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Preparation and characterisation of calcium-phosphate porous microspheres with a uniform size for biomedical applications

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Abstract In the present work, a novel route for the preparation of porous ceramic microspheres is described. Two ceramic powders, calcium-titanium-phosphate (CTP) and hydroxyapatite (HAp), were mixed with a sodium alginate solution that enabled the preparation of spherical particles, using the droplet extrusion method combined with ionotropic gelation in the presence of Ca^{2+}. The spherical particles were subsequently sintered, to burn-off the polymer and obtain calcium-phosphate microspheres with a uniform size and an interconnected porous network. CTP microspheres with diameters ranging from 513 $\pm$ 24 μm to 792 $\pm$ 35 μm and with pores of approximately 40 μm were obtained. HAp microspheres presented diameters of 429 $\pm$ 46 μm and 632 $\pm$ 40 μm and pores of ca. 2 μm. Depending on the formulations tested, the structure of both calcium phosphates may become altered during the sintering process, suggesting that the ratio between the ceramic phase and the polymer solution is a critical parameter. Porous microspheres prepared using the described methodology are promising candidates as bone defect fillers and scaffolds for bone tissue regeneration.

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1. Introduction

Due to the large number of orthopaedic surgical interventions performed each year, bone repair has become a subject of intensive investigation. The use of calcium phosphate ceramics in bone regeneration, either alone or in combination with a polymeric phase, has become a common practice, since these materials generally provide good biological responses and adequate mechanical properties. Although calcium phosphate ceramics have traditionally been used as dense or porous three-dimensional scaffolds, increasing efforts have been devoted to the development of injectable formulations for bone defects filling applications, since they can be applied through minimally invasive techniques [1]. Most injectable ceramic materials described in the literature consist of micro or nanoparticles suspended in appropriate vehicles [2–7]. In such systems, the shape of the microparticles determines their packing characteristics, spherical particles being more suitable for implantation than non-homogeneous granules, since they conform better to irregular implant sites. Moreover, uniform particles present more predictable flowing properties during injection [8–11].

In certain applications, the effectiveness of these microparticulate materials can be highly improved if they can act simultaneously as carriers for biologically active molecules. In this sense, porous materials are advantageous, since they present additional surface area, an important parameter that strongly influences the loading capacity and release rates that can be obtained.

In the present work, novel ceramic porous microspheres are proposed, which could find applicability in the field of bone regeneration, both as injectable bone-filling materials and drug delivery matrices. Two ceramic powders, calcium-titanium-phosphate (CTP) and

hydroxyapatite (HAp), were tested. CTP, a bioactive ceramic currently under investigation in our laboratory [12], is a potential material to be used in the biomedical field as it presents interesting properties, namely the ability to act as an immobilization matrix for several enzymes [13–16]. HAp, which possesses a chemical structure similar to bone mineral, has long been recognized in the biomedical field for its osteoconductivity and capacity to act as matrix for drug delivery [17–18] and was used as a reference material. Alginate was chosen due to its suitable rheology and ability to form relatively stable hydrogels under mild conditions [19].

CTP-alginate and HAp-alginate microspheres were first prepared using the droplet extrusion method as described in a previous work [16]. The ceramic powders were mixed with alginate, which enables the preparation of spherical particles through instantaneous crosslinking in the presence of Ca^{2+} [16]. The obtained microspheres were then dried and subsequently sintered to burn-off the polymer and aggregate the ceramic granules, allowing the preparation of ceramic microspheres with a uniform size and interconnected porous network.

Compared to other methods of preparing ceramic microspheres described in the literature [8–11], this novel process presents the advantage of simplicity and of being carried out in the absence of organic solvents or oils, thus enabling the recovery of the spherical particles without the need of fastidious washing processes. Moreover, the obtained microspheres present a regular size distribution, even without a subsequent fractionation by sieving.

In this study, the preparation and physical-chemical characterisation of CTP and HAp microspheres prepared according to the described methodology are presented.

2. Materials and methods

2.1. Characterisation of the ceramic powders

CTP was synthesised by solid-state reaction as described elsewhere [12]. HAp was kindly donated by CAM Implants. Both ceramics were analysed by X-ray diffraction (XRD, Philips PW 1710 diffractometer), and their granulometry characterised using a laser scanner particle size analyser (Coulter Electronics Incorporation). The specific surface area of each powder was measured by gas adsorption according to the BET (Brunauer, Emmel and Teller) method. Both powders were observed by scanning electron microscopy (SEM). Samples were sputter coated with gold using a JEOL JFC-100 fine coat ion sputter device, and observed using a JEOL JSM-6301F scanning microscope, an accelerating voltage of 15 kV and an working distance of 15 mm.

2.2. Preparation of CTP and HAp microspheres

Ceramic powders were dispersed in a pre-filtered (0.8 μm) 3% w/v sodium alginate solution (Pronova Biopolymers) under gentle stirring until a homogeneous paste was obtained. Different ceramic-to-polymer solution ratios (0.1, 0.2 and 0.4) were tested. These will be designated as 10/3, 20/3 and 40/3 (different ceramic-to-polymer solution ratios, using a 3% w/v sodium alginate solution). Preliminary assays were conducted to determine the maximum ceramic-to-polymer solution ratio and it was observed that for ratios higher than 0.4 the pastes obtained became too viscous to be handled and extruded. The pastes were extruded drop-wise into a 0.1 M $CaCl_2$ crosslinking solution, where spherical-shaped particles instantaneously formed and were allowed to harden for 30 min. The size of the microspheres was controlled by regulating the extrusion flow rate using a syringe pump (Cole Parmer) and by applying a coaxial air stream (Encapsulation Unit Var J1– Nisco). At completion of the gelling period, microspheres were recovered and rinsed in water in order to remove the excess $CaCl_2$. Finally, they were dried overnight in a vacuum-oven at 30°C, and then sintered at 1100°C with a uniform heating rate of 5°C/min, and a 1h stage at the maximum temperature.

2.3. Characterisation of the microspheres

The diameter of the microspheres ($n = 20$) was measured using an inverted plate microscope (Olympus) equipped with an ocular micrometer with an accuracy of 10 μm.

Morphological and physico-chemical characterisation was carried out using SEM and Fourier transform infrared spectroscopy (FT-IR). For FT-IR analysis, microspheres were reduced to powder and analysed as KBr pellets using a Perkin Elmer System 2000 spectrometer.

In order to identify the composition of the residue that results from burning the polymer during sintering, calcium alginate microspheres, produced as described, were submitted to the same heating cycle used for preparing CTP and HAp microspheres. The obtained residue was analysed by XRD and FT-IR.

3. Results

3.1. Characterisation of the ceramic powders

XRD of the ceramics indicated the presence of monophase crystalline compounds. Granulometric analysis of the powders revealed that 90% of the CTP particles are smaller than 25.32 μm, with a volume average diameter of 11.00 μm, and that 90% of the HAp particles are smaller than 20.51 μm, with a volume average diameter of 7.96 μm. How-

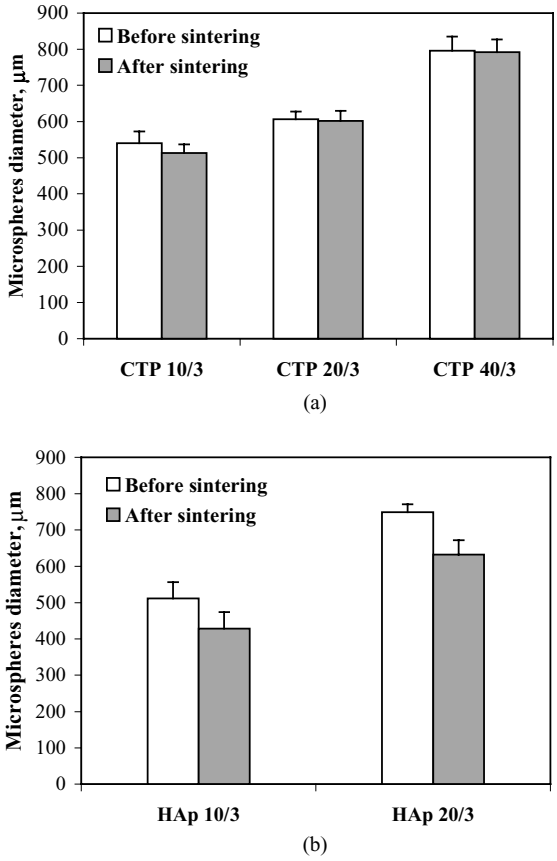

Fig. 1 Diameters of CTP (a) and HAp microspheres before and after sintering.

ever, surface area measurements indicated that HAp particles have a much higher surface area (76 cm^2/mg) than CTP (9.8 cm^2/mg) suggesting that the results obtained in the HAp granulometric analysis are overestimated, probably due to particle aggregation. Both powders were observed by SEM, which confirmed that CTP particles are in fact much larger than the HAp particles (data not shown).

3.2. Characterisation of CTP and HAp microspheres

To produce CTP and HAp microspheres, CTP-alginate and HAp-alginate mixtures of different compositions were prepared and drop-wise extruded into a 0.1 M $CaCl_2$ solution. As soon as the ceramic-polymer droplets contacted with the crosslinking bath, spherical particles of approximately 1000 μm were instantaneously formed, due to the rapid establishment of calcium-mediated junctions between polyguluronate chains on the polymer backbone [18]. An exception was observed for the 40/3 HAp formulation, with which it was not possible to obtain spherical particles due to the high viscosity of the ceramic-polymer mixture.

During drying, the volume contractions varied with the formulation as shown in Fig. 1 which presents the diameters of dried CTP and HAp microspheres, that were obtained before (ceramic-polymer microspheres) and after sintering (ceramic microspheres).

It can be concluded that the higher the ceramic-to-polymer solution ratio used, the lower the particles contraction during drying. Upon sintering, microspheres maintained their original spherical shape. In the case of the HAp microspheres their diameter further decreased approximately 20%, while CTP microspheres practically maintained their original dimensions. Such differences are probably related to the different particle size distributions and, in particular, to the higher percentage of fine particles in HAp that results in a more effective packing during sintering. With the described methodology, microspheres with a regular size distribution were obtained without a subsequent fractioning by sieving being necessary.

Microspheres with average final diameters of 513 $\pm$ 24 μm, 602 $\pm$ 28 μm, and 792 $\pm$ 35 μm were obtained for the CTP 10/3, 20/3 and 40/3 formulations, respectively; while with HAp microspheres diameters of 429 $\pm$ 46 μm and 632 $\pm$ 40 μm were obtained for the 10/3 and 20/3 formulations, respectively.

3.2.1. SEM analysis

SEM pictures of CTP and HAp microspheres, before and after sintering, are presented in Fig. 2. Only the results obtained with the 20/3 formulation are given, as similar results were obtained for the other formulations tested. The analysis revealed that in both cases microspheres were homogeneous, in terms of size and shape. No evidence of cracks was found.

CTP microspheres (Fig. 2 a, b, c) present a rather rough surface, while HAp microspheres (Fig. 2 d, e, f) are smoother. Such differences are mainly attributable to the differences in granulometry of the two ceramics, since the CTP particles are much larger than those of HAp. The volume contraction that occurred upon sintering in the case of HAp microspheres is evident (Fig. 2 a, b).

Non-sintered microspheres revealed, at higher magnifications, a homogeneous distribution of the ceramic phase in the alginate matrix (Fig. 3 a, d). It should be noted, however, that since the particles of HAp powder are much smaller than those of CTP, the former appear to be more densely packed. Upon sintering the polymer phase is substituted by a porous network (Fig. 3 a, b, e, f). The interconnectivity of the pores and their homogeneous distribution are clearly observed in Fig. 3b, 3c and 3f, respectively. Pores of approximately 40 μm were obtained when using CTP as the ceramic phase, while HAp resulted in pores of approximately 2 μm. Results were similar for all the formulations tested. Differences in porosity may be attributed to the different granulometries of the two compounds, which result in different packing of the

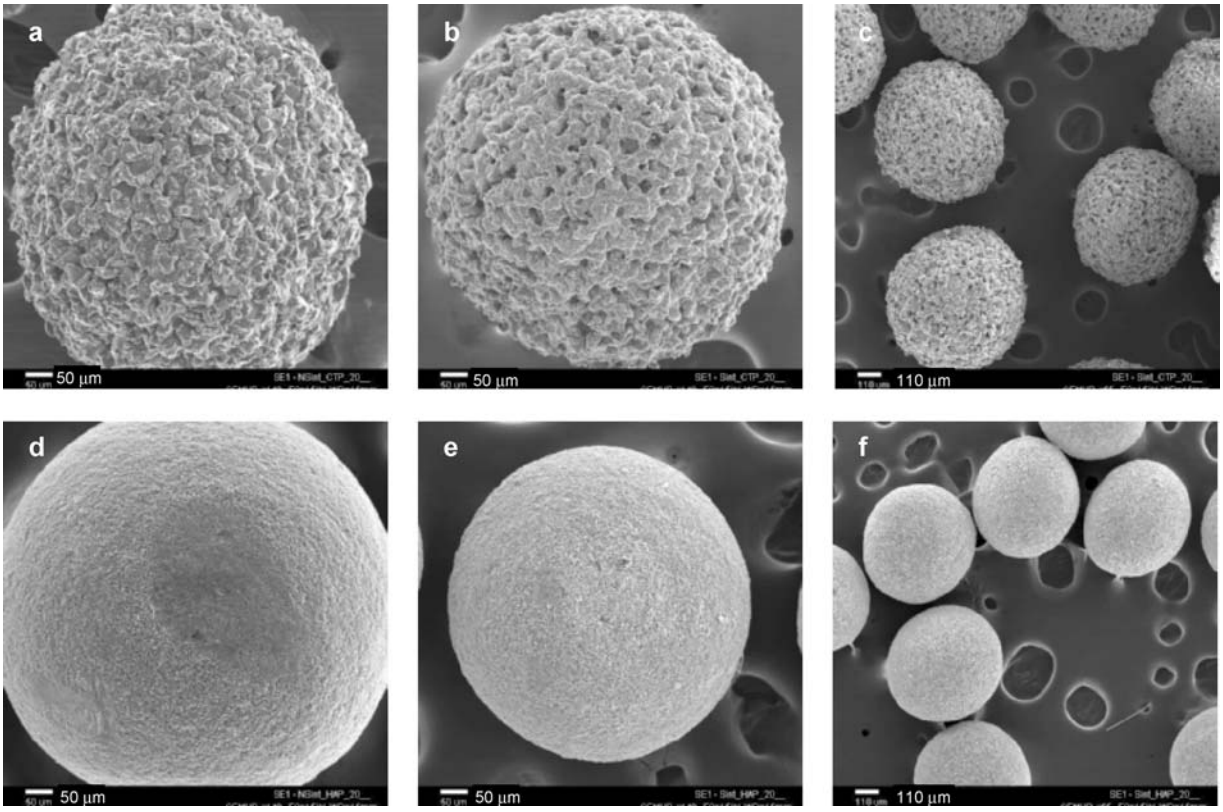

Fig. 2 SEM images of CTP and HAp microspheres before and after sintering: (a) Non-sintered CTP microsphere, (b, c) sintered CTP microspheres; (d) non-sintered HAp microsphere, (e, f) sintered HAp microspheres.

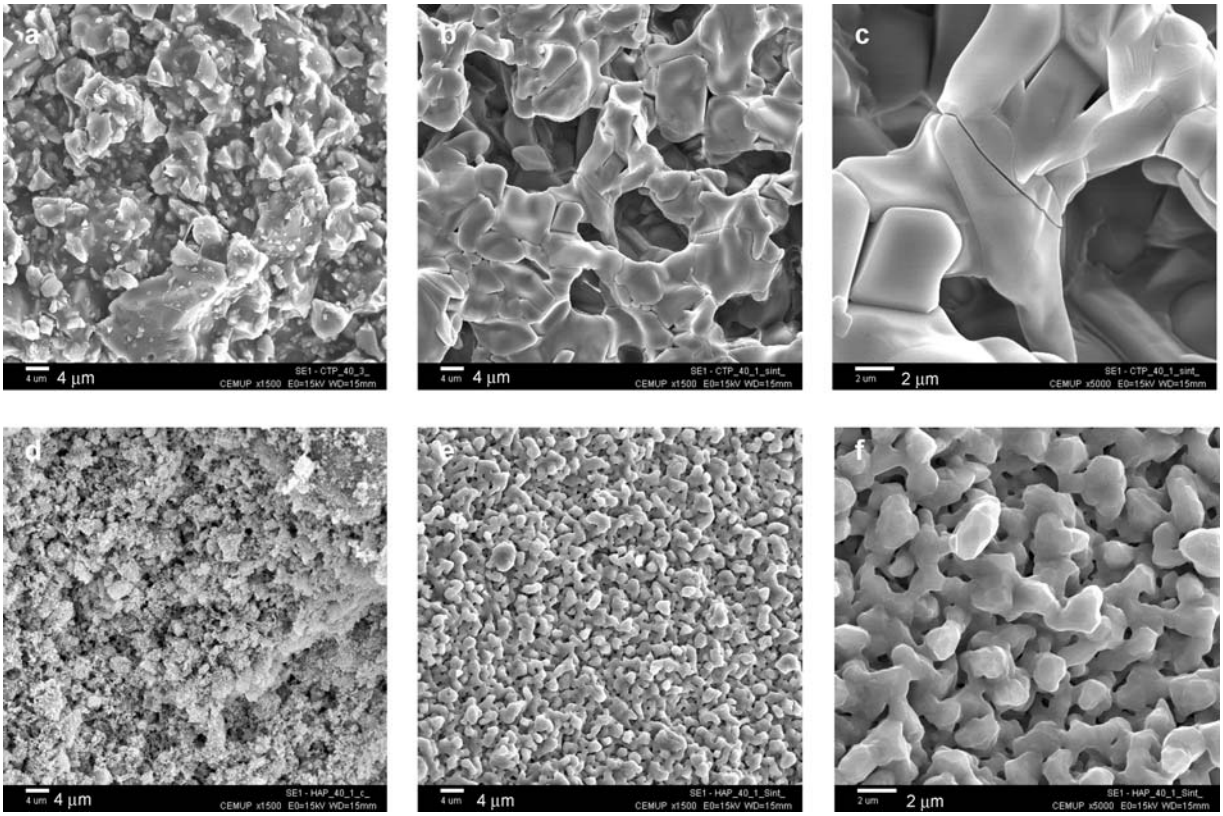

Fig. 3 SEM images of CTP and HAp microspheres before and after sintering: (a) Non-sintered CTP microsphere, (b, c) sintered CTP microspheres; (d) non-sintered HAp microsphere, (e, f) sintered HAp microspheres.

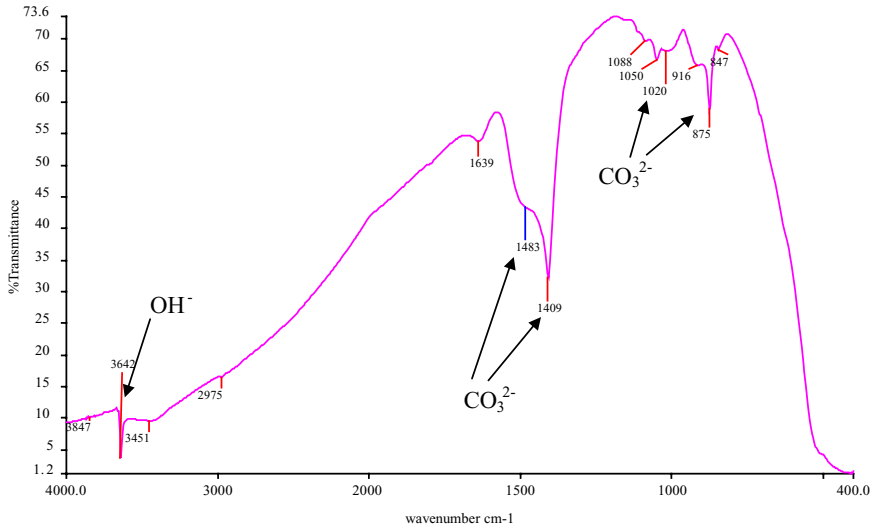

Fig. 4 FT-IR spectrum of the alginate-burning residue.

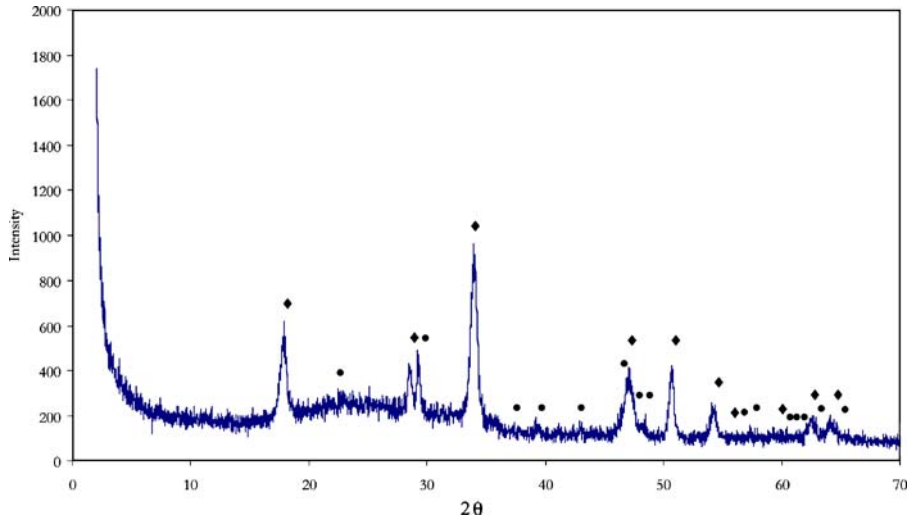

Fig. 5 XRD spectrum of the alginate-burning residue, ♦- Portlandite $Ca(OH)_2$, ● - Calcite $CaCO_3$.

ceramic particles during drying and sintering, as suggested already.

3.2.2. FT-IR and XRD analysis

The FT-IR spectrum of the residue powder obtained after subjecting the calcium-alginate microspheres to sintering of the ceramic-alginate microspheres (Fig. 4), showed the presence of bands characteristic of carbonates, namely the ones in the intervals 1409–1483 cm^{-1}, 1020–1100 cm^{-1} and 800–890 cm^{-1} [20, 21]. The band at 3642 cm^{-1} is assigned to O-H vibrations, suggesting the presence of a hydroxide compound [20, 22]. XRD analysis of the residue (Fig. 5) identified it as a mixture of $Ca(OH)_2$ (portlandite) and $CaCO_3$ (calcite), confirming the results obtained by FT-IR.

FT-IR spectra of CTP powder and CTP microspheres are presented in Fig. 6. The changes that are observed in the spectra of CTP microspheres when compared to those of the CTP powder, suggest that a reaction between CTP and calcium alginate must have occurred during sintering, leading to structural modifications that resulted in the appearance of new bands in the ν_3PO_4 and ν_2PO_4 phosphate zone, as well as a band at 726 cm^{-1}. These bands are more evident in the spectrum of the sample with the lowest ceramic-to-polymer solution ratio (10/3), and becomes less defined as the ceramic-to-polymer solution ratio increases, being the FT-IR spectrum of the 40/3 formulation very similar to the one of CTP powder. Fig. 7 shows the FT-IR spectrum of (a) 10/3 CTP microspheres, (b) commercial calcium pyrophosphate ($Ca_2P_2O_7$ - Sigma Aldrich) and (c) titanium pyrophosphate synthesised in our laboratory. Several bands which are characteristic of pyrophosphate groups, namely the ones in the ranges: 900–1200 cm^{-1}, 700–770 cm^{-1} and 500–600 cm^{-1}, are common to spectra (a) and (b), suggesting the formation of a calcium pyrophosphate phase in the CTP microspheres as a consequence of the sintering process. However, the existence of that phase could not be detected by XRD analysis

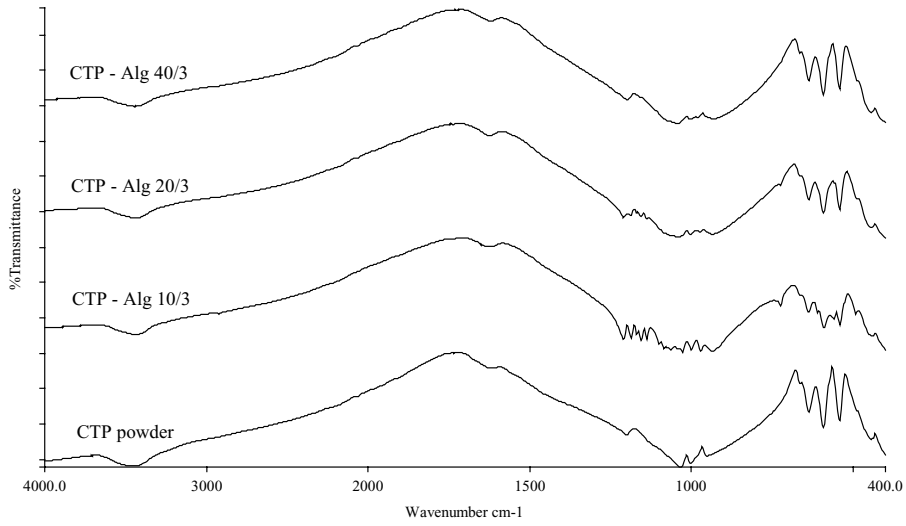

Fig. 6 FTIR spectra of CTP powder and of different formulations of CTP microspheres.

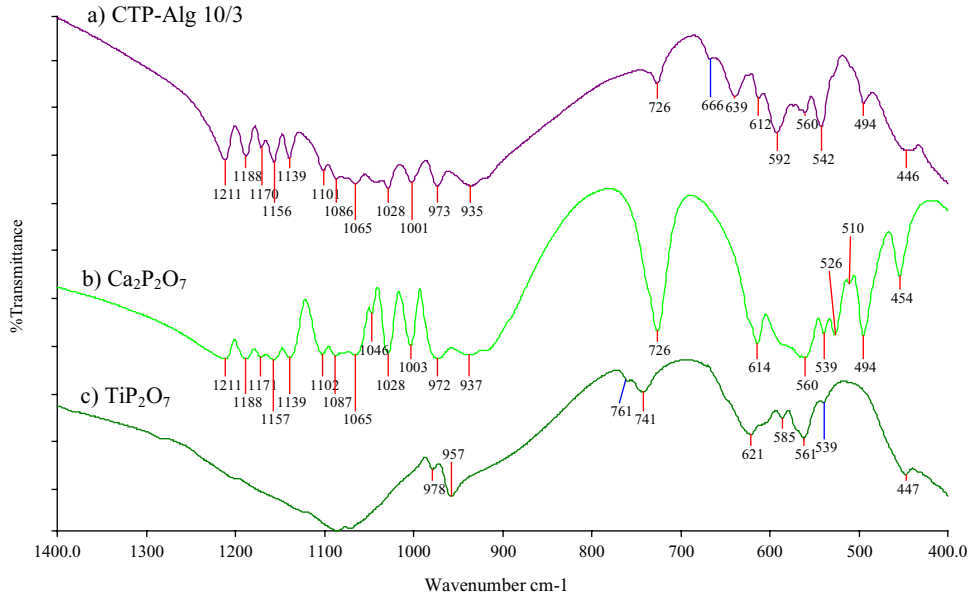

Fig. 7 FTIR spectra of CTP-Alg 10/3 microspheres, calcium pyrophosphate and titanium pyrophosphate.

(Fig. 8) leading to the assumption that the phase is either amorphous or present in residual concentrations. In addition, XRD analysis showed the presence of small quantities of titanium oxide (rutile) in the sintered CTP microspheres, its concentration being higher in the formulation 10/3. This was not unexpected considering the hypothesis described above. If a calcium pyrophosphate is formed, probably some titanium will become available to form an oxide.

In what concerns HAp microspheres, XRD analysis of the HAp powder and HAp microspheres revealed that after sintering no decomposition of the ceramic occurred, since no extraneous phases could be identified. Fig. 9 shows the FT-IR spectra of HAp powder and 10/3 HAp microspheres. It is worth noting the appearance of carbonate bands at 1440 cm^{-1} and 876 cm^{-1} in the samples with the 10/3 ceramic-to-polymer solution ratio, suggesting the presence of a carbonated hydroxyapatite. This may have practical implications, since it is well established that the presence of carbonates in calcium phosphate materials is an important factor contributing to the *in vivo* integration of the implant. For the 20/3 and 40/3 formulations, the FT-IR spectra of the microspheres are practically identical to the one of HAp, but the presence of carbonate bands is less evident. No signs of calcium hydroxide were observed in the sintered CTP-alginate and HAp-alginate microspheres.

In order to clarify if a carbonated apatite has been obtained, or if the carbonate bands were due to formation of a calcium carbonate residue, resultant from the burning of alginate, 10/3 HAp microspheres were subjected to a second sintering cycle at 1100°C for 1 h. When heated to this high temperature, any free calcium carbonate would disappear since calcium carbonate (calcite) decomposes at

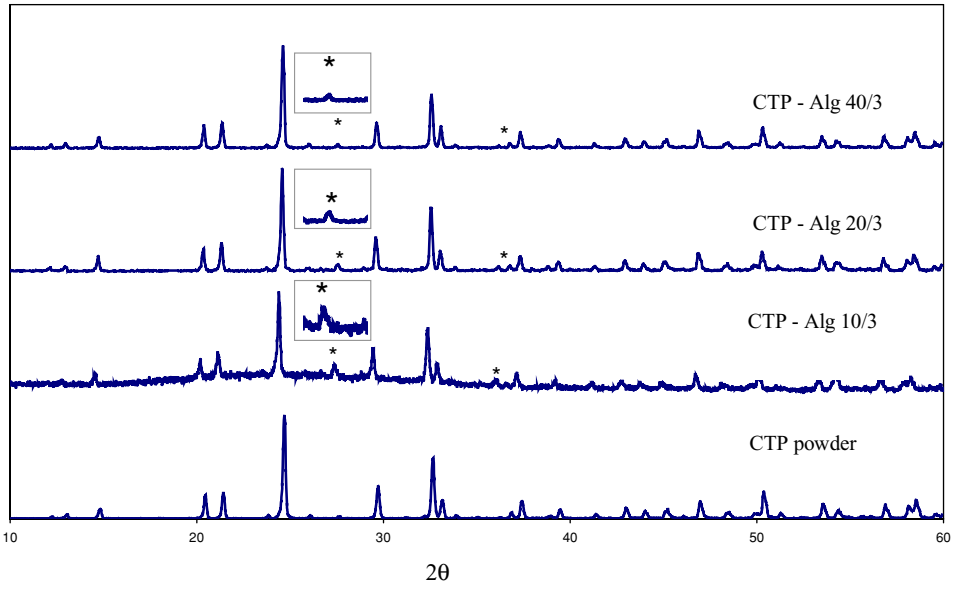

Fig. 8 XRD of CTP powder and of different formulations of CTP microspheres, * - Rutile TiO_2.

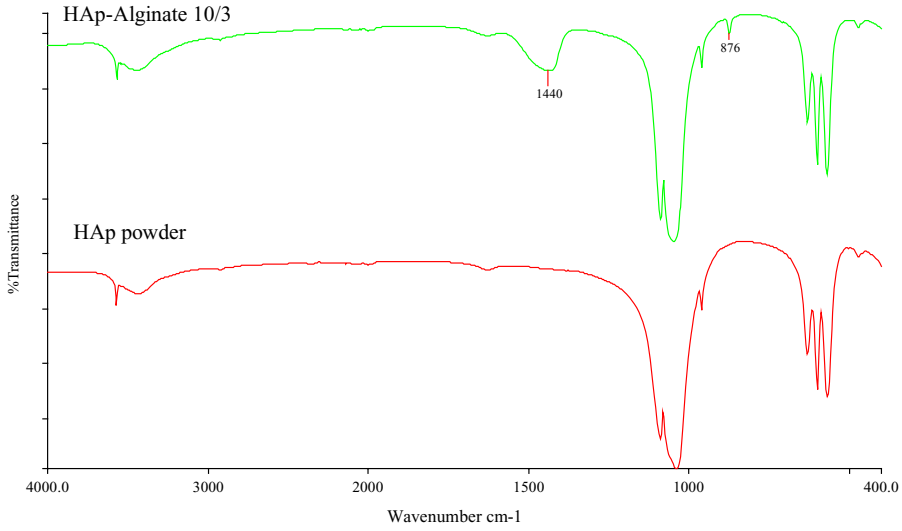

Fig. 9 FTIR spectra of HAp powder and of microspheres with HAp-Alginate 10/3 formulation.

approximately 825°C into CaO and CO_2 [23]. The FT-IR spectra of the re-heated HAp microspheres still presented the carbonate bands, confirming the presence of a carbonated apatite.

4. Discussion

There are several reports described in the literature concerning the development of HAp or HAp-polymer granulates with spherical geometry [8–11, 24–28]. Most of the methods used to produce microspheres have the disadvantage of using organic solvents or oils thus needing several washing stages to eliminate them. For instance, Sunny *et al.* [10] produced HAp/chitosan microspheres by the dispersion of HAp/chitosan slurry in liquid parafin, with the addition of gluteraldehyde to harden the spheres. Sivakumar et al [11, 28] developed a method to produce coralline HAp/chitosan and coralline HAp/gelatin composite microspheres using a PMMA dispersion solution containing toluene as a crosslinking agent. The experimental method proposed in this study has the advantage of not using such reactants, thus allowing the recovery of the microspheres without the need of fastidious washing processes.

Another advantage of the present methodology is the possibility of producing spherically shaped particles with uniform sizes. Irregularly or densely packed granules have been described in the literature as being the cause of

inflammatory reactions and slower bone formation [29]. Uniformly packed spherical particles would lead to a regular inter-particle porosity, thus contributing to easier migration of bone cells. Moreover, since our aim is to use the microspheres in an injectable form, their flowing properties during injection will be more predictable if their shape and size are more regular.

Since these microspheres may also be used to deliver growth factors and other proteins, their porosity is a critical parameter since it may strongly influence their loading capacity. The increase in porosity that occurs upon sintering will significantly increase the surface area and allow higher delivery efficiency.

This investigation has shown that the ratio between the ceramic phase and the polymer solution is of critical importance in the composition of the sintered microspheres. In the case of the CTP-alginate microspheres, a calcium pyrophosphate was formed after sintering, and its concentration increases as the ceramic to polymer ratio decreases. When subjected to the same heating cycle used for sintering the microspheres, CTP powder does not suffer any change in composition. These observations suggest that the formation of the pyrophosphate phase is related to the presence of alginate. Sodium alginate forms a relatively stable hydrogel of calcium alginate through ionotropic gelation in the presence of Ca^{2+}. A possible mechanism to explain the formation of the pyrophosphate phase after sintering is the interaction between calcium ions from the alginate and phosphate groups from the ceramic, possibly forming an acid salt. When a solid orthophosphate, which contains hydrogen bonds (e.g. $CaHPO_4$) is heated to an appropriate temperature, there is a loss of water accompanied by pyrophosphate formation [30, 31]. The amount of pyrophosphate formed is related to the number of hydrogen bonds present in the system. Pyrophosphate ion is a well-known inhibitor of apatite formation [31–33]. LeGeros et al [33] have demonstrated that the presence of pyrophosphate ions is efficient in promoting the formation of amorphous calcium phosphates. With the microspheres prepared in this investigation promotion of mineralization is an objective. Therefore, a formulation with a high ceramic to polymer ratio will be more adequate.

With HAp-alginate microspheres it was observed that the sintering process leads to the formation of carbonated hydroxyapatite. The carbonate ions were probably incorporated in the HAp structure according to the following reaction [34]:

$$2OH^-(\text{apatite}) + CO_2 \rightarrow CO_3^{2-}(\text{apatite}) + H_2O$$

that was probably favored by an increase of the local concentration of CO_2 resulting from the burning of alginate. This is in agreement with the fact that carbonate bands are more intense in the low ceramic to polymer ratio formulations. The substitution of carbonate in well and poorly crystallized hydroxyapatite is well documented in the literature. It has been suggested that planar CO_3 ion substitutes for tetrahedral PO_4 groups in hydroxyapatite prepared at room (or body) temperature while in high temperature preparations (about 1000°C), CO_3 substitutes for OH [31].

5. Conclusions

Porous ceramic microspheres with an uniform size were prepared using a novel methodology consisting of sintering spherical particles, obtained by droplet extruding a mixture of ceramic powders and sodium alginate followed by ionotropic gelation in the presence of Ca^{2+}. Microspheres with different porosities may be produced by this method, by varying the granulometry of the ceramic powders. It was also found that the ratio between the ceramic phases and the polymer solution is a critical parameter in the composition of the sintered microspheres and must be carefully selected. These materials could find applicability in the field of bone regeneration, both as injectable bone-filling materials and drug delivery matrices.

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