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Development and Characterization of 5% mol Zn Bioceramic in Granular Form

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Hydroxyapatite (HA) is capable of accepting substitute ions within its lattice, including zinc ions. Zinc is a trace element that activates the osteogenesis of osteoblastic cells and therefore plays an important role in the activity of alkaline phosphatase enzyme. The purpose of this work was to produce and characterize 5% mol Zn bioceramic in granular form (Zn-granules) for clinical applications and compare it with granules made from HA by using the same production route. Granules with addition of porogen agents were produced from powders of HA and zinc-containing HA by uniaxial pressing and heat treatment. The granules were subsequently ground and sieved. The results indicated that zinc contributed to the reduction of sample crystallinity and formed a biphasic structure after calcination at 1200 °C. Additionally, zinc release from granular material may have clinical applications as bone graft.

Keywords: *hydroxyapatite, zinc, granules, bone graft*

1. Introduction

The need to substitute lost body structures sparked interest in materials capable of integrating with the human body. Such materials should be able of mimetizing lost structures, to be biocompatible and should not be antigenic nor carcinogenic[1]. Hydroxyapatite (HA) is one of the most popular biomaterials as it fulfills these conditions. The use of synthetic HA [$Ca_{10}(PO_4)_6(OH)_2$] in granular form has found increasing applications in dentistry and medicine in the last few decades[2,3].

The mineral part of bone is composed of carbonated and non-stoichiometric HA structure containing substitutions of Na^+, K^+, Mg^{2+}, Sr^{2+}, Cl^-, F^-, HPO_4^{2-} ions originated in the extracellular matrix-mediated process of mineralization found in *in vivo* body fluid medium. Moreover, c-axes of HA crystals are parallel to the long axis of Type I collagen fibrils, which reveals the interplay of biomaterial and tissue. On the other hand, *in vitro* experiments using simulated body fluid revealed the presence of Na^+ and Mg^{2+} ions on the biomimetic coatings of titanium after a two-week soaking exposure, rather similar to the biological apatites of human bones[2,4]. These HA substitutions offer the possibility of using hydroxyapatite as a means to transfer essential ions directly to where is needed.

Zinc may activate the osteogenesis of osteoblastic cells, and it participates in the activity of more than 300 types of enzymes, including alkaline phosphatase[5,6]. These enzymes are essential because they take part in bone metabolism. It would therefore be desirable to obtain a hydroxyapatite with a certain amount of zinc so as to enhance several biochemical processes such as osteogenesis and increase the production of hundreds of enzymes.

HA powders can be partially decomposed into tricalcium phosphate (TCP) by heat treatment[7]. In addition, the calcination temperature and stoichiometry of hydroxyapatite affect HA→TCP transformation[8]. This transformation can be useful for the development of materials with biphasic structure and a degradation rate between HA and TCP[9]. Zinc seems to decrease the thermal stability of powder HA and, indeed, zinc-containing a TCP phase was identified in ZnHA in powder form calcined at temperatures higher than 800 °C[10]. Regarding the solubility of these phases, the literature shows that TCP is more soluble than HA when immersed in Tris-buffer (tris(hydroxymethyl)aminomethane)/HCl medium[11], and ZnTCP powder is more soluble than ZnHA in milliqui water[9].

Zinc plays an important role in proliferative effects on osteoblastic cells apart from inhibiting osteoclastic resorption. Zinc-substituted calcium phosphates has thus received considerable attention. In addition, hydroxyapatite solubilizes high fractions of Zn, approximately 15%mol[10], which is a necessary condition for delivering efficacy. The possibilities of increasing Zn releasing rate by controling the surface area and/or phase transformation are a promising means to achieve such purposes. However, clinical applications demand materials in blocks or granular form. Thus, the aim of this paper is to produce a Zn-containing bioceramic in granular form (Zn-granules) and compare its physicochemical properties and *in vitro* degradation with those of granular material produced from stoichiometric hydroxyapatite (HA-granules).

2. Materials and Methods

Hydroxyapatite (HA) and HA with 5% mol of zinc (ZnHA) powders were prepared by the precipitation method, according to a procedure described elsewhere[9]. In order to produce porous tablets, 1.000 g of each powder was mixed with 40 wt. (%) of porogen agent followed by uniaxial cold pressing at 27.8 MPa and calcination treatments at 400 and 1200 °C. The samples calcined at 400 °C were kept

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for 2 hours at this temperature (heating rate was 1 °C min^{-1}) followed by furnace cooling. Part of the samples calcined at 400 °C were maintained for 1 hour at 1200 °C (heating rate from 400 to 1200 °C was 20 °C min^{-1}) and also furnace cooled.

The porogen agent was pearl-shaped stearic acid of analytical grade (Vetec) with the size of powder ranging from 0.25 to 1.50 mm. The calcined tablets were ground and sieved to obtain HA-granules and Zn-granules within the 250–1,000 µm range. The porous structure facilitates the grinding and sieving step and the pores remaining in the granules can increase the surface area.

The physicochemical characterization of the calcined samples (HA-granules and Zn-granules) included X ray diffractometry (XRD), Fourier-transform infrared spectrometry (FT-IR), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For XRD, TEM and FT-IR characterizations, the granular calcined material was ground and sieved in order to obtain a homogeneous powder.

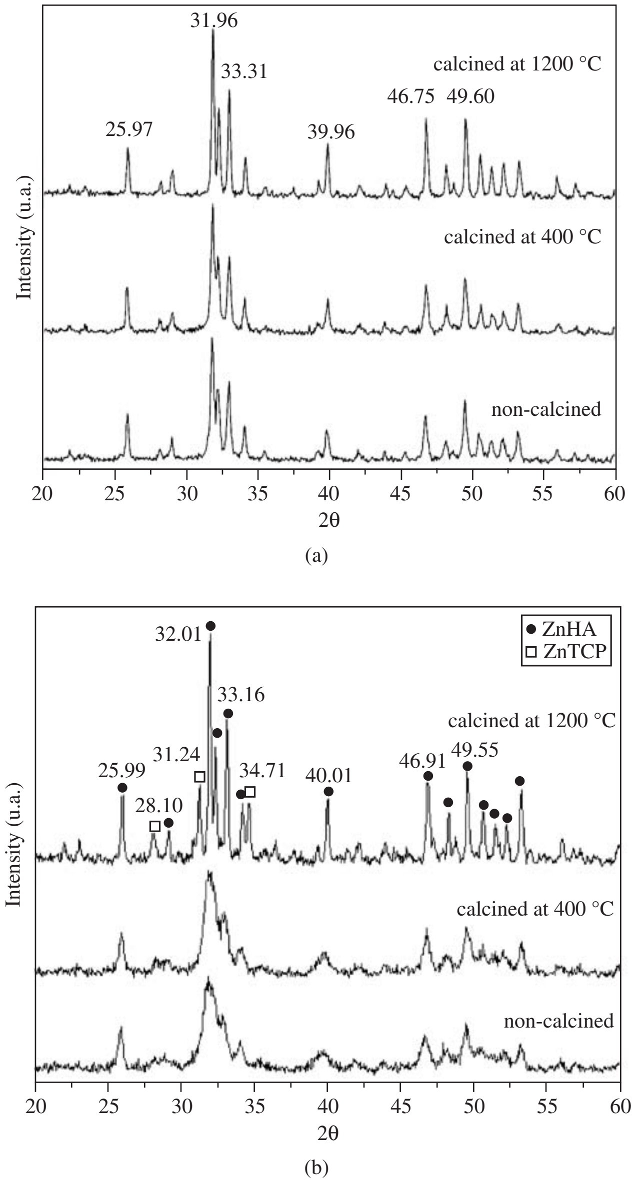
The crystalline structure of the samples was investigated by conventional XRD (Rigaku Miniflex) operating at 30 KV and 15 mA with CuKα radiation, and the data were acquired using the double angle range 10-100°. A diffusion reflectance FT-IR spectrometer (ABB Bomem Inc.) was used to identify the vibration modes of species such as phosphate, carbonate and O-H bonds of granules. The spectra were collected at room temperature at a nominal resolution of 4.00 cm^{-1} with number of scans equal to 100. The spectra were recorded from 400 to 4000 cm^{-1}. A scanning electron microscope (ZEISS DSM 940A) was employed to image the topography and porosity of granules.

The samples calcined at 1200 °C were further examined by using a transmission electron microscope (TEM, JEOL 2000 FX), operating at 200 kV. The samples analyzed by TEM were prepared by crushing small quantities of granular material, dispersed in ethanol and subsequently dropped on a carbon-coated copper grid.

A degradation test of the calcined samples at 1200 °C was carried out in accordance to the ISO 10993-9 standard. The samples of 1.000 g of HA-granules and Zn-granules were immersed in 20 mL of Tris-buffer (tris(hydroxymethyl)aminomethane)/HCl solution in an isothermal bath at 30 °C for 120 hours, under gentle shaking. According to the ISO standard, the pH of degradation medium was maintained at 7.4 ± 0.1. The experiments were run in triplicate and the concentrations of calcium and zinc in solution were measured by atomic absorption spectrometry (AA). This technique shows a limit of detection (approximately µg/L) suitable for these experiments[18]. The differences in the results from both types of samples were analyzed by the statistic ANOVA method.

3. Results and Discussion

Figures 1a and 1b show the diffraction patterns of the granules produced from HA and Zn-HA powders before and after calcination at 400 and 1200 °C, respectively. The XRD pattern of non-calcined HA-granules showed good correlation with stoichiometric hydroxyapatite (JCPDS 09-0432 card), with a small shift to higher angles, probably due to zinc substitution. Apart from hydroxyapatite, no other phases were identified, even after calcination at 1200 °C. In fact, the calcination temperature had little effect on crystallinity. On the basis of the great similarity between the standard pattern and the experimental results, we suggest a Ca/P ratio close to 1.67 and high chemical purity for these samples.



(a)

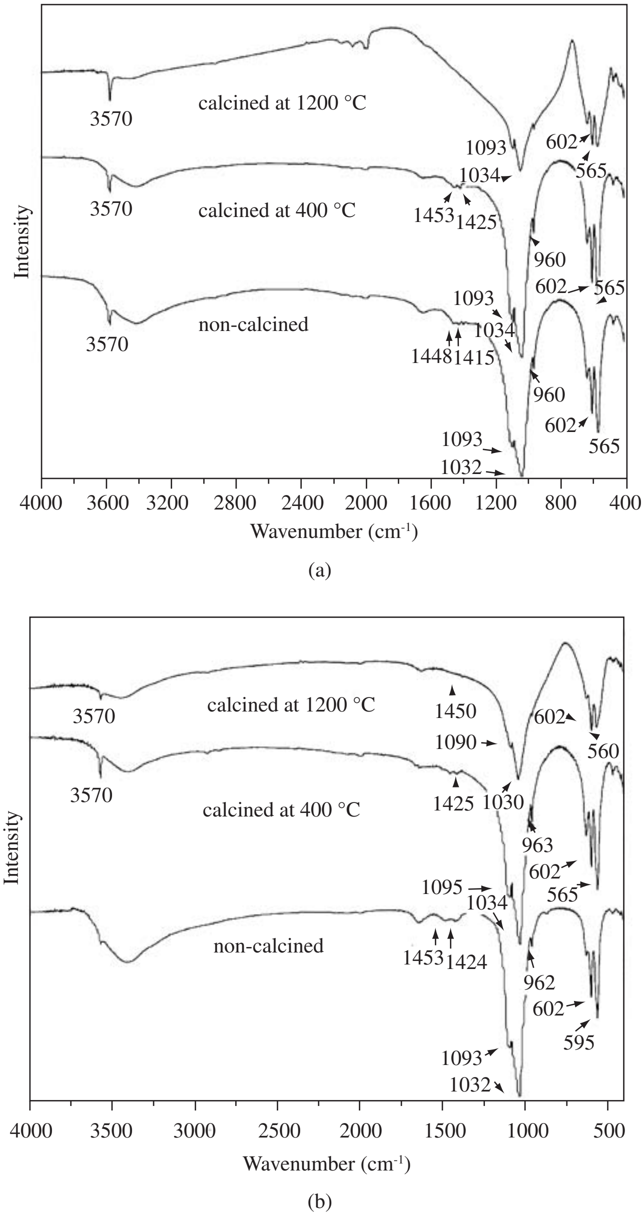
(b)

Figure 1. X ray diffraction patterns of samples with and without calcination: a) HA-granules; and b) Zn-granules.

The non-calcined Zn-granules were characterized as low-crystalline hydroxyapatite since the diffraction peaks have low intensity and are relatively broad. In this case, zinc affects the structure when it partially replaces calcium in the hydroxyapatite crystal sites. Zinc substitution reduces markedly the crystallite size[9,10] and causes, therefore, a reduction of cristallinity. As expected, heating at 400 °C did not change significantly the structure of the crystals nor altered XRD pattern. Therefore, a second phase was not observed. However, with calcination at 1200 °C, two phases were identified: a Zn-containing HA (JCPDS 09-0432 card) and a Zn-containing β-TCP (tricalcium phosphate, JCPDS 09-0169 card). Comparing the divalent ion substitutions of Mg^{2+} and Ca^{2+} in apatite we believed that the heat-treatment above 700 °C produces the same biphasic mixtures[23]. In the present study they are ZnHA and ZnTCp, as shown in Figure 1b. The presence of zinc in the hydroxyapatite lattice affects its stability, inducing HA thermal decomposition. Indeed, this phenomenon is observed for hydroxyapatite with carbonate substitution or for those calcium deficient when calcined above approximately 1000 °C[8]. According to Miyaji et al.[10], the diffraction peaks of β-TCP shifted to higher angles compared with those present in JCPDS data.

As for infrared spectroscopy, the spectra of HA-granules and Zn-granules (Figure 2a and 2b, respectively) present similar bands, though, with different relative intensities. These results can be attributed to small variations in the sample surface compositions. The bands of OH^- absorption characteristics of HA[9] at 3570 and 633 cm^{-1} and bands at 1093[22], 1034[20], 960[21], 602[20], 565[20] and 475[20] cm^{-1} corresponding to PO_4^{3-} groups were identified. Small peaks related to C-O vibration bands of CO_3^{2-} groups[20] at 1414-1450 cm^{-1} suggest that some carbonate was incorporated during the low temperature HA processing. When the samples were calcined at 1200 °C, these vibration bands disappeared. For the Zn-granules treated at 1200 °C, we did not clearly identify β-tricalcium phosphate (β-TCP) bands, albeit they were revealed by XRD. A possible reason for that could be the sample region covered by each technique, i.e., XRD is a bulk technique whereas diffusion reflectance FT-IR obtains information especially from the surface of samples.

Figure 3 shows SEM images of HA-granules and Zn-granules for the two different temperatures of calcination. The presence of



(a)

(b)

Figure 2. Infrared spectroscopy spectra: a) HA-granules; and b) Zn-granules.

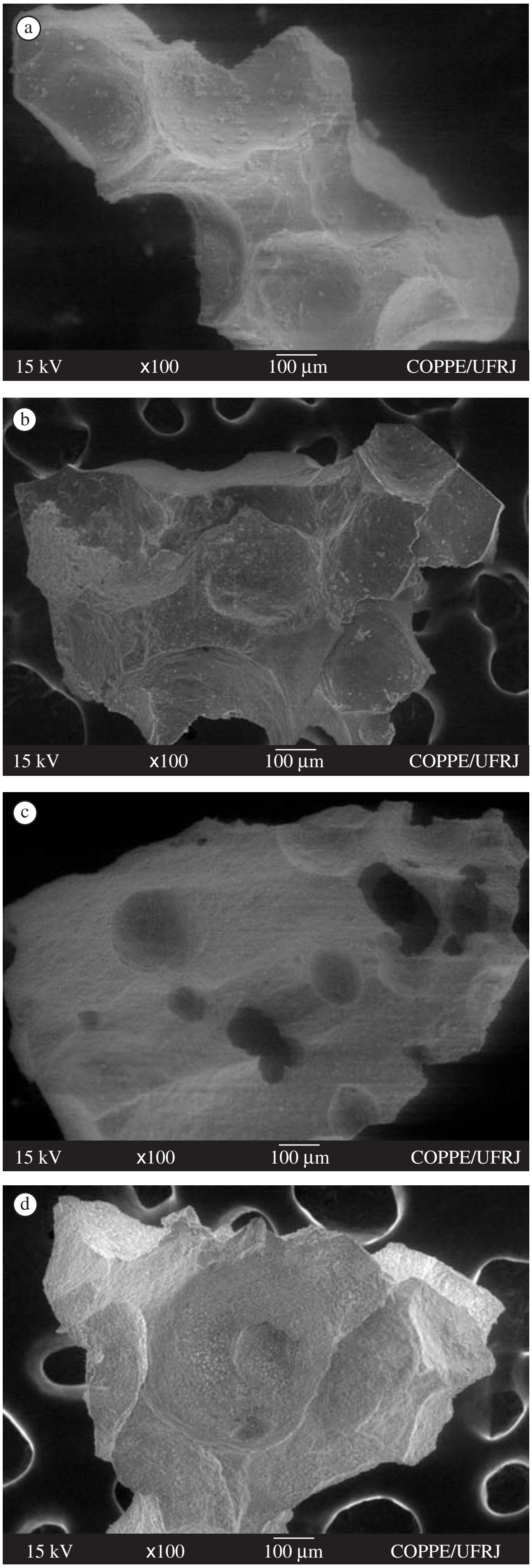


Figure 3. SEM images for two temperatures of calcination: a) HA-granules, 400 °C; b) HA-granules, 1200 °C; c) Zn-granules, 400 °C; and d) Zn-granules, 1200 °C.

superficial concavities due to the porogen agent can be observed. This morphology can be attributed to the size of the granules, which was close to that of porous. Moreover, no significant morphological differences between both granules were observed from SEM images. However, it is worth noting that the material calcined at 400 °C exhibited low mechanical integrity when evaluated by simple finger touch and was, therefore, discarded before the degradation test. This high brittleness precludes it from being used for *in vivo* application. Therefore, it was not tested in the degradation solution.

Figure 4 shows TEM micrography for Zn-granules calcined at 1200 °C and the correspondent electron diffraction pattern. The TCP particles exhibit a massive morphology whose particles are bigger than the usual needle-like HA crystals[12]. The diffraction lattices depicted in the inset of Figure 4 highligths the crystallinity of TCP phase, as already detected by XRD analysis. In an unpublished work we have identified zinc in both hydroxyapatite and TCP crystals.

The solution composition of calcium and zinc content after the 120 h degradation test is shown in Table 1. The average Ca content for HA-granules is 17.1 mg/L and for Zn-granules is 12.7 mg/L. Significant differences ($p < 0.05$) among calcium concentrations were observed for both set of samples. The reduction of calcium content after dissolution of Zn-granules can be associated with the competition between calcium and zinc to be dissolved.

Zinc replaces calcium in the hydroxyapatite crystal lattice and affects a- and c-axis unit cell parameters of hydroxyapatite due to difference of ionic radii[10]. The radius of Ca is 0.099 nm and for Zn, 0.074 nm, and zinc presence steadily reduces c-axis. For a-axis, there is a decrease up to 5% mol Zn, and above this fraction the a-axis size increases. In the present study, the choice of 5% mol was made to obtain a bioceramic material of small crystal size and consequently large surface area[9], which accelerates surface reactions, such as those occurring in the degradation test.

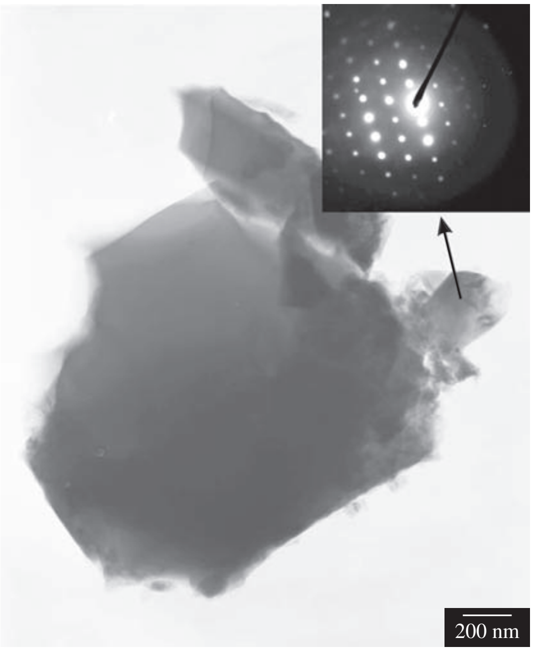


Figure 4. TEM micrography of Zn-granules calcined at 1200 °C. Inset: electron diffraction pattern.

Table 1. Chemical analysis of solution after the degradation test. Three samples were used for each condition.

	Ca (mg/L)	Zn (mg/L)
HA-granules	17.1 ± 0.6	-
Zn-granules	12.7 ± 1.1	40.1 ± 7.6

The conjoint effect of double dissolution (Ca and Zn) results in bigger averaged dissolution of Zn-granules than of HA-granules. This result is consistent with data available in the literature[11,13]. This behavior encourages the use of synthesized Zn-granules as an effective zinc deliver agent. On the other hand, the types of dissolution media play an important role because they can promote a selective dissolution of atoms. In this sense, two standardized media[19], pure water[9] or Tris (present work), have different effects on apatite dissolution. The Tris is present in high concentration (1 $molL^{-1}$), approximately 20 times its content in simulated body fluid[17]. Therefore it is relevant to know the values of the stability constant of $M(Tris)^{2+}$ in aqueous media. Fisher et al.[14] reported $\log K_{ML}^{M}$ as 1.94 for $Zn(Tris)^{2+}$ and less than 0.7 for $Ca(Tris)^{2+}$. In this equation M stands for the metal and L for the ligand (Tris). Hence, Zn complex is more stable than that of Ca, and this difference could explain the high Zn content depicted in Table 1 for Zn-granules. The AA technique quantifies the element concentration in solution regardless of its presence as a metallic ion or complex, i.e, Ca^{2+} or $Ca(Tris)^{2+}$. These complexations do not occur when milliqui water is used as degradation medium.

The recommended tests[15] of calcium salts encompass elemental analyses, dissolution/solubility and biocompatibility for characterization of the candidate biomaterials[3]. However, the link between *in vitro* and *in vivo* responses is not straightforward[16,17], as the *in vitro* tests are an inert closed environment. Consequently, before carrying out *in vivo* tests, at least cytotoxicity of Zn-granules must be evaluated, even for bioceramics that show good solubility, such as the Zn-granules studied in the present paper.

4. Conclusions

Zn-granules can be easily produced by cold pressing zinc-containing HA powders followed by heat-treatment and ground-sieving steps. The temperature of calcination was found to increase the crystallinity of the granules especially above 400 °C. The presence of ZnTCP phase in Zn-granules calcined at 1200 °C can contribute to its higher solubility. Moreover, zinc, rather than calcium, dissolves in Tris medium, resulting in zinc release when *in vitro* tested. Thus, the route herein employed seems to be effective to produce biphasic granules with zinc release for clinical applications.

Acknowledgments

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