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In vitro testing of surface-modified biomaterials

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Cell substrata

2.1.1. Surface-modified materials

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

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

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

2.1.2. Untreated materials

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

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

2.2. Cell culture

The samples were steam sterilized before cell culture for 20 min. A primary droplet rat bone-marrow culture (RBMC) was performed according to the method described by Maniatopoulos *et al.* [14] which induces an osteoblast-enriched cell population.

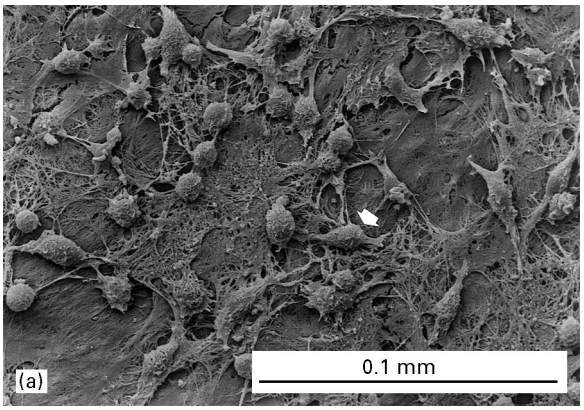
Young adult male Wistar rats, of about 100–120 g, were sacrificed. The epiphyses were removed and the bone marrow was flushed out from each dyaphise with α-minimum essential medium (Gibco), 15% foetal calf serum (Gibco) and 10% antibiotics (amphotericin B (Sigma), penicillin G (Sigma) and gentamycin (Gibco)). The cells were then grown on this medium to which 1% 1 M Na-β-glycerophosphate (Sigma), 1% 5 mg/ml ascorbic acid (Sigma) and 1% 10^{-8} M dexamethasone (Sigma) were added. A droplet culture of the bone-marrow cell suspension was placed on every sample. The cell culture was performed either in 6- or 24-well tissue culture plates (Costar) depending on the sample's dimension and kept in a humidified atmosphere of 95% air–5% CO_2 at 37 °C. The cultures were observed, every 48 h, with a light microscope. The cultures were maintained for 19 d and then prepared for observation with SEM.

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

3. Results and discussion

3.1. Thermanox™ culture

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



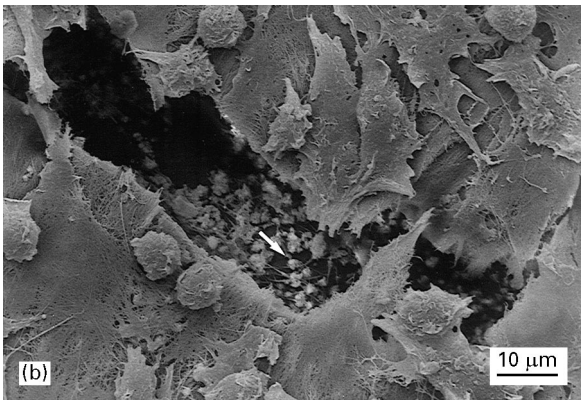


Figure 1 Scanning electron micrographs of osteoblasts cultured on Thermanox™. (a) The surface of the cell layer where osteoblasts (arrow) and collagen fibres can be identified. (b) Higher magnification micrograph showing the interface between the cell layer and the substratum where globular deposits (arrow) can be easily identified.

as negative imprints from the globules that were detached with the cell layer.

3.2. Untreated materials

Figs 2–5 represent scanning electron micrographs of several aspects of the RBMC on 316 L stainless steel, Ti–6Al–4V and Ti–5Al–2.5Fe. The cell cultures performed on these metals showed a similar morphology to each other, and also to those observed for the cultures performed on Thermanox™. The upper layer of the cell culture showed the presence of polygonal and round cells with extensive cell processes. The ECM seems to be composed mainly of collagen fibres. The detachment of the cell layer from the substratum showed the presence of the calcium and phosphorus-rich globules trapped in a complex net of collagen. Flattened cells were also visible. The structure of the RBMC on Ti–5Al–2.5Fe showed, in certain areas, clusters of polygonal cells, with a pronounced cell body and intense filapodia well attached to the ECM. From their morphology they appear to be osteocytes. The exposure of the metal/cell layer interface showed the presence of a high density of calcified globules.

3.3. Ion-implanted materials

Figs 6–10 show various aspects of the histology of the RBMC culture performed on the ion-implanted

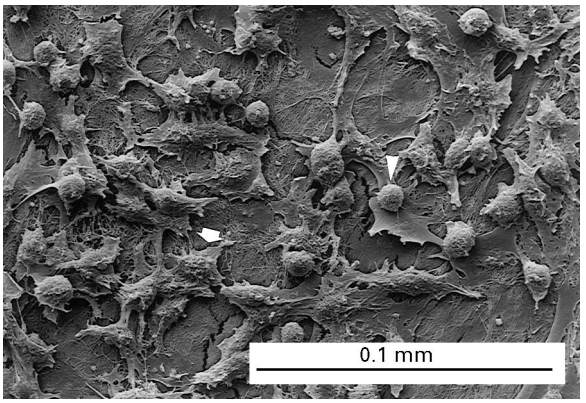


Figure 2 Scanning electron micrograph of the cell layer surface on 316 L stainless steel after 19 d culture showing polygonal cells (arrow), round cells (arrow head) and collagen fibres.

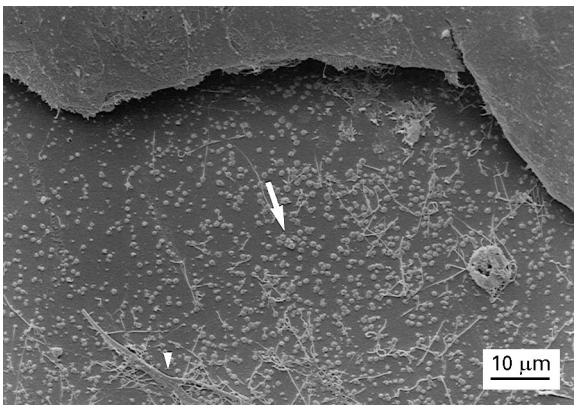


Figure 3 Scanning electron micrograph of Ti–6Al–4V after 19 d cell culture. The detachment of the cell layer shows the existence of globular deposits (arrow) where collagen fibres are incorporated (arrow head).

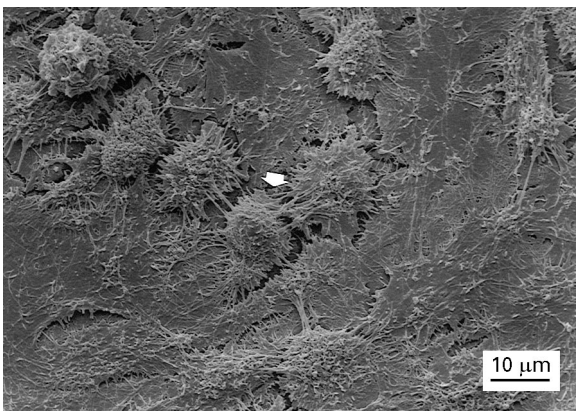


Figure 4 Scanning photomicrograph of a RBMC cell layer surface on Ti–5Al–2.5Fe showing the presence of osteocytes (arrow).

metals. All the cell cultures showed the same surface morphology as those obtained for the untreated materials: osteoblast-like cells with dorsal ruffles and filapodia in an extracellular matrix mainly composed of collagen where globular material is trapped. The detachment of the cell layer allowed the observation of the interface between the cell layer and the material's

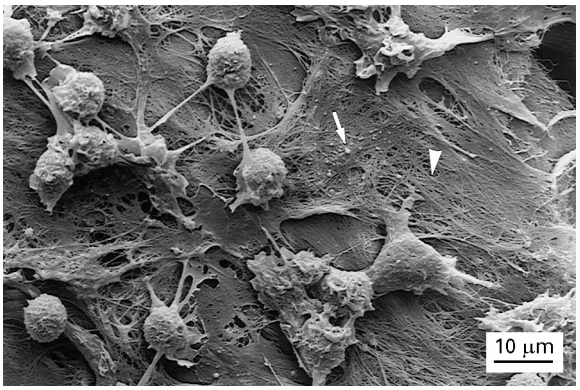


Figure 5 Scanning electron micrograph of a RBMC on Ti–5Al–2.5Fe showing the cell layer surface. The ECM is mainly composed of collagen fibres (arrow head). Note the lower density of globular deposits (arrow) trapped in the collagen fibres when compared with the density observed at the interface with the substratum.

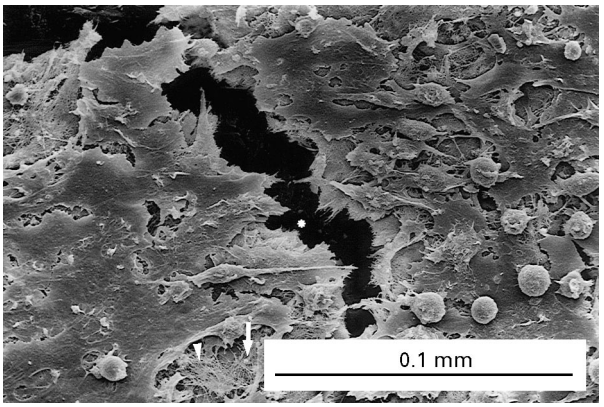


Figure 6 Scanning electron micrograph of a cracked cell layer on a N^+-ion implanted with 10^{15} ions cm^{-2} 316 L stainless steel. Collagen fibres (arrow head) and globular deposits can be identified in the ECM (arrow) and on the substratum (∗).

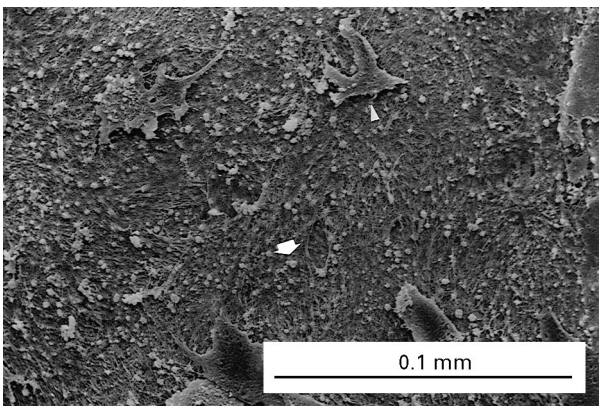


Figure 7 Scanning electron micrograph from a cell layer that has been detached from a N^+-ion implanted with 10^{16} ions cm^{-2} 316 L stainless steel sample. Many globular deposits (arrow) surrounded by collagen fibres can be observed, as well as flattened cells (arrow head). Note the high density of globules.

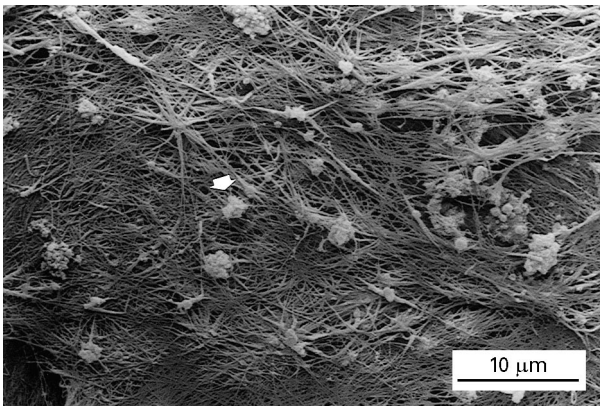


Figure 8 Scanning electron micrograph from a cell layer that has been detached from a N^+-ion implanted with 10^{17} ions cm^{-2} Ti–6Al–4V sample showing many globular deposits (arrow) trapped in a net of collagen fibres.

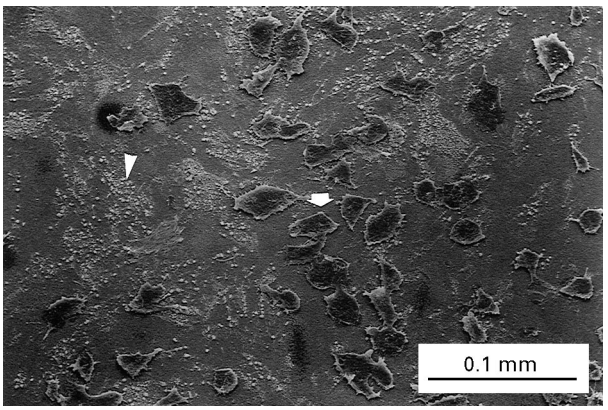


Figure 9 Scanning electron micrograph from a N^+-ion implanted with 10^{17} ions cm^{-2} Ti–6Al–4V sample showing the metal substratum after the removal of the cell layer. Osteoblast-like cells (arrow) and globular deposits (arrow head) can be identified.

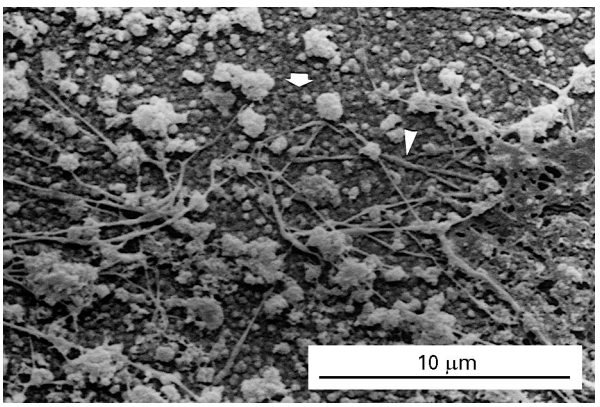


Figure 10 Scanning electron micrograph at a higher magnification of a N^+-ion implanted with 10^{17} ions cm^{-2} Ti–6Al–4V sample showing the substratum after the removal of the cell layer. A layer of globular deposits (arrow) covers all the surface. Calcified collagen fibres (arrow head) can also been seen.

surface. The layer adjacent to the substratum was composed of an intricate net of collagen fibres containing trapped calcified globules. Osteoblast-like cells were also observed. They displayed a flat shape and cell processes indicating that they were well attached to the substratum. Comparing the ion-implanted material's surface after the cell layer detachment with equivalent surfaces of the untreated materials, it is possible to observe that the metal surface in the former

case is covered with a continuous layer of globules where calcified fibres of collagen can be seen.

3.4. Sputter-coated 316 L stainless steel

The cell layer grew heterogeneously on these materials. The nitrogen sputter coating induced worse *in vitro* cell response. Fig. 11 shows a nitrogen sputter-coated sample after 19 d cell culture. Most of the surface was only covered by widely dispersed polygonal cells. Contacts between cells could easily be identified but no ECM was produced. Nevertheless, small clusters of cells with a morphology similar to that obtained on Thermanox™ could be observed. Fig. 12 shows a photomicrograph of a surface cell layer formed on a carbon sputter-coated stainless steel. Fig. 13 presents a photomicrograph of the same material in which the cell layer has been mechanically detached to expose the substratum surface. In the nitrogen sputter-coated samples, not all the surface was completely covered with a cell layer. However, in this case, where the cell layer was present its aspect seemed to be smoother than the cell layer surfaces previously described, as no individual cells are protruding. At higher magnifications it was possible to identify fibres of collagen as the main constituent of the ECM. Cracks induced by the critical point drying procedure allowed the observation of the inner layers. It was possible, in some areas, to observe the presence of calcified globules associated with calcified collagen fibres. Osteoblast-like cells that initially colonized the surface were also observed.

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

Based on the light microscopy and SEM observations, it seems that the culture evolves in three stages, in the same way as the model proposed by Davies *et al.* [5]: cell colonization and multilayering, initial multicellular mineralized matrix production and bone formation on the substrata. After cell inoculation the cells start to spread on the substrata and to multilayer. According to the studies performed by Maniatopoulos *et al.* [14], the multilayering of these cultures, prior to confluence, is an indication that bone nodule formation will occur. By observing the surface of the substrata and the tissue immediately adjacent, either by mechanically detaching the cell layer or by observing the cracks induced by the critical point drying process, it was possible to detect the presence of calcium- and phosphorus-rich globular deposits which seem to be formed first followed by the production of collagen fibres. These deposits were identified by Davies *et al.* [17] as spheritic foci of calcification and their origin was proved not to be a substratum-mediated process but one which is dependent on the cells expressing their osteoblast phenotype. The observation of these globular accretions was

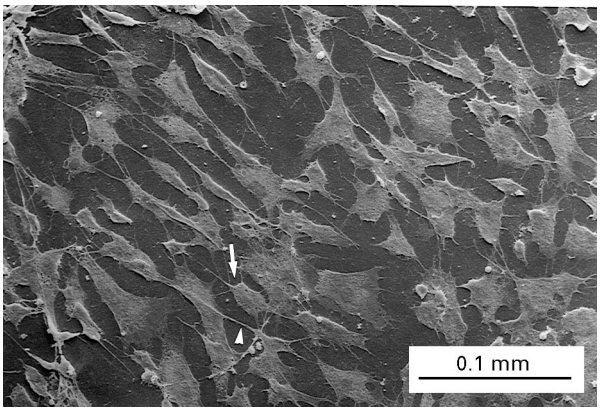


Figure 11 Scanning electron micrograph of a nitrogen sputter-coated sample after 19 d RBMC. No ECM was formed. Spread polygonal-shaped cells (arrow) with cell processes (arrow head) can be identified.

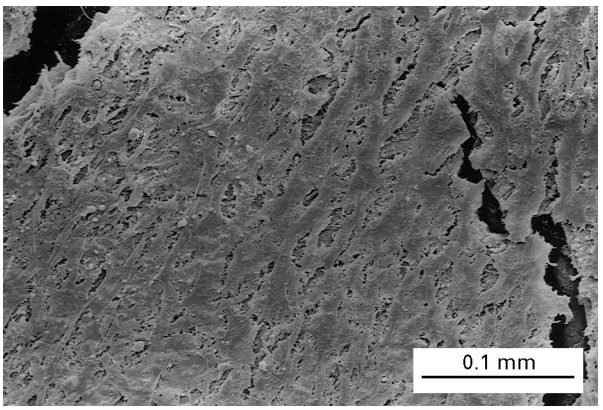


Figure 12 Scanning electron micrograph of a carbon sputter-coated sample after 19 d RBMC. Note the smoother appearance of the cell layer surface.

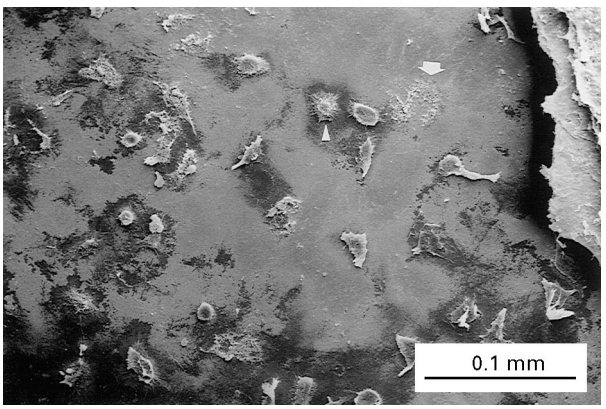


Figure 13 Scanning electron micrograph of a carbon sputter-coated sample after the removal of the cell layer. Clusters of globular deposits (arrow) and osteoblast-like cells (arrow head) were observed.

also achieved by other groups [5, 21, 23]. Further studies [17] on the constitution of the interfacial zone between Ti c.p. and the nearest bone cell suggested that the interface cell layer/metal substratum was composed of at least two layers: a proteoglycan-rich layer and a bonding zone between the proteoglycan-rich layer and the metal beneath.

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

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

4. Conclusions

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

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

3. The study of the interface substrata/cell layer showed the presence of calcium- and phosphorus-rich globular deposits in higher density than on the cell layer surface.

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

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

Acknowledgements

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