

# Substrate geometry directs the in vitro mineralization of calcium phosphate ceramics

Citation for published version (APA):

Bianchi, M., Edreira, E. R. U., Wolke, J. G. C., Birgani, Z. T., Habibovic, P., Jansen, J. A., Tampieri, A., Marcacci, M., Leeuwenburgh, S. C. G., & van den Beucken, J. J. J. P. (2014). Substrate geometry directs the in vitro mineralization of calcium phosphate ceramics. *Acta Biomaterialia*, 10(2), 661-669. <https://doi.org/10.1016/j.actbio.2013.10.026>

## Document status and date:

Published: 01/02/2014

## DOI:

[10.1016/j.actbio.2013.10.026](https://doi.org/10.1016/j.actbio.2013.10.026)

## Document Version:

Publisher's PDF, also known as Version of record

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## Substrate geometry directs the in vitro mineralization of calcium phosphate ceramics



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### ARTICLE INFO

#### Article history:

Received 4 July 2013

Received in revised form 20 September 2013

Accepted 23 October 2013

Available online 31 October 2013

#### Keywords:

Calcium phosphates

Hydroxyapatite

$\beta$ -Tricalcium phosphate, Concavities

Mineralization

### ABSTRACT

Repetitive concavities on the surface of bone implants have recently been demonstrated to foster bone formation when implanted at ectopic locations in vivo. The current study aimed to evaluate the effect of surface concavities on the surface mineralization of hydroxyapatite (HA) and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) ceramics in vitro. Hemispherical concavities with different diameters were prepared at the surface of HA and  $\beta$ -TCP sintered disks: 1.8 mm (large concavity), 0.8 mm (medium concavity) and 0.4 mm (small concavity). HA and  $\beta$ -TCP disks were sintered at 1100 or 1200 °C and soaked in simulated body fluid for 28 days at 37 °C; the mineralization process was followed by scanning electron microscopy, energy-dispersive spectroscopy, X-ray diffraction and calcium quantification analyses. The results showed that massive mineralization occurred exclusively at the surface of HA disks treated at 1200 °C and that nucleation of large aggregates of calcium phosphate started specifically inside small concavities instead of on the planar surface of the disks. Regarding the effect of concavity diameter size on surface mineralization, it was observed that small concavities induce 124- and 10-fold increased mineralization compared to concavities of large or medium size, respectively. The results of this study demonstrated that (i) in vitro surface mineralization of calcium phosphate ceramics with surface concavities starts preferentially within the concavities and not on the planar surface, and (ii) concavity size is an effective parameter to control the spatial position and extent of mineralization in vitro.

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### 1. Introduction

Commonly employed biomaterials in bone tissue engineering and regenerative medicine include bioactive ceramics and glasses, natural and synthetic polymers and composites thereof [1]. In particular, calcium orthophosphates (CaPs) are the most widely investigated materials for the substitution of lost or damaged hard tissue due to their similarity to the inorganic phase of bone, as well as having excellent biocompatibility and bioactivity [2–4]. Notably, several CaPs recently were demonstrated to exhibit osteoinductive capacity, i.e. the ability to induce bone tissue formation at an ectopic location, which in turn requires the differentiation of osteoprogenitor cells into bone-forming osteoblasts [5–7].

CaPs are available in a number of chemical compositions and crystalline phases, among which hydroxyapatite (HA),  $\beta$ -tricalcium

phosphate ( $\beta$ -TCP) and biphasic HA/TCP ceramics are the most common for bone replacement. The bioactivity of these CaPs is strongly related to their solubility in the physiological environment, which increases with increasing amount of  $\beta$ -TCP (i.e.  $\beta$ -TCP > biphasic CaP > HA) [2]. In addition to compositional effects, surface morphologies have been shown to affect biological performance [8,9]. It is now clear that micro- and nanoscale morphologies of the CaP surface can strongly influence osteoblast and stem cell adhesion, proliferation and differentiation [10–12]. Ripamonti and co-workers have extensively investigated the osteogenic effect of repetitive surface concavities on either bulk ceramics [13] or CaP-coated titanium implants [14]. The starting hypothesis for these studies was that concavities, by resembling the hemi-osteon trench observable at different stages of osteoclastogenesis, are able to initiate the ripple-like cascade of bone induction and morphogenesis [15]. Ripamonti and co-workers in fact found that the presence of concavities (diameter 800–1600  $\mu$ m, depth 400–800  $\mu$ m) affected the osteoinductive capacity notably without the need for exogenous osteogenic soluble

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molecules [13]. More recently, Scarano et al. [16] showed that  $500 \times 500 \mu\text{m}$  hemispherical concavities prepared at the surface of CaP-coated titanium implants inserted into the tibias of rabbits led to a significant increase in bone formation inside the concavities compared to the convex areas.

Despite this increasing number of *in vivo* studies demonstrating a positive effect of concavities on osteogenesis, no attempts have been carried out to rationalize the mineralization process through *in vitro* tests. Consequently, the aim of the current study was to evaluate the role of concavities on the *in vitro* mineralization of different bioceramic materials. To this end, HA and  $\beta$ -TCP disks were prepared with hemispherical concavities of different diameters and sintered at 1100 or 1200 °C. Mineralization at the surface of these bioceramic disks upon immersion in simulated body fluid (SBF) for up to 28 days was assessed by means of scanning electron microscopy (SEM), energy-dispersive spectroscopy (EDS), X-ray diffraction (XRD) and calcium quantification assays. Our starting hypotheses were that: (i) concavities prepared at the surface of bioceramic disks could effectively guide the surface mineralization process *in vitro*; and (ii) concavity size represents a key parameter for controlling the extent of surface mineralization *in vitro*.

## 2. Materials and methods

### 2.1. Fabrication of heat-treated calcium phosphate disks

The HA starting powder used in this study was obtained from a commercial source (Merck, Germany). It had a Ca/P molar ratio of 1.67, contained Al, Fe, Mg and Mn as the main impurities, and had a specific surface area ( $S_{\text{BET}}$ ) of  $73 \text{ m}^2 \text{ g}^{-1}$  and a particle size distribution of around 3–4  $\mu\text{m}$ . The  $\beta$ -TCP was obtained from a commercial source (CAM Bioceramics BV, The Netherlands). This  $\beta$ -TCP powder had a Ca/P ratio of 1.5, contained Mg as the main impurity, and had a value of  $S_{\text{BET}}$  of  $60 \text{ m}^2 \text{ g}^{-1}$  and a particle size distribution of around 30  $\mu\text{m}$ . Ceramic disks were prepared from these two powder source materials. Powders were uniaxially pressed at 103 MPa (15,000 psi) for 10 min in a cylindrical steel mold (internal diameter  $\sim 21 \text{ mm}$ ). Subsequently, cylindrical HA and  $\beta$ -TCP green ceramics were heat-treated in a furnace at 800 °C for 6 h ( $1.67 \text{ }^\circ\text{C min}^{-1}$ ) to provide suitable strength to the ceramic to withstand the stresses applied during the machining process. Subsequently, heat-treated HA and  $\beta$ -TCP cylinders were cut into disks (thickness  $\sim 4 \text{ mm}$ , diameter  $\sim 21 \text{ mm}$ ).

### 2.2. Role of chemical composition and sintering temperature on mineralization process

For a preliminary evaluation of the role of chemical composition and sintering temperature on the onset of the mineralization process, drill tips with different diameter sizes (2.1, 1 and 0.5 mm; Horico, Germany) were used to prepare the hemispherical

concavities (four of each diameter) at the surface of heat-treated HA and  $\beta$ -TCP disks (Table 1 and Fig. 1a) with diameters of 1.8 mm (large concavities), 0.8 mm (medium concavities) and 0.4 mm (small concavities). The center-to-center distance between similar concavities was set at twice the concavity diameter in order to display the maximum number of concavities on the same disk while minimizing possible mutual effects on the deposition of calcium phosphate. Subsequently, the disks were sintered in a furnace for 6 h ( $1.67 \text{ }^\circ\text{C min}^{-1}$ ) at either 1100 °C (HA11\_LMS and TCP11\_LMS; LMS: large, medium and small concavities) or 1200 °C (HA12\_LMS and TCP12\_LMS).

The average crystallite size of the CaP ceramics was calculated using the Scherrer formula and the full width at half maximum of the (002) reflection of apatitic depositions. Lanthanum hexaboride (LaB6; NIST standard reference material #660) was employed to determine the instrumental broadening under similar measuring conditions.

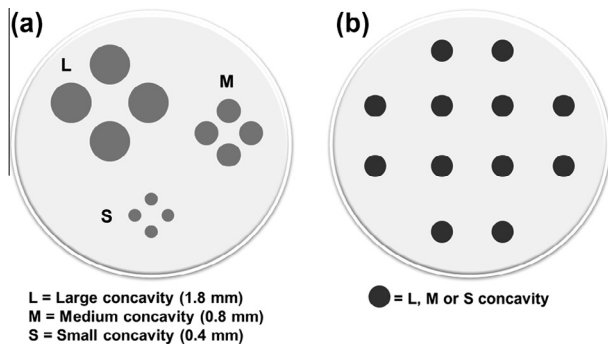
Freshly prepared, filter-sterilized simulated body fluid (SBF; pH 7.4, ionic composition: 142.0 mM  $\text{Na}^+$ , 5.0 mM  $\text{K}^+$ , 1.5 mM  $\text{Mg}^{2+}$ , 2.5 mM  $\text{Ca}^{2+}$ , 147.8 mM  $\text{Cl}^-$ , 4.2 mM  $\text{CO}_3^{2-}$ , 1.0 mM  $\text{HPO}_4^{2-}$  and 0.5 mM  $\text{SO}_4^{2-}$ ) was used to assess the surface mineralization capacity of the experimental CaP disks [17]. HA and  $\beta$ -TCP disks ( $n = 3$  for each experimental group) were transferred into 6-well plates and immersed in SBF solution (10 ml per well) for 7, 14, 21 and 28 days. The plate was kept at 37 °C and shaken moderately (1 Hz). Additionally, controls ( $n = 3$ ) consisting of empty wells filled with only SBF were used for each time point in order to detect any homogeneous nucleation in the solution or heterogeneous nucleation on the walls of well plates. The SBF was changed daily, and the supernatant was collected to determine calcium levels using the ortho-cresolphthalein complexone (Sigma–Aldrich) method. To this end, 100  $\mu\text{l}$  of the supernatant was mixed with 100  $\mu\text{l}$  of 1 N acetic acid (Boom BV, Meppel, The Netherlands) and incubated overnight on a shaking table. Calcium concentrations were calculated with respect to the controls (empty wells) and averaged for three samples per group. At 7, 14, 21 and 28 days, disks were removed from the SBF, gently washed with distilled water and dried at 40 °C for 24 h.

### 2.3. Role of concavity dimension on mineralization process onset

For the quantitative evaluation of the effect of concavity size on the mineralization process, a different surface geometry was adopted: 12 concavities of 1.8, 0.8 or 0.4 mm diameter were prepared on the surface of HA green ceramic disks heat-treated at 800 °C (Table 1 and Fig. 1b). The center-to-center distance was set at 4 mm in order to maximize the distance between adjacent concavities. Finally, disks were sintered for 6 h at 1200 °C ( $1.67 \text{ }^\circ\text{C min}^{-1}$ ). For surface mineralization experiments, two HA12 disks containing 12 surface concavities of large (HA12\_L), medium (HA12\_M) or small diameter (HA12\_S) were immersed

**Table 1**  
Overview of the experimental setup.

Study	Calcium phosphate ceramics investigated	Concavity dimension investigated	Number of concavities on each disk	Distribution of concavities on each disk	Sintering temperature	Sample acronym
Role of chemical composition and sintering temperature on mineralization process	HA, $\beta$ -TCP	Large (L, 1.8 mm)	12	4 L + 4 M + 4 S	1100 °C	HA11_LMS
		Medium (0.8 mm)			1200 °C	TCP11_LMS
Role of concavity size on mineralization process onset	HA	Small (0.4 mm)	12	12 L or 12 M or 12 S	1200 °C	HA12_LMS
						TCP12_LMS
						HA12_L
						HA12_M
						HA12_S



**Fig. 1.** Schematic representation of disks surface geometry employed in the current study. (a) Schematic distribution of the concavities at the surface of the disks for the preliminary experiment on the effect of chemical composition and sintering temperature on the mineralization process in vitro. Four concavities of each size (1.8 mm (large concavity, L), 0.8 mm (medium concavity, M) and 0.4 mm (small concavity, S)) were prepared on the surface of the disks; the center-to-center distance was kept at twice the concavity diameter. (b) Scheme of the distribution of the concavities at the surface of the disks for the experiment on the effect of concavity size on mineralization process in vitro. Disks were machined with 12 concavities of the same size (L, M or S).

in SBF for 14 days according to the protocol described above. Additionally, the disks were weighed before and at the end of the experiment, after being gently washed with distilled water and dried at 40 °C for 24 h.

#### 2.4. Disk characterization

The mineralization process on the surface of the CaP disks was followed through SEM, EDS and XRD analyses. Surface morphology and microstructure of the specimens were observed using a scanning electron microscope (JSM6310, JEOL, Japan; operating at an accelerating voltage of 10 kV and a current of ~10 mA) after coating the samples with a thin gold layer (5–10 nm). The elemental analysis was carried out using a scanning electron microscope (Philips XL30, The Netherlands) equipped with an energy-dispersive spectrometer (EDAX, AMETEK Materials Analysis Division, USA) at an accelerating voltage of 10 kV, a working distance of 10 mm and different magnifications. To improve the surface conductivity of the samples, a thin gold layer was deposited on the samples using a common sputtering instrument, which did not affect the measurement of the phosphorus content by EDS. The crystal phase composition of the specimens was characterized by means of a PW3710 X-ray diffractometer (Philips, The Netherlands; thin film configuration (fixed angle of incidence of 2.5°), with Cu  $K_{\alpha}$  radiation, a voltage of 40 kV, a current of 30 mA, a step-size  $2\theta$  of 0.02°, a scanning speed of 0.005  $2\theta$  s<sup>-1</sup> and a sample time of 4 s per step).

#### 2.5. Statistical analysis

All quantitative data were expressed as mean  $\pm$  SD and statistical analyses were performed with GraphPad® Instat 3.05 software (GraphPad Software Inc, San Diego, CA, USA), using one-way analysis of variance (ANOVA) with Tukey Kramer multiple comparison post hoc tests. Results were considered significant at  $p < 0.05$ .

### 3. Results

#### 3.1. Role of chemical composition and sintering temperature

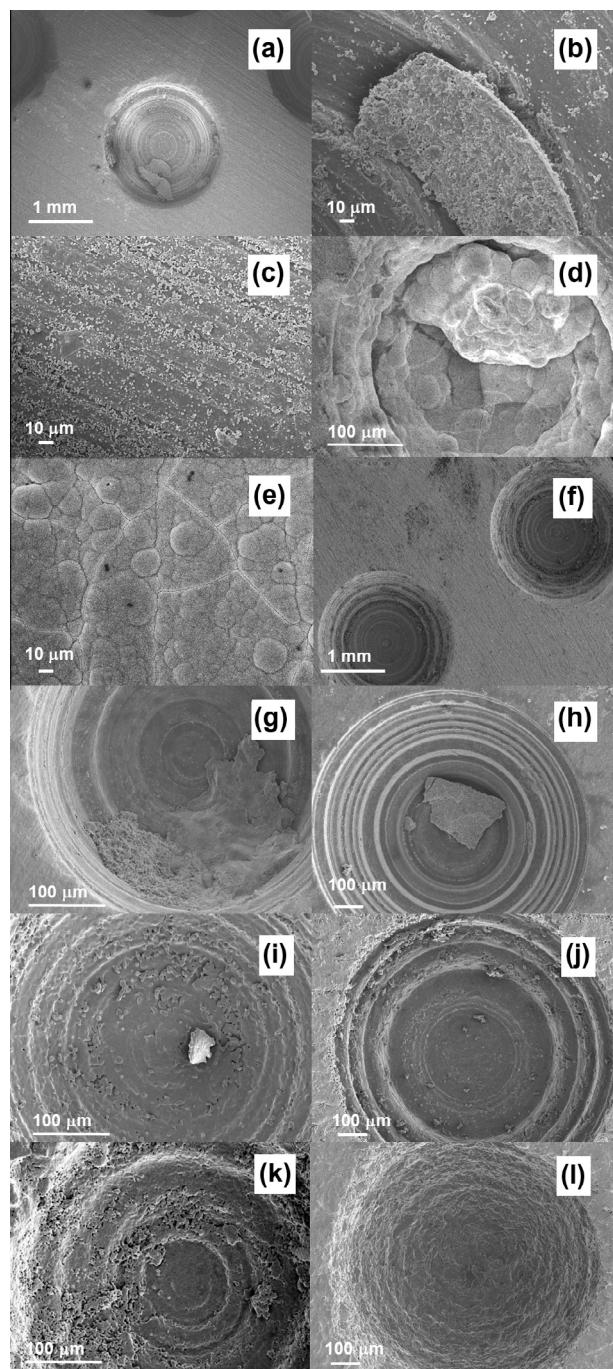
The effect of sintering temperature on crystallite size in base materials and sintered disks is presented in Table 2.

Initial evaluations focused on the effect of disk chemical composition and sintering temperature on the mineralization process in vitro. SEM images of the surface of HA and  $\beta$ -TCP disks containing four large, four medium and four small (LMS) concavities on their surface at different experimental time points are depicted in Fig. 2. After the first week of soaking in SBF, large CaP aggregates up to several hundred micrometers long were observed only within the concavities of HA12\_LMS disks, not on the planar surface (Fig. 2a and b). In contrast, only small isolated CaP particles of about 1–5  $\mu$ m were detected on the planar surface of HA12\_LMS disks (outside concavities; Fig. 2c). From the second to the third week of immersion in SBF, a globular CaP layer was formed inside concavities (Fig. 2d), while the planar surface between HA12\_LMS disks was homogeneously covered with CaP after 3 weeks of soaking (Fig. 2e), which finally dissolved during the fourth week (Fig. 2f). Analysis of the HA11 samples revealed no formation of such a globular layer, only the presence of CaP aggregates inside large, medium and small concavities and not at the planar surface at any of the experimental time points (Fig. 2g,h). Fewer, smaller aggregates were found inside concavities of TCP12\_LMS (Fig. 2i,j), whereas hardly any CaP aggregates were detected within the concavities of TCP11\_LMS disks (Fig. 2k,l). Further, the surface of TCP11\_LMS showed partial degradation after 3 weeks of soaking in SBF, as evidenced by the decreased intensity of machining features inside concavities, whereas the surface of TCP12\_LMS was only slightly changed and the surfaces of HA11\_LMS and HA12\_LMS retained their original appearance.

Calcium quantification analysis revealed that all experimental disks induced calcium uptake during immersion in SBF (Fig. 3). Similar calcium uptake profiles were observed for TCP11\_LMS and TCP12\_LMS; a significantly ( $p < 0.001$ ) higher calcium uptake was observed for HA11\_LMS compared to the calcium uptake of the  $\beta$ -TCP disks, whereas the largest calcium uptake was observed for HA12\_LMS. From the second week onward, a significant difference ( $p < 0.001$ ) between the calcium uptake of HA12\_LMS and HA11\_LMS was observed. The final cumulative calcium uptake for HA12\_LMS was 2-fold higher compared to the uptake for HA11\_LMS and 4.6- and 5.4-fold higher compared to the uptake for TCP12\_LMS and TCP11\_LMS ( $p < 0.001$ ). Calcium assay

**Table 2**  
Crystallite size of the ceramics used, obtained via the Scherrer equation.

Material	Temperature (°C)	$2\theta$	Crystallite size (nm)
HA powder	–	25,888	44.3
$\beta$ -TCP powder	–	25,822	46.6
HA	1100	25,920	140.6
HA	1200	25,885	151.0
$\beta$ -TCP	1100	25,801	140.5
$\beta$ -TCP	1200	25,856	262.9



**Fig. 2.** SEM evaluation of the mineralization process on HA and  $\beta$ -TCP disks sintered at 1100 and 1200 °C at different experimental time points. (a) Low-magnification image of the surface of HA12\_LMS after week 1, showing macroscopic CaP aggregates inside a large concavity; (b) detail of a CaP aggregate inside a large concavity of HA12\_LMS after week 1; (c) planar surface between concavities at the surface of HA12\_LMS after week 1; (d) small concavity at the surface of HA12\_LMS completely filled by CaP between weeks 2 and 3; (e) planar surface between concavities at the surface of HA12\_LMS after week 3; (f) low-magnification image of a small concavity at the surface of HA11\_LMS after week 1; (g) CaP aggregate inside a small concavity at the surface of HA11\_LMS after week 1; (h) CaP aggregate inside a medium concavity at the surface of HA11\_LMS after week 3; (i) small CaP aggregate inside a small concavity at the surface of TCP12\_LMS after week 1; (j) medium concavity surface of TCP12\_LMS after week 3; (k) small concavity surface of TCP11\_LMS after week 1; (l) medium concavity surface of TCP11\_LMS after week 3.

performed on well plates filled only with SBF revealed no calcium uptake on the walls of the well plates at any time point for every disk (data not shown). Due to the observed superior ability to

induce surface mineralization for HA12\_LMS, further studies were carried out using HA12\_LMS disks in order to rationalize the mineralization process and the effect of concavity size on CaP mineralization at the surface.

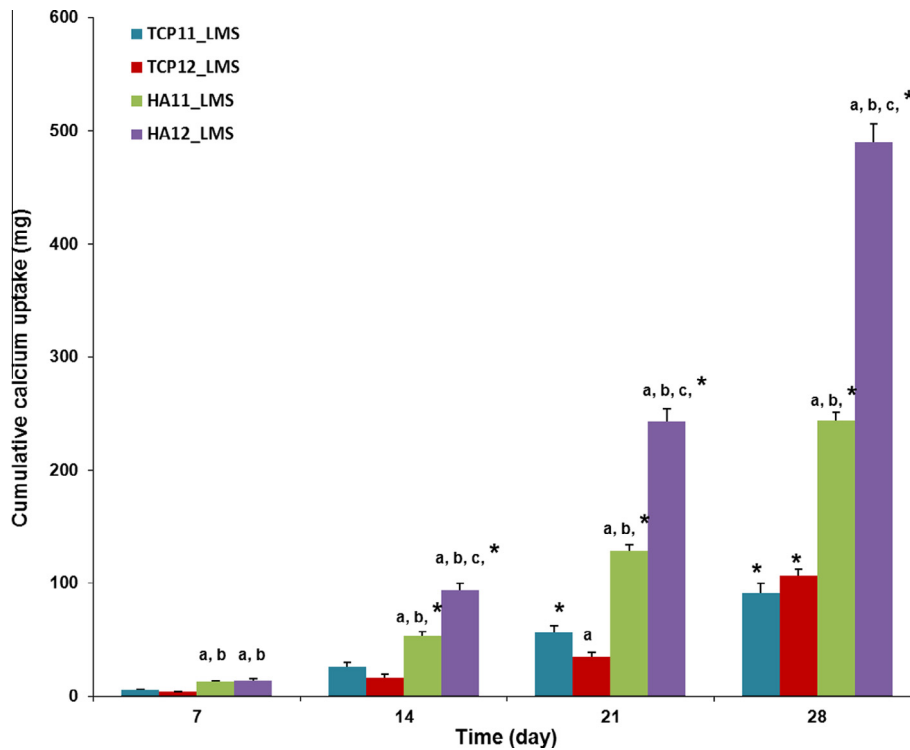
### 3.2. Mineralization process

SEM and XRD analyses were performed to follow the onset of the mineralization process. SEM images of CaP deposition onto HA12\_LMS disks after different immersion times in SBF are presented in Fig. 4. The large aggregates found within concavities after the first week of immersion were composed of micron-sized CaP spherical-like cohesive particles (Fig. 4a). EDS analysis (Fig. 5a) revealed a molar ratio of calcium to phosphorus (Ca/P) of 1.9–2.0 for these CaP spherical-like particles. At 2 weeks' immersion in SBF, a globular CaP deposition layer had grown within the concavities that gradually covered the underlying spherical-like CaP particles (Fig. 4b and c). This globular CaP deposition layer was composed of ~50 nm thick crystals (Fig. 4d) with a Ca/P ratio of 1.4–1.5 arranged into a reticular porous structure. EDS analysis (Fig. 5b) revealed a slightly higher atomic percentage of carbon (~15%) compared to spherical-like particles (9–10%), along with small amounts of sodium (1–1.5%) and magnesium (1–1.1%) and traces of potassium (<1%) and chlorine (<1%). The SEM images presented in Fig. 4e and f show that two small concavities on the same HA12\_LMS disk after a 2-week incubation period in SBF are completely filled by the globular CaP. Specifically, in Fig. 4e initial outgrowth of CaP from the concavity can be detected (black arrows), while in Fig. 4f the globular CaP has already spread over the planar surface close to the concavity (the black arrowheads show the directions of the CaP outgrowth).

XRD diffractograms of HA12\_LMS after different SBF immersion times are presented in Fig. 5c. The intensity of all of the main reflection peaks of the diffractogram of HA12\_LMS virgin disks decreased from week 0 (before soaking) to week 1, until a minimum was reached at 2 and 3 weeks. After a 4-week SBF immersion period, the spectrum showed an increase in intensity again. Fig. 5d shows the HA12\_LMS diffractograms at  $2\theta$  of 31–33° ((211), (112) and (300) crystal planes), where the main HA peaks can be found, in order to observe the above-mentioned behavior in more detail. Several secondary reflection peaks were observed at  $2\theta$  of 43–46° ((113), (400) and (203) crystal planes), corresponding to the CaP deposited on the disks (Fig. 5d, inset). The intensity of these peaks corresponding to the deposited CaP increased from week 0 to week 3, followed by a decrease at week 4.

### 3.3. Role of concavity dimension on SBF mineralization

In order to quantify the effect of concavity dimension on CaP nucleation in vitro, HA12\_L, HA12\_M and HA12\_S disks were soaked in SBF at 37 °C for 14 days and characterized for morphology, crystal phase and calcium uptake. The final time point of 14 days was selected in order to study the mineralization process before the spreading of CaP deposits over the planar surface of the disks. Fig. 6a displays representative SEM images of the concavities of HA12\_L, HA12\_M and HA12\_S after a 2-week immersion period in SBF. From detailed SEM investigation, it was observed that nearly all (23 out of 24) of the concavities at the surface of HA12\_S disks were filled with spherical-like CaP. In contrast, the concavities at the surface of HA12\_M and HA12\_L disks were only partially and scarcely filled with CaP deposits, respectively. Similar to the results described above, substantial CaP aggregates were only observed inside concavities and not at the planar surface. This absence of a planar surface CaP layer was confirmed by XRD spectra of HA12\_L, HA12\_M and HA12\_S, which did not show



**Fig. 3.** Calcium uptake of TCP11\_LMS, TCP12\_LMS, HA11\_LMS and HA12\_LMS disks during SBF immersion. Each data point represents the average and standard deviation of values obtained from three disks: (a) significantly different compared to TCP11\_LMS within the same experimental time; (b) significantly different compared to TCP12\_LMS within the same experimental time; (c) significantly different compared to HA11\_LMS within the same experimental time; (\*) significantly different compared to the same group (same disk type) between one experimental time and the previous one.

differences between the various experimental disks or as compared to the non-soaked HA12 control samples (Fig. 6b).

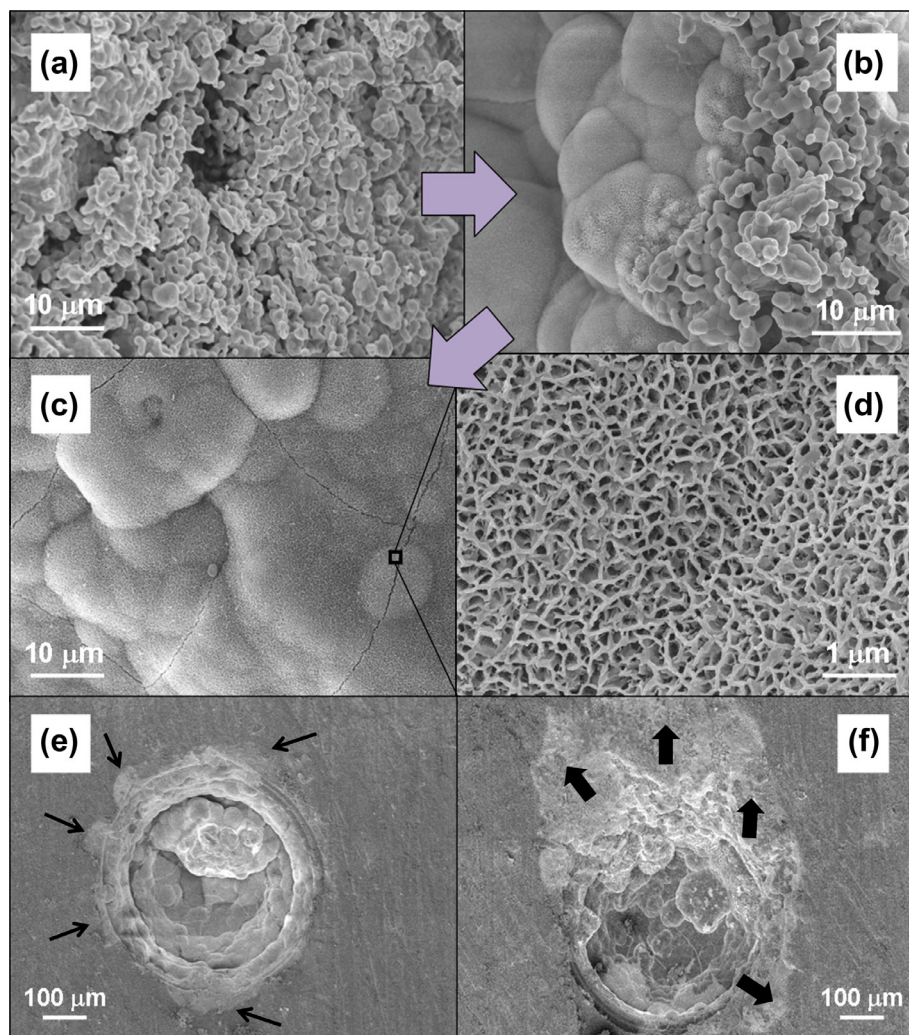
Differences in the extent of calcium uptake by HA12\_L, HA12\_M and HA12\_S revealed a clear effect of the concavity dimension on the extent of mineralization (Fig. 7a). The calculated amounts of calcium uptake relative to the concavity dimension are presented in Table 3. The total calcium uptake of HA12\_S was ~1.7- and ~1.8-fold higher compared to HA12\_M and HA12\_L, respectively. Direct weight measurements of the disks before and after the SBF immersion experiment revealed a weight increase ( $\Delta W$ ) of 0.36% for HA12\_S, a weight increase in 0.33% for HA12\_M and a weight increase in 0.24% for HA12\_L. The total calcium uptake per volume unit ( $\text{mg mm}^{-3}$ , calculated by assuming that all CaP was deposited in the concavities) of the concavities of HA12\_S (Fig. 7b) was ~123- and ~10-fold higher compared to HA12\_L (S/L) and HA12\_MC (S/M), respectively, whereas  $\Delta W$  per volume unit of the concavities ( $\text{mg mm}^{-3}$ ) of HA12\_S was ~109- and ~7-fold higher compared to HA12\_L (S/L) and HA12\_MC (S/M), respectively.

#### 4. Discussion

The aim of this study was to evaluate the effect of surface concavities on the surface mineralization of different CaP ceramics in vitro. Our hypotheses were that: (i) concavities prepared at the surface of bioceramic disks could induce surface mineralization in vitro, as surface concavities have been previously demonstrated to induce bone formation at ectopic locations in vivo [13]; and (ii) concavity dimensions affect the extent of surface mineralization. The results of this study clearly demonstrated a strong effect of both disk chemical composition (HA >  $\beta$ -TCP) and sintering temperature ( $1200^\circ\text{C}$  >  $1100^\circ\text{C}$ ) on CaP deposition from SBF. Further, it was shown that CaP deposition was initiated exclusively within

concavities and not on the planar surface of the disks. Finally, concavity dimensions showed a strong effect on surface mineralization, with a 4-fold reduction of concavity dimensions resulting in an ~123-fold increase in calcium uptake.

The results of the present study showed that only HA disks sintered at  $1200^\circ\text{C}$  supported abundant CaP deposition. Sintering has a strong effect on the density, grain size, porosity, crystalline phases and surface charge of ceramics [18,19]. HA sintered at lower temperature is known to be composed of grains of smaller size, thus exhibiting a higher porosity and lower crystallinity compared to HA sintered at higher temperature [19]. Consequently, it can be envisaged that dissolution rates were higher for HA11\_LMS compared to HA12\_LMS, which in turn limited the extent of nucleation and growth of stable CaP aggregates for HA11\_LMS compared to HA12\_LMS. Similarly, the faster dissolution of the  $\beta$ -TCP surface compared to the HA most likely prevented the nucleation of stable CaP nuclei, despite locally (i.e. at the surface) increased ionic concentrations of calcium and phosphate ions; hence it could be inferred that the negative contribution to CaP nucleation from surface destabilization was higher than the positive contribution due to the local ion concentration, this phenomenon being more important as the sintering temperature decreased.  $\beta$ -TCP disks sintered at lower temperature were even more unstable, as partial degradation of the surface (evidenced by the loss of regularity of the circular lines following the drilling step) without CaP nucleation on the surface was observed for these disks, corroborating previously reported results [20]. The results of the present study suggest the occurrence of a two-step mineralization process on the HA disks heat treated at  $1200^\circ\text{C}$ , represented by an initial aggregation of spherical-like CaP particles within the concavities (no large aggregates were found on the planar surface of the disks), which were subsequently replaced by a globular CaP phase

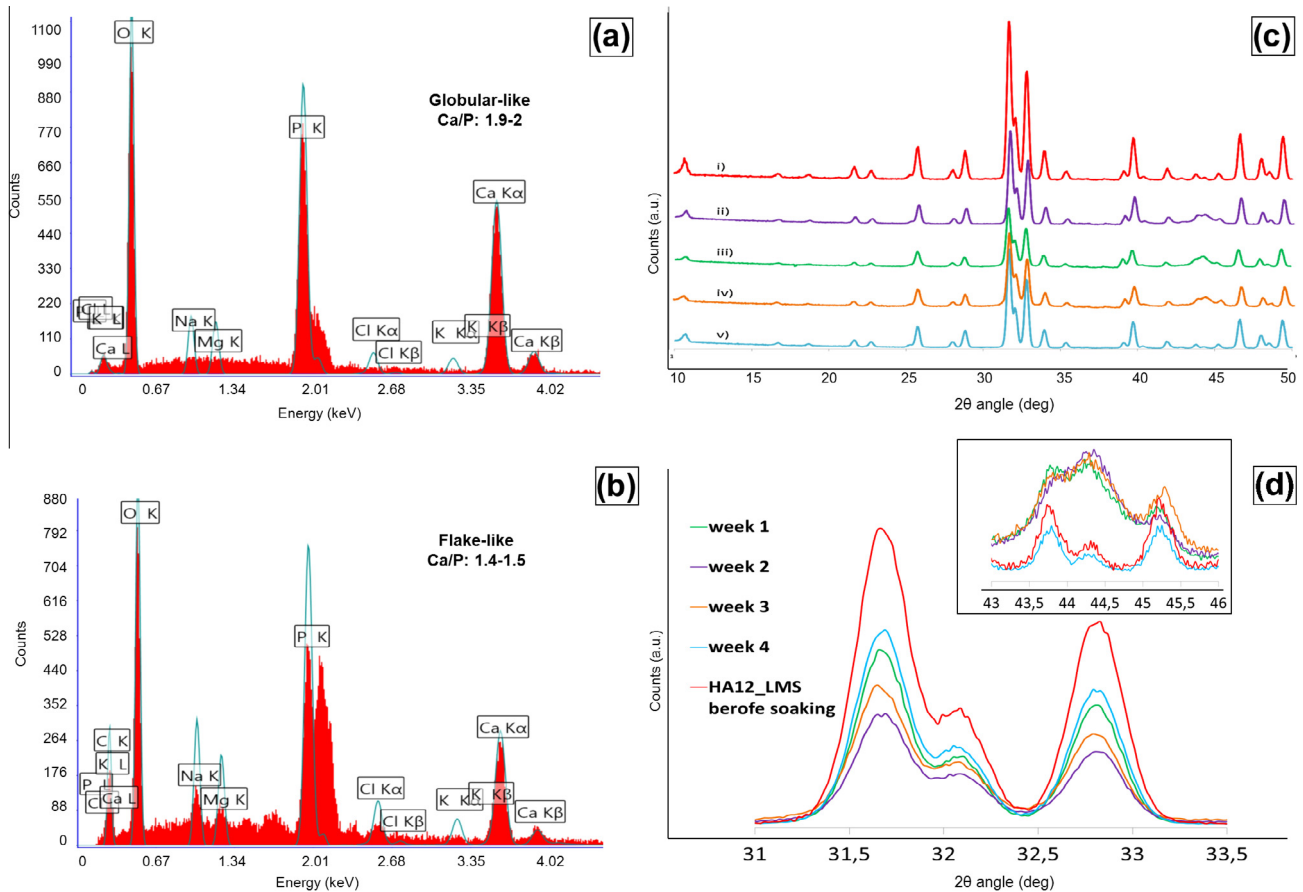


**Fig. 4.** SEM images of different morphologies of CaP deposits on HA12\_LMS disks observed during SBF immersion. (a) Detail of the spherical-like structure of a CaP aggregate (large concavity, week 1); (b) transition phase between the spherical-like and the globular macroporous structure (medium concavity, week 3); (c) globular CaP deposits (medium concavity, week 3); (d) detail of globular CaP deposits (medium concavity, week 3); (e) small concavity completely filled by globular CaP deposit (week 3); arrows indicate the initial outgrowth of CaP outside the concavity; (f) spreading of CaP deposits outside a small concavity onto the planar surface (week 3, same disk of (e)); arrowheads indicate the outgrowth direction of the CaP outside the concavity on the planar surface.

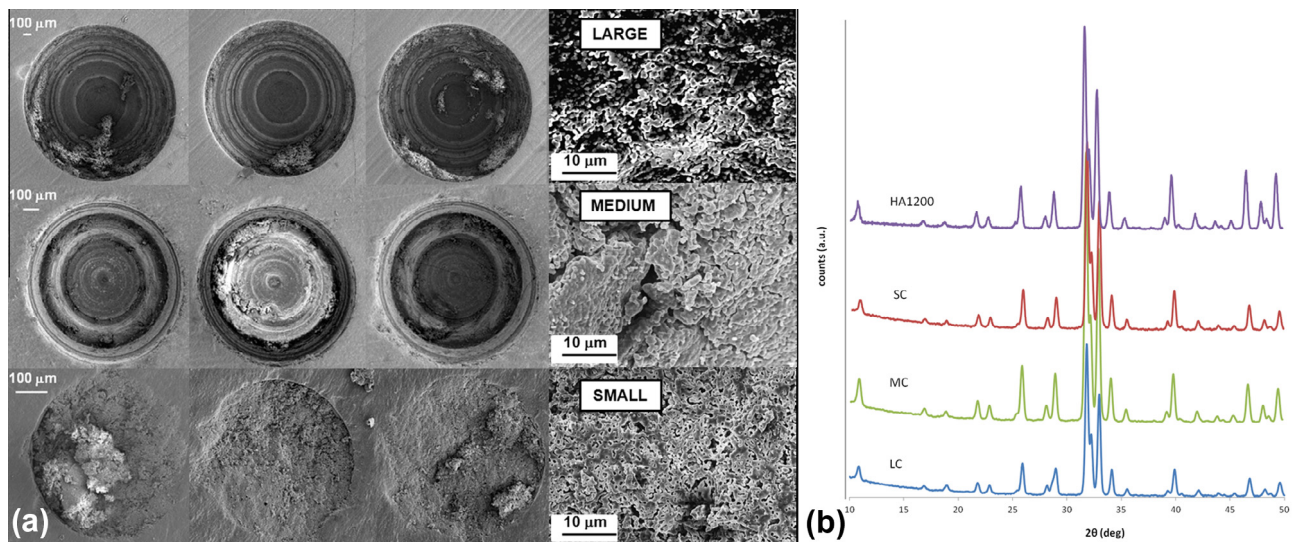
consisting of crystals arranged into a reticular macroporous structure, exhibiting a chemical composition which resembled the inorganic phase in bone tissue more closely [21]. Accordingly, semi-quantitative EDS data suggested the formation of a Ca-rich (Ca/P ratio  $\sim 1.9$ – $2.0$ ) amorphous calcium phosphate (ACP), eventually replaced by a Ca-poor (Ca/P ratio  $\sim 1.4$ – $1.5$ ) carbonate calcium phosphate of general formula  $\text{Ca}_{10-x}(\text{PO}_4)_{6-y}(\text{CO}_3)_y(\text{OH})_2(\text{X})_y$ , in which a part of  $\text{PO}_4^{3-}$  was substituted by  $\text{CO}_3^{2-}$  and a small fraction of  $\text{Ca}^{2+}$  was replaced by  $\text{X} = \text{Mg}^{2+}$  and  $\text{Na}^+$  and  $\text{OH}^-$  by  $\text{Cl}^-$  [22]. These findings corroborate several studies reporting the formation of a transient amorphous calcium phosphate phase before the establishment of a globular more crystalline apatitic phase [18,23]. A possible explanation resides in the fact that, in pH 7.4 SBF solution, HA possesses a negative surface charge; this negatively charged surface attracts cationic calcium ions from the SBF solution, forming a Ca-rich ACP. Consecutive calcium ions accumulation makes the Ca-rich ACP on the surface of the HA acquire a positive charge. At that stage, the Ca-rich ACP attracts negative phosphate ions in the SBF to form a Ca-poor ACP, which eventually crystallizes into a bone-like apatite by incorporating also other ions from SBF, such as sodium, magnesium, carbonate and chloride

[18]. At the stage of complete filling of small concavities, globular CaP started to spread out from the concavities, ultimately covering the planar surface of the disks completely, as suggested by SEM images like Fig. 4e and f. The formation and subsequent dissolution of the globular CaP coating on the HA12 disk surface could also be monitored by XRD analysis: specifically, the intensity of the main peaks of the HA substrate decreased with soaking time, indicating the establishment of a layer of lower crystallinity on the surface of the disks that limited X-ray diffraction from the more crystalline underlying substrate; after week 4, the peak intensity increased again toward the starting conditions, which most likely suggests a partial redissolution of the globular CaP layer.

Concavities resemble the hemi-osteonic trenches (from few to several hundreds of micrometers) generated during osteoclastogenesis [24,25]. The effect of concavities on bone growth in ectopic extraskeletal sites *in vivo* has already been well documented [26], whereas no study attempted to rationalize this phenomenon *in vitro*. The results of the current study evidenced a very strong templating effect of surface concavities on CaP surface mineralization, whereas no significant CaP deposition occurred on the planar surface. Although no solid explanation could be found to account



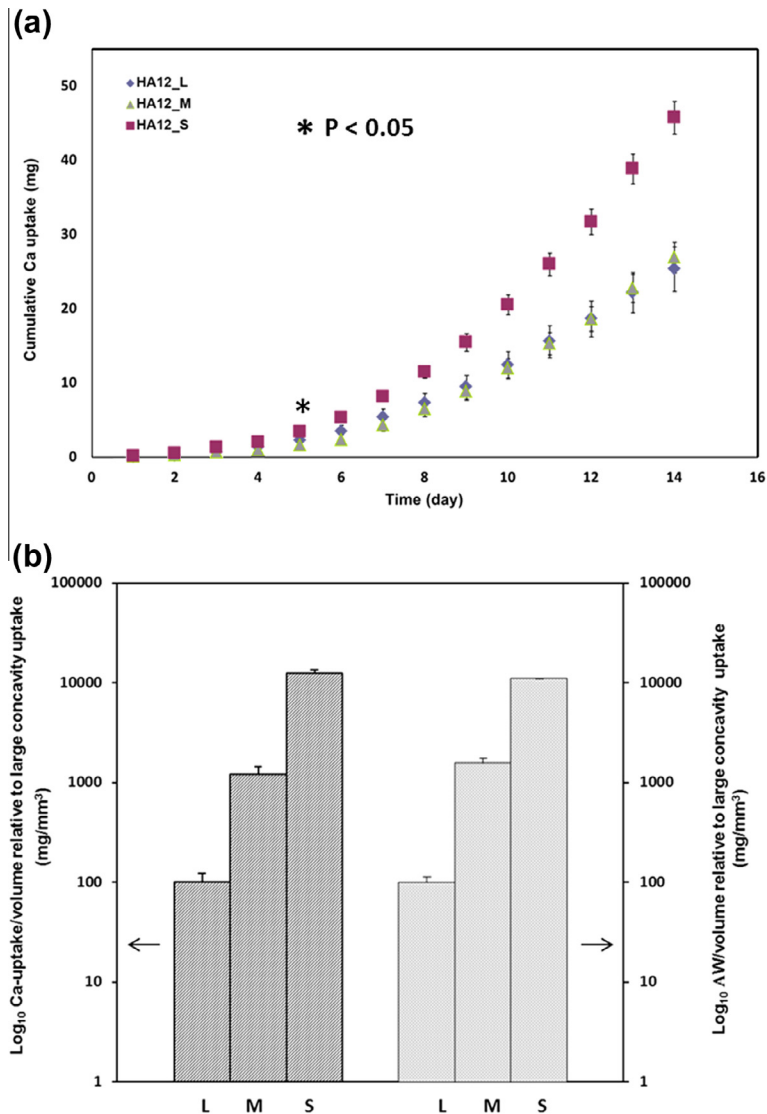
**Fig. 5.** EDS and XRD spectra of HA12\_LMS. (a) EDS spectrum of spherical-like CaP deposits at week 1; (b) EDS spectrum of globular CaP deposits at week 3; (c) XRD spectra diffractograms of HA12\_LMS at different SBF soaking times: 0 (i), 7 (ii), 14 (iii), 21 (iv) and 28 (v) days; (d) detail of the XRD diffractograms of (c) in the range 31–33.5°; (d, inset) detail of the XRD diffractograms of (c) in the range 43–46°.



**Fig. 6.** SEM images of large, medium and small concavities at the surface of HA12 disks after 14 days immersion in SBF. (a) Top-view SEM images of large cavities (top), medium cavities (middle) and small cavities (bottom) on HA12\_L, HA12\_M and HA12\_S, respectively, after 14 days of immersion in SBF at 37 °C. Representative concavities for each dimension are displayed. The panels on the right show magnified images of the mineralization inside the concavities. (b) XRD spectra of planar surface of HA12\_L, HA12\_M and HA12\_S (and a reference HA1200 sample).

for this intriguing phenomenon, we speculate that the main contribution to CaP nucleation inside concavities compared to the flat surface can be ascribed to the lower fluid flow rate inside the concavities, which allowed the deposition of calcium and phosphate

ions, unlike the more turbulent environment outside concavities: i.e. nuclei deposited on the flat surface redissolved in a shorter time than the time required to reach the critical stable nucleus size. In a recent paper, Kasiteropoulou et al. [27] demonstrated that the



**Fig. 7.** Quantification of the mineralization process at the surface of HA12 disks after 14 days immersion in SBF. (a) Cumulative calcium uptake for HA12\_L (rhombus), HA12\_M (triangle) and HA12\_S (square) after 14 days immersion in SBF. From day 5 to the end of the experiment,  $p < 0.05$  between HA12\_S and HA12\_M or HA12\_L. (b) Graph showing the calcium uptake per concavity volume unit of the disks normalized to the uptake of HA12\_L (left bars, log scale) and the weight variation for concavity volume unit of the disks normalized to the uptake of HA12\_L (right bars, log scale).

**Table 3**  
Quantification of the in vitro mineralization process.

	Concavity diameter (mm)	Mean disk surface area (mm <sup>2</sup> )	SBF volume to surface area (mL/mm <sup>2</sup> )	Volume of 1 concavity (mm <sup>3</sup> )	Volume of 12 concavities (mm <sup>3</sup> )	Total Ca uptake (mg)	Ca uptake per concavity volume (mg/mm <sup>3</sup> )	ΔW (mg)	ΔW per concavity volume (mg/mm <sup>3</sup> )
LC	1.79 ± 0.03	376 ± 39	0.027 ± 0.003	1,5 ± 0.1	18 ± 2	25 ± 3	1.4 ± 0.3	5.0 ± 0.2	0.28 ± 0.04
MC	0.80 ± 0.02	352 ± 35	0.028 ± 0.003	0,13 ± 0.01	1.6 ± 0.1	27 ± 2	17 ± 3	7.1 ± 0.2	4.4 ± 0.5
SC	0.44 ± 0.01	348 ± 34	0.029 ± 0.003	0,022 ± 0.001	0.27 ± 0.01	46 ± 2	172 ± 14	8.1 ± 0.2	30.5 ± 0.9

LC = large concavity; MC = medium concavity; SC = small concavity; ΔW = (Weight)<sub>final</sub> – (Weight)<sub>initial</sub>.

velocity of the fluid inside microchannels with protrusions was lower inside the cavities (i.e. between two protrusions) than on the flat surface, and the higher the protrusion the lower the velocity. They also studied pressure maps inside microchannels, the pressure being fluid pressure that was strictly related to the density of the particles (calcium and phosphate ions in our case) inside

the fluid. The authors found that inside the cavities “there is a high pressure region (high particle density region) whose length depends on the protrusion size, being larger as the protrusion size decreases”. Even if extrapolation of this behavior from the micron to the submillimeter regime cannot be underestimated, we found that smaller concavities provided lower flows and higher

particulate density compared to larger concavities, which in turn led to an accelerated increase in local ionic concentrations of  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  compared to larger concavities, where higher flow rates are expected due to the larger size. Finally, since mineralization is a unique process involved in bone tissue formation, the data presented herein represent a first attempt to link mineralization to osteoinduction, which by itself is a completely novel approach.

## 5. Conclusions

The results of the present study showed that the in vitro surface mineralization process of CaP ceramics with surface concavities starts preferentially within the concavities and not on the planar surface of the ceramics, indicating a strong templating effect of the concavities on CaP surface mineralization. Further, concavity size was revealed to be an extremely effective parameter for controlling the extent of in vitro surface mineralization, with small concavity dimensions resulting in considerably more surface mineralization.

## Acknowledgement

This research forms part of the Project P2.04 BONE-IP of the research program of the BioMedical Materials Institute, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

## Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 3–7, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi: <http://dx.doi.org/10.1016/j.actbio.2013.10.026>.

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