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Importance of dynamic culture for evaluating osteoblast activity on dense silicon-substituted hydroxyapatite

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ABSTRACT

This paper reports an investigation on human osteoblast-like cells (SaOs-2) seeded onto pure hydroxyapatite (HA) and silicon-substituted HA (SiHA) tablets under static and dynamic culture conditions. The biological characterizations were conducted in classical static conditions in multi-wells plates, and in a perfusion bioreactor that permits continuous circulation of culture medium at 2 mL/h. The morphology, proliferation and differentiation of osteoblastic cells were examined for the two types of samples in the both culture conditions after 1, 3 and 8 days. Under dynamic conditions, cells cultured on SiHA surfaces showed a faster adhesion process and the formation of longer and thinner focal adhesions than in static conditions. The number of cells grown onto both ceramic surfaces was higher in dynamic conditions when compared with static conditions. Moreover, a higher activity of alkaline phosphatase was found for cells seeded under dynamic conditions. Our findings suggest that the application of perfusion culture system on cells cultured on dense substrates is valuable for predicting *in vivo* behaviour of cells on biomaterials.

1. Introduction

Synthetic hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) has a chemical and phase composition similar to the inorganic component of bone. It presents excellent biocompatibility and is commonly used in many biomedical applications [1].

Actually, bone mineral is essentially a non-stoichiometric hydroxyapatite, with various ion substitutions in the anionic sites: OH^- (F^-, Cl^-), and PO_4^{3-} (SiO_4^{4-}, CO_3^{2-}) and/or cationic site Ca^{2+} (Na^+, Mg^{2+}, K^+, Sr^{2+}, Zn^{2+}, Ba^{2+}, Al^{3+}). One approach for improving the biological properties of synthetic HA is to adjust its chemical composition to more closely approximate that one of bone mineral [2,3].

Silicon, in particular, is known as an essential element for normal bone and cartilage formation as it is found in areas of calcification. Silicon has also a structural role as component of all glycosaminoglycans and polyuronides: chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate. The hyaluronic acid is also enriched in silicon. Finally, it was found a correlation between the amount of Si in the diet and the mineralization level of young bones of rat and chicken [4,5]. Since physiological levels of silicon have a beneficial role in bone calcification and metabolism, it may be hypothesised that the incorporation of comparable levels of silicate ions in HA may also enhance the bioactivity of these samples [6,7].

Results of *in vitro* tests of hydroxyapatite substituted by silicon (SiHA) showed that a Si content of 2.2 wt% may be the optimum loading to improve the bioactivity of human osteoblasts [8,9]. Furthermore, in bioactivity studies, the level of osteoblast markers expressed on 0.8 wt% SiHA granules was higher than that expressed on 1.5 wt% SiHA sample. It was also demonstrated a higher osteoclastic resorptive activity on the 1.5 wt% SiHA [10].

In vivo studies also proved a better performance of SiHA in the ovine model. Percentage of bone ingrowth for 0.8 wt% SiHA was greater than for HA granules, when implanted into the femoral condyle of sheep for 6 weeks. Additionally, organized collagen fibrils were found at the bone SiHA interface after 6 weeks whereas they were found only after 12 weeks around the pure HA sample [11,12].

In order to understand the influence of immersion conditions (static/dynamic) on ceramic surface transformation, we have recently proposed a new biomimetic approach based on the use of a bioreactor for dynamic immersion of ceramics under constant physiologic flow. Also, to be more close to the *in vivo* fluid composition we proposed to compare the surface transformation of ceramics after soaking in cell culture medium added, or not, with fetal bovine serum. The influence of these biomimetic conditions was validated using Si-substituted hydroxyapatite tablets

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compared to pure hydroxyapatite controls. Si-substituted hydroxyapatite presented a higher capacity of protein adsorption [13].

The human osteoblasts are classically used to investigate *in vitro* the biocompatibility and the bioactivity of biomaterials for bone replacement. Both, the cytotoxicity and the quality of cell adhesion at the initial contact stage are considered as very important process for the following steps such as cell proliferation and cell differentiation [14]. It has been shown that dynamic culture improves the homogeneity of the distribution of cells and matrix, and that shear stresses applied by medium stimulate the cell proliferation and differentiation. Furthermore, the systems enhance mass transport, ensuring continuous nutrition of cells and removal of waste products [15].

The goal of this study was to investigate the effect of dynamic culture conditions on human osteoblast SaOs-2 cells activity, cultured on HA and SiHA tablets. Basic osteoblast reactions were investigated on these surfaces by determination of the cytoskeleton organization, focal adhesion formation, cell proliferation and cell differentiation. The results obtained for dynamic conditions were compared to those obtained in 24-wells plates, the classical static incubation conditions.

2. Materials and methods

2.1. Production of hydroxyapatite tablets

Stoichiometric hydroxyapatite (HA – $Ca_{10}(PO_4)_6(OH)_2$) powder was obtained by wet precipitation [16] in the Brazilian Center for Physical Research (CBPF). Silicated hydroxyapatite powder (SiHA – $Ca_{10}(PO_4)_{6-x}(SiO_4)_x(OH)_{2-x}$) with 1.13% in weight of silicon ($x = 0.4$) was also obtained by the wet precipitation method in SPCTS/University of Limoges [3]. These two powders were used to produce HA and SiHA samples, respectively. The powders were calcined at 650 °C and then uniaxially pressed under a compressive stress of 125 MPa. After pressing, the obtained tablets were sintered at 1200 °C for 1 h in order to produce dense surface structure. The tablets were polished with a sequence of SiC paper (1000–4000 meshes). This procedure was relevant to standardize the morphology and roughness of both ceramics (HA and SiHA).

2.2. Ceramic surface characterization

Atomic force microscopy (AFM) was used to characterize surface roughness by measuring the average roughness (Ra). Images were taken from five different regions using the tapping mode in a Multimode Nanoscope IV AFM (Veeco (DI), United States).

2.3. Cell culture

The present study used the human osteosarcoma cell line SaOs-2. The cells were cultured in McCoy's 5A medium (Eurobio, France) supplemented with 10% fetal bovine serum (Eurobio, France) and 100 U/mL of Penicillin and 100 μg/mL of Streptomycin (Eurobio, France). The cells were kept in an incubator at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. At confluence, the cells were detached by trypsinisation with 0.25% crude trypsin and 0.02% EDTA, pH 7.2.

2.4. Static and dynamic cell cultures

After trypsinisation, 4.0×10^4 SaOs-2 cells at the 9th passage were seeded on each sample into a 24-wells plate coated with agar gel (2% in water) to avoid cell growth on the bottom of the well. After 24 h of incubation, half of the samples were transferred in the Minucells® perfusion chamber (MINUCELLS and MINUTISSUE, Germany). The comparative study was done in parallel with the other half of samples let into the static 24-wells culture plates.

Dynamic cell culture was performed inside the Minucells® flow perfusion bioreactor (MINUCELLS and MINUTISSUE, Germany), as illustrated in Fig. 1. The system consists of a chamber, supplied by medium by a peristaltic pump at a 2 mL/h flow rate. Medium (McCoy's, Sigma, France), flows through the ceramic samples vertically in a bottom-up direction.

Before each experiment, the system was sterilized at 105 °C and 0.5 bar pressure in an autoclave apparatus. At the end of each period, the cells on samples were either treated for immunofluorescence, for proliferation assay and for differentiation assay. A total of three independent assays were carried out for each experiment, with each assay performed in triplicate.

2.5. Focal adhesion and stress fiber formation

After a fixed time point, the cells were fixed with 2% paraformaldehyde for 30 min. They were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and washed again with PBS. The samples were then incubated in 1% albumin solution (in PBS) for 20 min at room temperature to block nonspecific adsorption, washed three times with PBS (5 min each). After these steps, a part of samples was stained for focal adhesion and the other part was stained for visualisation of cytoskeleton.

After rinsing, 200 μl of a mouse monoclonal anti-human vinculin antibody (Sigma) were incubated with the cells for 30 min at room temperature followed by three rinses with PBS. Secondly, the cells were labelled with TRITC-anti-mouse IgG antibody (Sigma) at room temperature for 30 min and rinsed again three times with PBS. The cells were then mounted on a microscope slide under glass cover slips using 50% glycerin (in PBS). The labelled cells were examined using an Olympus BX51 microscope equipped for epifluorescence.

The length and the aspect ratio (length/width) of vinculin-labelled focal adhesion clusters were measured on digital images using Global Lab Image J (ref. 1.42q) software. The analysis of focal adhesion clusters was obtained from at least 10 cells/surface.

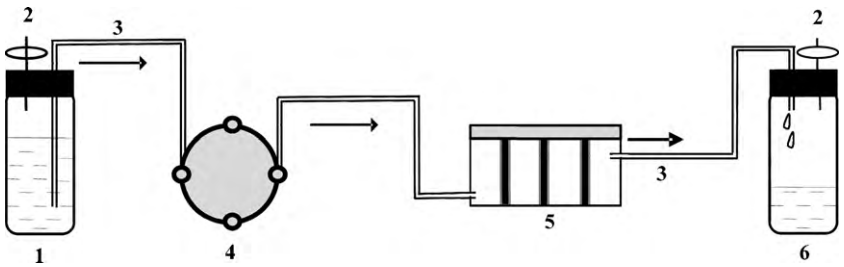

Fig. 1. Schematic diagram of the bioreactor system placed in the constant temperature oven (37 °C). A silicone tube (3) makes the connection between fresh medium bottle (1), which supports an air filter (2), bioreactor (5) and waste medium bottle (6). A multi-channel peristaltic pump (4) placed just before the bioreactor is adjusted to a constant flow fluid at 2 mL/h.

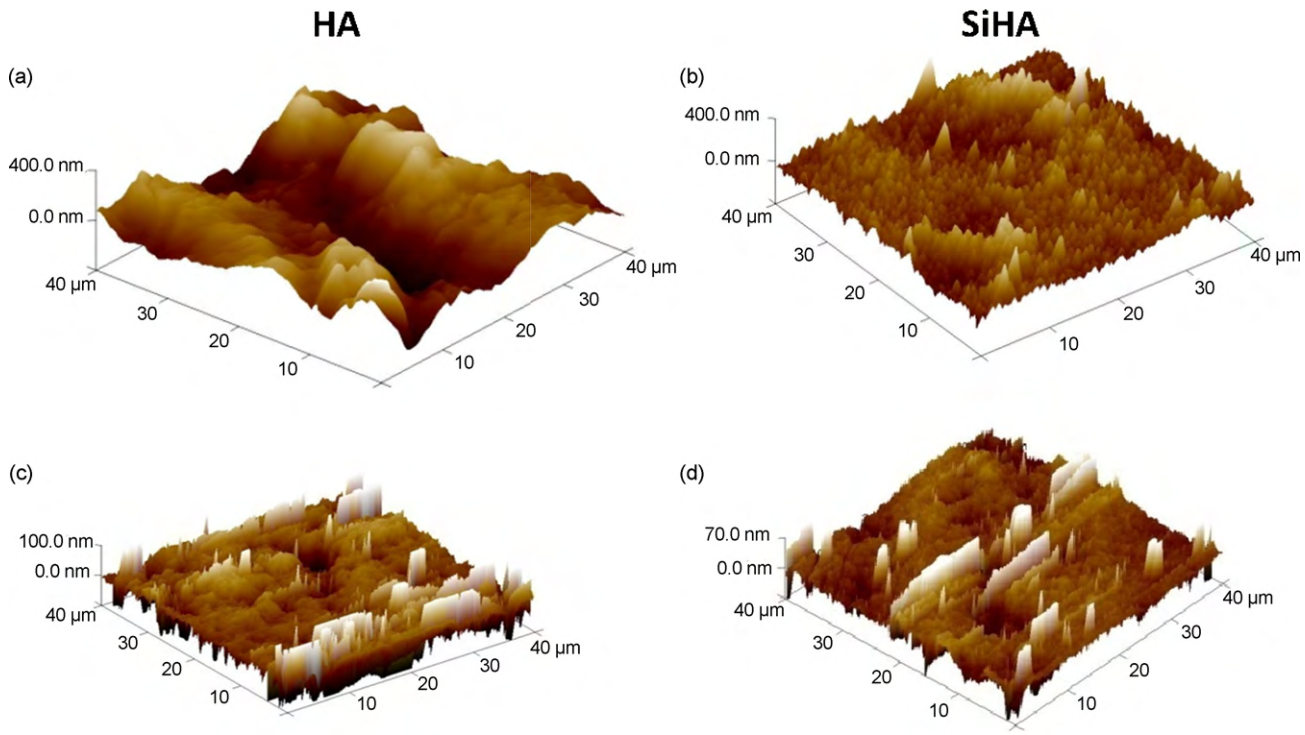

Fig. 2. AFM images of original (a) HA and (b) SiHA surfaces and of polished (c) HA and (d) SiHA surfaces.

The cells were incubated with FITC-phalloidin (0.4 µg/ml, Sigma, France) for 1 h at room temperature and washed again with PBS. Then, the cells were incubated with a DAPI solution (100 ng/mL) for 20 min at room temperature. Samples were examined with a microscope Olympus BX 51 equipped with epifluorescence (Olympus, France).

2.6. MTT assay

The cells were monitored after 1, 3, and 8 days in culture. At each time point, the samples were slightly rinsed with PBS solution to remove nonadhered cells. 1000 µL of 5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, France) were added to the adherent cells and incubated for 3 h at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. Afterwards, the MTT was removed and the cells were immersed in 300 µL of acidic isopropanol under shaking to dissolve intracellular formazan crystals produced by viable cells. Absorbance was determined at 570 nm using an ELISA plate reader. Cell number was obtained using a linear correlation between absorbance and SaOs-2 cell concentration (from 0.5×10^4 up to 2×10^4 cells/mL). The cell number was adjusted to the surface of samples and was expressed in cells/mm^2. The results from three individual experiments (in triplicate) were averaged.

2.7. Alkaline phosphatase

Alkaline phosphatase (ALP) activity was assessed by the hydrolysis of *p*-nitrophenyl phosphate in alkaline buffer solution (substrate). After 8 days in culture, the cells plated on the surfaces were previously permeabilized in 0.5% Triton X-100 (octylphenol ethoxylate) in water and incubated for 30 min with the substrate. Colorimetric determination of the product (*p*-nitrophenol) was carried out at 405 nm (ELISA reader). ALP activity was calculated from a standard curve, and the results were expressed in nanomoles of *p*-nitrophenol produced per cell (nmol L^{-1}/cell).

2.8. Statistical significance

The statistical significance of the obtained data was assessed using one-way ANOVA variance analysis and the Tukey test. Level of significance was set at $p < 0.05$.

3. Results

3.1. Physico-chemical characterization

Fig. 2 shows AFM images of original and polished surfaces. The calculated roughness before polishing was 49 ± 12 and 88 ± 7 nm for HA and SiHA samples, respectively. After polishing, similar roughness ($Ra = 12 \pm 1$ nm) was achieved for both materials.

3.2. Focal adhesion formation

Fig. 3 represents vinculin staining after 3 days of incubation under static and dynamic conditions. Cells seeded onto SiHA tablets presented differences of focal adhesions according to the culture condition. In static conditions, the focal adhesions appeared like short and thick patches whereas in dynamic conditions they were thinner and longer. Moreover, the number of focal adhesions was higher in dynamic than in static conditions. This was confirmed quantitatively after image analysis of focal adhesions.

Figs. 4 and 5 present histograms, in which *y*-axes are equal to the frequency (%) of vinculin-positive focal adhesion length and vinculin-positive focal adhesion aspect ratio, respectively. Using dynamic flow, the distribution of vinculin-positive clusters length followed a normal curve with a mean point around 4.5, while for static condition, the distribution was not normal and the mean point was smaller than for dynamic conditions, Fig. 4. Also, vinculin-positive clusters on SiHA tablets displayed a higher aspect ratio under dynamic culture compared with static culture, Fig. 5.

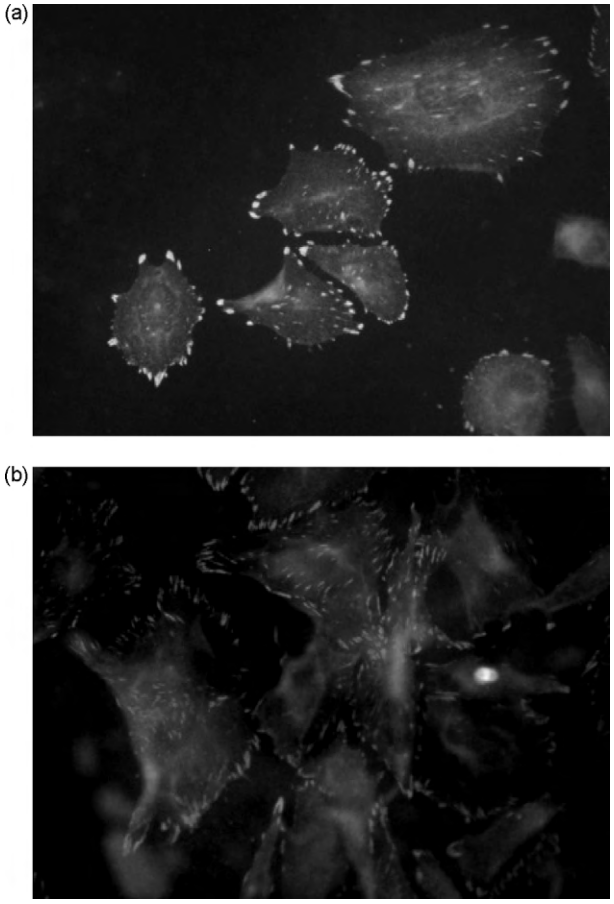

Fig. 3. Vinculin-positive focal adhesions (FA) after 3 days of incubation under (a) static and (b) dynamic conditions on SiHA sample.

3.3. Stress fiber formation

Figs. 6 and 7 show fluorescence images of actin fibers of cells cultured on HA and SiHA during 3 days, respectively, under static and dynamic conditions. A denser actin cytoskeleton was seen for cells cultured under dynamic conditions.

3.4. Cell spreading

After 1 day, a larger size of cells was observed on both surfaces under dynamic condition (Fig. 8). After 3 days, the cells on HA surface under dynamic condition were larger than in static condition while for cells seeded on SiHA, a similar spreading was observed for both culture conditions.

3.5. Cell proliferation and differentiation

Fig. 9 presents the proliferation of SaOs-2 cells seeded onto ceramic surfaces under static and dynamic conditions. The proliferation under both conditions showed that the number of cells seeded for 1, 3 and 8 days was higher for HA than for SiHA. Under dynamic conditions, the number of cells seeded onto both ceramic surfaces was higher when compared with those under static conditions.

In our study, ALP was investigated at 8 days of culture. At this time point, ALP level on HA was lower than on SiHA samples, in both static and dynamic conditions, as shown in Fig. 10. However, for both materials, the dynamic condition increased the ALP activity of cells.

4. Discussion

The goal of this study was to investigate the effect of dynamic culture conditions on human osteoblast-like SaOS-2 cells activity on HA and SiHA dense samples. Silicon substitution has been shown as an essential element for bone formation and development as Si is found in areas of calcification, even, until now, the precise underlying mechanism is uncertain. To investigate basic osteoblast reactions on these surfaces, cytoskeleton organization, focal adhesion formation, cell proliferation and cell differentiation

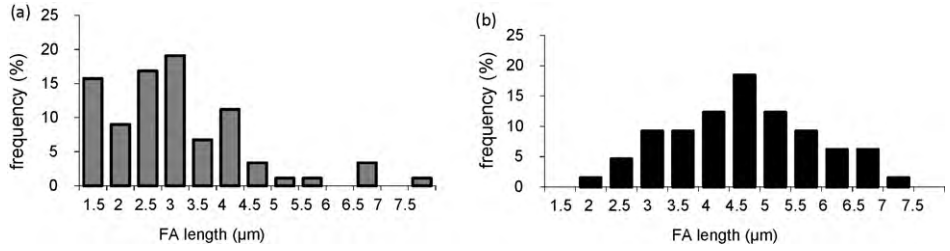

Fig. 4. Distribution of vinculin-positive focal adhesions (FA) length on SiHA under (a) static and (b) dynamic conditions calculated with Image J software.

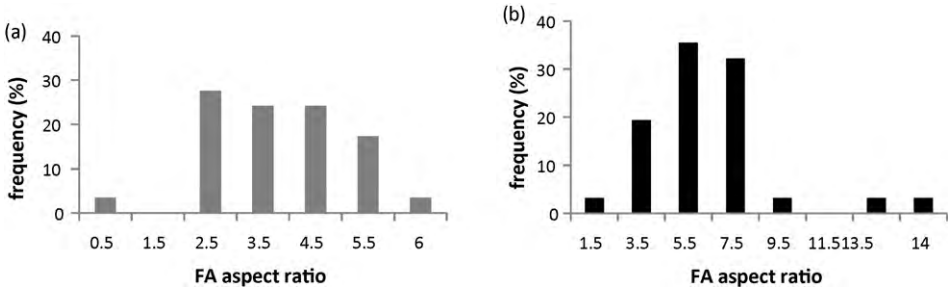

Fig. 5. Distribution of vinculin-positive focal adhesions (FA) aspect ratio on SiHA under (a) static and (b) dynamic conditions calculated with Image J software.

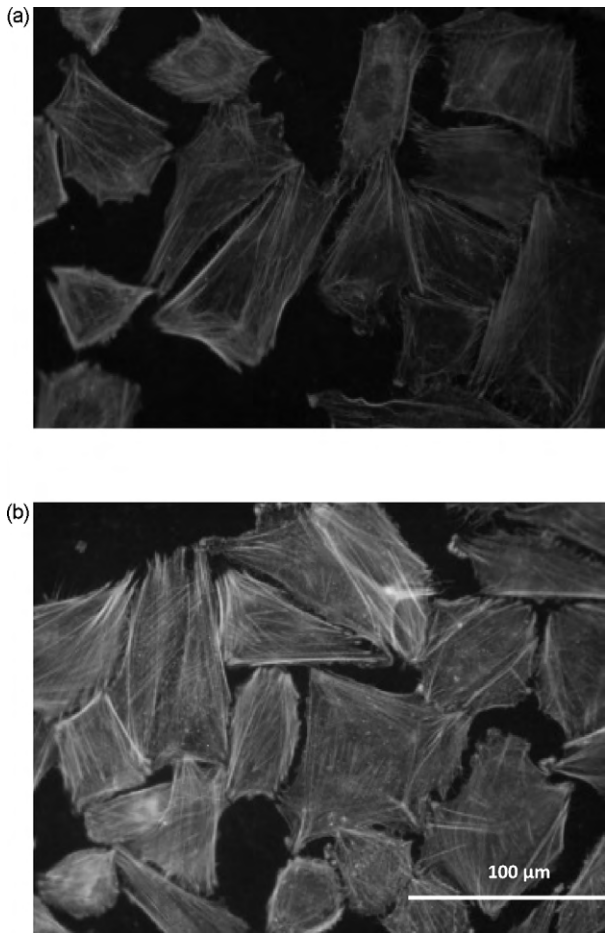

Fig. 6. Phalloidin-labelled actin fibers of cells cultured on HA for 3 days under (a) static and (b) dynamic conditions.

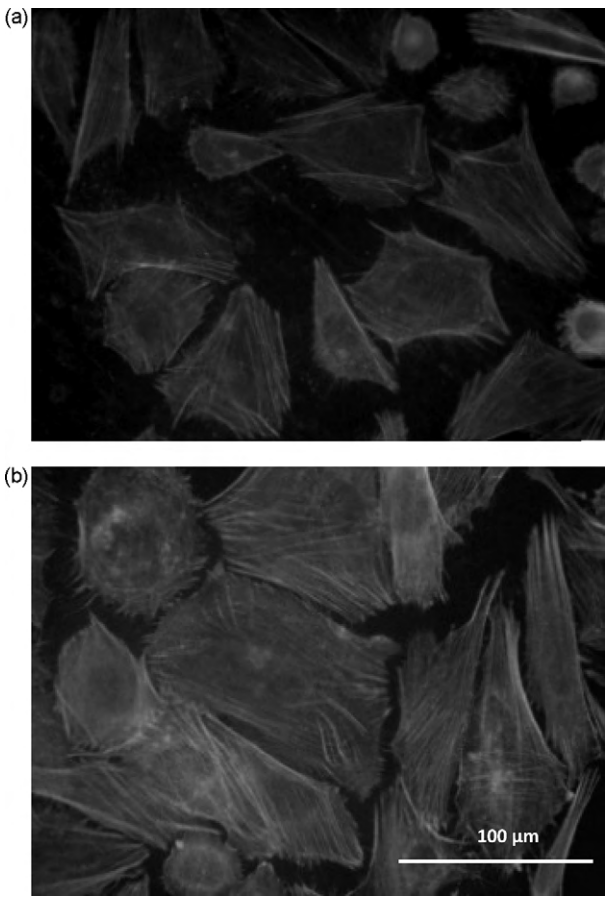

Fig. 7. Phalloidin-labelled actin fibers of cells cultured on SiHA for 3 days under (a) static and (b) dynamic conditions.

were determined. The results obtained in dynamic culture conditions were compared to those obtained in classical static incubation conditions in 24-wells plates.

After sintering, tablets of both ceramics exhibited different grain size, due to Si incorporation in apatite structure. Surface roughness of these surfaces was also different, roughness of HA being higher than the one of SiHA samples ($p > 0.05$). Polishing procedure was used to standardize the morphology and roughness of both ceramics. Consequently, the two HA and SiHA samples used in this study displayed comparable roughness. The differences observed in cell behaviour were due mainly to the different chemical compositions, namely Si introduction in the apatite structure. XRD patterns of HA and SiHA after sintering confirmed the presence of monophasic ceramics with crystalline structure of hydroxyapatite, matching the ICDD standard for HA (PDF 9-432) (data not shown).

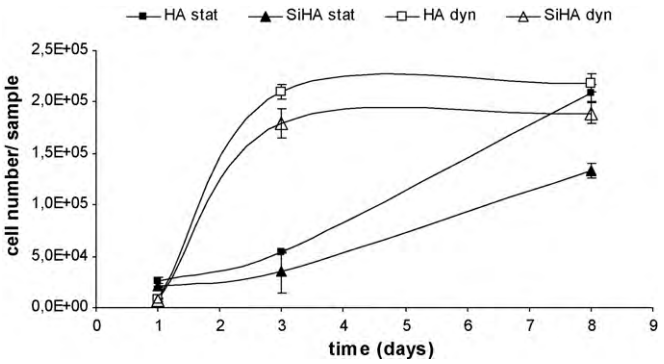

Fig. 9. Proliferation of SaOS-2 cells cultured on HA and SiHA after 1, 3 and 8 days of cell culture under static and dynamic conditions.

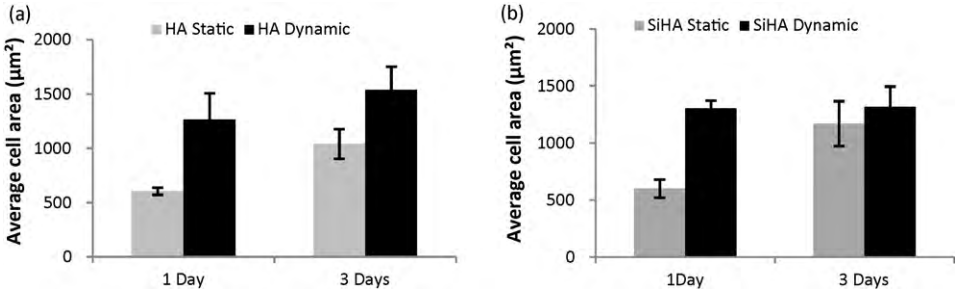

Fig. 8. Average cell area on (a) HA and (b) SiHA surfaces for 1 and 3 days in culture.

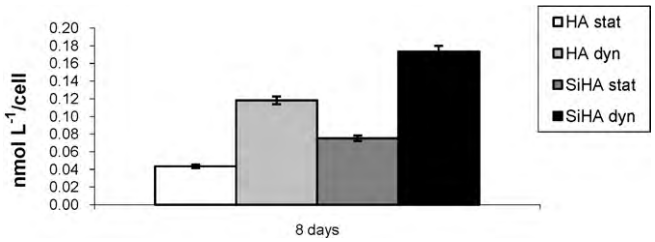

Fig. 10. ALP activity of SaOS-2 cells after 8 days of culture under static and dynamic conditions.

The substitution with Si did not affect the phase composition, as no secondary phases, such as tricalcium phosphate or calcium oxide, were formed. The FTIR and XPS analysis confirmed the presence of Si in the apatite structure and the absence of undesired impurities [13].

The bone, like all organs, is continuously perfused by interstitial fluid because of both vascular pressure and mechanical loading. When an implant, like HA, will replace a damaged bone zone, it will be in contact with the complex composition of bone interstitial liquid that circulates at 2 mL/h [17]. That is why, the currently used *in vitro* static culture conditions, are not the optimum for studying the bioactivity of a materials in contact with the cells. The metabolism of cells is coupled to the secretion of acidic waste products, like lactate and CO_2, and to the consummation of oxygen. These wastes products could influence the behaviour of cells and wrong conclusions could be drawn concerning the biocompatibility of materials.

The bioreactor that we proposed to use in this study perfused constantly fresh medium. So, the osteoblasts on HA and SiHA samples received constant nutrition and the metabolic waste products were removed swiftly.

During cell spreading, specific adhesion structures between cell and substratum are formed and the cellular skeleton is reorganized to change and maintain the shape of cells. Cell membrane receptors, like integrins, and many kinds of cytoskeleton proteins (vinculin, talin, paxillin, etc.) are involved in this process. The vinculin and other cytoskeleton proteins locate at the cytoplasmic face of focal adhesions and are involved in linking actin filaments to the membrane at these sites.

Studies developed by Tsuruta and Jones indicate that the size of focal adhesion is related to shear stress applied on cells. Under shear stress, size of focal adhesions increases leading to stronger cell-surface linkages [18]. Seemingly, our results indicate that under dynamic condition, focal adhesion clusters of cells seeded on SiHA were more elongated in dynamic than in static condition, due to the shear stress applied by medium.

The cells on both ceramics seemed to be larger for 1 day under dynamic culture than for static, which suggests that the continuous nutrition of cells speed their spreading during the first 24 h of culture. However, the morphology of cells seeded onto SiHA for 3 days were found similar for both culture conditions. In fact, it appears that cell spreading on SiHA was achieved in 24 h under dynamic condition whereas for static condition, cells needed 3 days to complete their spreading. Comparing both culture conditions, the actin fibres network appeared more dense under dynamic than static condition. This is coherent with the more elongated morphology of focal adhesions in dynamic than static conditions.

As MTT assays showed, both ceramics are not toxic. After 3 days in culture, it was noted a significant increase of cell number in dynamic condition. This is probably due to the constant renewal of the medium and higher mass transferring, which favours the cell proliferation as it was observed also previously by other authors [19]. However, after 8 days under dynamic condition, there was no increase in cell number surely because cells have reached confluence. For the same time in culture, under static condition, it was observed more cells onto HA surface that on SiHA. This could be related to the higher phosphate precipitation process on SiHA than on HA observed previously [13].

Alkaline phosphatase (ALP) is an ectoenzyme, produced by active osteoblasts involved in the early initiation of mineralization of newly formed bone tissue. Therefore, ALP is a useful marker for osteoblast activity [20]. Our results showed a high activity of this enzyme under dynamic conditions, surely because of the constant renewal of media. Moreover, actin fibres organization has a direct influence on cellular differentiation [21]. Stressed fibres change their biophysical properties generating several types of signalling molecules [8]. Therefore, the higher cell differentiation observed on ceramic surface could be related to the modification of actin fibres morphology exhibited by cells cultured under dynamic conditions.

Under both static and dynamic conditions, ALP activity strengthened rapidly for cells seeded onto SiHA surface, compared with those seeded onto HA. The reason for this could be the presence of Si in hydroxyapatite structure. Indeed, silicon is essential for normal bone growth and development, because collagen and proteoglycans are cross-linked by this element. Moreover, silicon has also been shown to act directly in the bone mineralization process [22].

5. Conclusions

This study demonstrates the interest of dynamic culture conditions as an alternative to standard methods for studying the response of cells to biomaterials. Effectively, the culture under dynamic conditions exacerbates the differences in cell behaviour in function of surface chemistry observed in static culture. For instance, under dynamic conditions, cells cultured on SiHA surface showed a faster adhesion process and the formation of longer and thinner focal adhesions than in static conditions.

Finally, it is likely that the results given by an *in vitro* study in dynamic conditions are more representative of what occurs *in vivo* than culture in static conditions. However, studies comparing results obtained *in vitro* in static and dynamic conditions with *in vivo* tests are still needed to clearly demonstrate that.

Acknowledgements

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