Focus ion beam / Scanning electron microscopy characterisation of osteoclastic resorption of calcium phosphate substrates

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Abstract

This paper presents the application of dual Focused Ion Beam-Scanning Electron Microscopy (FIB-SEM) imaging for pre-clinical testing of calcium-phosphates with osteoclast precursor cells and how this high resolution imaging technique is able to reveal microstructural changes at a level of detail previously not possible. Calcium phosphate substrates, having similar compositions but different microstructures, were produced using low and high temperature processes (biomimetic calcium deficient hydroxyapatite and stoichiometric sintered hydroxyapatite, respectively). Human osteoclast precursor cells were cultured for 21 days prior to evaluate their resorptive potential on varying microstructural features. Alternative to classical morphological evaluation of osteoclasts (OC), FIB-SEM was used to observe the subjacent microstructure by transversally sectioning cells and observing both the cells and the substrates. Resorption pits, indicating OC activity, were visible on the smoother surface of high temperature sintered hydroxyapatite. FIB-SEM analysis revealed signs of acidic degradation on the grain surface under the cells, as well as intergranular dissolution. No resorption pits were evident on the surface of the rough calcium deficient hydroxyapatite substrates. However, whereas no degradation was detected by FIB sections in the material underlying some of the cells, early stages of OC-mediated acidic degradation were observed under cells with more spread morphology. Collectively, these results highlight the potential of FIB to evaluate the resorptive activity of OC, even in rough, irregular, or coarse surfaces where degradation pits are otherwise difficult to visualize.

Introduction

In recent years, Focused Ion Beam (FIB) systems have gained interest as a technique for the evaluation of biological samples. Although microscopic evaluation of biological matter is challenging, FIB equipped with scanning electron microscopy (SEM) or energy dispersive X-ray spectroscopy (EDX/EDS) provides an enhanced working environment for studying biomaterials.[1]

The regenerative potential of bone substitute materials is usually evaluated *in vitro* by assessing how cultures of relevant cells interact with them. Briefly, at the early stages of implantation, inflammation occurs and a cascade of chemical signals which drive the monocyte/macrophage phenotype are triggered.[2] In an ideal scenario, these cells will fuse to form osteoclasts (OC), that is, bone resorbing cells.[3,4] Recent research indicates that osteoclasts can establish a cross-talk with osteoblasts leading to subsequent bone remodeling.[5]

Several pre-clinical studies of bone substitutes have focused on these early interactive stages and, particularly, on the biomaterial-osteoclasts interactions in terms of OC adhesion, gene expression and evaluation of resorption pits.[6-8] Usually, these investigations require, on the one hand, the study of OC differentiation and activity, such as gene expression or marker identification, and on the other hand, the evaluation of morphological changes in the substrates, which implies staining or removal of cells in order to visualize the underlying structure. In this context, FIB-SEM technique offers the possibility of evaluating both cells and subjacent microstructural changes simultaneously. Various studies have used FIB for biological sample evaluation,[9-15] and the interest in the field has been increasing over recent years. Nevertheless, studies on osteoclast resorption on substrates of challenging topography are lacking. Thus, this work aims to assess the potential of FIB-SEM technique as a tool to evaluate cell morphology together with the microstructural changes caused by OC-mediated degradation beneath the cells. Three substrates with similar composition and different nano-microstructures are investigated to disclose to what extent FIB-SEM analysis may allow assessing the resorption activity of the cells on substrates with different topographies.

Materials and Methods

Three types of calcium phosphate substrates were prepared. Low temperature biomimetic calcium deficient hydroxyapatite (CDHA) was obtained through the hydrolysis of alpha-tricalcium phosphate (α-$Ca_3(PO_4)_2$, α-TCP) powders at 37°C. Powders with two different particle sizes, coarse (C: 5.2 μm median size) and fine (F: 2.8 μm median size), were mixed with a 2.5 wt% solution of sodium hydrogen phosphate (Na_2HPO_4, Merck) at a liquid to powder ratio of 0.35 mL/g, to produce materials with microstructure consisting of an entangled network of plate-like (CDHA-C) and needle-like (CDHA-F) CDHA crystals (both with a Ca/P ratio of 1.5) respectively.[16] High temperature stoichiometric sintered HA (sin-HA) was obtained by solid state reaction of a mixture of calcium hydrogen phosphate ($CaHPO_4$, Sigma–Aldrich C7263) and calcium carbonate ($CaCO_3$, Sigma–Aldrich C4830) with a calcium to phosphorous ratio of 1.67 at 1100 °C for 11 h. All materials were molded into discs of 14 mm diameter and 0.25 mm thickness in Teflon moulds.

Cell cultures were performed using human peripheral blood mononuclear cells (PBMC), from healthy 30-35 year old male voluntary blood donors. Donations were anonymous, so institutional review board (IRB) approval was not required. Mononuclear cells were isolated by centrifugation with Ficoll-Histopaque (Sigma-Aldrich) and seeded at a density of $6x10^6$ cells per cm^2 on calcium phosphate discs. PBMC were differentiated into osteoclast precursors with RANKL-containing cell culture medium (DMEM) from human osteoblast supernatants. Cells were seeded on two discs for each substrate. The experiment was repeated twice using cells from two independent donors. OC precursors were cultured for 21 days. Afterwards, the cells were rinsed with phosphate buffered saline (PBS, Gibco, UK), fixed with paraformaldehyde/glutaraldehyde in 0.1M sodium cacodylate buffer, and post-fixed in 2% osmium tetroxide (OsO_4) at room temperature for 2 hours in order to achieve higher contrast. Then, samples were dehydrated in an ethanol series followed by hexamethyldisilazane (HMDS) drying to preserve osteoclasts morphology.[17, 18] A thin gold-palladium film was sputtered before FIB/SEM examination to impart conductivity to samples.

SEM in combination with FIB (Zeiss Neon 40) was used to examine both the surface of cells/materials and the microstructural features underneath the cells. As a first step conventional SEM was used to evaluate both the microstructure of the substrates and the morphology of the

cells. Two representative cells were then selected for FIB cross-sectioning using gallium ions (Ga+). Prior to cutting, a thin layer of protective platinum (Pt) was deposited on the surface by ion-beam assisted deposition in order to reduce the curtaining effect. This effect can result in image artefacts due to changes in the sputtering yield, as the beam passes over different composition regions, resulting in the appearance of parallel patterns in the ablated zones. The deposition of Pt helps obtaining smoother cross-sections, thus minimising the curtaining effect.[13] Afterwards, a rough (coarse) milling was performed with a maximum current of 10 nA to quickly remove most of the material up to a depth of approximately 20 µm nearby the region of interest. Finally, a polished cross-section was attained by subsequently reducing the Ga^+ beam current from 2 nA down to 500 pA when approaching the Pt layer. This procedure reduces the ion beam damage on the sample and any re-deposition effect, and allows the observation of smoother cross-sections of the cell and the underlying microstructure with minimal modifications.

Results and Discussion

Sintered and biomimetic calcium phosphates have long been used in bone regeneration applications.[19,20] Despite biomimetic CaP substrates can better mimic the microstructure and composition of the mineral phase of bone than sintered CaP, *in vitro* assays on biomimetic materials are often more challenging.[21,22] The rough microstructure that results from the precipitation of nano/submicron crystals compared to the smoother textures of the high-temperature processed materials can have great impact on cell behavior. When challenged with osteoclasts the role of surface topography is particularly relevant, as osteoclastic resorption is dependent on the formation of an actin-rich sealing zone that precedes degradation.[23,24] *In vitro* studies had shown that too rough textures can hinder the formation of a proper sealing zone, impairing material degradation.[6,25]

The possibility offered by FIB, to closely look at the cell-substrate interface can help shedding new light on the evaluation of resorption events on challenging biomimetic substrates compared to traditional sintered substrates. But FIB is a complex technique that requires accurate setting of the processing parameters to minimize the generation of artifacts during sectioning. Only artifact-free sections will allow drawing accurate conclusions. FIB tomographic studies have successfully been performed to analyze at different depths the delicate nature of biomimetic

CaP consisting of an interlocked network of thin plate-like crystals, proving that artifact-free sections can be obtained.[26] The situation is more complex, however, when FIB is used to section cells cultured on these substrates. Drobne *et al.,* as well as other authors, extensively described the potential of FIB milling to process biological samples and shed light on the optimal processing parameters in order to avoid shrinkage, melting effect, Ga^+ implantation or side-wall artefacts.[18, 27-30] These effects can be effectively reduced by a first Pt layer deposition which protects the sample surface against re-deposition of ablated atoms, provides mechanical stability and reduces curtain effects. In addition, damage can be minimized by working with low ion beam currents and low acceleration voltages, especially during the final steps of the cross-section polishing.[29] All these strategies have been applied in the present study to investigate a series of three apatitic substrates consisting of two biomimetic formulations and a sintered material.

Figure 1 shows the SEM images of the three different substrates in the absence (Figure 1a, c and e) and in the presence (Figure 1b, d, f, and g) of cells after 21 days of culture. Biomimetic substrates obtained at 37 ^{o}C, i.e. CDHA-C and CDHA-F, consisted of an entangled network of plate-like crystals (Figure 1a) and needle-like crystals (Figure 1c), respectively, whereas sin-HA presented a smoother polyhedral grain surfaces (Figure 1e) typical of high temperature ceramics.

SEM evaluation of the seeded substrates showed similar cell morphologies consisting of flat and well spread cells ranging 20-30 µm with various connecting filopodia (Figure 1b, d, and f). As illustrated in Figure 1g, resorption pits were easily visualized on some substrates (white arrows), namely sintered HA, where typical 30-50µm sized resorption lacunae were observed. In contrast, no clear resorption pits were evident on the rough and tortuous surfaces of biomimetic CDHA samples. In this case, FIB cuts could provide useful information to assess if any potential osteoclastic degradation is taking place locally, at the surface underneath the cell.

The sequence to obtain the vertical section of a cell and the underlying substrate by FIB is shown in Figure 2, for biomimetic CDHA-F. No signs of resorption were observed in this case in the material under the cell (Figs. 2 c and d), as the needle-like crystals appeared intact, when compared with the microstructure of the pristine material (Fig. 1c, insert). One possible

explanation is that the high roughness of the substrate prevented the OC from sealing the substrate, thus hampering the degradation process, as described in previous works.[6,25] One aspect worth noticing is the fact that the microstructure of the pristine material was revealed intact beneath the cell, thus confirming that there was no damage associated to FIB-sectioning.

Interestingly, when on the same substrate a different cell of bigger size and with a more spread morphology, compatible with an osteoclastic or macrophage phenotype was analyzed, FIB/SEM images revealed that resorption was taking place in the underlying material (Figure 3). Thus, acid etching was evident in the subjacent microstructure where the needle-like crystals were no longer visible (Figure 3b* and c). Few needle-like crystals were only visible in the outer part of the cell (Figure 3d). The presence of both, resorptive and non-resorptive cells could evidence the multiple and reversible macrophage phenotype.[31]

FIB-SEM images for the CDHA-C substrate are shown in Figure 4. As for CDHA-F, no resorption pits were found on the surface, and no signs of degradation even underneath the cells were detected by FIB cuts (Figure 4c and d), as revealed by the presence of the original plate-like crystals in the areas subjacent to the cell (*).

As observed by traditional SEM imaging, numerous resorption pits and several flat and spread cells were observed on the sin-HA substrate (Figure 1f and g). Previous works have shown the resorptive capacity of osteoclast-like cells derived from mice bone marrow on similar substrates.[32,33] However, the study of osteoclast activity using primary human cells is less common, despite providing a closer understanding of the physiology of human bone resorption. FIB-SEM images of a cell on sin-HA are shown in Figure 5. Figure 5a shows a few cavities on the substrate at low magnification, corresponding to osteoclastic resorption pits (white arrows) clearly different from the intrinsic porosity of the material (+). Intergranular dissolution of the ceramic was displayed just under the cell (Figure 5b), with excavated cavities penetrating several micrometers into the substrate. This preferential dissolution in the grain boundaries is a frequently observed phenomenon, due to the higher reactivity of these regions that have a high free energy. Moreover, the surface of the polyhedral grains under the cells presented a rougher aspect compared to the smoother appearance of the pristine ceramic, consistent with an acidic etching process (Figure 5c and d).

Conclusions

The FIB-SEM technique has been shown to be a useful technique to assess the *in vitro* cell resorption activity, even at early stages of the process. The present study on different calcium phosphate substrates proved the potential of this technique to evaluate both the cell morphology and the microstructure of the substrate underneath. Indeed, this technique may disclose the initial activity of resorbing cells when not evident yet using other analytical methods, or may confirm and support morphological and biochemical evidences of osteoclast degradation capability *in vitro*. Sintered HA surfaces showed visible resorption pits and FIB cuts across the cell demonstrated the initial degradation of the ceramic as a consequence of acid etching promoted by the cells, which resulted in intergranular dissolution and roughening of the grain surface. In contrast, in low temperature biomimetic HA substrates, where no clear signs of degradation on the surface were visible by common (seen using standard?) SEM imaging, possibly due to the higher roughness of these materials, different situations were found by (following) FIB cuts. For some cells, no signs of degradation of the underlying material were observed, probably due to the lack of a sealing actin ring and a resorbing lacuna formation. However, at different sites where large flattened cells and more spread cells were observed, signs of degradation were clearly seen in the material underneath the cells, with the etching of the needle-like crystals. These findings underline the potential of the FIB-SEM technique to evaluate cell-mediated resorption in rough, irregular or coarse substrates. Indeed, FIB milling of resorbing cells allows exploring their underlying structure to unravel early degradation processes at stages when resorption pits have not yet occurred or are virtually impossible to detect.

Disclosure Statement

No competing financial interests exist.

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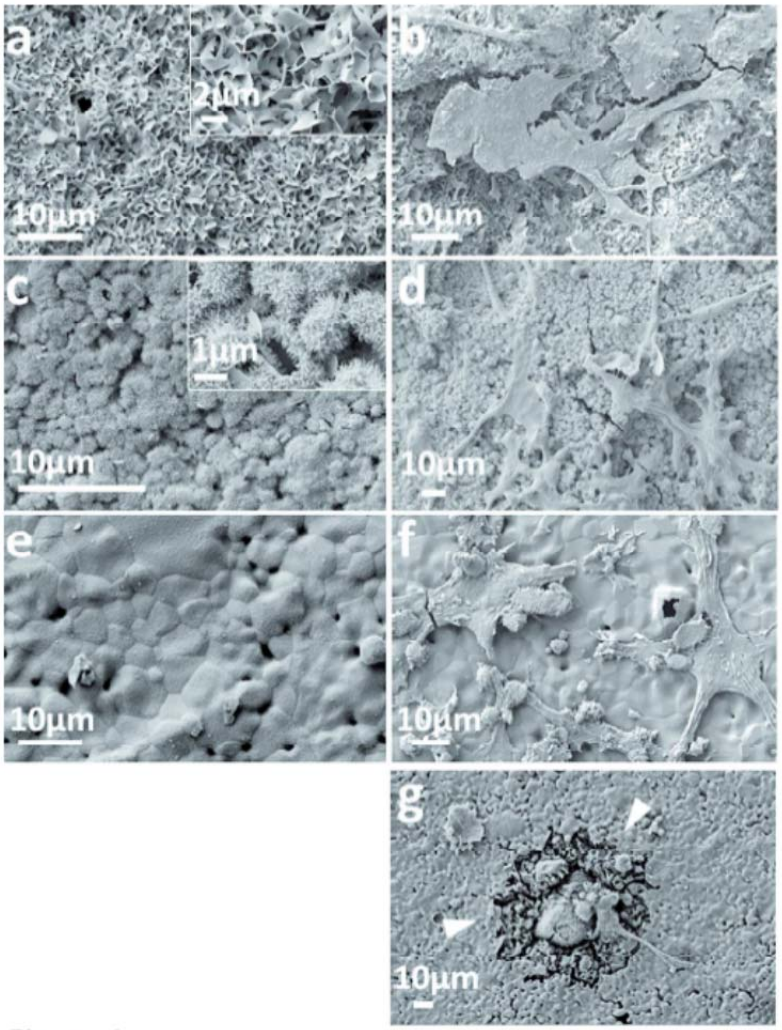

Figure 1

Figure 1. SEM images of the surface of pristine CaP substrates (a, c and e) and cells on the substrates (b, d, f and g): CDHA-C (a, b); CDHA-F (c, d) and sin-HA (e, f, g).White arrows indicate a resorption pit.
Figure 1
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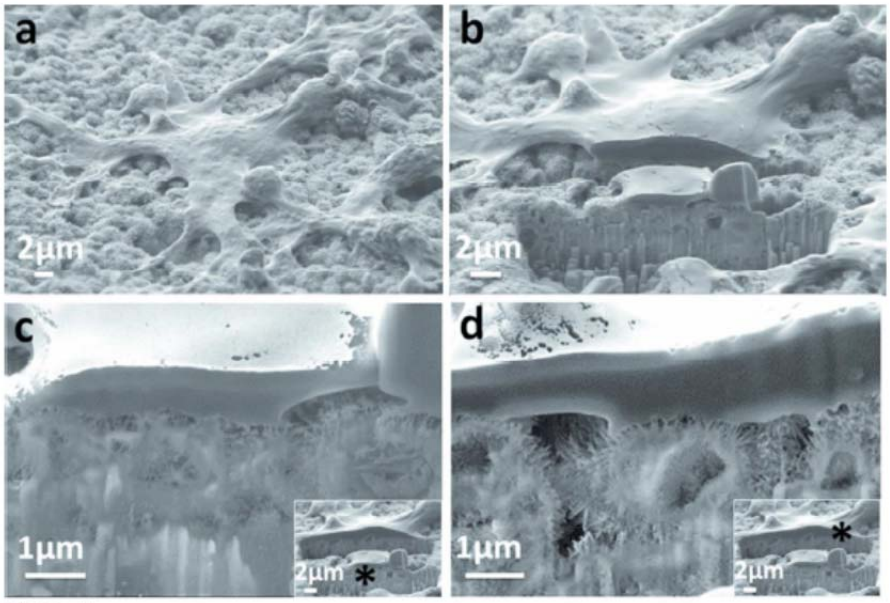

Figure 2

Figure 2. Sequence to obtain vertical sections of a cell on the CDHA-F substrate (a-b). No signs of degradation are visible in the material underlying the cell, where the needle-like crystal morphology can be clearly observed (c and d; the asterisk in the insert indicates the region that has been magnified in the image)

Figure 2

70x50mm (300 x 300 DPI)

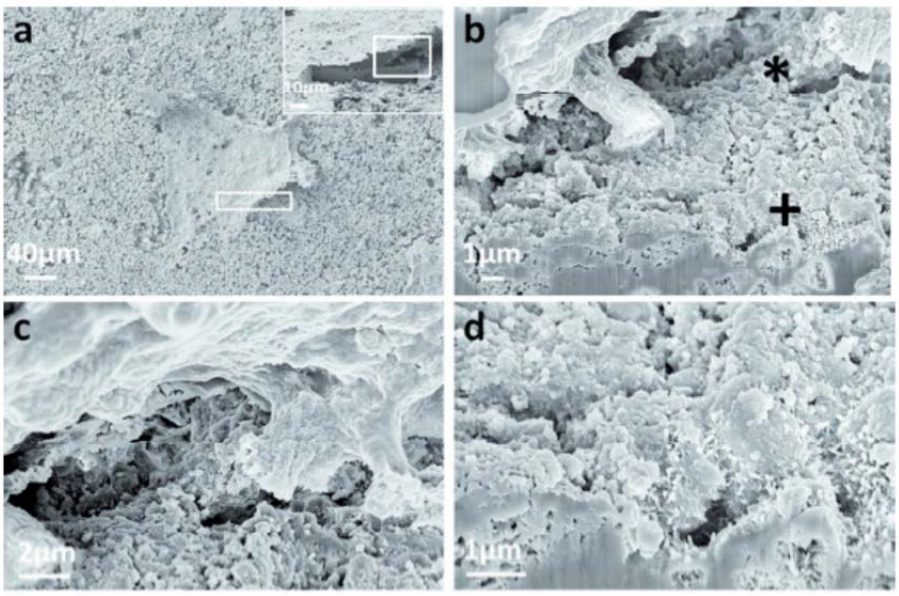

Figure 3

Figure 3.FIB-SEM images of a well spread cell on CDHA-F substrate with the insert showing the FIB cut performed (a).Higher magnification of the cell sectionand the subjacent area (b). A region with degraded crystals, marked with *is shown at a higher magnification in (c), whereas non-degraded crystals in the periphery of the cell, marked with +are shown at a higher magnification in (d).

Figure 3

68x47mm (300 x 300 DPI)

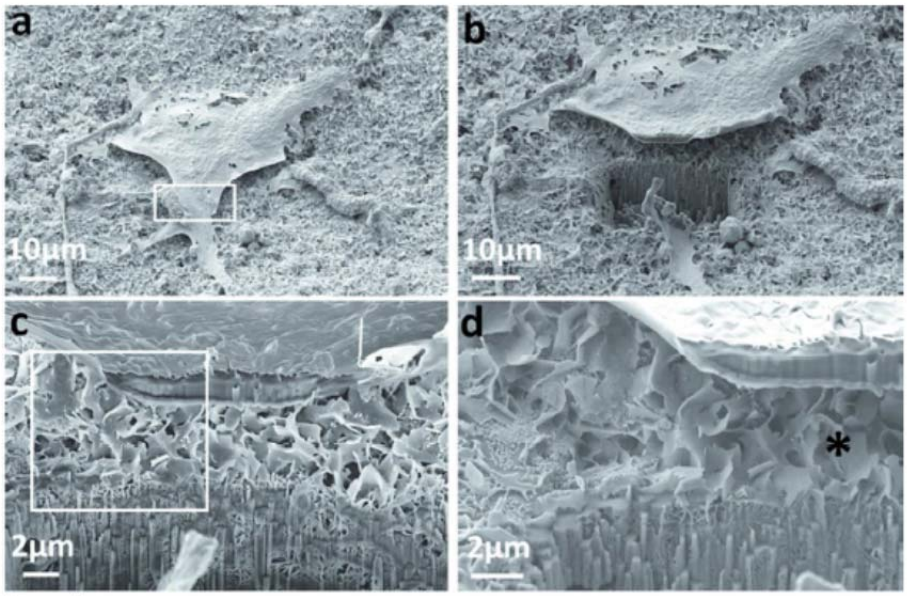

Figure 4

Figure 4.FIB-SEM images of a cell on CDHA-C. The FIB section(b-d) show that the pristine microstructure, consisting of plate-like crystals, was not degraded under the cell (c and d, marked with *).

Figure 4

68x46mm (300 x 300 DPI)

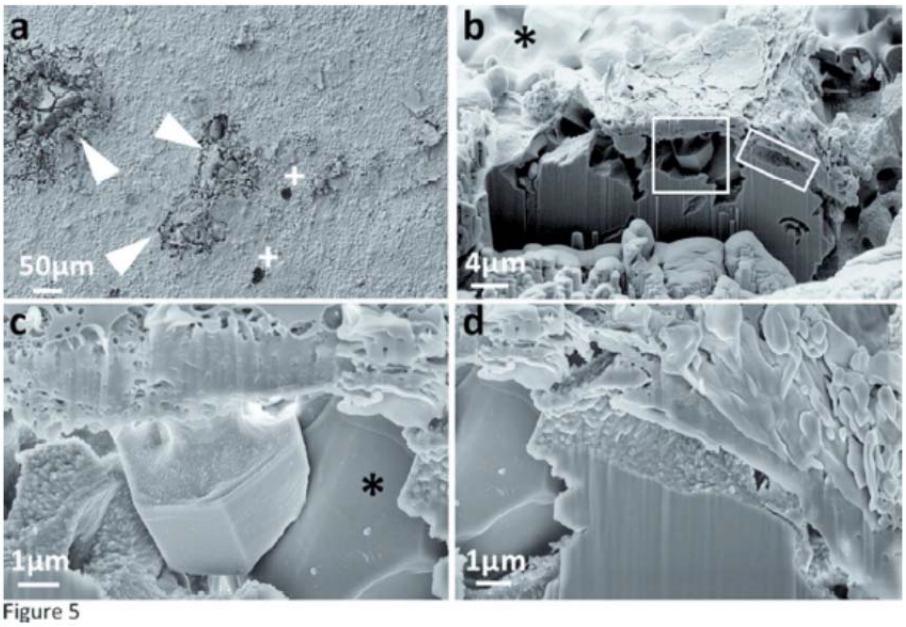

Figure 5

Figure 5.SEM imageshowingvarious resorption pits (arrows) and intrinsic pores marked with + on sin-HA (a).FIB sections at higher magnification are shown (b-d), illustrating the underlying microstructure. Rougher grains are visible in (c) and (d) compared to the smooth original grains visible in (b), marked with *.

Figure 5

68x47mm (300 x 300 DPI)