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Surface transformation of silicon-doped hydroxyapatite immersed in culture medium under dynamic and static conditions

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ABSTRACT

A comparative study of *in vitro* bioactivity of hydroxyapatite (HA) and silicon-doped hydroxyapatite (SiHA) has been carried out by immersion in a cell culture medium with or without fetal bovine serum during 14 days in static and dynamic conditions. A specific bioreactor was developed for the experiments in dynamic conditions. Ceramic surface transformations were characterized by electron microscopy, atomic force microscopy and X-ray photoelectron spectroscopy before and after immersion. The monitoring of calcium, phosphate and proteins in immersion medium was also done during the experiment. The two hydroxyapatite surfaces immersed in cell culture medium under dynamic conditions were found to be more probably covered by a new Mg-enriched Ca-deficient apatite layer than surfaces immersed under static conditions. These results suggest that dynamic procedure and medium with serum macromolecules seem to be more adequate to predict the in vivo activity of bioceramics. Moreover, SiHA presented a higher capacity of protein adsorption.

1. Introduction

Bioceramics based on calcium phosphate have received important attention as bone substitutes due to their chemical similarity to natural bone. One of the most common materials used as bone graft is hydroxyapatite (HA), due to its biocompatibility properties [1,2]. One way to speed up the rate of osseointegration of HA is to adjust its chemical composition to more closely approximate that of the bone mineral. Then some ions, which are found in bone mineral, have been substituted into the HA structure, such as carbonate, magnesium, fluorine, sodium and silicon. Silicon was identified as a possible factor enhancing bone calcification [3]. Similarly, the role of silicon substituting part of the phosphorus atoms present in the hydroxyapatite lattice seems to be an important factor influencing the bioactive behaviour of the material [4]. Create a silicon substituted hydroxyapatite (SiHA) as bioactive implant is an attractive and original idea for enhancing bone tissue growth rate because the *in vivo*, [5–8] and *in vitro* [9,10] studies report this substitution to be favourable. Many synthetic routes have been developed for obtaining SiHA coating [11], particles [7] or disks [12–16]. Moreover, solutions with high Si concentration induce osteoblast proliferation, cellular vacuole formation, and the expression of mRNA in osteoblast-like cells [17,18].

To date, the bioactivity of CaP materials for orthopaedic implants has been studied *in vitro*, especially in Kokubo's simulated body fluid (SBF) solutions. But it is well known that the bone tissue environment is more complex especially because of the presence of proteins and other macromolecules coming from blood capillaries and surrounding tissue. Some studies have concerned the influence of proteins on the process of bio-mineralisation of materials surface, but they have been performed in general by adding only one protein to SBF (albumin, collagen, fibronectin, sialoprotein, phosphorin, etc) [19–23], glycosaminoglycans [24,25] or glucose [26]. The concentrations of proteins introduced in SBF vary also from an author to another which makes difficult the interpretation of results. Generally, the addition in SBF of one of these macromolecules (proteins, glucose or glycosaminoglycans) was found to inhibit growth crystal nucleation on solid substrate [27].

One of the key challenges in bone tissue engineering is the development of new biomaterials and new culture methods to provide living constructs associating cells and scaffolds forming hybrid materials that possess the ability to integrate the surrounding tissue. Bone is a porous tissue that is continuously perfused by the interstitial fluid. Fluid flow, driven by both vascular pressure and mechanical loading, may generate significant shear stresses through the canaliculi as well as along the bone lining at the

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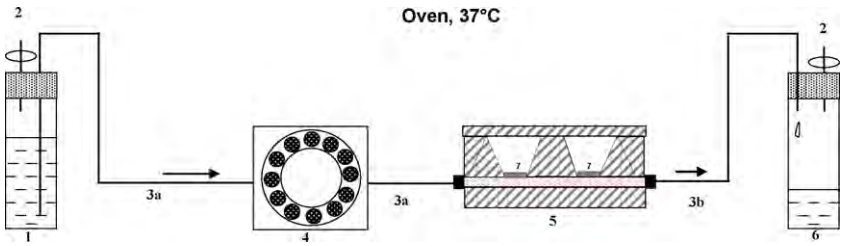

Fig. 1. Schematic diagram of the bioreactor system placed in the constant temperature oven (37 °C). A silicone tube (3) makes the connection between fresh medium bottle (1), which supports an air filter (2), bioreactor (5) and waste medium bottle (6). A multi-channel peristaltic pump (4) placed just before the bioreactor is adjusted to a constant flow fluid at 2 mL/h. The medium is oxygenated by diffusion via the silicone walls of the connecting tubes. Two samples (7) were used for each experiment and the analysing face (below side) was immersed permanently with the medium.

endosteal surface. So, when an implant will be in contact with bone it will be submitted to a bone interstitial liquid flow order of 2 mL/h [26].

For developing hybrid materials for bone engineering, the culture method is of great importance. Static cultures (flasks, Petri dishes, multi well plates), suffer from accumulated waste products, limited diffusion and often result in inhomogeneous cells and extracellular matrix distribution. In order to overcome the drawbacks associated with static systems but also to be close to *in vivo* dynamic conditions, several different bioreactors have been investigated for tissue-engineering applications: spinner flask, rotating wall vessel reactor, pulsatile flow bioreactor, eggshell bioreactor etc. [28]. Among these bioreactors, the flow perfusion cultures presents particular interest for bone substitutes application because the shear stress induced by fluid dynamic across the scaffold is believed to be the most important mechanical stimulus in activating the mechanotransduction signalling of osteoblasts. [29].

In order to make clearer the role of proteins and the role of immersion conditions (static/dynamic) on ceramic surface transformation, we propose a new biomimetic approach based on the use of a bioreactor for dynamic immersion of ceramics under constant physiologic flow. To be also more close to the in vivo fluid composition we propose 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 will be validated on surface transformation of Si-substituted hydroxyapatite ceramics compared to pure hydroxyapatite controls.

2. Materials and methods

2.1. Production of hydroxyapatite tablets

Stoichiometric hydroxyapatite (HA – $Ca_{10}(PO_4)_6(OH)_2$) powder was obtained by the wet precipitation [30] 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 [13]. These two powders were used to produce HA and SiHA samples, respectively. The powders were calcined at 650 °C and then uniaxial 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 to 4000 meshes). This procedure was relevant to approximate the morphology and roughness of both conditions (HA and SiHA).

2.2. Immersion in static condition

In order to study the surface transformation of materials in static condition, the samples were put in classical 24-wells plates for cell culture and incubated into a sterile CO_2 cell culture incubator (Hera Cell, France) at 37 °C. The samples were immersed in McCoy's 5A non-complete medium (Sigma, France) or complete medium (added with 10% foetal bovine serum) (VWR, France) for 1, 3, 8 and 14 days.

2.3. Immersion in dynamic condition

For dynamic conditions, it was used a home-made bioreactor (Fig. 1), incubated into a closed oven at 37 °C in atmospheric air. The samples were immersed in McCoy's 5A non-complete medium (Sigma, France) or complete medium (added with 10% foetal bovine serum) (VWR, France) for 1, 3, 8 and 14 days.

A peristaltic pump (Type IPC-N8 from ISMATEC) assured a constant flow of 2 mL/h in all system. The pH of solutions was verified to be constant (7.4) every day. The system was commercialised by Minucells™ (Germany) except the bioreactor. The whole system was sterilized before each experiment at 105 °C and 1 Bar pressure in an autoclave. The whole system was assembled under a sterile laminar flow and the first bottle was filled with fresh, sterile complete or non complete McCoy's medium.

2.4. Surface characterisation

2.4.1. Topographical aspects

The initial surface morphology of HA and SiHA tablets was examined by scanning electron microscopy (SEM) in a JEOL JSM 6460LV microscope to investigate if some undesired entities (cracks, grooves) were present on the surface. The digital images were processed by using Global Lab Image software.

Atomic force microscopy (AFM) was used to characterize surface roughness by measuring the average roughness (R_a). Images were taken from five different regions using the tapping mode in an AFM Multimode Nanoscope IV.

2.4.2. Crystalline phases identification

The phases presented after sintering were investigated using X-ray diffraction (XRD). The patterns were performed from $2\theta = 10°$ to 100° (step size of 0.05° and scanning speed of 1° per minute) in a RIGAKU MINIFLEX powder diffractometer.

2.4.3. Surface chemical composition

The surfaces were analyzed by X-ray photoelectron spectroscopy (XPS). Analysis was carried out using a Gammadata Scienta SES 2002 X-ray photoelectron spectrometer under ultra high vacuum ($P < 10^{-9}$ mbar). The monochromated Al Kα source (1486.6 eV) was operated at 420W (30 mA, 14 kV), with a nominal take-off angle of 90° (i.e. photoelectrons ejection normal to the surface). The samples were outgassed into ultra high vacuum chamber with isolated pumping system until transfer to the analysis chamber. No further cleaning process was made to avoid carbon contamination. During acquisition, the pass energy was set

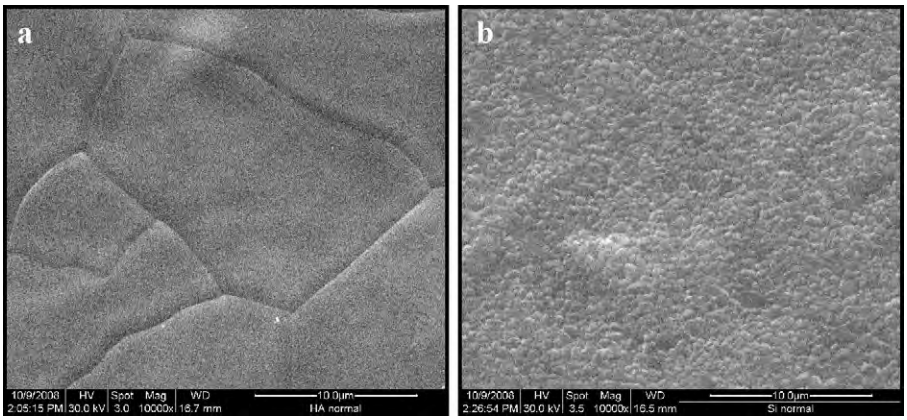

Fig. 2. SEM micrographs of (a) HA and (b) SiHA before immersion.

to 500 eV for survey spectrum with a step of 500 meV. The overall resolution of the spectrometer can be estimated to 0.4 eV.

For quantification purpose, raw area of each photoelectron peaks was determined on survey spectrum using Shirley background and 30% Gaussian–Lorentzian shape with CasaXPS software (Casa Software Ltd., Teignmouth, UK – www.casaxps.com). Raw areas were further modified using classical sensitivity factors and transmission factor of the spectrometer leading to a chemical composition expressed in atomic percentage in the article.

2.5. Calcium and phosphorus content in medium

The concentration of calcium and phosphorus in the immersion medium after contact with the HA and SiHA tablets was evaluated at the end of each immersion time (1, 3, 8, 14 days) by colorimetric methods using a Calcium AS FS kit and Phosphorus UV FS kit purchased by Diasys Diagnostic Systems (France). Additionally this analysis was performed at early immersion times: 30 min, 1, 4, and 18 h.

2.6. Protein concentration in medium

The concentration of total proteins in the immersion medium after contact with the HA and SiHA tablets was evaluated at the end of each immersion time (1, 3, 8, 14 days) by the Micro BCA™ kit using the supplier instructions (Pierce, USA). Protein concentration was obtained by comparison with BSA standards. Additionally this analysis was performed at early immersion times: 30 min, 1, 4, and 18 h.

2.7. Statistical significance

The statistical significance of the obtained data was assessed using one-way ANOVA variance analysis and the Turkey Test. Differences at $P \geq 0.05$ were considered statistically not significant.

3. Results and discussion

3.1. Initial sample characterization

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).

After sintering at 1200 °C, HA and SiHA tablets exhibited different grain size, due to Si incorporation on apatite structure (Fig. 2). However, as the surfaces were polished, no significant roughness difference between them was observed, with R_a equal to 13 ± 1 nm and 11 ± 1 nm for HA and SiHA, respectively.

3.2. Calcium and phosphate concentrations in immersion medium

The time-dependent variation of calcium and phosphate concentration in the complete and non-complete media after

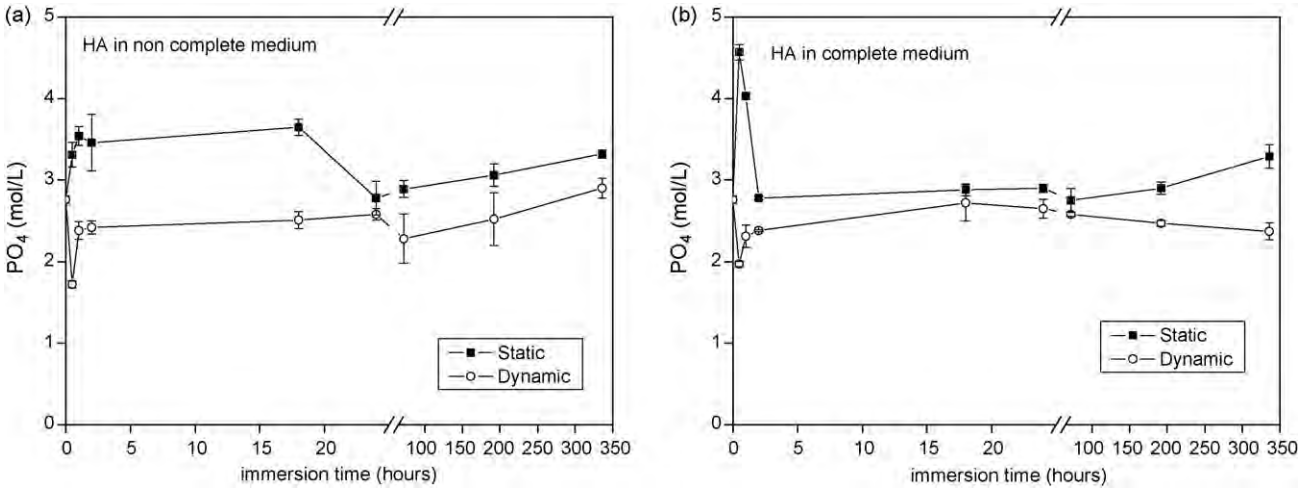

Fig. 3. Variation of concentration of phosphate for HA in static and dynamic conditions in: (a) non-complete and (b) complete culture medium.

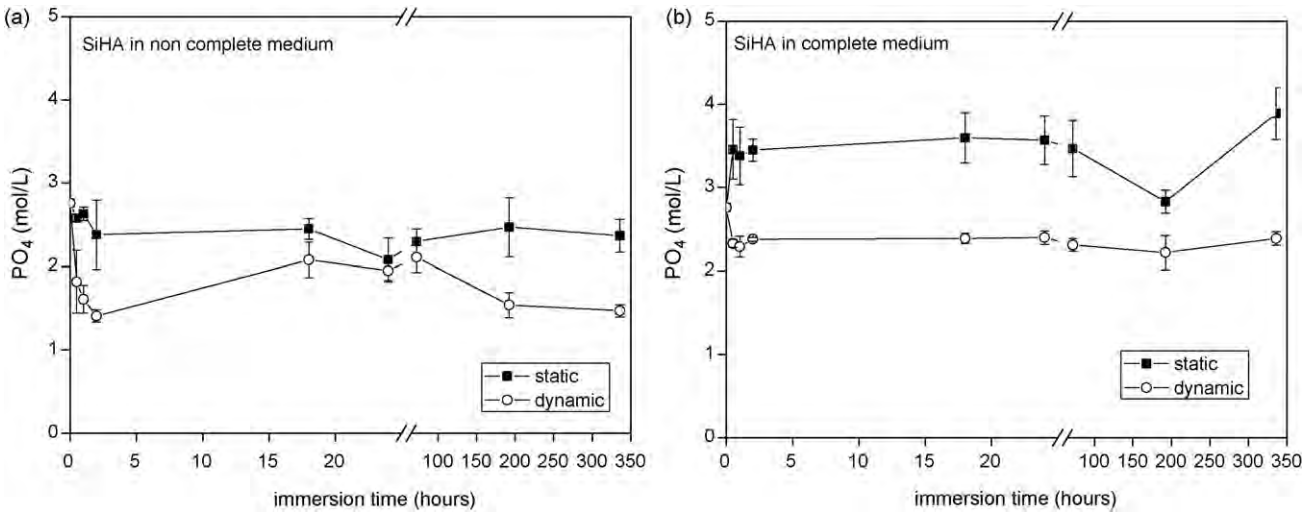

Fig. 4. Variation of concentration of phosphate for SiHA in static and dynamic conditions in: (a) non-complete and (b) complete culture medium.

immersion in dynamic and static conditions of HA and SiHA samples was determined. No difference in calcium concentration in non complete and complete media was noticed for HA and SiHA immersed until 14 days in static and dynamic conditions (data not shown).

By the other side, the concentration of phosphate was significantly different comparing static and dynamic conditions, and even more for SiHA compared with HA. For HA, the concentration in phosphate was systematically lower in dynamic conditions (compared with static conditions) (Fig. 3).

In non complete medium (Fig. 3a) the phosphate concentration increased significantly during the first 18 h but significantly decreased in dynamic conditions. This increase in phosphorus concentration demonstrates rather a fast initial dissolution of HA surfaces. On the contrary, except the strong decrease after 30 min, the phosphate concentration after dynamic immersion of HA remained relatively stable. After 24 h, the phosphate contents were comparable in static and dynamic condition. Later, the phosphate concentration increased slightly with time demonstrating a regular late dissolution of HA.

In complete culture medium (Fig. 3b) there was no marked variation with immersion time in dynamic conditions but like previously, a strong increase in phosphate concentration was observed during the first 30 min in static conditions whereas a decrease was observed in dynamic conditions. After 2 h in static condition, the phosphate concentration had reached again the initial concentration and stayed constant until 72 h but an increase in phosphate concentration was finally observed after 14 days. Again, this can be explained by a dissolution process divided in two phases, a fast initial dissolution phase occurring in the first 2 h and a second slower phase starting after 3 days of immersion.

Finally it appears that static immersion favours a dissolution process of HA whereas the dynamic condition initially induces a precipitation of phosphate but later a slight dissolution.

For SiHA (Fig. 4), similarly, the concentration in phosphate was systematically lower in dynamic conditions than in static ones. In non complete medium, the phosphate concentration decreased significantly after 30 min in dynamic conditions but increased again after and reached the values of static condition after 24 h (Fig. 4a). The phosphate concentration decreased again after 72 h demonstrating a higher precipitation volume than in static conditions.

In complete culture medium (Fig. 4b) there was no strong variation with immersion time in static and dynamic conditions but like previously, an increase in phosphate concentration was observed after 30 min in static conditions whereas a slight decrease was observed in dynamic conditions. Again an increase in phosphate concentration was observed after 14 days of immersion in static condition.

Finally, contrary to HA, SiHA showed a slight dissolution in static condition in complete culture medium but not in non complete medium. As previously observed with HA but in a larger extent, SiHA induced the precipitation of phosphate in dynamic condition notably in complete culture medium.

These in vitro results indicated an approximately constant calcium content but mostly a decrease in phosphate concentration in culture medium. Therefore we can expect the precipitation on samples of an apatite layer with a Ca/P ratio lower than 1.67, in accordance with a Ca-deficient apatite, called bone-like apatite.

Furthermore, during these immersions it is believed that there is a process of precipitation and dissolution. The dissolution process of SiHA surfaces produces a Ca- and Si-rich environment, which can stimulate cell proliferation and subsequent differentiation, suggesting better tissue regeneration, and may be suitable for orthopaedic and dental applications.

3.3. Protein concentration

Fig. 5 shows the variation of concentration in proteins for both samples, under static and dynamic conditions. All results referred to the complete medium with fetal bovine serum and the initial protein concentration was equal to 5.96 mg/mL for all experiments.

After 30 min of immersion of HA in dynamic conditions, a decrease of 2 mg in protein concentration in the immersion

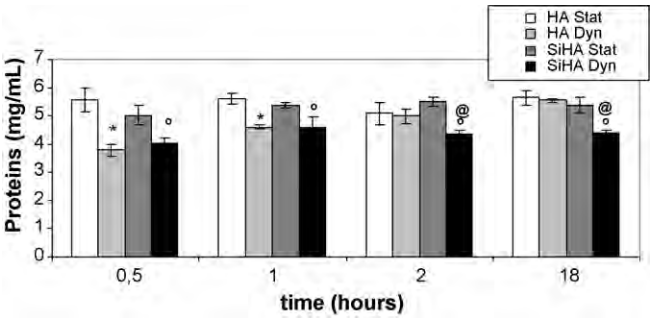

Fig. 5. Variation of concentration of the proteins for (a) HA and (b) SiHA under static and dynamic conditions in complete culture medium. (*) and (°), denote significant difference between static and dynamic conditions for HA and SiHA, respectively, and @ for significant difference between HA and SiHA under dynamic condition, $p < 0.05$.

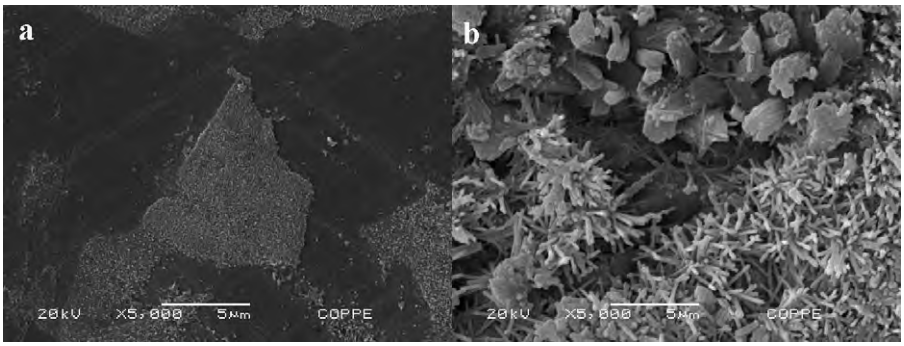

Fig. 6. SEM micrographs of HA immersed for 3 days in non complete medium under (a) dynamic and (b) static conditions.

medium was observed suggesting a faster protein adsorption on HA samples. After 2 h no significant differences was observed any more between static and dynamic conditions. On the contrary, for SiHA, the difference between static and dynamic conditions persisted after 30 min, with a lower value for dynamic conditions. This indicated that the adsorption of proteins on SiHA was higher in dynamic condition compared to static condition. Comparing both ceramics it is possible to note a higher protein adsorption for SiHA under dynamic condition after 2 and 18 h of immersion.

3.4. Surface microscopic analysis after immersion

After immersion in non complete medium, the ceramic surfaces presented different aspects depending on immersion time and condition. For instance, for 3 days of immersion, the SEM images presented in Figs. 6 and 7 show that both ceramics present areas covered with precipitates and dissolution aspects depending on the surface and the culture condition. Under dynamic condition, HA surface immersed for 3 days (Fig. 6a) presents different and less precipitates in comparison to static condition (Fig. 6b); this could be explained by the precipitation/dissolution process that is accelerated for dynamic condition, due to the constant renewed fluid. Under dynamic condition (Fig. 7a), SiHA surface presents different kinds of precipitations, whereas, under static condition (Fig. 7b), it is noted a dissolution aspect. The reason for the different precipitations found on SiHA surface under dynamic condition is still unclear, but we believe that it is probably linked to silicon present on surface and the constant flow of the non complete medium.

On the contrary, the surface morphology of the ceramics in contact with complete medium does not show any differences in function of immersion condition (pictures not shown).

The AFM observations (data not shown) confirmed the presence of much less precipitation onto surfaces incubated in complete medium comparing with non complete medium. At scale of 1 μm and 300 nm it was observed that the constituents of serum "dress" the ceramic grains with a smooth layer. Therefore it was assumed that the precipitation of calcium-deficient apatite *in vitro* was slowed by organic constituents. The cause of this delay could be the following: (i) proteins adsorb on the samples surface and occupy the sites where the precipitation occurs and (ii) calcium and/or phosphorous ions are masked by the macromolecules of serum such as proteins, enzymes, glucose creatinine, urea, etc. Many authors [23,27,31] reported that the serum components adsorbed at the surface of hydroxyapatite delay the precipitation of the superficial layer, indicating the important role of organic constituents in *in vivo* ceramic transformation. This hypothesis is confirmed by the low variation of phosphate concentration we observed after immersion in complete culture medium in static and dynamic conditions.

The surfaces incubated with non complete medium present much more precipitates, especially for SiHA samples. This can be attributed to an increase of hydroxyl function on the ceramic surface due to the presence of silicate groups [32].

The roughness amplitudes (R_a) after exposure of HA and SiHA samples during 3 days in complete and non-complete medium are shown in Fig. 8. Samples immersed in complete medium in static and dynamic conditions exhibited lower R_a values compared to samples immersed in non complete culture medium. The presence of a layer of adsorbed proteins could be at the origin of this smoothing effect. We can also note that the R_a increased after immersion in non complete medium compared to control in the two culture conditions whereas, for complete medium, this was observed only in dynamic condition, according to Turkey test ($p < 0.05$). This could be related either to a dissolution effect that could remove amorphous phases and let apparent well crystallized grains or to a precipitation effect that could cover the surface with spherules. As seen previously, these two hypotheses are valuable depending on the culture conditions and composition of the ceramic.

In the static assay the precipitates are absent or, if they are present, they are larger measuring about 1 μm in diameter. These morphological observations are in accordance with the results of

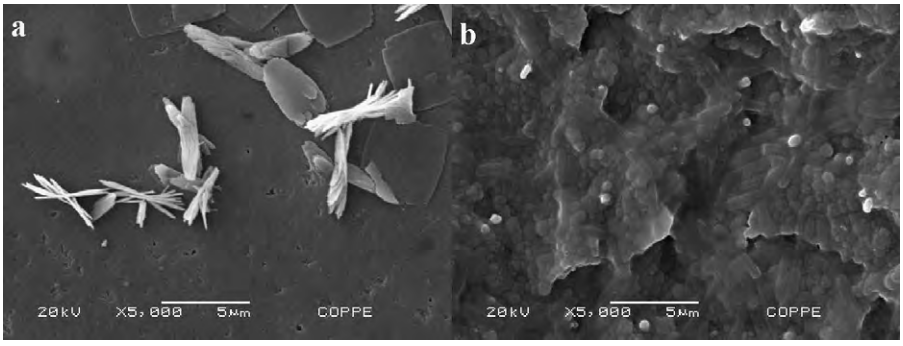

Fig. 7. SEM micrographs SiHA immersed for 3 days in non complete medium under (a) dynamic and (b) static conditions.

Table 1
Variation in magnesium (at%) evaluated by XPS and EDS in function of experimental conditions after 3 days of incubation of samples in complete and non complete medium.

Sample	Non complete medium		Complete medium	
	XPS Mg (at%)	EDS Mg (at%)	XPS Mg (at%)	EDS Mg (at%)
HA control	0.63	0	0.63	0
HA static condition	0.43	0	0	0
HA dynamic condition	4.02	0.21 ± 0.12	2.4	0.07 ± 0.02
SiHA control	0.60	0	0.60	0
SiHA static condition	0.79	0	0	0
SiHA dynamic condition	2.51	0.22 ± 0.19	1.9	0.14 ± 0.01

Table 2
Summarized results for HA and SiHA in static and dynamic conditions, NCM: non complete culture medium, CM: complete culture medium and NA: not applicable.

			Ca	P	proteins	SEM	AFM	Ra	Mg	Conclusion
HA	Static	NCM	=	+	NA	Dissolution aspects	Dissolution aspects	+	=	Dissolution
		CM	=	+	=	No modification	No modification	=	=	No modification or homogenous protein layer
	Dynamic	NCM	=	+/−	NA	Precipitation aspects	Precipitation aspects	+	+	Precipitation of Ca-deficient HA + Mg
		CM	=	−	−	No modification	No modification	+	+	Precipitation of Ca-deficient HA + Mg + proteins
SiHA	Static	NCM	=	=	NA	Dissolution aspects	Dissolution aspects	+	=	Dissolution
		CM	=	+	=	No modification	No modification	=	=	No modification or homogenous protein layer
	Dynamic	NCM	=	−	NA	Precipitation aspects	Precipitation aspects	+	+	Precipitation of Ca-deficient HA + Mg
		CM	=	−	−	No modification	No modification	+	+	Precipitation of Ca-deficient HA + Mg + proteins

=, +, −, +/−: change in concentration in immersion medium compared to initial concentration or change in R_a or magnesium content of surfaces compared to initial state.

Ramila and Vallet-Regi [33], which showed the same surface transformation in static and dynamic assay for sol–gel glass samples in presence of SBF. For static assay they found also large spherical particles of about 1 μm diameter while for dynamic conditions the glass was fully covered by a layer in which spherical particles were present.

3.5. Surface chemical analysis after immersion

The surface chemical composition was determined by XPS analysis of HA and SiHA samples after 3 days of immersion and results are summarized in Table 1. Only modification in magnesium content was observed and only in dynamic conditions (Table 1). The increase in magnesium content was true for the two ceramics and after immersion in dynamic condition in the two media. This increase is seen soon after 1 day in complete medium and 3 days in non complete medium. After these delays, the magnesium content is maintained until 8 days. The source of magnesium in our experiments would be the inorganic $MgSO_4$ salt present in McCoy's culture medium. The serum components (proteins, enzymes, lipids, etc.), could also contain a small quantity of magnesium.

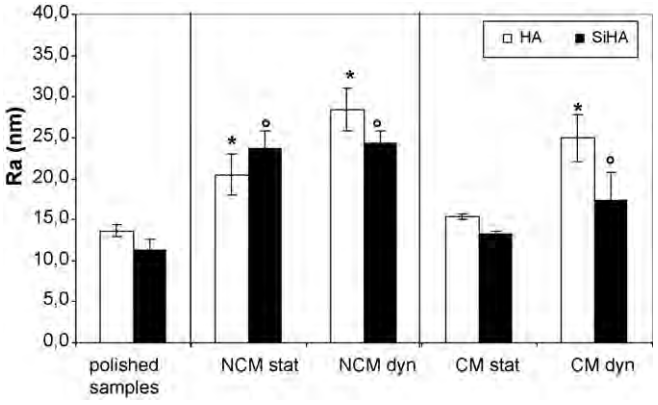

Fig. 8. Surface roughness measured by AFM after 3 days of incubation in non complete (NCM) and complete medium (CM) under static and dynamic conditions (image area 1600 μm^2). (*) and (°), denote significant difference from the polished samples, HA and SiHA, respectively, $p < 0.05$.

The EDS data confirmed the change in magnesium content on samples surface and were also included in Table 1. Again an increase in magnesium content was observed only in dynamic conditions for the two ceramics and in the two media. We can note here that it was possible to detect this variation in magnesium content by EDS although EDS analyses on a larger depth (1 μm) than XPS (10 nm). This confirms that this magnesium surface precipitation is highly significant.

The reason of this precipitation of magnesium on surfaces in dynamic conditions is unclear but we could hypothesize that the continuous flow of culture medium on samples during 14 days is needed to overpass a critical threshold in magnesium concentration on the surface inducing its precipitation.

The XPS analysis detects magnesium but not sulphur that normally is linked to magnesium in non complete medium, in the form of $MgSO_4$ salt. Thus, the magnesium precipitations could be present under carbonates and phosphates forms if they would have precipitated with the PO_4^{3-} and CO_3^{2-} salts of the medium. Okazaki [34] reported that the presence of magnesium caused decrease of the cristallinity of hydroxyapatite accompanied with a great reactivity. It is known that biological apatites are poorly crystalline and contain cationic and anionic substitutions. Thus our results show that apatite obtained in dynamic condition, could be more bioreactive, thanks to magnesium presence, compared with supports immersed in static conditions.

Finally all our results are summarized on Table 2. From this, it appears few differences between HA and SiHA behavior after immersion. For these two ceramics the immersion in non complete culture medium in static conditions induced a dissolution of surfaces whereas in complete culture medium the surface was less modified or covered by a homogenous protein layer. On the contrary, the immersion in dynamic conditions induced systematically a precipitation of a Mg-enriched Ca-deficient HA layer that could increase the bioreactivity of ceramics.

4. Conclusions

This *in vitro* study showed that hydroxyapatite surfaces immersed in cell culture medium under dynamic conditions are found to be more probably covered by a new Mg-enriched Ca-deficient apatite layer than surfaces immersed under static con-

ditions. Moreover, the Si-substituted hydroxyapatite presented a higher capacity of protein adsorption, which suggests good properties concerning bioactivity. Further *in vitro* studies with cells are now under investigation to explore under dynamic condition the applicability of these ceramics for hybrid materials preparation and bone tissue engineering.

Acknowledgements

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