Effect on growth and osteoblast mineralization of hydroxyapatite-zirconia (HA-ZrO2) obtained by a new low temperature system

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Abstract

Ceramics and bioceramics, such as hydroxyapatite and zirconium, are used in bone tissue engineering. Hydroxyapatite has chemical properties similar to bone while zirconium offers suitable mechanical properties. The aim of this article is to evaluate the ability to support cell growth and osteoblastic mineralization of hydroxyapatite-zirconium obtained by a new system based on different low temperatures, such as 873 K (HZ600), 923 K (HZ650) and 973 K (HZ700). Hydroxyapatite-zirconia obtained by this new system was examined in terms of thermogravimetric features and x-ray diffractograms. Furthermore, the ability for supporting osteoblast growth and mineralization were analyzed. By x-ray diffraction analysis, we clearly demonstrated that no high-temperature processing was required. Moreover, it is possible to form tetragonal-zirconium at 923 K. Proliferation assays showed that osteoblast growth was not influenced by any of the composite evaluated. Regarding the osteogenic marker ColI, a 2-fold increase in expression was observed for HZ650 compared to HZ600 and HZ700.

Interestingly, osteoblasts grown on HZ650 showed globular accretions covered with collagen bundles and calcium-rich extracellular matrix whereas HZ600 and HZ700 showed no phosphate or calcium deposits. This study demonstrated that at 923 K it is possible to generate stable tetragonal-zirconium and the resulting HZ650 composite is able to promote a suitable osteoblast mineralization process.

Introduction

Scaffolds play an important role in cell attachment, proliferation and guidance of new bone tissue formation in bone tissue engineering [1,2]. Scaffolds from several materials, including biopolymers [3], bioactive ceramics [4] and high-strength composites [5], have been used. Bioceramic scaffold materials have been developed from various natural sources, including coral [6] and bone [7], wherein the main inorganic component is calcium phosphate, which binds closely with the structure of hydroxyapatite (HA).

HA has attracted interest as a biomaterial for use in orthopedic applications due to the coincidence of its crystallography and chemical composition to that of human bones [8–12]. However, its major weakness of HA lies in its low mechanical strength which makes it inadequate for load-bearing applications.

An alternative way of producing tougher HA is to use composites of stabilized tetragonal zirconium and HA, where the bioactivity comes from the apatite phase and the high strength is derived from the zirconium oxide (ZrO2) phase [13–16]. Nonetheless, HA may release calcium which interacts with zirconium, promoting the formation of cubic zirconium (calcium zirconate), which restrains the toughening mechanism [17–19].

Based on temperature, zirconia exists in three phases, monoclinic (M), cubic (C) and tetragonal (T). Tetragonal zirconia has high strength, toughness, and
biocompatibility [21]. M-phase is frail at room temperature, and therefore requires stabilization to prevent tetragonal (T)-to-monoclinic (M) transformation in technical applications [20]. To minimize this transformation and achieve the stabilization of the tetragonal phase at low temperatures, different strategies can be applied and one of these consists of obtaining particles in the nanometer range. It has been shown that in nano-particles with average sizes in the range of 5–16 nm, the tetragonal phase is conserved at room temperature and in some cases only traces of the monoclinic phase can be present [22].

Likewise, scaffolding with hydroxyapatite-zirconium should be biologically tested to study its ability to support osteoblast maturation. Osteoblast differentiation from mesenchymal stem cells (MSCs) progress through three cell stages: proliferation, extracellular matrix maturation, and matrix mineralization [23]. The first stage is defined by an increase in cell number and accumulation of procollagen. An increase in ALP activity and collagen accumulation in the extracellular matrix (ECM) define the second stage. Finally, osteoblast maturation is featured by expression of extracellular matrix proteins such as osteocalcin (OCN) and osteopontin (OPN), and accumulation of mineral deposits [24].

The aim of this study was to compare the effects of hydroxyapatite-zirconium obtained at different low temperatures, such as 873 K (HZ600), 923 K (HZ650) and 973 K (HZ700) on phenotypic markers of osteoblastic maturation. In vitro studies were performed to forecast the effect of HA-ZrO₂ obtained at low temperature on osteoblasts during bone regeneration, which could determine its potential application as a bone filling material.

**Materials and methods**

**Synthesis and characterization of HA-ZrO₂**

The chemical synthesis starts from a 0.05 M solution of ZrOCl₂·8H₂O (Sigma-Aldrich, St. Louis, MO) which was hydrolyzed at room temperature for 90 min to achieve complete hydrolysis. The pH was adjusted to 3.5 to generate an acidic environment and the sample was dried in air at 100 °C for 30 min. Afterwards, the amorphous Zr(OH)₄–ZrO₂ obtained was mixed with hydroxyapatite (Sigma-Aldrich) 1:3, respectively. The hydroxyapatite was obtained from a suspension consisting of hydroxyapatite powder dispersed in propylene glycol with the ratio 7:3, to obtain at 21 000 cP viscosity. Finally, sintering was carried out [25]. Samples without thermal annealing were thermogravimetric (TG). These samples were analyzed by differential scanning calorimetry (DSC) using a STDQ600 TA Instruments Thermal Analyzer (Newcastle, England) at a heating rate of 30° min⁻¹ at air atmosphere up to 873, 923, and 973 K for each sample (HZ600, HZ650 and HZ700, respectively). The temperatures were maintained until the analyzer did not detect any change.

**X-ray diffraction (XRD)**

Samples with thermal annealing were characterized by XRD in powder mode using a Philips diffractometer; model X’pert (Paris, France) at 2θ from 20° to 80°, with 0.3 s of integration time and 5° incidence angle. Samples were taken at 0.02° intervals.

**Isolation of hMSCs and osteoblast cell culture**

hMSCs were isolated as described by Leyva et al [26]. IRB approval from the Ethics Committee of the UANL University Hospital was obtained for the collection of human placenta after written informed patient consent (Registration number MI15-008). For cell sorting, a single suspension of hMSCs (7 × 10⁶) was stained with anti-human specific antibodies CD73-phycocerythrin (PE) (BioLegend; San Diego, CA), CD44-biotin (Serotec; Kidlington, UK) and CD105-APC (BioLegend). The sorted CD44⁺/CD73⁻/CD105⁻ cells were expanded in DMEM (GIBCO, Scotland, UK) supplemented with 10% FBS.

For osteoblastic differentiation, cells were plated at 2 × 10³ cells cm⁻² in osteogenic medium, (DMEM (GIBCO) supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.25 mM ascorbic acid, and 10⁻⁸ M dexamethasone (Sigma-Aldrich)), and maintained for two weeks; the phenotype was confirmed by bone ALP activity assay.

**Cell seeding on scaffolds**

Microspheres of HA-ZrO₂–alginate were prepared in two steps: 0.075 g of natural bone matrix fine powder (NBM) and 0.15 g of HA-ZrO₂ (HZ600, HZ650 or HZ700) were mixed with the osteoblast (1 × 10⁶ cells) resuspended in a solution of 1.2% alginate/DMEM. The mixture was extruded dropwise with a 0.65 mm diameter needle into a CaCl₂ solution (0.102 M). To allow gelation of implants, the mixture was dispersed under stirring for 15 min to create a uniform mixture. As a control scaffold, microspheres were prepared by the same method, but without the addition of zirconium. The implants were incubated with the osteogenic medium in 24 well-plates for 10 days for in vitro maturation, and the medium was changed every three days.

**Assessment of cell proliferation by the Alamar blue (AB) method**

Osteoblasts seeded in the HA-ZrO₂–alginate scaffolds as described in the previous section were grown for nine days at 37 °C and with 5% CO₂. Each day, the culture media was removed and DMEM (GIBCO, Scotland, UK) containing 10% AB (Invitrogen, Carlsbad, CA) was added to the cell culture. After 4 h, the reduction of AB was quantified at 570 nm using an
ELISA reader (iMark Microplate Absorbance Reader, BioRad).

**Scanning electron microscopy**

*In vitro* matured HA-ZrO$_2$–alginate scaffolds (HZ600, HZ650 or HZ700) were fixed in 12.5% glutaraldehyde in phosphate buffer pH 7.2, then washed in the same buffer, and post-fixed by osmium tetroxide 2%. The fixed specimens were dehydrated in graded alcohol solutions. After the cells were dried to a critical point, the samples were sputtered with a 100 nm thick layer of gold using an ion coater and viewed in a JEOL, JSM-6510LV model in a high vacuum.

**Mineralized matrix formation**

To detect calcium deposition, HA-ZrO$_2$–alginate scaffolds (HZ600, HZ650 or HZ700) were incubated with the osteogenic medium in 24-well plates for 18 days. After this, the implants were incubated with 2% Alizarin red S solution adjusted to pH 4.1 for 30 min at room temperature. Mineralization was demonstrated by the presence of red deposits observed through a bright field light microscope (Nikon Model E600; Nikon Corporation, Tokyo, Japan).

**Western blotting**

Western blotting was performed using standard methods. HA-ZrO$_2$–alginate scaffolds (HZ600, HZ650 or HZ700) implants were washed with cold PBS and cells were lysed using RIPA buffer (20 mMTris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate) containing Complete™ (ROCHE, Molecular Biochemicals, Mannheim, Germany). To ensure efficient protein extraction from the HA-ZrO$_2$–alginate scaffolds, the samples were sonicated for 30 s (Fisher Scientific Model 120). Lysates were centrifuged at 14 000 rpm for 15 min and the proteins in the supernatant were analyzed. Immunoblotting was performed with one of the following antibodies: rabbit anti-human COL1, anti-human Osteopontin (both from Santa Cruz Biotechnology, CA) and rabbit anti-human actin (Abcam, Cambridge, UK).

**Statistical analysis**

Data were analyzed with SPSS 13.0 statistical software and the results presented as means ± standard deviation. ANOVA was applied and differences with a p-value < 0.05 were considered significant.

**Results**

**Thermogravimetric analyses of HZ600, HZ650 and HZ700 composites**

Figure 1 shows the HZ600, HZ650, and HZ700 thermo-gravimetric plots. Plot 1A shows the HZ600 sample in which endothermic peaks at 376.21 K correspond to water evaporation. Moreover, the typical martensitic zirconium transformation at 439.64 K is also shown. The inflection curve at 705.33 K has a direct relationship with a monoclinic zirconium formation. The negative slope around 792.81 K is characteristic of water evaporation at high temperature. The same thermogram shows an exothermic peak at 741.47 K that corresponds to a tetragonal zirconium formation. Figure 1(B) shows the HZ650 annealing graph with endothermic peaks at 368.06, 434.58, 707.45 and 788 K. These peaks represent features such as the characteristic to martensitic zirconium transformation, those related to monoclinic zirconium formation and also those related to the high temperature of water evaporation. The following exothermic peaks are also depicted: at 304.87 K HA decomposition into octa-calcium-phosphate (OCP); at 656 K characteristic amorphous calcium-phosphate (ACP); and at 728 and 749 K, tetragonal zirconium formation. Figure 1(C) shows the HZ700 thermal analysis. In this, the endothermic peaks at 459.70 and 788.75 K are detected; these correspond to monoclinic zirconium formation, due to the evaporation of water at high temperature. The exothermic peaks shown within plot 1c at 344.13 K correspond to HA decomposition.

The process at 632.53 K shows characteristic amorphous calcium phosphate crystallization. The peak at 676.30 K is related to dicalcium-phosphate-dihydrate (DCPD) and dicalcium-phosphate-anhydride (DCPA) formation. Finally, at 760.19 K the peak is due to tetragonal zirconium formation. According to those results, it is possible to formulate the following chemical reaction:

\[
\begin{align*}
\text{Zr(OH)}_4 + \text{ZrO}_2\text{(am)} + 3\text{Ca}_4\text{(PO}_4)_6\text{(OH)}_2 & \rightarrow 2\text{ZrO}_2 + \text{Ca}_4\text{H}_2\text{(PO}_4)_6\text{H}_2\text{O} + \text{CaHPO}_4 \\
& + \text{CaHPO}_4 \cdot 2\text{H}_2\text{O} + 2\text{Ca}_4\text{(PO}_4)_6\text{H}_2\text{O} \\
& + 4\text{CaO} + \text{Ca}_4\text{(PO}_4)_6\text{H}_2\text{(OH)}_2.
\end{align*}
\]

For this reaction, the calcium oxide resulting from HA decomposition contributes to stabilize the tetragonal zirconia phase. This reaction also indicates the formation of bioreactive material OCP, ACP, dicalcium phosphate and calcium phosphate, which are able to form HA in physiological conditions.

**Synthesis of HA-ZrO$_2$ by a low-temperature process**

To determine the effect of high temperature (1473 K) on the formation of HA-ZrO$_2$, a full set of new experiments were performed. The following procedure was settled at high temperature, low heat rate (1 K min$^{-1}$) and under inert atmosphere (Ar) conditions in order to verify the range of temperatures at which the reactions occur. The results obtained at 1473 K are summarized in figure 1(D), which shows early endothermic and exothermic peaks. These peaks together correspond to monoclinic and tetragonal zirconium formation as well as HA decomposition in calcium phosphates. It is clear that no chemical
reactions are foreseen over 923 K. In order to support the idea that the formation of HA-ZrO2 is possible at a temperature lower than 1473 K, x-ray diffraction analysis was performed in samples obtained at low temperatures (873, 923 and 973 K). Figure 1(E) shows the DRX plot in which the diffractograms of HZ600, HZ650 and HZ700 are clearly present. In each one of those diffractograms, the characteristic peaks of x-ray diffraction are: HA (22.90, 25.35, 31.77, 34.05, 35.48, 39.82, 40.45, 45.31, 46.71, 48.10, 48.62, 53.14, 63.01, 64.07, 66.38, 77.17, 78.22), monoclinic zirconia (24.05, 28.17, 54.06, 77.56 and 78.62), tetragonal zirconia (30.26, 50.37, 60.20, 73.46, and 74.53), OCP (31.66 and 37.65) ACP crystalized (40.86, 71.70, and 75.52), DCPD (42.65) and DCPA (22.03, 45.40, and 51.53). These data clearly show that the HZ600, HZ650, and HZ700 obtained correspond to HA-ZrO2, indicating that no high-temperature processing is required.

Proliferation of osteoblast cultured on scaffolds dotted with HZ600, HZ650, and HZ700

The cellular compatibility of the material for tissue engineering applications is demonstrated by its ability...
to support cell attachment, and cell proliferation and differentiation. To assess whether the composites obtained under this new system at low temperature provide an environment that maintains proliferation of osteoblasts, HZ600, HZ650 or HZ700 were mixed with NBM and alginate and then human osteoblasts were embedded into the scaffold to analyze their growth. Osteoblast proliferation on NBM/alginate (control), HZ600, HZ650 and HZ700 scaffolds was assessed using the AB assay. The proliferation rate was evaluated during 9 days. As shown in figure 2, osteoblast cultured on control and also HZ600, HZ650 or HZ700 scaffolds increased with time. A discrete proliferation peak at day 6 and a slight decrease towards day 9 was observed for all of the groups. It is should be noted that the differences observed among the different composites during the analysis showed no significant differences ($P < 0.05$). Cell proliferation was not influenced by any of the composites tested, and no signs of toxicity were observed.

HZ600, HZ650, HZ700 composites analyzed by SEM

Figure 3 shows SEM micrographs of osteoblasts cultured on HA-ZrO$_2$ by 10 days. Non-coated zirconia control ((A) × 1000, (E) × 4000), HZ600 scaffold ((B) × 1000, (F) × 2000), HZ650 scaffold ((C) × 1500, (G) × 3000), HZ700 scaffold ((D) × 1000, (H) × 3000). EDAX spectra shows the chemical composition of the cell surface and the ECM of the area within white square in SEM micrographs; non-coated zirconia control (I), HZ600 (J), HZ650 (K) and HZ700 (L).
mesh was observed. The fibers had a solid, but irregular surface. Fibers were randomly oriented and large interconnected gaps were present (figures 3(C) and (G)). For HZ700, cellularity was scarce and the extracellular matrix was absent, although cells preserved their morphology. While in the HZ650 scaffold osteoblasts covered the surface abundantly, in the HZ700 scaffold, surfaces were inadequately covered (figures 3(D) and (H)).

The formation of calcium phosphate or mineral deposition, is a key function of osteoblasts. In order to explore the surface chemical composition of cells on the different scaffolds, energy-dispersive spectroscopy (EDAX) was determined. This identifies calcium and phosphate-rich particles. The EDAX spectra in figure 3 were taken from the cell surfaces, likewise the scaffold matrices (indicated with white square frames in figures 3(E)–(H)) are shown in the SEM images. Interestingly, the EDAX cell surface spectra of osteoblasts grown on the HZ650 sample showed extracellular matrix rich in calcium and phosphate after the 15th day in culture. In contrast, cells on the HZ600 and HZ700 scaffolds showed no signs of phosphate or calcium.

Expression of osteoblast maturation markers and mineralization

To assess whether the enrichment of scaffolds with the HZ600, HZ650 or HZ700 improved osteoinductive properties, markers associated with the formation of ECM as type 1 collagen (COL1) and osteopontin, were evaluated. After osteogenic induction by 14 days, the expression of type I collagen for HZ600 and HZ700 was lower compared to the control without zirconia. In contrast, a 2-fold increase in collagen expression ($P < 0.05$) was observed for HZ650 enriched scaffold as compared with HZ600, HZ700, and the control (figures 4(A) and (C)). Since osteopontin is one of the main proteins involved in calcium fixation, its expression was also analyzed. The trend in osteopontin expression among composites was very similar and no significant differences were found (figures 4(B) and (D)).

Calcium accumulation in the extracellular space and its organization into hydroxyapatite crystals are an important marker of osteoblast maturation. Calcium deposition analyzed by alizarin red staining showed that the osteoblasts cultured for 18 days on alginate scaffold control, HZ600, HZ650 or HZ700 scaffolds produced red stains of scattered calcium deposits around the cell clusters; however, interestingly, the HZ650 scaffold showed a more intense and uniform staining. This mineralization data agrees with the results from the EDAX analysis showed before (figure 4(E)).

Discussion

The process of densification or sintering of HA usually involves pressure-less or hot-pressing. Pressure-less sintering usually requires temperatures above 1473 K [27–29]; moreover, HA–ZrO$_2$ powders require sintering treatment over 1773 K [20, 30].

Since sintering HA at high temperatures can result in the formation of oxyhydroxyapatite and decomposition into tetracalcium phosphate (TTCP), which can further degrade to tri-calcium phosphate (TCP) [31, 32] both of which are unfavorable for cellular response, strategies involving temperatures below 1273 K result in suitable compounds for biological applications.

Curran et al in 2010 described a method based on the use of microwaves for the sintering process of HA–ZrO$_2$; with this strategy a decrease to 700 °C (973 K) was reached obtaining powders of HA–ZrO$_2$ dispersed homogeneously at the nanoscale; however, this was insufficient to produce densified bodies. Under this method, a sintering temperature of 1200 °C (1473 K) produces dense bodies with open porosity sufficient for biological applications; nonetheless, biological tests were missing [33].

In this paper, HA–ZrO$_2$ was synthesized at 973 K, 923 K and 873 K to produce HZ700, HZ650 and HZ600, respectively. Significantly, with this method, the formation of bioactive material, such as OCP, ACP, dicalcium phosphate and calcium phosphate, which are able to form HA and promote mineralization in physiological conditions, was evidenced [34].

According to our data, we obtained tetragonal and monoclinic zirconia phases stabilized with CaO at low temperatures. Also, HA was decomposed into other biocompatible calcium phosphates; this statement is supported by other authors. Zong et al analyzed the thermal behavior of Zr(OH)$_4$ obtained from ZrOCl$_2$ hydrolysis at low temperature. Endothermic peaks at 361 K and 433 K were attributed to water evaporation and martensitic zirconia. Peaks at 713 K and 733 K were related to monoclinic zirconia and tetragonal zirconia formation, respectively [35]. Likewise, Spalski et al performed the thermal analysis of HA and OCP without ZrO$_2$ and established differences between HA and OCP [36].

Slepko et al [37] analyzed thermally HA as a reference material. They showed that HA decomposes exothermally at 303 and 633 K, forming calcium phosphate precipitates. On the other hand, Miller et al reported that DCPA is obtained at 567 K as a product of DCPD dehydration [38]. Lin et al demonstrated that it is necessary to generate a basic environment with hydroxides or air in order to prevent HA decomposition into calcium phosphates [39]. HA decomposition at low temperature is possible due to the excess of OH– ions from Zr(OH)$_4$ and HA. During thermal annealing, the powders dehydrate leading to ZrO$_2$ crystallization and the decomposition of HA into calcium phosphates from less than 348 K [36]. This process is facilitated by the formation of an acidic environment in the powder during thermal annealing.
The influence of the acidic environment in HA-ZrO₂ formation has been recognized by other researchers. For instance, Curran et al. reported that it is possible to stabilize zirconia with calcium oxide at low temperature (599–699 K) [33]. Vasanthavel et al. obtained Zr(OH)₄ powder from ZrOCl₂ hydrolysis [40]. In this case, the powder potassium phosphates and thermal annealed during 1 h and as a result, tetragonal zirconia was obtained. Giocondi et al. obtained HA decomposition in OCP, ACP, DCPD y DCPA under similar acidic conditions [41].

Zirconium oxide exists in three polymorphic forms: monoclinic, tetragonal and cubic; m-ZrO₂ being the thermodynamically most stable phase at room temperature [20]. On the other hand, the tetragonal phase t-ZrO₂, used at high temperature has high hardness and wear resistance. Due to their applications zirconia can be stabilized at room temperature, as mentioned above, by doping with small amounts of stabilizing oxides. Monoclinic zirconium dioxide, by reversible crystalline inversion, is converted into a tetragonal variety at 1443 K [42]. The stabilization of the tetragonal phase at low temperatures can be achieved through different strategies and one of them lies in the decrease in particle size. It has been shown that in nanoparticle with average sizes in the range of 5–16 nm, the tetragonal phase conserved at room temperature and in some cases only traces of the monoclinic phase can be present [22]. Through the procedure described here, it is possible to fabricate
HA-ZrO₂ with a particle size of 8 nm as a result of a low temperature sintering process [43], contributing to the stabilization of the tetragonal phase.

The homogeneous and nanostructured tetragonal structure mainly obtained in HZ650 generates on the scaffold, the arrangement of a more stable reticular surface compared to monoclinic zirconia predominant in HZ600. Since cells are inherently sensitive to their supporting substrate [44] this arrangement could positively influence cell adhesion. This behavior is evidenced by SEM analysis, where we could observe a suitable number of cells on the stable tetragonal structure of zirconia; even the presence of filopods was evidenced, which are a signal of an adequate adhesive process (figure 3(G)).

Song et al demonstrate that the topography is able to promote osteoblastic differentiation and mineralization in the MSCs [45]. In this line of thought, the expression of type I collagen observed by Western blot was 2-fold higher in HZ650 compared to HZ600 and HZ700, in response to better cell attachment (figures 4(A) and (C)).

The last step of ECM mineralization depends on the presence of collagen [46]. The increase in the expression of type I collagen observed in HZ650 is in agreement with the fact that the HA sintered at 923 °K (condition for HZ650) does not decompose; those events create synergy to result in a better mineralization process. The controlled expression of type I collagen in HZ650 directly promotes the deposition of new HA in the bone matrix and therefore, the mineralization process becomes more efficient (figure 4(E–g)).

By contrast, as observed by SEM, the adhesion of pre-osteoblasts to scaffolds constructed from HZ600 and HZ700 is less efficient and consequently the maturation process is shifted. This also agrees with type I collagen expression data. Regarding the integrity of the HA, HZ700 presents a decomposed HA due to the high sintering temperature (973 °K); concerning HZ600, even though the HA remains functional, the zirconia report a transformation from the tetragonal to the monoclinic phase. It should be noted that the monoclinic phase generates a poorly stable network unable to support cell adhesion.

Although numerous biocompatible materials have been identified as suitable fillers for bone defects, the long-term effectiveness of these materials in vivo may depend on their ability to stimulate favorable host responses, such as proliferation, and osteoblast maturation.

Pae et al studied the effects of zirconia coated with calcium phosphate and HA compared to smooth zirconia after bone marrow-derived osteoblast culture. No significant difference between smooth and surface-coated zirconia was found in the MTT assay [47]. Our results are in line with Pae’s results since the addition of zirconia has no effect on the rate of proliferation of osteoblasts cultured on the scaffolds (P > .05), independently from the zirconia materials synthesized at different temperatures (figure 2).

Osteoblast cell maturation is characterized by differential expression of osteogenic protein markers during the process [48, 49]. According to human osteoblast differentiation models, COL1 and ALP are early differentiation markers in the osteoblast lineage. Transcription of mRNAs for these protein markers is high in pre-osteoblasts and decreases during osteoblast maturation. The on the other hand, OPN is a late differentiation marker and its mRNA is initially expressed at very low levels, but its transcription is enhanced in later differentiation stages [48, 49].

By SEM analysis, HZ650 sample showed the formation of a dense network of collagen fibers (figure 3), which was also confirmed by Western blot assays (figures 4(A) and (C)). Since the maturation stage of osteoblastic differentiation is marked by collagen accumulation in the extracellular matrix, the ability of HZ650 to promote osteoblast maturation was remarkable.

OPN is a phosphoprotein that mediates integrin binding and has phosphorylated serine residues that bind calcium phosphate crystals to modulate crystal growth. OPN expression was significantly increased in the HZ650 matrix, compared to HZ600 and HZ700. The similar OPN levels in all tested materials suggest that expression of this phenotypic marker is modestly sensitive to the zirconia properties.

Since calcium and phosphate ions are regulators of in vitro osteoblastic differentiation and mineralization [50–52], ceramic scaffold material capable of releasing these ions may also provide suitable osteoinductive properties for accelerating bone healing. According to the EDAX analysis, HZ650 showed high levels of calcium and phosphate. This confirms that HZ650 promotes better conditions for maturation and bone mineralization.

This study indicates that HA-ZrO₂, synthesized at 923 K (HZ650) contributes to generating a micro-environment that promotes osteoblastic mineralization and maturation since increases in COL1 expression and mineralization were evidenced (figures 4(A) and (C)).

Our findings demonstrate that the concentration of Ca²⁺ and P released from the coatings was higher in the HZ650 than in HZ600 and HZ700 composites. This implies that the temperature of synthesis influences adequate stabilization of nodules of mineralization.

Moreover, we demonstrated that tetragonal zirconium synthesized at low temperature (at 923 K) doped with hydroxyapatite is able to enhance the osteoblastic process and therefore, HZ650 might be used as a new bioactive material for bone regeneration applications.

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