

Synthesis and Characterization of Double Calcium Iron Phosphate from Chicken Eggshell

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With the increase in life expectancy and the significant incidence of lesions of the musculoskeletal system, both the public and private sectors seek improvements in their products and processes in order to contribute to the health of the population. Every year tons of chicken eggshell (CES) from domestic consumption are released in environment and the present work aims the production of a synthetic ceramic bone substitute using the eggshell as a source of calcium ions. According to the methodology, 5, 10, 15 and 20 mol% of iron ions were used and the characterization assays demonstrated the formation of a double calcium iron phosphate - $\text{Ca}_9\text{Fe}(\text{PO}_4)_7$. MG63 cells were cultured on test specimens to cell viability assay and to each doped group analyzed, verifying cell compatibility with the studied compounds, as well as a good adhesion profile. Scanning electron microscopy (SEM) of the cells showed that material surface favored cell adhesion and the results demonstrated that the production of the compounds were viable to $\text{Ca}_9\text{Fe}(\text{PO}_4)_7$ phase. So, in the future, it will be possible to have a final comprehensive biological compatibility of the prototype for further *in vitro* and *in vivo* assays.

Keywords: double calcium phosphates, iron ions, chicken eggshell, bone substitutes

Introduction

The increase in life expectancy of the population has been observed in the last years and according to the World Health Organization (WHO), the world's average is 71 years old. It has also been increased more than 6 years between 2000 and 2019 (from 66.8 in 2000 to 73.4 in 2019) as well as Healthy Life Expectancy (HALE) increased by 8% from 58.3 in 2000 to 63.7, in 2019.¹ With this trend, health industry and universities keep searching for new medical devices that can meet such needs, like orthopedic implants, prostheses, synthetic blood vessels, dental implants, among others that nowadays can be manufactured by 3D printing technology (additive manufacturing).² These data highlight the significance of this sector for the global

economy, while also showcasing the formidable task of assessing and delivering innovative medical devices or biomaterials to the population, ensuring their proven efficacy and safety. This responsibility is under domain of regulatory sciences and the impact attributed to taxes, and the very particular logistics of this economical sector are important variables that must also be considered.³

These technologies present a significant challenge for chemical and biological sciences in terms of biological behavior. In addition, considering important properties such as the microstructure and mechanics of the biomaterial, it is crucial to assess the local tissue response. This evaluation helps to classify the biomaterials as bioinert, bioactive, or biodegradable and determine their potential applications, such as bone loss treatments.⁴ The exploration of these compounds for treating bone loss traces back to the mid-16th century when calcium phosphate was discovered in bones. Subsequently, the chemical composition of the

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constituents of mineralized tissue was elucidated. Similarly, in 1906, the first ionic substitution in calcium phosphates, now recognized as carbonate-apatite, was accomplished.⁵

The possibility of using synthetic bone substitutes, especially calcium orthophosphates, contributes to the development of clinical protocols in the most diverse indications of bone defects. In these terms, it is worth highlighting the main characteristics of two major representatives of this class: hydroxyapatite (HAP) and beta tricalcium phosphate (β -TCP).⁶ Regarding the mechanism of action, *in situ* formation of calcium phosphates occurs through the release of Ca^{2+} , PO_4^{3-} , HPO_4^{2-} ions into the medium, promoting saturation of the body fluid present and causing precipitation of a carbonated apatite with incorporation of Mg^{2+} , Na^+ and CO_3^{2-} ions, in addition to proteins and other inorganic compounds in the structure of the precipitate. Both HAP and β -TCP compounds present a set of characteristics, which classify them as synthetic bone substitutes with marked bioactivity.^{7,8} The β -TCP structure allows the replacement by various complexes and metals like Mg^{2+} , F^- , Fe^{3+} , Sr^{2+} and Ag, being widely used in maxillofacial surgery and orthopedics, due to its similarity with the mineral phase of bones and excellent biocompatibility. Its bioactivity profile is related to the physicochemical properties, and the affinity of the compound with the mineral phase of bone tissue.⁹ In addition, there are distinct allotropic forms of the compound such as α and β and not all forms are of technological interest, for example, alfa tricalcium phosphate (α -TCP) due to their chemical instability during synthesis, processing and degradation rate.^{10,11}

Calcium phosphates currently used in medicine and dentistry are classified as synthetic osteoconductive bone substitutes, as they allow the migration, adhesion and proliferation of cells forming bone tissue on its structure, acting as a scaffold.¹² Although these compounds present excellent biocompatibility, they do not have osteoinductive properties and, therefore, many research groups in biomaterials field have been concentrating efforts to promote structural changes in calcium phosphates, such as the inclusion of mono, bi and trivalent cations, to increase the biological response of calcium phosphates synthetic bone substitutes.¹³ There is a lack of studies in the literature relating the activity of trivalent substitutes in the crystalline structure of ceramic compounds with their respective biological activities *in vitro* or *in vivo*. However, it is worth mentioning that the trace ions present in bone tissue are under current investigation relating both physicochemical and biological aspects. Among trivalent metals (M^{3+}), some elements such as boron (B^{3+}), manganese ($\text{Mn}^{2+/\beta+}$) and iron (Fe^{3+}) stand out.^{14,15}

Iron and its oxidation states (Fe^{2+} and Fe^{3+}) participate in several organic functions, among which are, transport of oxygen in the blood of mammals (hemoglobin), storage of oxygen in the muscles (myoglobin), transport of electrons in plants and animals (cytochromes and ferredoxins) and iron storage and absorption (ferritin and transferrin), respectively.¹⁶ In the biology of living beings, iron ions have been highly investigated with therapeutic proposal due to the intrinsic magnetic property of their formed oxides, like in cancer therapy, promoting hyperthermia of the involved tissue under the application of a magnetic field.¹⁷ Specifically in the orthopedic field, there has been an evolution in scientific publications using calcium phosphates doped or substituted with iron ions, not only related to physical and chemical aspects, but also to their impact on mechanical strength, crystallographic aspects and on bone tissue formation.¹⁸ It is worth noting that the increase in iron concentration alters the arrange of atoms and the substitution in calcium sites by iron ions leads to an increase in degradation rate, which is in some cases an important characteristic considering the time of resorption of the compound and type of bone lesion.^{19,20} In this way, research in the field of ceramic materials indicates that ionic substitutions in specific regions in the structure of compounds such as HAP and TCP, can promote changes in their physicochemical properties and in the cellular response, targeting, for example, for imaging studies, in magnetic resonance, drug delivery systems, cell separation and treatment of bone tumors by hyperthermia.²¹ Considering the mechanism of action of iron in cell differentiation, there is still a lack of definition in the literature regarding the biochemical pathway, although a major concern is focused on the effectiveness of cell stimulation and its toxic effect. The aim of this work is to evaluate the synthesis, characterization and cytotoxicity profile of different concentrations of iron substituted calcium orthophosphate.

Experimental

Chemical reagents

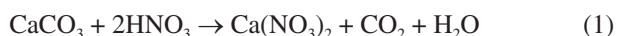
To carry out all the chemical reactions, different solutions were prepared from the reagent grade for analysis (P.A.): nitric acid (HNO_3 , Merck Millipore, Darmstadt, Germany), ammonium hydrogen phosphate ($[(\text{NH}_4)_2\text{HPO}_3]$, Merck, Millipore), ammonium hydroxide (NH_4OH , Merck Millipore) to correct the pH of the reactions, calcium carbonate (CaCO_3 , Merck Millipore) and ferric chloride (FeCl_3 , Merck Millipore). In addition to these reagents, the solutions used in all reactions had distilled water (FreseniusKabi Ind. Bras.), 70° alcohol (Via

Pharma Ind and Com.) and CES (chicken eggshell) as source of calcium ions. As culture medium, Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for cytotoxicity assay.

Preparation of chicken eggshells (CES) and calcium nitrate synthesis

CES were washed in water and kept in a 20% descaling solution (Higimed[®], Biomechanica Ind. Com. natural descaling agent) for 2 h at 60 °C. Then, they were washed again with water to remove the descaling solution, followed by manual removal of the inner membrane.

At the end of this process, the CES were crushed and washed again with distilled water and 70° alcohol. Finally, the starting material was dried at 70 °C for 3 h and stored for later use. Calcium nitrate, the starting reagent for the production of the bone substitute proposed in this work, was obtained from the calcium carbonate present in the CES.²² The chemical equation (equation 1) describes the route used. For the production of the stock solution, a previously prepared mass of CES was used. The slow dripping of 1 mol L⁻¹ nitric acid started at 40 °C under moderate stirring for 24 h, obtaining a nitrate solution corresponding to 0.36 mol L⁻¹.



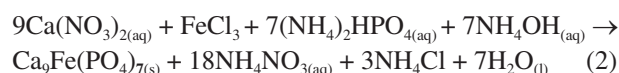
Synthesis of tricalcium phosphates (TCP) from CES

The reaction started from the theoretical concentrations contained in the calcium nitrate stock solution obtained in the previous step and under strong agitation, the system was heated to 40 °C. Upon reaching the planned temperature, the phosphate source [(NH₄)₂HPO₄] was slowly dripped (3 mL min⁻¹) to obtain the precipitate (TCP), respecting the Ca/P ratio of 1.5. Ammonium hydroxide solution was used to adjust the pH to 7.0 for the compound formation and after completing the dripping, the system was kept under stirring and heating conditions for 60 min.²³ At the end, the precipitate was washed, dried and underwent heat treatment at 1000 °C for 2 h.

Synthesis of iron substituted calcium phosphate

Four groups of compounds were produced to carry out the ionic substitutions. Each group respected the substituent metal + Ca/P molar ratio at 1.5 and different molar percentage ratios for each metal were calculated in order to maintain, as close as possible, TCP structure considering

rhombohedral geometry (space group *R3c*), unit cell parameters like *a* = *b* (10.435 angstroms), *c* = 37.4029 Å and angles (*α* = *β* = 90° and, *γ* = 120°). Using the same reactor just like in the previous step (unsubstituted compound), a second separating funnel was adapted for the insertion of the Fe³⁺ donor at the following concentrations (5, 10, 15 and 20 mol%). At the end of dripping, the suspension was aged for 24 h with subsequent washing, filtration and drying (60 °C) for 24 h. Thus, the compounds were stored and taken to the final heat treatment. Equation 2 demonstrates the synthesis route using iron chloride as iron ions donator.



Material processing

The specimens were produced in the form of pellets using a hydraulic press (Bovenau P10ST, São Paulo, Brazil), loading the matrix with 150 mg of the powder synthesized under 2 tons. All samples produced were subjected to heat treatment in a muffle furnace (Graphite Furnace Atomizer, Shimadzu-GFA.EX7, Kyoto, Japan) to convert the powder (green) into the crystalline phase of the calcium phosphate produced. The sintering temperature was standardized at 1000 °C with an increase of 5 °C min⁻¹ and a plateau of 2 h. All the samples obtained were conducted for phase characterization tests and chemical analysis.

Characterization techniques

The Fourier transform infrared spectroscopy (FTIR) technique (Shimadzu, IR Prestige-21, Japan) was used to identify the functional groups present in the compounds. Spectra were collected using potassium bromide (KBr) pellets (2 cm⁻¹ resolution).

The powder X-ray diffraction (XRD) measurements were performed in XRD Multiuser Laboratory at Fluminense Federal University using the Bruker D8 Advance diffractometer with cobalt anode tube operating at 40 KV voltage and 30 mA current, resulting in a wavelength of *λ* = 1.79 Å (Co K_α). Data scan was carried out ranging 2θ from 10° to 80° with step of 0.04° and the qualitative analysis was performed with HighscorePlus version 3.1.²⁴

The background was refined using 10-terms of Chebyshev polynomial, peak profile was fitted using the modified Thompson-Cox-Hastings pseudo-Voigt function. Preferential orientation was adjusted using an eight-term spherical harmonics function and zero shift and specimen displacement was also refined. Crystal structure parameters as unit cell and crystallite size

were individually refined for each sample and using International Centre for Diffraction data (ICDD) card and Crystallographic Information File (CIF) for all the X-ray diffraction analysis.

Biological assays

In vitro cytotoxicity assay-indirect

For the indirect cytotoxicity assay, 0.2 mg of the synthesized compounds were placed in 1.5 mL microtubes and taken to sterilization in an autoclave (121 °C for 1.5 h). Then, 1 mL of DMEM medium (not supplemented) was added to each tube for a period of 72 h in an oven at 37 °C with 5% CO₂, 95% humidity (Panasonic, model MCO-19AIC-PA, Gunma, Japan) for the extraction of soluble components that could present toxic effects to the cells (ISO 10993-5).²⁵ The human osteosarcoma lineage - MG63 in third passage was used for the indirect assay, being expanded with the period of extraction of soluble components from the samples. 3×10^3 cells *per* well were seeded in a 96-well flat-bottomed plate in DMEM medium supplemented with 10% fetal bovine serum and at 80% confluence, the cells were incubated with the extraction media in an oven at 37 °C with 5% CO₂ for 24 h under the following conditions: group 1-negative control (only with DMEM medium); group 2-iron 5 mol%; group 3-iron 10 mol%; group 4-iron 15 mol%; group 5-iron 20 mol%; group 6-CaCO₃ (CES) and group 7-TCP.

After the incubation period, cell viability was evaluated by the MTT reduction method. Initially, 10 µL of a 5 mg mL⁻¹ MTT solution were added to 100 µL of culture medium in each well and incubated for 2 h at 37 °C. Subsequently, the medium was discarded and 50 µL of dimethyl sulfoxide (DMSO-P.A.) were added *per* well, followed by stirring for 5 min, with subsequent analysis at a wavelength of 570 nm (Glomax-multiR Promega Biotecnologia, São Paulo-SP, Brazil). Finally, the reading data were processed in GraphPad Prism 5.0 software.²⁶

In vitro cytotoxicity assay-direct

For the direct cytotoxicity assay, the synthesized powders formed into pellets were sterilized in an autoclave (121 °C for 2 h). Subsequently, MG63 cells were grown on them (1×10^4 cells) for a period of 24 h in a 24-well plate. Only in the case of CES, it has been decided to use it directly (crushed), through previous cleaning and sterilization processes, since CES in powder form does not present good aggregation during compression. It is worth noting that the natural raw material could not undergo heat treatment due to its degradation to calcium oxide (CaO), which is not desired for the planned experiments.

After the incubation period, the fragments of CES were submitted to scanning electron microscopy (SEM), (Quanta 250, Thermo Fisher Scientific, Oregon, USA) - 30 kV with detection of secondary electrons, previously applied fixation procedure. The cells grown on materials surfaces were fixed in 2.5% glutaraldehyde and subsequently the specimens were subjected to serial dehydration in ethanol, then covered with a thin layer of gold by the gold-sputtering method (Bal-tec, model: SCD 005, Germany) to improve the secondary electron signal.

In vivo test

For *in vivo* assays, the synthesized powders were sterilized in an autoclave and then incubated with 10⁶ of mesenchymal stem cells. About 30 mg of the powders were associated with cells and incubated for 12 h under agitation at 37 °C. After this period of association, nude mice were anesthetized by inhalation, using isoflurane (Biochimico, Itatiaia-RJ, Brazil) at a rate of 5% for induction and 2.5% in volume (L) of oxygen for maintenance in a vaporizer (Insight Equipamentos Ltda, Ribeirão Preto-SP, Brazil).

Immediately after anesthesia, the animals received analgesic and anti-inflammatory induction of ketoprofen 5 mg kg⁻¹ (Artrínid®, União Química, São Paulo-SP, Brazil) and tramadol 5 mg kg⁻¹ (Tramadol hydrochloride, Laboratório Teuto, Anápolis-GO, Brazil) in the subcutaneous tissue of the cervical region. With the mice positioned in dorsal decubitus, an incision was made in the skin at the level of the lumbar vertebrae, and, with the aid of forceps, the skin was lifted so that the grafts could be positioned in the subcutaneous region. Two grafts were introduced *per* animal, one in the upper right region and another in the lower left region. After 90 days, tissue was fixed in 10% formalin for 3 h and decalcified with 10% nitric acid for 72 h. In addition, the liver of the animals were collected for histological evaluation. Finally, samples were embedded in paraffin, 3 µm histological sections were performed and slides were stained with hematoxylin and eosin (HE).

All the animal surgical procedures were performed in accordance with the Institutional Animal Care and Use Committee (002/2014) and Institutional Ethics Committee protocol number (41660415.3.0000.5273)

Statistical analysis

The comparison between groups in the indirect cytotoxicity assay was evaluated by Dunn's multiple comparison method (GraphPad Prism version 5.1), considering $p < 0.05$ as statistically significant.²⁶

Results and Discussion

All groups related to substitutions presented a similar FTIR spectrum (Figure 1). The vibrational modes related to the O–H stretching bonds of adsorbed water were identified by broad peaks spanning from 2500 to 3500 cm^{-1} , as well as a distinct peak at approximately 1625 cm^{-1} .²⁷ Pronounced bands localized between ca. 990–1140 cm^{-1} are related to PO_4^{3-} vibrational modes (symmetric and asymmetric stretching modes). The bands at lower wavenumber (ca. 550–600 cm^{-1}) are attributed to PO_4^{3-} bending modes. Weak bands related to CO_3^{2-} from eggshell precursor were identified near 712, 875 and 1427 cm^{-1} .²⁸

The phosphate region (range comprising 480–560; 600–900 and 1000, 1150 cm^{-1}) may present eventual displacements due to the entry of iron ions, however, absorption bands of the group Fe–O very close to the phosphate region may have contributed to the response of the broadening of the absorption bands presented in the range of 442, 540, 750, 1070, 1080 and 1150 cm^{-1} characteristic of the phosphate region as well.²⁹ A previous work²⁹ analysis indicate that all compounds substituted with iron ions are in the reduced state, $\text{Ca}_9\text{FeH}_{0.9}(\text{PO}_4)_7$, however, the X-ray diffraction results of this work present data corresponding to the oxidized state. Thus, complementary analyzes are necessary to elucidate this question. Another important factor in relation to the synthesis is the fact that pH plays an important role in the formation of phases, such as iron oxides, impacting the behavior of the infrared bands. As observed during the doping processes, a band is identified at 2930 cm^{-1} , suggesting a correspondence to the O–H band related to the intercalation of protons in the phosphate network of the reduced compound $[\text{Ca}_9\text{FeH}(\text{PO}_4)_7]$.³⁰

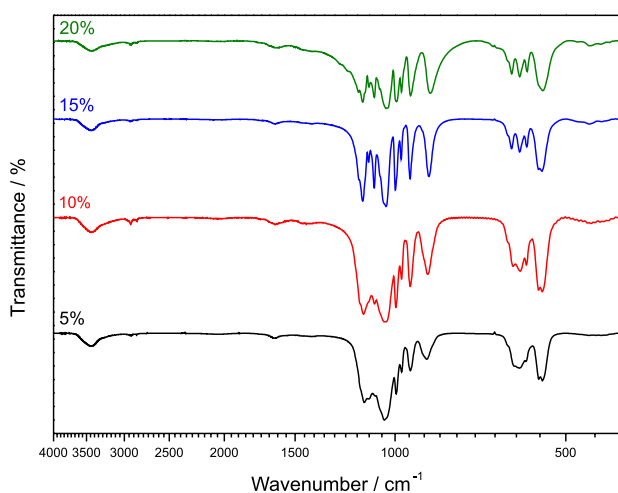


Figure 1. Fourier transform infrared (KBr) spectra for samples containing 5, 10, 15 and 20 mol% Fe^{3+} .

Regarding to chicken eggshell characterization, XRD analysis (Figure 2) shows the presence of calcium carbonate (calcite-ICDD 00-005-0586/CIF file 2100189)³¹ with rhombohedral geometry, space group $R\bar{3}c$ and lattice parameters a , b (4.9890), c (17.0620). Some papers^{32,33} report the existence of other polymorphic phases such as aragonite, vaterite and even hydroxyapatite in the inner cuticle, however, no other phases were detected for the CES employed in this study. The chemical composition of the CES vary in this work from 97 to 98.2% of calcium carbonate.

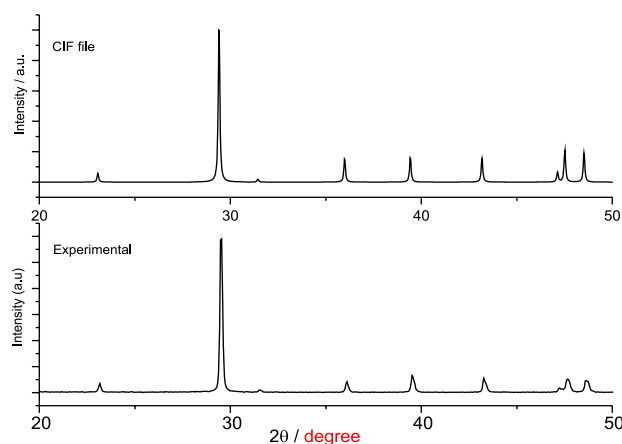


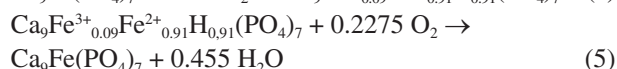
Figure 2. X-ray diffractogram of calcium carbonate from CES. Experimental CES and theoretic files (ICDD 00-005-0586/CIF file 2100189).

The iron ions substitution processes (Figure 3) resulted in the formation of a double calcium iron phosphate $[\text{Ca}_9\text{Fe}(\text{PO}_4)_7]$ with rhombohedral crystal system, $R\bar{3}c$ space group, $a = b = 10.3576$ and $c = 37.1429$, angles (α , $\beta = 90^\circ$ and $\gamma = 120^\circ$) (ICSD 96623/CIF file 1655973), for the groups with 5, 10, 15 mol%, as the main crystalline phase. However, when analyzing the inclusion of iron ions with 20 mol%, other two phase were obtained, an orthorhombic iron phosphide (FeP_4 -ICDD 00-032-0470) with $P2_1/c$ space group, at 29, 20° (2θ) and an iron phosphate $[\text{Fe}_7(\text{PO}_4)_6]$, which can be presented as $[\text{Fe}_3^{2+}\text{Fe}_4^{3+}(\text{PO}_4)_6]$ ICDD 00-49-1088 at 28.01° (2θ). These data indicate that there is a limit from 10 to 15 mol%, according to the synthesis conditions adopted, to obtain the double phosphate as the majority phase. It is noteworthy that it is possible to have different maximum limits of a single phase of double calcium iron phosphate according to the synthetic method adopted and thermal processing.³⁴ Equation 3 demonstrates the theoretical dehydroxylation mechanism from a calcium deficient species, to achieve through thermal treatment the final compound, calcium iron phosphate phase with a TCP like structure.³⁵ Figure S1 (Supplementary

Information (SI) section) depicts the Rietveld refinements and Table S1 (SI section) shows the crystal structures unit cell parameters and crystallite sizes.



The synthesis of double calcium phosphate enables the planning to achieve distinct oxidation states (Fe^{2+} and Fe^{3+}) under mild synthesis conditions. Considering the coexistence of both oxidation states, stoichiometric analysis reveals the maintenance of the dual-state. Even under a reducing environment, it shows incomplete reduction, with approximately 9% of the iron ions remaining in the ferric state (equations 4 and 5).³⁶



Due to crystalline defects and the flexibilization of 3d electrons configurations, the most favorable geometry for Fe^{2+} is the hexagonal distorted, along the Fe–O bond. In contrast, because of Fe^{3+} oxidation state and defects present in the crystal structure, tetrahedral geometry is assumed at Ca(1) site and pyramidal geometry at Ca(2) site. This geometric variation can influence directly general materials properties. It must also be taken into account the influence of atomic rays in bond lengths, since the atomic radius of Fe^{2+} is 0.78 Å, Fe^{3+} is 0.64 Å, and for Ca^{2+} ions it is 0.99 Å, and can also occupy Ca(4) and Ca(5) sites.³⁷

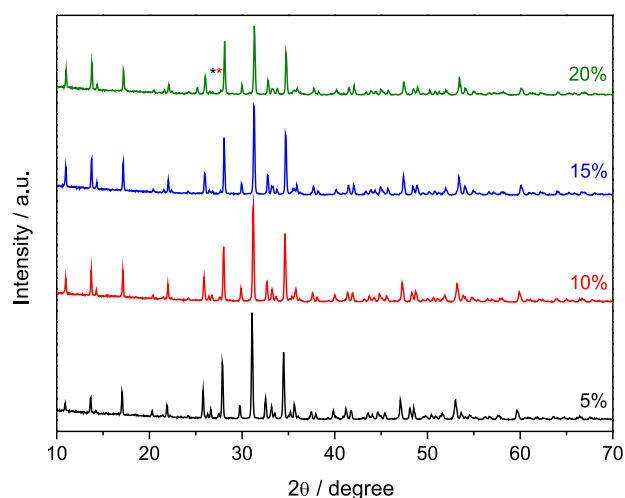
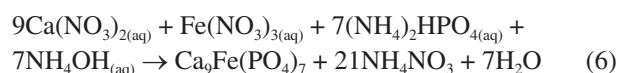


Figure 3. X-ray diffraction of iron substitution reactions. (a) 5 mol%, (b) 10 mol%, (c) 15 mol%, (d) 20 mol% (inside: * FeO_4 and * $\text{Fe}_7(\text{PO}_4)_6$).

Iron ions do not appear randomly in the structure of calcium phosphates. Advanced techniques in structural

refinement help the mapping of the compound, identifying specific sites of cation entry. Throughout advanced techniques in X-ray diffraction, factors for calcium and iron should be verified, identifying corresponding reference values for the entry of iron ions. Furthermore, for the entry of iron into the hexa-coordinated site of calcium (5) for tricalcium phosphate, the structural refinement values were positive.³⁸ Equation 6 demonstrate the reaction proposed where iron ions are entering in the tricalcium phosphate structure, via iron nitrate, obtaining unreduced orthophosphate (equation 6).³⁹



The changes of the powder colors were observed as the ferric chloride concentrations were increased, Figure 4. Starting with a red-orange hue at 5 mol%, to a darker tone at 20 mol%. The electrons of the material, when absorbing white light, perform the electronic transition mechanism of the d-d type, from t_{2g} to e_g orbitals. Thus, when exposed to white light, absorption occurs in the visible field, leading to color changes.

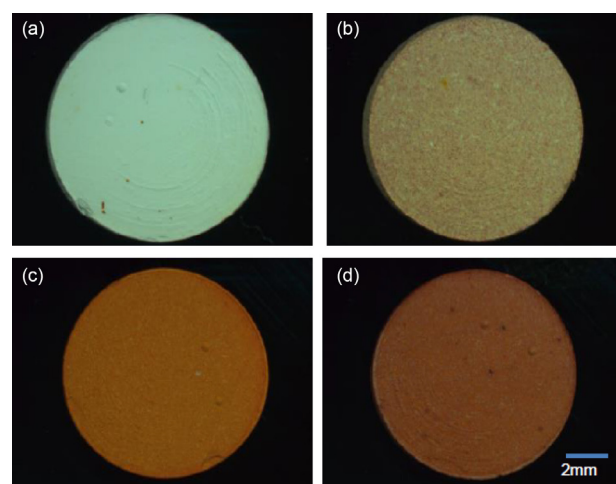


Figure 4. Specimen of substituted calcium phosphates obtained from CES. Synthesis with different percentages in mol of iron after uniaxial pressing. Fe^{3+} 5 mol% (a), Fe^{3+} 10 mol%; (b), Fe^{3+} 15 mol% (c), Fe^{3+} 20 mol% (d). Scale bar corresponds to 2 mm.

Biological assays

Indirect cytotoxicity assay

Regarding to some literature data, the excess of iron ions promotes inhibition of osteoblast proliferation and differentiation in cells of the MC3T3-E1 lineage,³⁹ when the concentration of 0.1 $\mu\text{g mL}^{-1}$ of ferric ammonium citrate is used. On the other hand, the positive effect of 200 $\mu\text{g mL}^{-1}$ of hydroxyapatite doped with iron ions on

the viability of mesenchymal cells after 7 days of testing has already been demonstrated.⁴⁰ Despite not being the objective of this work to explore all the cytotoxic parameters related to iron ions, it is worth to mention that there is a correlation of iron (Fe^{3+}) in cell death through a dependent mechanism via glutathione peroxidase 4 (GPx4) known as ferroptosis, which can promotes cell contractions through the accumulation of perinuclear lipids and their subsequent diffusion into the cell cytoplasm, that could raise the levels of reactive oxygen species (ROS) leading to stages of cytotoxicity.⁴¹

Considering the literature data that show the influence of ionic substitutions in the crystalline structure of ceramic bone substitutes on the cellular response, we have not verified negative influence of the active extraction medium on the MG63 cells, compared to the control group (cell + culture medium). The experimental stage of this work was carried out according to criteria already established in international standards (clause 10.3.5 of the ISO 10993 standard)²⁵ and the statistical analysis revealed no significant difference in toxicity between the groups doped with different levels of iron ions ($p = 0.423$) (Figure 5).

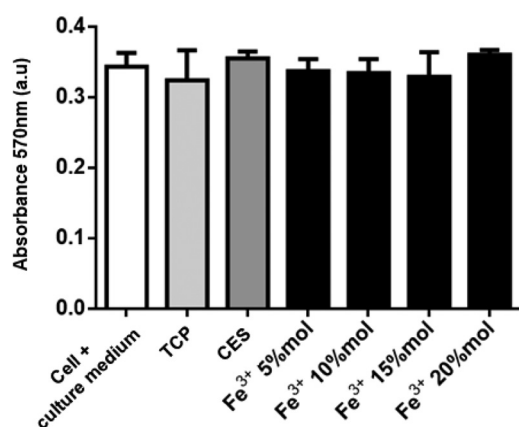


Figure 5. MTT cell viability assay. Viability of MG63 cells exposed for 24 h to different extraction media containing iron-doped calcium phosphate (5, 10, 15 and 20 mol%), cells treated with DMEM medium supplemented with 10% fetal bovine serum (cell + medium), undoped tricalcium phosphate (TCP) and chicken eggshell (CES), analysis of variance (ANOVA) $n = 3$.

Simultaneously with the viability assays, the cell morphology of the experimental conditions was evaluated. All images were obtained in the central region of the well and no significant morphological changes were noted in the treated cells (Figure 6).

Direct cytotoxicity assay

The material surface images were performed by SEM, and we sought to evaluate the surface observing

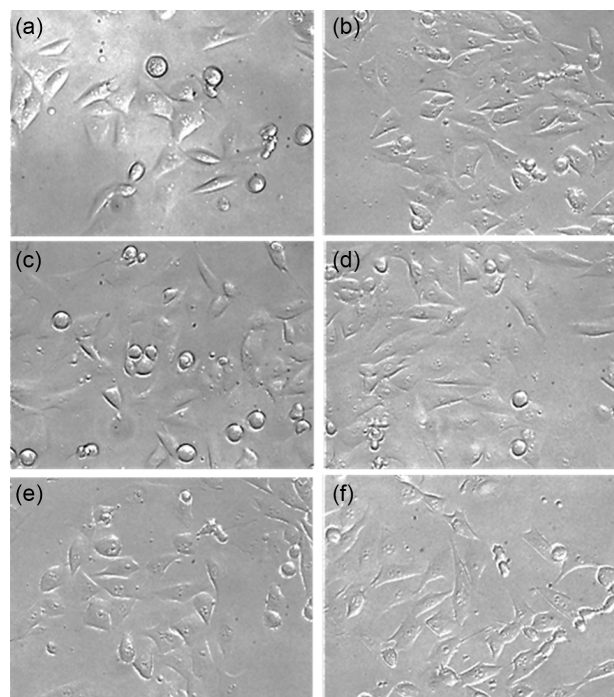


Figure 6. Photomicrograph of MG63 cell culture treated with DMEM media exposed to doped calcium phosphates. MG63 cells cultured on tricalcium phosphate (a), chicken eggshell (b), calcium phosphate doped with iron 5 mol% (c), 10 mol% (d), 15 mol% (e) and 20 mol% (f).

the apparent roughness, since the topography, chemical composition of the surface are parameters that will dictate the cell-biomaterial relationship in cell adhesion and proliferation.⁴² In this way, the morphological evaluations of the cells cultured on the calcium phosphate specimens, substituted with 5 and 10 mol% iron, revealed an excellent cell adhesion profile on all samples through SEM analysis, evidencing the spreading of the cells, flat and elongated morphology, and their cytoplasmic extensions on the material (Figures 7a-7d) in relation to the effect on the surface of the compounds. After the culture period, there is solubilization of the sample, generating the formation of surface pores (Figures 7e-7f).

Complementing the direct assay (ISO 10993-5), SEM evaluations of MG63 cells cultured on substituted calcium iron phosphate, 15 and 20 mol%, were performed. There was excellent adhesion of MG63 cells on both groups, in addition to maintaining the surface solubilization profile after 24 h of culture (Figures 8a-8f).

In vivo analysis

After three months, the animals were euthanized according to the criteria of good animal experimentation practices and after collection of the material, the bone neoformation was evaluated using the histological technique. Figure 9 highlights the three-month evolution as described.

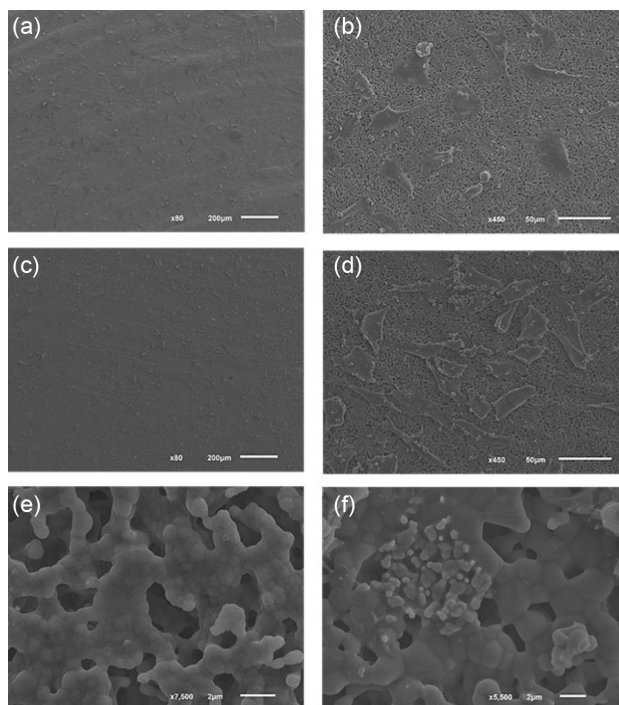


Figure 7. Cell culture on a calcium phosphate specimen substituted with 5 and 10 mol% iron. (a) Photomicrograph of calcium phosphate specimen with 5 mol% iron, at lower magnification; (b) 5 mol% iron, at higher magnification; (c) 10 mol% iron, lower magnification; (d) 10 mol% iron, at higher magnification; (e) and (f) show solubilization effect on the surface after the cell culture period.

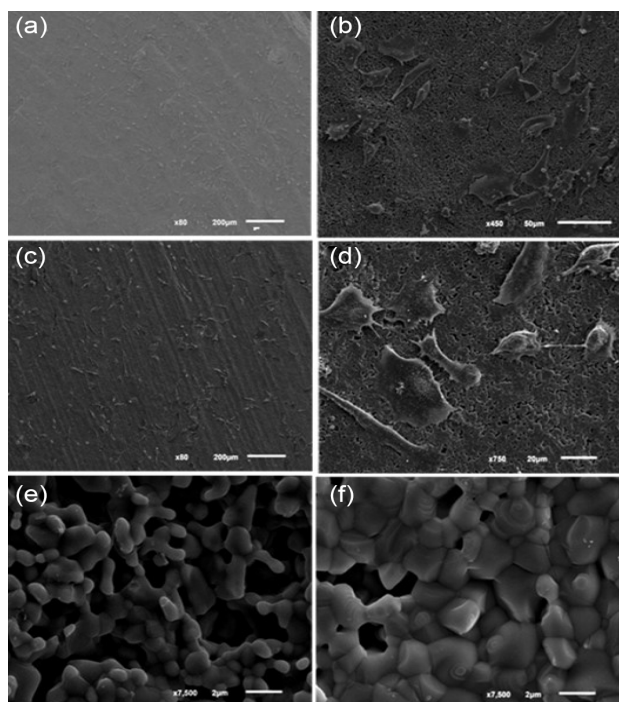


Figure 8. Cell culture on a calcium phosphate specimen substituted with 15 and 20 mol% iron. (a) Photomicrograph of calcium phosphate specimen doped with 15 mol% iron, at lower magnification; (b) 15 mol% iron, at higher magnification; (c) 20 mol% iron, lower magnification; (d) 20 mol% iron, at higher magnification; (e) and (f) effect of surface solubilization after the cell culture period.

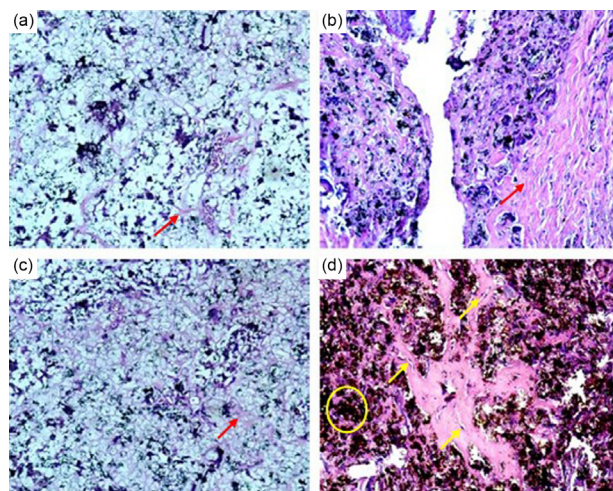


Figure 9. Histological evaluation of implants with different amounts in mol of iron. (a) 5 mol%, (b) 10 mol%, (c) 15 mol%, (d) 20 mol%. (a, b and c) Presence of fibrous tissue (red arrows); (d) yellow arrows indicates the presence of an osteocyte, and the pink region shows the new bone formation. The yellow circle presents the remaining $\text{Ca}_9\text{Fe}(\text{PO}_4)_7$.

In the animals that received 5, 10 and 15 mol% of iron, the formation of a fibrous tissue was observed but only the animal that received 20 mol% of iron showed the formation of an osteoid matrix and bone tissue, as observed with the presence of osteocytes in different points of the pink region (yellow arrow), Figure 9. Finally, considering the total amount of the compound introduced into the animal, the hepatotoxicity profile was evaluated by histological analysis (Figure 10) and as a result, it has not been observed any alteration pattern in the liver parenchyma cells of the evaluated animal.

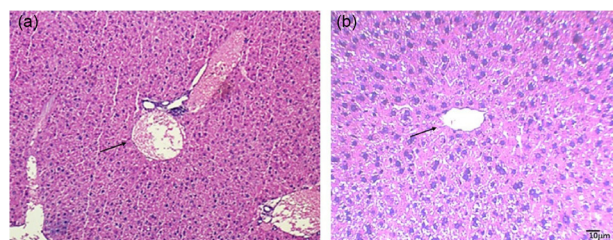


Figure 10. Histological evaluation of the liver of animals. (a) Nude mice that received implants with different amounts of iron (5, 10, 15, and 20 mol%). (b) control group. Black arrows show the port vein.

Conclusions

The proposed synthesis of calcium phosphates substituted with different concentrations of iron ions from the chicken eggshell allowed the formation of calcium iron double orthophosphate $[\text{Ca}_9\text{Fe}(\text{PO}_4)_7]$ as the main phase. The concentrations of 5, 10, 15 mol% led to the formation of the substituted compound, stabilizing the TCP like crystallographic phase, while at the concentration of 20 mol% there have been formation of double calcium iron

orthophosphate, iron oxide and ferric phosphate, indicating that there may be a maximum concentration allowed for the entry of iron ions into the crystal structure. In addition to the synthesis results, all synthesized groups showed good cell adhesion capacity on the surface of the specimens, as well as low cell cytotoxicity when submitted to cell viability assays. There was a preliminary profile of no liver toxicity when analyzing the histological data and the beginning of bone tissue formation in the 10 and 20 mol% groups. Therefore, the presence of iron oxide and calcium iron double phosphate phases, as they do not have negative effects on cells, suggests a biocompatible behavior. Future analyses, such as the expression of alkaline phosphatase, type I collagen, osteoprotegerin and RUNX-2, which are associated with differentiation, as well as the expression of Vascular Endothelial Growth (VEGF) and nitric oxide, should be performed, to evaluate all biocompatibility profile.

Supplementary Information

Supplementary information (additional characterizations) is available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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Luiz Claudio S. Gomes was responsible for conceptualization, data curation, formal analysis, investigation, methodology, writing original draft; Rhayra Braga for investigation, validation; Diego P. Aguiar for investigation, validation, writing review and editing; Leonardo R. Andrade for investigation, validation, writing review and editing; Amanda S. Cavalcanti for investigation, validation, writing review and editing; Marcos V. Colaço for investigation, validation, writing review and editing; João Antonio M. Guimarães for conceptualization, formal analysis, funding acquisition, investigation, project administration, writing review and editing; Juliana F. de Lima for conceptualization, formal analysis, funding acquisition, investigation, project administration, supervision, writing original draft, review and editing.

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