Thermanox (TMX, Life Technologies) at a density of 1×10^6 cells/ml, and incubated for 3, 7 and 14 days at 37 °C in humidified air with 5% CO_2. The culture medium (Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, L-ascorbic acid (150 μg/ml), 0.02 M L-glutamine, 0.01 M HEPES, 100 units/ml penicillin and 100 mg/ml streptomycin) was changed at appropriate time intervals, taking care to minimize disturbance to the culture conditions.

Cell growth and proliferation were assessed using total DNA and tritiated thymidine ([^{3}H]-TdR) incorporation, and cell phenotype was determined by the measurement of alkaline phosphatase (ALP), with measurement carried out on cells incubated for 3, 7 and 14 days, as described in detail elsewhere [21, 22]. Briefly, a Hoechst 33285 DNA specific fluorescent dye was reacted with cell lysates and DNA standards and the fluorescence was measured using a Fluoroscan fluorimeter (Ascent, Life Science International). The DNA content of the different samples was calculated from the standard curve. Tritiated thymidine incorporation was measured by incubating the cells with 1 μCi/ml [^{3}H]-TdR for 24 h before lysis and [^{3}H]-TdR incorporation was measured using a scintillation counter. The ALP activity was determined by using a COBAS-BIO (Roche, UK) centrifugal analyzer. Using *p*-Nitrophenol phosphate in a diethanolamine buffer as a substrate for ALP, the reaction product of *p*-Nitrophenol could be quantified at a wavelength of 405 nm. Tritiated thymidine incorporation and ALD expression values were normalized for total DNA.

To study the cell morphology and attachment at the earliest time point (day 3), the culture medium was removed and the samples were fixed in 2.5% gluteraldehyde buffered in 0.1 M sodium cacodylate (pH 7.2–7.4) at 4 °C for one day. The fixed samples were washed with 0.1 M sodium cacodylate buffered solution, post-fixed in 1% osmium tetroxide buffer and 1% tanic acid, then dehydrated through a sequence of alcohol concentrations (20, 30, 40, 50, 60, and 75%). The samples were then stained in 0.5% uranyl acetate then dehydrated further in a series of ethyl alcohol concentrations (90, 96 and 100%), with a final dehydration made with hexamethyl-disilazane, followed by drying in air overnight. The samples were then sputter-coated with Au–Pd, and observed using a JEOL SEM microscope with an accelerating voltage of 10 kV.

3. Results

The R_a, R_q and R_z roughness parameters of the four different surfaces produced in this study are listed in Table I. The Ti plasma-sprayed and the HA electrochemically coated surfaces had very comparable values for all of the three roughness parameters. The as-machined surface produced the lowest values for roughness parameters, indicating it was the smoothest surface finish. The surface produced by Al_2O_3 blasting had considerably higher values of both R_q and R_z, in particular the latter value, compared to the other three surface finishes.

SEM images of the surfaces of the four different substrates prior to cell culture are presented in Fig. 1 (a)–(d). The as-machined surface appeared to have the lowest level of visible roughness; the surface appeared to contain parallel grooves or scratches, which would have been introduced during the machining stage. The morphology of the Al_2O_3-blasted and the Ti-plasma sprayed surfaces appeared to be quite similar, with each displaying relatively rough surfaces that contained areas of porosity. The Al_2O_3-blasted surface (Fig. 1(b)) contained small amounts of imbedded alumina particles, which resulted from the blasting process. The Ti-plasma sprayed surface (Fig. 1(c)) showed features corresponding to melted Ti particles which had formed flattened particles or ''splats'' on the surface, which then cooled to produce relatively flat but pronounced features. The hydroxyapatite electrochemically coated surface had a very regular morphology of hydroxyapatite crystals of between 1 and 5 μm in length, which were randomly orientated across the surface. The HA-coated surface did not appear to have any defects or large areas of porosities.

The change in tritiated thymidine ([^{3}H]-TdR) incorporation (expressed per μg/ml of DNA) at incubation times of 3, 7 and 14 days are presented in Fig. 2.

TABLE I Surface roughness parameters for the four different surfaces produced on c.p. titanium substrates (results are the mean ± SD, $n = 5$)

Surface finish	R_a (μm)	R_q (μm)	R_z (μm)
As-machined	1.8 ± 0.9	2.0 ± 1.0	22 ± 5
Al_2O_3-blasted	3.4 ± 0.2	8.9 ± 0.1	151 ± 3
Ti-plasma sprayed	5.0 ± 1.0	6.0 ± 1.0	32 ± 1
HA-electrochemically coated	5.0 ± 0.6	6.5 ± 0.7	34 ± 3

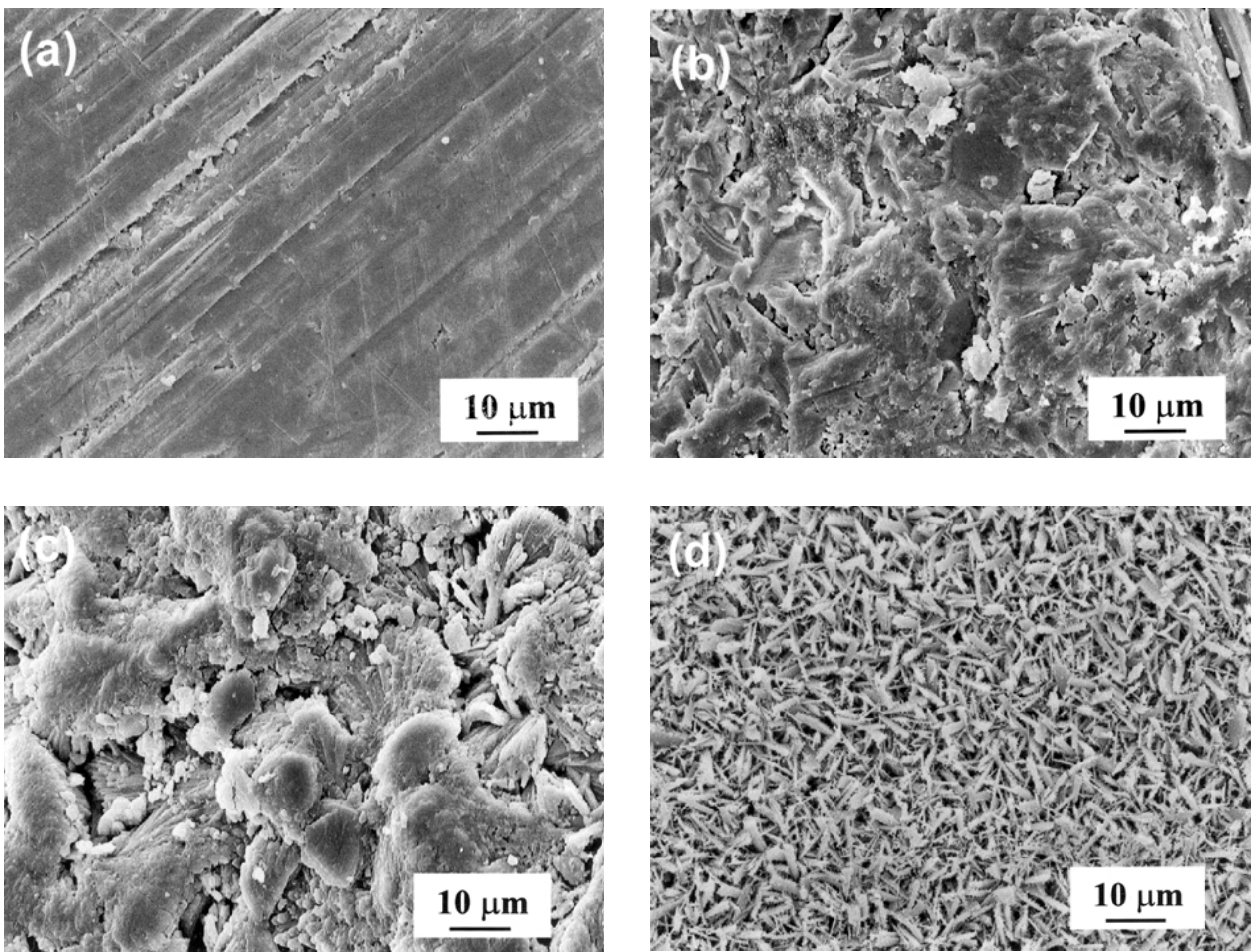

Figure 1 Scanning electron microscopy images showing the surface morphology of the titanium substrates with four different surface treatments, prior to cell culture: (a) as machined, (b) blasted with Al_2O_3 particles, (c) plasma-sprayed with titanium powder particles and (d) electrochemically coated with hydroxyapatite.

After 3 days of incubation, the Ti-plasma sprayed and the hydroxyapatite electrochemically coated surfaces showed comparable levels of [^{3}H]-TdR incorporation to the Thermanox control. At the same time point, the as-machined and the Al_2O_3-blasted surfaces resulted in lower levels of [^{3}H]-TdR incorporation. At the 7 day time point, all the surfaces except the Al_2O_3-blasted surface produced a significant drop in [^{3}H]-TdR incorporation, whereas the level of incorporation for the Al_2O_3-blasted surface was comparable to the level observed at the 3 day time point. At day 14, the [^{3}H]-TdR incorporation corresponding to the Al_2O_3-blasted surface reduced to the same level as the other surface finishes.

The values of ALP activity, normalized for DNA, appeared to increase with increasing incubation time from 3 to 14 days, Fig. 3, following normal osteoblast behavior, although there was no statistical significance to the changes observed.

The morphology of human osteoblast cells cultured for 3 days on the Al_2O_3-blasted, Ti-plasma sprayed and the HA-coated surfaces at a low magnification (500 ×) are presented in Fig. 4 (a)–(c), respectively. At this magnification, it appeared that a dense layer of cells were covering the surfaces of the Al_2O_3-blasted and the Ti-plasma sprayed surface, but the HA-coated surface was still visible between the individual cells. At higher magnification (2000 ×), the differences in the response of the osteoblast cells to the various surfaces were much clearer, Fig. 5(a)–(d) (N.B. the magnification used to view cells on the as-machined Ti surface, as indicated by the scale bar, was slightly different to the magnification used for the other surfaces). Cells on the as-machined Ti surface displayed a flattened, osteoblast-like morphology, with cell processes attaching to either the material surface or a dense cell layer, Fig. 5(a). Cells attached directly to the Al_2O_3-blasted surface, Fig. 5(b),

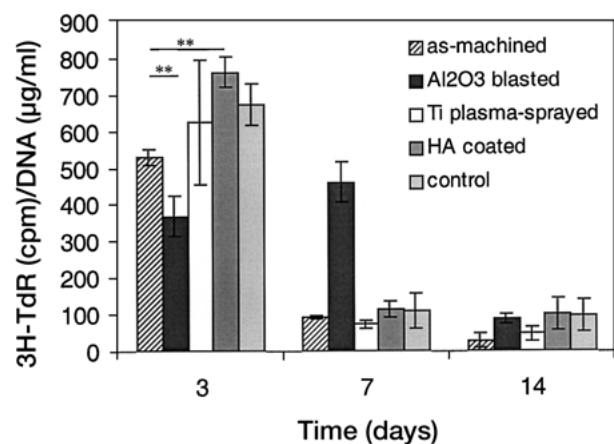

Figure 2 Results of biochemical assay for tritiated thymidine [^{3}H]-TdR incorporation (per μg/ml of DNA) for the evaluation of HOB cell proliferation on different surface finishes, at 3, 7 and 14 days incubation (results are the mean ± SD, $n = 3$, t-test; $^{**}p < 0.01$).

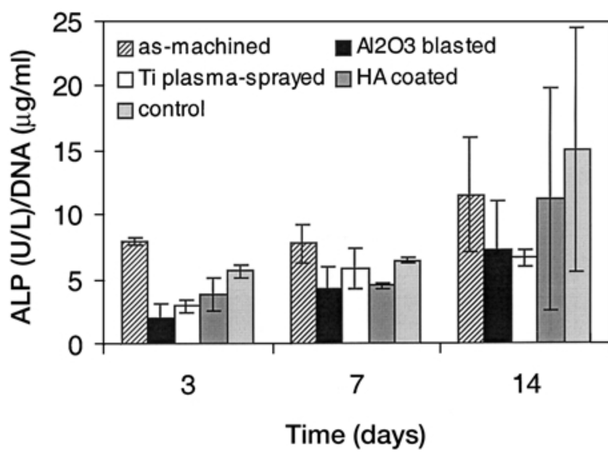

Figure 3 Measurement of the alkaline phosphatase activity (measured as ALP activity per µg/ml of DNA) of HOB cells cultured on samples with different surface finishes, at 3, 7 and 14 days incubation (results are the mean $\pm$ SD, $n = 3$).

but the number of cell processes observed was considerably less than observed with cells attached directly to the HA-coated surface, Fig. 5(d). Some of the surface features of the Al_2O_3-blasted surface prior to cell culture, Fig. 1(b), were apparent between attached cells in Fig. 5(b) (marked with white arrows), indicating that the surface had not yet been covered by a complete cell layer. This was not observed with cells cultured on the Ti-plasma sprayed surface, as a complete dense layer of cells had formed on the substrate surface, with subsequent cells attaching to this cell layer, Fig. 5(c).

Cells incubated on the HA-coated surfaces exhibited a large number of filopodia in contact with the substrate surface, but did not exist as a dense cell layer as observed for the Ti plasma-sprayed surface. Examining cells attached to various areas of the HA coated sample revealed a clear difference in the substrate morphology. This was viewed more clearly at higher magnification

Figure 4 Low magnification (500 ×) scanning electron microscopy images showing the morphology of the human osteoblast cells incubated for three days on titanium surfaces (a) blasted with Al_2O_3 particles, (b) plasma-sprayed with titanium powder and (c) electrochemically coated with hydroxyapatite. Black scale bar denotes 40 µm.

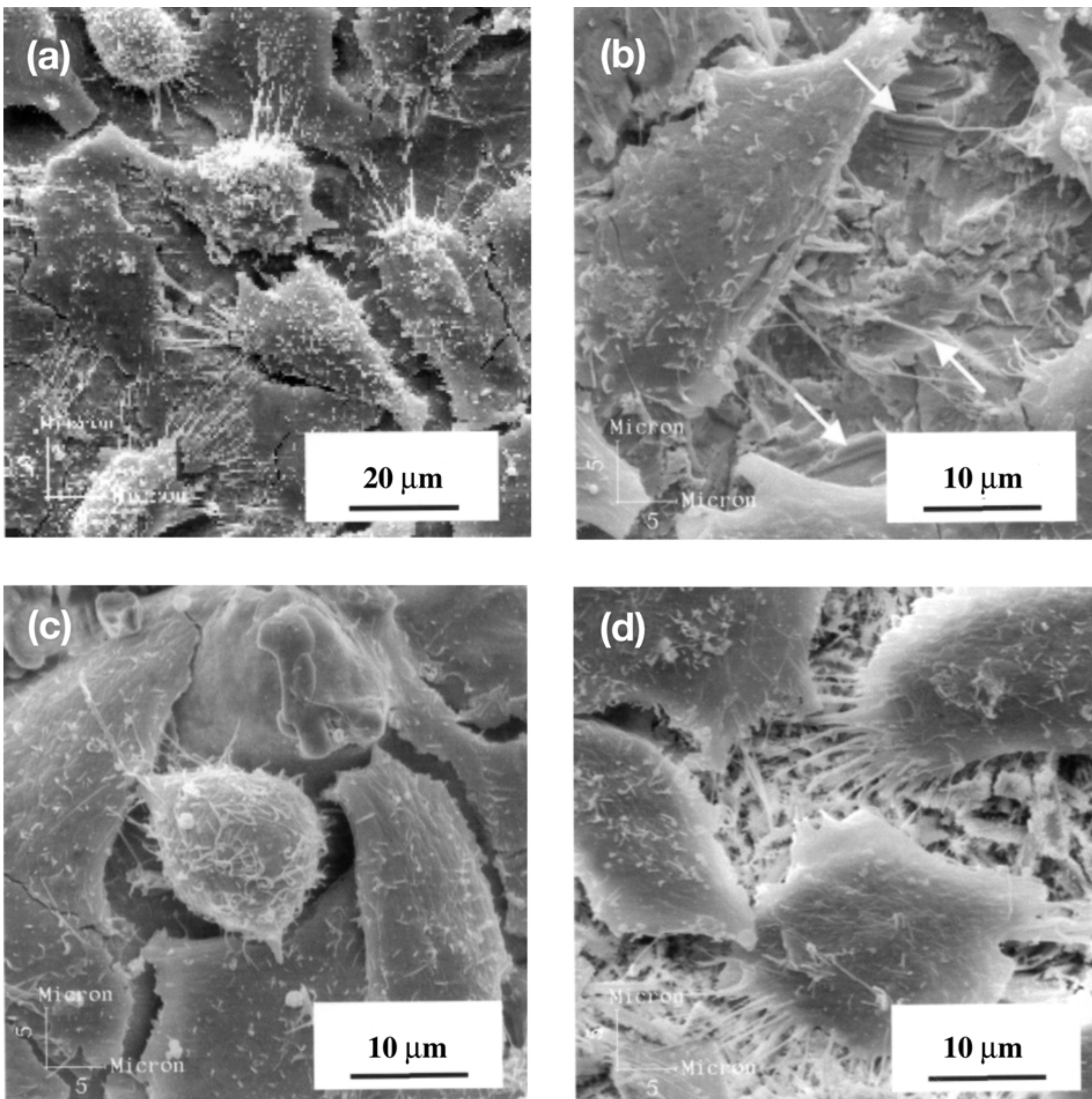

Figure 5 Scanning electron microscopy images (2000 ×) showing the morphology and adhesion of the human osteoblast cells incubated on titanium substrates for three days with different surface treatments: (a) as-machined, (b) blasted with Al_2O_3 particles, (c) plasma-sprayed with titanium powder and (d) electrochemically coated with hydroxyapatite. The white arrows on (b) indicate surface features that are still visible from the Al_2O_3-blasting process (see Fig. 1(b)). Black scale bar denotes 10 μm (except for Fig. 5(a), where scale bar denotes 20 μm).

(3000 ×), where the normal HA coating morphology consisting of acicular needles and plates of HA crystals was observed, Fig. 6(a), compared to a morphology that consisted of regions of spherical deposits, Fig. 6(b). These deposits were not observed on any of the other surfaces. A dense continuous cell layer covered all of the different surfaces after 7 and 14 days of incubation (data not shown).

4. Discussion

The different surface treatments that were used to produce four different substrates resulted in a range of surface topographies. Most noticeably, the changes in the value of the parameter R_z, which provides a measure of the most extreme peaks and valleys produced by a surface treatment, showed that the Al_2O_3-blasted finish gave a much higher value than the other three surface finishes. The as-machined surface had the smoothest topography of all the samples studied. Although the different surface treatments all changed the surface topography, it must be noted that all of the treatments will, in some way, alter the surface chemistry of the titanium substrate. Although this is most obvious with the application of an HA coating, Al_2O_3-blasting can result in contamination with an aluminum oxide layer [23] and the process of plasma-spraying with titanium can also result in some chemical contamination [22]. The surface topography and the surface chemistry can both, therefore, affect the cellular response. The purpose of this study was, however, to compare the cellular response of a HA coating produced by a novel, non-plasma spraying method with a variety of surface finishes produced by processes typical of commercial titanium implants.

At the earliest time point in this study (three days), the

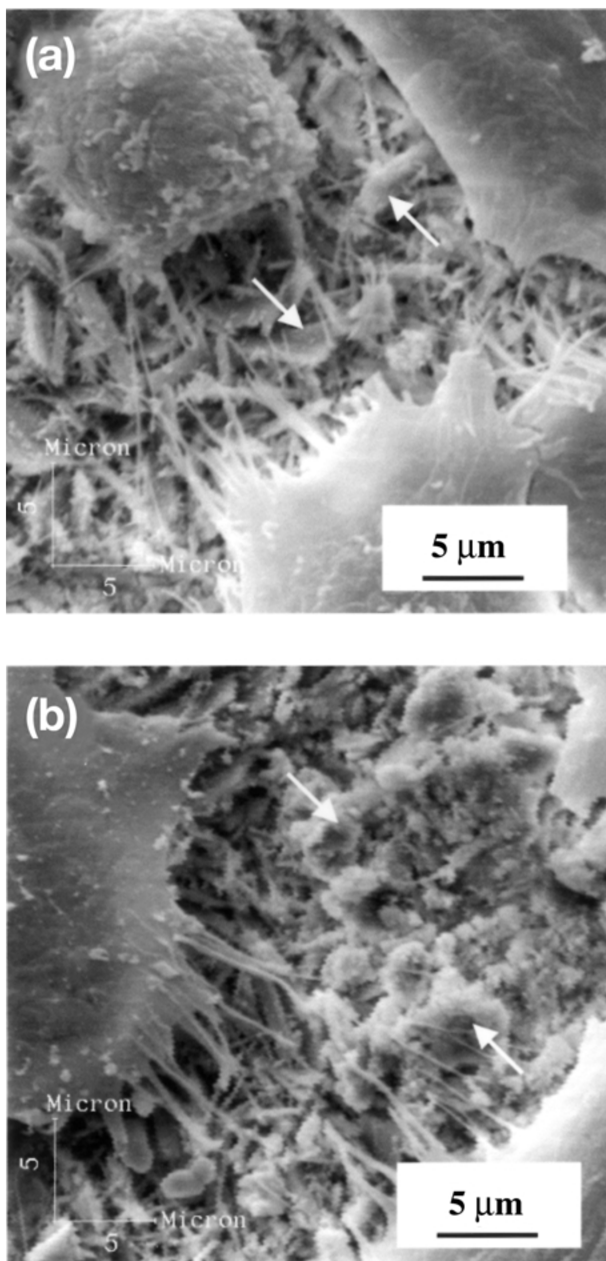

Figure 6 Scanning electron microscopy images (3000 ×) that show variations in the coating morphology throughout different areas of the HA-coated surface, incubated with HOB cells for three days. The white arrows on (a) indicate the typical hydroxyapatite needle or plate-shaped crystals produced by the electrochemical deposition process, and on (b) indicate the presence of nodular deposits that have formed on top of the HA coating. Black scale bar denotes 5 μm.

results suggested that the level of HOB cell proliferation was lowest on the Al_2O_3-blasted surface, with the highest levels of cell proliferation being observed for the Ti-plasma sprayed and the HA electrochemically coated surfaces. The as-machined surface showed an intermediate level of proliferation. The as-machined surface had the lowest level of surface roughness, but surfaces with greater degrees of roughness either showed greater proliferation (Ti-plasma sprayed and the HA electrochemically coated surfaces) or less proliferation (Al_2O_3-blasted surface). Clearly, cell attachment and proliferation is enhanced by the creation of a certain degree of increased roughness of the substrate but this is not a direct relationship as surfaces with a very high degree of roughness can inhibit the level of proliferation, as observed for the Al_2O_3-blasted surface. An interesting feature of the results obtained for cell proliferation was that the level of [^{3}H]-TdR incorporation remained almost constant at day 3 and day 7 for the Al_2O_3-blasted surface, indicating that a significant number of cells were still proliferating at day 7. This was in contrast to the other surfaces which showed a large decrease in [^{3}H]-TdR incorporation over this time period; a decrease in proliferation with increased incubation time, with a corresponding increase in ALP activity is typical of normal osteoblast growth and phenotype activity. This suggested that it took a longer period of time for cells to proliferate and reach confluence on the Al_2O_3-blasted surface, compared to the other three surfaces and the Thermanox control. There was no clear effect of this prolonged cell proliferation stage on the ALP production of the Al_2O_3-blasted surface, although the large standard deviations in the data in Fig. 3 prevent any detailed discussion or conclusions of the ALP activity of cells cultured on the different surfaces to be made. There was some evidence of imbedded alumina particles on the Al_2O_3-blasted surface, but it is unclear from this study if these particles had a direct effect on the different cell proliferation profile observed for this surface finish. This surface finish resulted in a R_z roughness parameter, which gives an indication of the height of the highest peaks and lowest troughs of the surface, that was significantly greater than any of the other surfaces studied, Table I. This difference may have a more significant effect on the attachment and proliferation of cells on the surface than the presence of a small number of imbedded alumina particles.

Anselme *et al.* [23, 25] studied the effect of different surface treatments of Ti implants on the adhesion and proliferation of osteoblastic cells and observed that the relative rate of cell proliferation decreased as the roughness of the surface increased after 3 days of culture. This is the opposite trend of enhanced cell attachment and proliferation with increasing titanium surface roughness that has been reported by a number of authors [24, 26, 27]. It should also be noted that in the present study the three different surface finishes produced directly on the titanium surface were formed by using a variety of methods. This is in contrast to the majority of studies, which produced different levels of surface roughness by polishing the substrate to different finishes.

The results obtained for cell proliferation and ALP production on the Ti-plasma sprayed and the HA coated surfaces showed that conventional surface roughness parameters alone, such as the arithmetic average roughness (R_a), are not sufficient parameters to interpret cell behavior. The roughness parameters for these two surfaces were, within the standard deviations, identical, Table I. Although the level of cell proliferation, determined bio-chemically, was similar for these two surfaces, suggesting a possible link to surface roughness, the physical appearance of cells was quite different. For the Ti-plasma sprayed surface the cells had appeared to reach confluence after three days of incubation, with the appearance of a dense cell layer, whereas cells on the HA coated had not reached confluence but appeared to be well attached to the HA surface. In addition to these

surfaces having very different chemistries, the HA coating contained a high degree of nano-topography [19]. In addition to their surface micro-topography, the importance of the surface nano-topography of biomaterials on cellular behavior has been reviewed recently by Curtis *et al*. [28] and Flemming *et al*. [29]. This has been related to the nano-topography observed in basement membranes [29] which have been shown to activate integrin receptors [30].

SEM observation of HOB cells cultured on the different surfaces for three days confirmed some of the observations from the measurement of cell proliferation. SEM of cells on as-machined titanium appeared to show better cell adhesion compared to the Al_2O_3-blasted surfaces at three days; this was also reflected in the [^{3}H]-TdR incorporation at three days. The HA-coated surface produced the highest level of [^{3}H]-TdR incorporation of all samples after three days; the difference in the level of [^{3}H]-TdR incorporation was, however, only statistically significant to the as-machined and the Al_2O_3-blasted surfaces (Fig. 2). The images of cells on the HA-coated surface illustrated a high level of cell attachment, with the greatest number of cell processes observed on this surface. The large number of cell processes observed on the HA coated sample confirmed findings reported in the literature that state surface roughness at the sub-micron scale, i.e., nano-topography, has a larger superficial area and has more sites for anchorage of the cellular processes than smooth surfaces. This has been related to enhanced adsorption of biological entities, such as proteins, which play an important role in cell adhesion [26]. Therefore, hydroxyapatite nano-crystals produced by methods such as electrochemical coating may act as micro anchors for the development of a direct bony connection [31].

Soaking the HA electrochemically coated titanium produced in the present study in a simulated body fluid (SBF) at 37 °C over a period of 14 days showed that the Ca^{2+} and PO_4^{3-} ion content of the SBF solution decreased with increasing soaking time, which was indicative of a bone-like apatite layer precipitating on the surface of the HA coating [32]. Also, immersion in the SBF solution for just one day revealed the presence of globular bone-like apatite on the HA electrochemically coated surface. This result supports the observation of a deposited phase on the HA-coated surface after three days of cell culture, Fig. 6(b), and suggests that this phase may be a calcium phosphate apatite that has precipitated from the culture medium. The results from soaking the HA-coated titanium in a SBF solution also demonstrated that the HA-coating was not itself dissolved/resorbed during soaking, which is in contrast to the dissolution of plasma-sprayed HA coatings *in vitro*, either in a SBF solution [33] or in cell culture medium [34].

The cellular response of the electrochemically coated HA compared to other HA coatings such as plasma sprayed coatings can not be assessed from the results of the current study. In addition to the effect of the different levels of roughness of the HA coatings [35], the level of crystallinity of HA coatings that are produced by very different techniques will have a major impact on the different cellular responses [36]. It would be of significant interest to compare both the *in vitro* and *in vivo* behavior of HA coatings produced by high temperature plasma-spraying and by low temperature electrochemical deposition, with particular attention to cell attachment and bone apposition.

5. Conclusions

The biochemical measurements of HOB cell proliferation and growth indicated that the HA coated samples resulted in the highest level of proliferation after three days of incubation. No direct correlation could be made between surface roughness of the four different substrates and cell proliferation/attachment. The roughest surface, produced by Al_2O_3-blasting, did however result in the lowest level of cell proliferation after three days of incubation, and examination by SEM revealed fewer cell processes attached to this substrate. While the HA coating, produced by a low temperature route, appeared to offer a more bioactive surface to the c.p. Ti substrate than some of the other Ti surface treatments, notably the as-machined and the Al_2O_3-blasted surfaces, it was not possible to tell from this study how this HA coating would behave compared to a plasma-sprayed HA coating. The phase purity and crystallinity of the electrochemically coated HA surface in this study would indicate a more stable, less bioresorbable, surface than a plasma sprayed HA coating, but it is not clear if this would be beneficial or damaging to the cellular response.

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