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Biomimetic Calcium Phosphates Derived from Marine and Land Bioresources

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.71489>

Abstract

This chapter aims to establish the key factors for technological optimization of biogenic calcium phosphate synthesis from marine and land resources. Three natural calcium sources—marble, seashell and bovine bone—were considered as raw materials. The proposed materials are suitable candidates for the synthesis of bone substitutes similar to the inorganic bone component. The synthesis processes were developed based on the investigations of thermal phenomena (TGA-DSC analysis) that can occur during thermal treatments. By this method, we were able to determine the optimum routes and temperatures for the complete dissociation of calcium carbonate as well as risk-free deproteinization of bovine bone. An exhaustive characterization, performed with modern and complementary techniques such as morphology (SEM), composition (EDS, XRF) and structure (FT-IR, XRD), is presented for each precursor. The final chemical composition of ceramic products can be modulated through a careful control of the key parameters involved in the conversion, in order to create long-term performant biphasic apatite biomaterials, with broad medical applicability. Identifying the suitable strategies for this modulation contributes to an appreciable advance in orthopedic tissue engineering.

Keywords: phosphate biomaterial synthesis and processing, marine and land bioresources, biomimetic calcium phosphates, modulated calcium carbonate-derived HA proportion, bovine bone-derived HA

1. Introduction

The fifth of twelve principles of Green Chemistry states that: *“The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary whenever possible and innocuous when used”* [1].

During the last years, the increased awareness of the scientific community regarding clean preparation and processing of bulk and powder biomaterials resulted in intense use of alternative precursors for preparing adequate materials for orthopedic medical applications. However, the development of a bone reconstruction material, completely biologically and mechanically compatible with the different types of bone tissue, is still an ongoing challenge.

Human bone anatomy has the architecture of a nanocomposite material, made of 60–70% mineral component, up to 30% organic components (mostly type I collagen) and approximately 10% water. The mineral component, usually defined as biological apatite and sometimes misrepresented as natural hydroxyapatite, incorporates multiple substitutions [2, 3]:

- Calcium (Ca^{2+}) can be substituted by Sr^{2+} , Ba^{2+} , Mg^{2+} , Na^+ or K^+ ;
- Phosphorus (P) can be substituted by C, As, V or S; and
- Hydroxyl groups (OH^-) can be substituted by carbonate groups (CO_3^{2-}), fluorine (F^-), chlorine (Cl^-) or their place can remain vacant.

The need for restoring damaged bone tissues leads to the development of various bone reconstruction and tissue engineering solutions. Currently, the most popular are various types of bone grafts but every type is confronted to disadvantages such as the risk of biological contamination, infection and fast absorption (for xenografts); difficult harvesting and storage, high risk of tumoral cells and pathogens transfer (for allografts); low availability, additional surgical procedures, scars and prolonged healing of harvested area (for autografts) [4, 5].

Alloplastic materials are intensively developed as alternatives for bone grafts. The current market offers a wide range of calcium phosphate-based biomaterials as substitutes for bone tissue. Most representative materials are hydroxyapatite (HA), beta-tricalcium phosphate (β -TCP) and different combinations of these, generally named biphasic calcium phosphates (BCP) [2, 3, 6–8]. The main reason for using calcium phosphate-based biomaterials is their resemblance with the bone tissue, so research and development of this area tends to reproduce more accurately the damaged tissue, with more efficient results. This scope involves firstly the preparation of a calcium phosphate with potential use in orthopedic bone reconstruction.

Currently, this trend is expressed by improving a relatively new concept, which combines advanced fabrication of bioceramics with the sustainable use of natural resources, namely functionalization of marine and land resources for preparing biogenic calcium phosphates [2, 9, 10]. Dedicated studies offer extensive information regarding [1] *marine resources* such as vertebrates bones—fish bones [5, 6, 11, 12] and calcified structures of invertebrates—coral, snail, seashell, cuttlefish, sea urchin, etc. [12–17], and [2] *land resources* such as animal bone tissue—preponderantly bovine bone [2, 4, 18, 19] or other calcified structures such as eggshells [9, 20], which could be used as cost-effective raw materials. Most of these resources are naturally available as various polymorphs of calcium carbonate (CaCO_3); by exception, vertebrate bones contain calcium phosphate closely related to the mineral component of human bones. Another CaCO_3 resource available on land is marble. This is, to our knowledge, an innovative precursor for preparing biocompatible calcium phosphates.

All these natural resources bring, besides the calcium resources required for calcium phosphate synthesis, many beneficial chemical elements such as Mg, Na, K, Cl, F, Si, Sr, which are found

in human bones in various amounts [2]. This provides a unique advantage to naturally derived biomaterials against conventional calcium phosphates because the synthesis and preparation methods do not need additional procedures for doping the stoichiometric compounds.

Various synthesis methods were developed so far for using CaCO_3 as a precursor for obtaining calcium phosphates, among which, two research directions being currently considered:

- *Direct synthesis*, which implies precursor treatment with phosphorus-based reagents. Although intensively studied [6, 14, 15, 21–24], the process parameters are incompatible with reproducible manufacturing, while the final products are susceptible to impurification with trapped intermediate products.
- *Indirect synthesis*, which requires thermal dissociation of CaCO_3 in calcium oxide (CaO) prior to phosphate-based reagent treatment. Initially proposed by Rathje in 1939 [25], the method was studied for different types of precursors in an attempt to obtain reproducible results [5, 6, 11, 13, 16–18, 26]. To date, the correlation between the synthesis parameters and the material characteristics is poorly understood, so further research is needed for adapting the method for advanced manufacturing.

For animal bones, isolation and processing of the existent calcium phosphate resource begins with chemical or thermal deproteinization; then, the resulted material is thermally treated in a controlled manner: thermal treatment could be performed by combining different temperatures (700–1400°C), heating environments (air, argon, nitrogen, carbon dioxide) and cooling conditions (air or water with ice) [27, 28]. Although methods for manufacturing bovine bone-derived medical devices are standardized [29] and their use is regulated [5], the current research strategies aim to align bovine bone processing to the fifth principle of *Green Chemistry* and to improve the existent methods by eliminating all reagents which could induce a risk, thus upgrading the quality management approaches related to bovine bone-derived products, their manufacturing and large-scale utilization.

This study aims to identify the key parameters for optimization of biogenic calcium phosphate synthesis and processing. The marine and land resources included in this study are marble, seashells and bovine bone. The proposed natural resources are convenient candidates for preparing bone substitutes, which resemble the inorganic (mineral) component of natural bone tissue. Moreover, by careful control of key parameters involved in CaCO_3 conversion and biological apatite isolation, the composition of final ceramic products could be modulated in order to create long-term performant biphasic calcium phosphates with larger biomedical applicability. Identifying the optimal routes for achieving this aims contributed to a substantial advancement of bone reconstruction materials.

2. Marine and land bioresources

The worldwide scientific community is aware of the negative environmental effects of human consumption. A continuous effort aims to reduce the impact of unsustainable use of limited resources by developing environment-friendly processing methods and applications [9]. The use of marine and land materials resulted after industrial processing of different animal species for producing performant biomaterials is a sustainable solution for reducing waste generation.

2.1. Marine resources

Different invertebrate organisms from marine environment contain considerable resources of CaCO_3 in different calcified structures such as thorns, shells, exoskeletons or bones. Some of the most popular marine precursors used for the preparation of hydroxyapatite and other calcium phosphates were corals, due to a well-established conversion procedure for coralline hydroxyapatite which was developed by Roy and Linnehan in 1974 [22]. However, current threats such as climate change, destructive fishing practices, overfishing, careless tourism, pollution or coral mining (for use as bricks, road-fill, cement or souvenirs) drastically limited the possibility of using these resources for producing hydroxyapatite. In this respect, different available alternatives can be used:

- Bone-like structures in cuttlefish (e.g., *Sepia officinalis*), generally known as cuttlebones, are organized in CaCO_3 (aragonite) pillars and organic membranes (3–4.5% organic matter) [30]. Synthesis of hydroxyapatite is usually achieved by direct synthesis with phosphorus-based reagents.
- Gastropods (snails) and bivalves (clams, mussels, oysters) shells are primarily composed of CaCO_3 . Many types of shells (e.g., *Strombus gigas*, *Tridacna gigas*, *Mytilus edulis*) are considered lamellar microcomposites. The inner layers of these shells consist of 95–99% of CaCO_3 as aragonite and different organic macromolecules [13]. Based on their environment, the different species of shells may contain variable amounts of oxides such as SiO_2 , MgO , Al_2O_3 , SrO , P_2O_5 , Na_2O or SO_3 [14–16].
- Echinoderm skeletons (e.g., *Heterocentrotus mamillatus*, *Heterocentrotus trigonarius*) are composed of CaCO_3 plates and spines, each skeletal element being a single crystal of CaCO_3 in form of calcite, very finely branched and structured, for which conversion in hydroxyapatite was reported [14].
- Crustacean wastes (crabs or lobsters such as *Portunus pelagicus*, *Nephrops norvegicus*, etc.) contain three types of valuable compounds—20–40% protein, 20–50% CaCO_3 and 15–40% protein. Current waste processing is destructive, wasteful and expensive, as the methodology required to separate these three types of compounds uses corrosive or hazardous reagents. Creative chemistry is summoned in order to fully benefit from this type of waste, which is largely available [31].

Besides CaCO_3 calcium phosphate sources are available in fish bones:

- Fish bones (e.g., *Thunnus obesus*, *Pseudoplatystoma corruscans*, *Pseudoplatystoma fasciatum*, *Oreochromis mossambicus*, *Paulicea lutkeni*, etc.) represent a significant part of the fish—10–15% of total fish biomass being bones from the head to vertebrae. Although interspecies variation of composition of fish bones is significant in the level of proteins and lipids, the mineral bone matrix contains similar amounts of Ca and P, giving a similar Ca/P ratio regardless of the species.

2.2. Land bioresources

Similar to marine bioresources, terrestrial or land raw materials can be divided into CaCO_3 and calcium phosphate resources:

- Land gastropod shells (snails such as *Helix pomatia* or *Helix aspersa*) contain mainly CaCO_3 and minor amounts of MgCO_3 and organic compounds, which were reported as raw materials for producing natural bioceramics [32, 33].
- Land crustaceans (e.g., *Orchestia cavimana*) contain amorphous CaCO_3 and minor amounts of amorphous calcium phosphate within their structures.
- Bird eggshells (*Gallus gallus domesticus*, *Struthio camelus*) contain up to 97% CaCO_3 as calcite and 3–4% organic components. Conversion of CaCO_3 in biocompatible calcium phosphates can be achieved by direct synthesis with phosphate-based reagents [9, 20].
- Large vertebrate bones are primarily composed of calcium phosphate (biological apatite) and are largely available worldwide. Procedures for isolating the mineral component of several bone species (*Cervidae*, *Ovis aries*, *Equus caballus*, *Crocodylinae*, *Struthio camelus*, *Anatidae*) were already reported. Use of bovine bones is considered a more practical approach in terms of size, availability and similarity with human bones [2, 4, 5]. The biological apatite of bovine bones also includes Mg^{2+} ions and CO_3^{2-} groups which further influence the characteristics of processed materials [2].

3. Synthesis and preparation

3.1. Precursor's preparation: impurities and organic components removal

Independent of the resource used for calcium phosphate preparation, the raw material shall be subjected to preliminary preparation procedures in order to ensure the quality of the final products. Generally, these procedures refer to the macroscopical impurities removal and organic components separation from the natural material's structure.

For invertebrates, cleaning of the precursors can be accomplished by brushing under water pressure and distilled water ultrasonication [15, 17]. The residual organic matter can be removed by immersion in hydrogen peroxide solution (50%), through boiling or in autoclave [13]. After drying, materials can be crushed and grounded in a ball mill or agate mortar and optionally sieved [15, 17].

Fish bones can be first mechanically cleaned to remove impurities/particles from the natural environment and then sectioned into small pieces [4, 12]. Further, the bone can be repeatedly boiled in distilled water to separate the organic tissue and bone marrow [4, 6, 11]. Degreasing and elimination of external hyaluronic acid and proteins can be achieved by bones immersion either in alcohol baths (ethanol 70%, v/v), followed by distilled water washing and hydrogen peroxide preservation (30%, v/v) or in alkaline sodium hydroxide (NaOH) solution (1 N) [4, 12]. After drying at 50°C in hot air oven, the bone pieces can be stored in formaldehyde solution (4%, v/v) if it is not immediately processed [4].

Bird or tetrapod bone preparation starts by freezing at -20°C for facile segmentation; otherwise, mechanical removal of macroscopical impurities may lead to local heating of the bone and therefore to inadequate separation of bone marrow and other potential antigenic substances [2].

Further deproteinization can be carried out in an autoclave at 100°C by boiling [2, 19]. Prolonged exposure to autoclaves or vapors reduces collagen in the form of gelatin and thus lowers the risk of coal black matter appearance at the end of processing [2]. This step can also be achieved with organic solvents such as methyl acetate or hydrogen peroxide [2].

For eggshells, cleaning with sodium hypochlorite (NaClO) solution (5%) was reported, followed by ball mill or agate mortar grinding for 2 hours and sieving. Further, the obtained powder was repeatedly washed with the same solution and then dried in conventional oven at 100°C for 24 hours [9, 20].

3.2. Synthesis by chemical precipitation

3.2.1. Direct method

Direct synthesis (**Figure 1**) is performed on CaCO_3 powder with phosphorus-based reagents. One commonly studied reagent is ammonium phosphate monohydrate ($(\text{NH}_4)_2\text{HPO}_4$), which

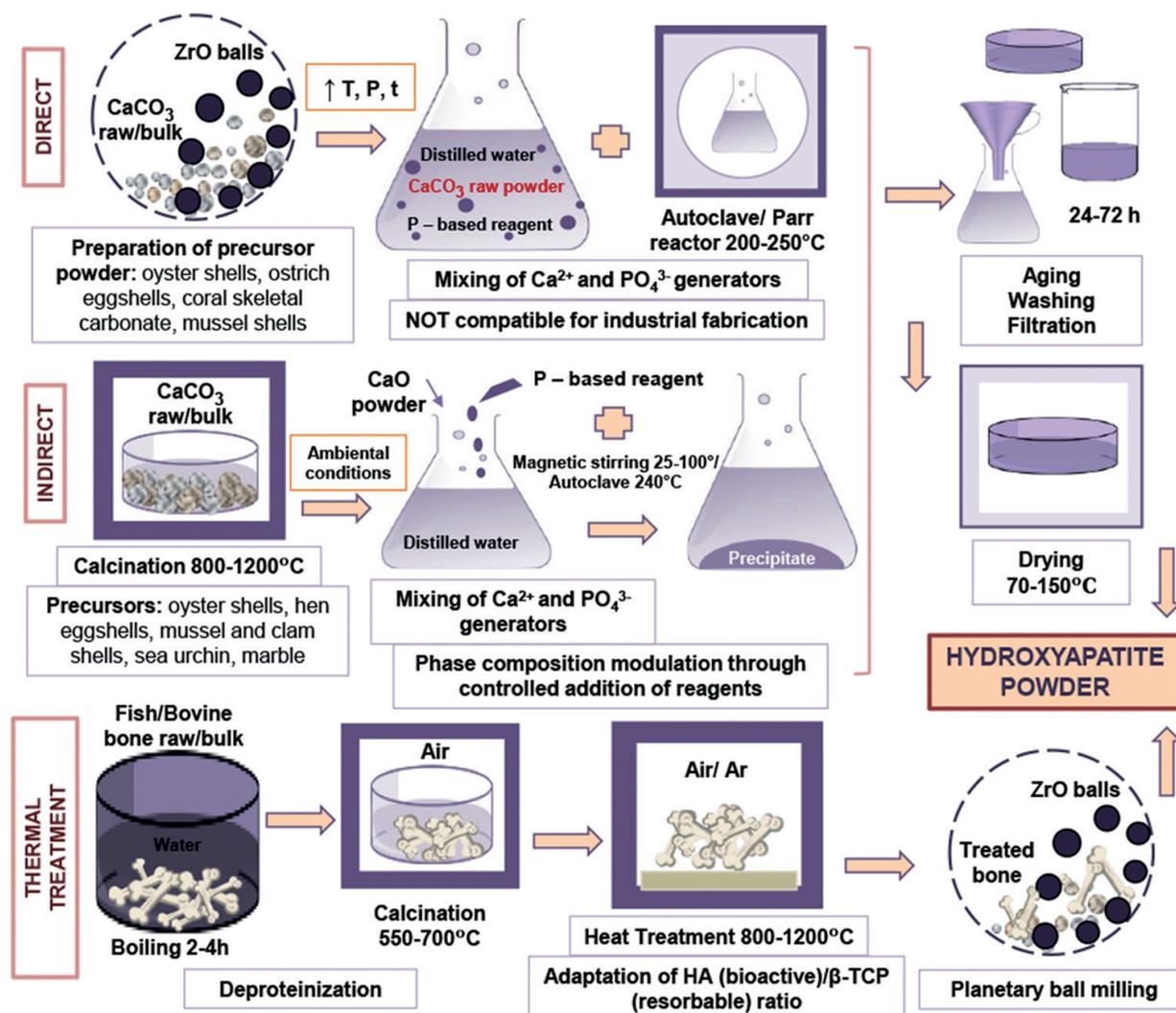


Figure 1. Schematic representation of the main routes for converting natural precursors such as marble, seashell, and bovine bone in biocompatible calcium phosphates.

can be mixed with CaCO_3 powder in Parr reactors at approximately 250°C [22] or in an autoclave. CaCO_3 powder can also be mixed with distilled water and then treated by controlled addition with phosphoric acid (H_3PO_4) in equivalent proportions for desired Ca/P molar ratio [20]. Another possible method concerns the direct treatment with calcium pyrophosphate ($\text{Ca}_2\text{O}_7\text{P}_2$) by wet grinding in a planetary ball mill [15]. In all cases, the synthesis is followed by distilled water washing and drying at temperatures between 70 and 150°C [15, 20, 22–24].

3.2.2. Indirect method

3.2.2.1. Thermal dissociation of calcium carbonate

Regardless of the CaCO_3 polymorphic form (aragonite, calcite or dolomite), thermal dissociation takes place through calcination at temperature of 800 – 1200°C , for at least 2 hours [5, 6, 16, 17, 34]. Calcination leads to carbon dioxide (CO_2) release, associated with a mass loss of $\sim 45\%$. The obtained product is calcium oxide powder (CaO), which can be involved in the chemical synthesis as prepared or sieved in advance [16, 34].

3.2.2.2. Chemical precipitation

Post calcination, CaO powder is usually mixed with distilled water and transformed into calcium hydroxide ($\text{Ca}(\text{OH})_2$); during hydration, an exothermic reaction occurs and its volume doubles [34]. After hydration, the $\text{Ca}(\text{OH})_2$ aqueous solution can be further treated with phosphorus-based reagents such as diammonium phosphate ($(\text{NH}_4)_2\text{HPO}_4$) [5, 13] or H_3PO_4 . CaO powder can also be dissolved in nitric acid (HNO_3), to obtain calcium nitrate ($\text{Ca}(\text{NO}_3)_2$) which would then react with $(\text{NH}_4)_2\text{HPO}_4$ [17] or EDTA solution, to convert CaO into Ca-EDTA complex, which reacts with disodium phosphate (Na_2HPO_4) [16].

Reactions take place for several hours by using magnetic stirring at temperature of 25 – 100°C [5, 16, 26] or several days, in autoclave, at maximum 240°C [13]. The reaction ends with formation of a white precipitate which can be further dried in a vacuum oven at 80°C for 6 hours [16] or in an electric one for 3 hours [5, 17, 35] to obtain the final calcium phosphate powder. Following this procedure, after sintering at 900°C , the obtained HA was reported to be pure and thermally stable.

Depending on the medical applicability, the product's final composition can be tuned during synthesis and through the final thermal treatment. This is the main reason why elevated synthesis conditions (direct method) are not necessarily adequate for synthesizing large amounts of powdery samples. Therefore, in case of calcium carbonate-based precursors, synthesis techniques were adapted to normal (room) conditions such as temperature, pressure and time (indirect method). On the other hand, for bone-like precursors, the synthesis route is completely different, given their compositional similarity to the human bone. The HA extraction and final composition adaptation is carried out only by thermal treatment.

3.3. Thermal treatment

The thermal treatment of marine resources can be performed at 160 – 1200°C for 2–8 hours [6, 12, 21]. A soaking time of 2 hours proved insufficient for complete transformation of

CaCO_3 , and small quantities of residual aragonite could be identified in the material structure. Extension of thermal treatment to 8 hours was reported to ensure the complete conversion of calcium carbonate into HA [14]. Even though it was reported that at 1000°C synthesized HA is stable and similar to the pure one with Ca/P molar ratio of 1.67 [21], HA preparation from marine resources and exclusively thermal methods is not reproducible.

On the other hand, thermal treatment of bone tissue aims for producing biphasic calcium phosphates with modulated content of HA and β -TCP, since β -TCP transformation into α -TCP was not identified in bovine bone-derived materials at temperatures lower than 1200°C [27, 36]. Adaptation of HA (bioactive)/ β -TCP (resorbable) ratio relies upon the precursor features such as substitutions in the crystalline structure of biological apatite, the elemental species embedded in the structure and the interactions between them during thermal processing. Apart from the biological apatite characteristics, an important role for processing is addressed to the thermal treatment parameters:

3.3.1. Heating rate and heating duration

Temperature and heating duration are dependent on the bone pieces' dimensions, the amount of oxygen present in the heating environment and precursor preparation methods [37]. At 600 – 1000°C , at least 2 hours is necessary for the removal of all organic component from 1 cm^3 of bone tissue. Optimum reported heating rate was $10^\circ\text{C}/\text{min}$; thermal treatments conducted below this rate ($5^\circ\text{C}/\text{min}$) could lead to the partial fixation of carbon and delayed decomposition reactions [28].

3.3.2. Treatment temperature

Thermal degradation of bovine bone begins with the evaporation of surface water. Collagen denaturation is carried out in parallel with the water loss and continues up to 500 – 600°C , with mass losses and carbon dioxide emissions [28]. Until complete degradation, the organic component acts as a protective shield for calcium phosphate found in the bone mineral component. For this reason, the mineral matter does not undergo thermal transformations up to 500 – 600°C . Above this temperature, the biological apatite is subjected to a recrystallization process, made in three stages: lattice diffusion (500 – 750°C), surface diffusion (750 – 900°C) and grain boundary diffusion (900 – 1000°C) [38]. Recrystallization is usually correlated with removal of carbonate groups from the crystalline structure. Thermal degradation of bone-derived HA is possible above 1000°C but the event is strongly influenced by the precursor's chemical composition (with compositional variations of bone tissue from different animals) and the thermal treatment environment. The main products obtained after HA decomposition include different forms of oxyapatites, which can subsequently decompose into β -TCP, CaO [36] or tetracalcium phosphate (TTCP).

3.3.3. Thermal treatment environment

Thermal treatment environment is responsible for the heat transfer and assuring/disposing of gaseous products and reactants. Thermal analyses performed in nitrogen atmosphere proved

that bovine bone-derived HA decomposition begins at approximately 1000°C and lead to the β -TCP and CaO traces formation. Other result obtained in air atmosphere and from different species of vertebrates pointed the beginning of HA transformation at around 800°C [39]. In argon atmosphere, bovine bone-derived HA decomposed into β -TCP (without any detectable CaO traces) at temperature of ~1200°C [36]. Heating in carbon dioxide atmosphere does not induce significant modifications of HA up to 1200°C.

3.3.4. Cooling conditions

The control of cooling conditions contributes to the modification/preservation of the bone-derived calcium phosphates' phase composition because the conversion β -TCP \rightarrow α -TCP is reversible through slow cooling. α -TCP (resorbable) conservation within the thermally processed calcium phosphates' structure was achieved by quenching.

4. Results and discussion

The investigations described in this section were made on samples prepared from natural precursors—marble, marine seashells and bovine bone—and from bioceramic materials derived from those precursors. For marble and seashells, an indirect Rathje-based method was optimized by means of magnetic stirring, reagent treatment and thermal treatment [40, 41]. In this study, the materials were evaluated in three stages: [1] raw marble and seashell precursors (after cleaning), [2] thermally treated marble and seashells (intermediate products), and [3] marble and seashell-derived bioceramics (named Marble-TT and Seashell-TT, respectively) resulted after treatment with H_3PO_4 and drying at 120°C.

Bovine bone samples were investigated in three stages: [1] raw bovine bone precursor; [2] intermediate product obtained after deproteinization at 500°C; and [3] final ceramic product obtained after thermal treatment at 1000°C in air atmosphere. Following this route, contamination risks were reduced by eliminating all reagents involved in processing and by performing thermal treatment at temperatures above 850°C, which are considered microbiologically safe [27, 28]. The preparation procedures for all precursor types are described in detail in Refs. [36, 40].

4.1. TGA/DSC analysis: thermal behavior of natural resources

Temperature-induced thermal transformations were evaluated by TGA-DSC analysis (SDT Q600 equipment) between 25 and 1200°C, with 10°C/min, in argon atmosphere. The results are presented in **Figure 2**.

Thermal degradation of calcium carbonates (marble and seashells) began with a thermal event associated with evaporation of surface water, at approximately 75°C, without significant mass loss (**Figure 2**). $CaCO_3$ thermal degradation included the decomposition of dolomite from marble and aragonite from seashells into calcite, a thermally stable phase. Decompositions occurred at approximately 300°C (endothermic peaks in **Figure 2**) and were accompanied by a mass loss of approximately 2%. Thermal dissociation of $CaCO_3$ continued until 850–900°C

temperature was achieved. This dissociation temperature is inferior to the one corresponding to pure CaCO_3 (963°C). Degradation was associated with mass losses of approximately 40%, corresponding to CO_2 emissions. The CaO resulted after carbonate degradation was stable until 1200°C was achieved, in agreement with previous studies [42].

Thermal degradation of bovine bone began with surface water removal, which occurred until approximately 300°C and was accompanied by a mass loss of approximately 10% (**Figure 2**). Combustion of the organic bone component began concomitantly with water loss. This phenomenon was accompanied by a significant mass loss of 20–30%, until 500°C was reached. The temperatures between 500 and 800°C usually induce the removal of carbonate groups within materials' structure; in the current study, this event was associated with a mass loss of approximately 5%. First major thermal event was identified in DSC results at approximately 800°C and corresponds to the partial transformation of HA in β -TCP, between 850 and 1200°C , with an exothermic peak at approximately 1000°C . The beginning of a new thermal event was observed at approximately 1200°C , which suggests that β -TCP was partially transformed into α -TCP. This result is in agreement with the previously reported results, which pinpoint the beginning of α -TCP at 1125°C [27, 28].

4.2. SEM-EDS analysis: morphocompositional characteristics

The morphocompositional characteristics of marble, seashell and bovine bone precursors and bioceramic products derived from those precursors were highlighted by SEM analysis (Philips Xl 30 ESEM TMP equipment) coupled with EDS (EDAX Sapphire equipment). The

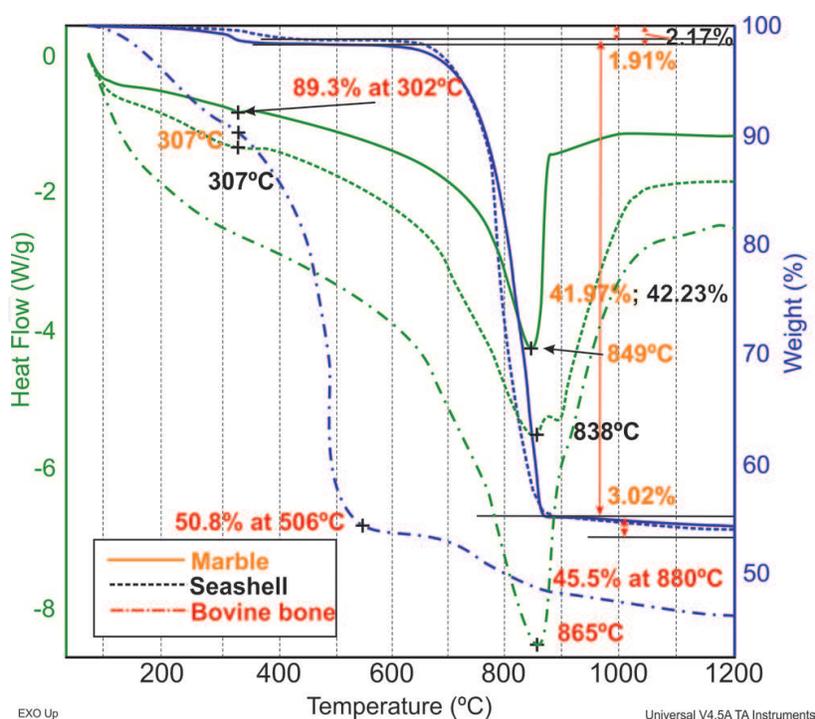


Figure 2. TGA-DSC analysis results for marble, seashell, and bovine bone precursors used for biocompatible calcium phosphate preparation.

results for precursors, intermediate synthesis products and final bioceramics derived from each type of natural resources are presented comparatively in **Figure 3**.

The raw marble (**Figure 3A**) exhibited a compact microstructure, with a separated phase arrangement. Considering the EDS results (in which magnesium presence was confirmed, shown in **Figure 3**), as well as the previously reported results, the fine white lines in the marble microstructure represent calcite microregions. This alternates with the broader regions of dolomite (magnesium and calcium carbonate), highlighted by darker gray shades. Isolated

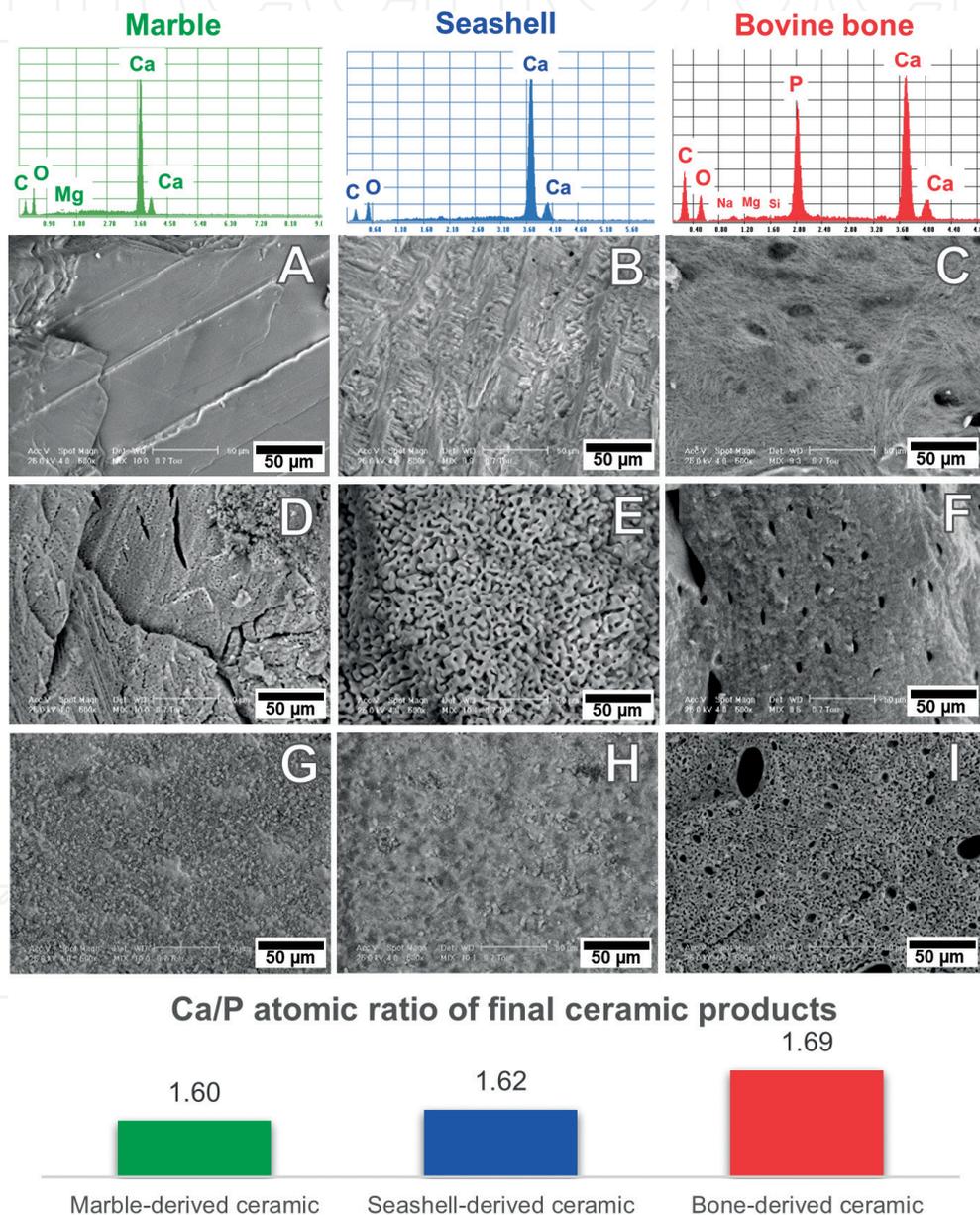


Figure 3. SEM-EDS results for marble, seashell, and bovine bone precursors and bioceramic products prepared from those precursors: EDS spectra of natural precursors; (A–C) SEM results for initial (raw) precursors; (D–F) SEM results for intermediate products: marble and seashell thermally treated at 1300°C and bovine bone thermally treated at 500°C. (G–I) SEM results for final bioceramic products (obtained after chemical treatment and drying of marble and seashell, and thermal treatment of bovine bone, respectively). Variation of Ca/P atomic ratio of final ceramic products.

calcite grains, with polyhedral shape and sharp edges were also observed. After the thermal treatment performed at 1200°C (**Figure 3D**), the material exhibits an acute cracking of the initial compacted microstructure. The final bioceramic product (**Figure 3G**), obtained after chemical treatment with H_3PO_4 and drying at 120°C exhibits a dense and uniform microstructure with no pores or defects.

The seashell precursor (**Figure 3B**) had a typical lamello-fibrillar microstructure, in which calcite layers alternated with perpendicular aragonite layers. After thermal treatment (**Figure 3E**), shells morphology was constituted from connected particles and many pores resulted after cracking and aeration of calcite layers. After synthesis and final thermal treatment (**Figure 3H**), the resulted bioceramic had a compact, uniform and defect-free microstructure.

The raw bovine bone microstructure (**Figure 3C**) is typical for cortical bone tissue, that is, an association of osteons with concentric lamellae arranged around haversian canals. The organic component of bone tissue (highlighted by darker gray shades in **Figure 3C**) was mostly present in the haversian canals and in the lacunae disposed along bone lamellae. In thermal treated bone (at 500°C—**Figure 3F** and at 1000°C—**Figure 3I**), both haversian canals and lacunae were transformed into different sized pores due to complete combustion of the bone organic component.

The composition of carbonate precursors includes Ca, C and O as major elements (EDS spectra in **Figure 3**). Traces of Mg were identified in the EDS spectra of the marble precursor. Based on their origin, these precursors may contain variable quantities of Na and/or Si, but were not identified in the current study. The EDS spectra of the bovine bone precursor includes, besides Ca, P, C and O (characteristic major elements), peaks of Na and Mg.

The compositional key performance indicator for the naturally derived bioceramic was atomic Ca/P ratio (graph in **Figure 3**), calculated based on EDS results. Ca/P ratio varied between 1.60 for marble-derived materials and 1.69 for bovine bone-derived ones.

4.3. XRD: structure and phase composition

The structure and phase composition of natural precursors and final ceramic products were evaluated by XRD (Bruker D8 Advance diffractometer equipped with a LynxEye detector), in Bragg-Brentano geometry, with $Cu K_{\alpha}$ ($\lambda = 1.5418 \text{ \AA}$). Analyses were performed for $2\theta = 10\text{--}50^\circ$, with 0.04°/1 s step. The results are presented in **Figures 4** and **5**.

The XRD patterns for marble and seashell precursors (**Figure 4**) indicated the presence of $CaCO_3$ by its characteristic peaks at $\sim 29.5^\circ$, $47\text{--}48^\circ$ (marble—calcite, ICDD: 01-086-2339) and $\sim 27^\circ$, 32.5° , 43° (seashells—aragonite, ICDD: 00-005-0453), respectively. In agreement with EDS results (**Figure 3**), XRD pattern for marble (**Figure 4**) signaled the presence of magnesium carbonate $CaMg(CO_3)_2$ (ICDD: 00-036-0426) in the material, by characteristic peaks located at 37° and 42.5° . XRD results for the bovine bone precursor indicate a low crystallinity due to the presence of the organic components within the bone tissue.

The peaks identified in the XRD pattern of the bioceramic obtained after bone thermal treatment (bovine bone-derived ceramic in **Figure 5**) confirm that the materials contain HA as single phase. The sharpness of the peaks suggests a high crystallinity. In comparison, the bioceramics derived from marble and seashells (marble-derived ceramic and seashell-derived

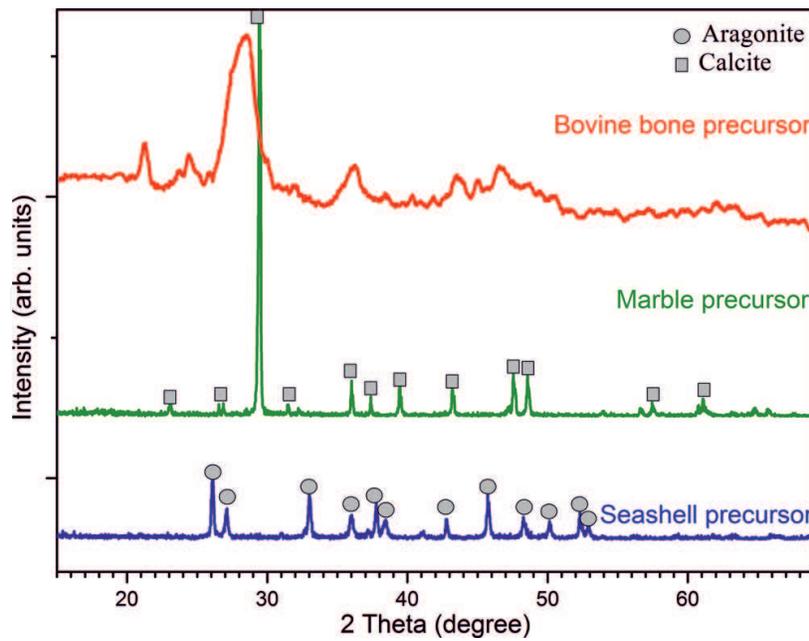


Figure 4. XRD patterns of marble, seashell, and bovine bone precursors used for biocompatible calcium phosphate preparation.

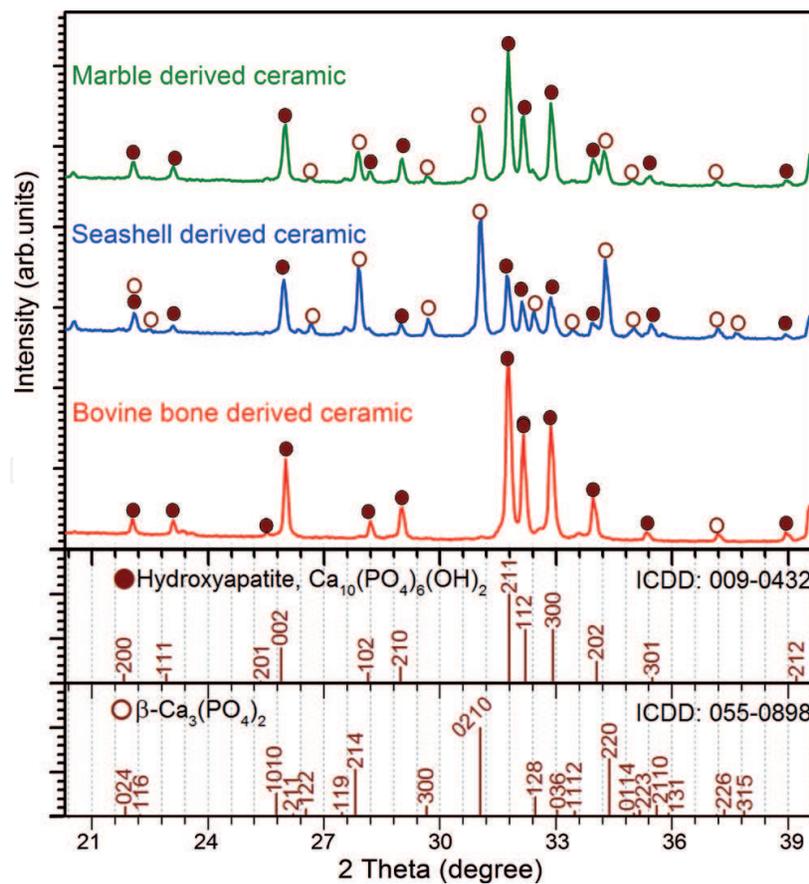


Figure 5. XRD patterns of final derived bioceramic products obtained after indirect chemical synthesis and thermal treatment of marble and seashell precursors (marble-derived ceramic and seashell-derived ceramic, respectively) and thermal treatment at 1000°C of cortical bovine bone (bovine bone-derived ceramic).

ceramic in **Figure 5**) included low-intensity peaks suggesting a lower crystallinity. The indirect synthesis of both precursor types led to the obtaining of a biphasic material consisting of different proportions of HA and β -TCP.

4.4. FT-IR analysis: functional groups architecture

Functional groups architecture was evaluated by FT-IR analysis (Perkin Elmer Spectrum BX II equipment) in attenuated total reflectance (ATR) mode (PikeMiracle head). IR spectra were recorded between 800 and 3600 cm^{-1} for the raw precursors (**Figure 6**) and between 500 and 1200 cm^{-1} (**Figure 7**) for the final bioceramic products, with 4 cm^{-1} resolution and 32 scans per experiment.

IR spectra of the marble and shell precursors (**Figure 6**) included the characteristic vibration bands of CO_3^{2-} groups in CaCO_3 , namely ν_2 asymmetric bending (870 cm^{-1}), ν_3 asymmetric bending ($\sim 1400 \text{ cm}^{-1}$) and ν_1 symmetric stretching (2312 cm^{-1} , 2968 cm^{-1}), as well as the peaks' characteristic for the vibrational mode of water molecules (3640 cm^{-1}) [43]. The IR spectra of the bovine bone precursor included a high-intensity peak at 1008 cm^{-1} , corresponding to ν_3 symmetric stretching of $(\text{PO}_4)^{3-}$ groups along with peaks of lower intensity, corresponding to CO_3^{2-} groups. The bone organic component is represented by peaks corresponding to amide in collagen at: 1645 cm^{-1} (amide I vibrations), $\sim 1550 \text{ cm}^{-1}$ (amide II vibrations) and $\sim 1200 \text{ cm}^{-1}$ (amide I vibrations).

After synthesis and thermal treatment, the IR spectra of all three precursors included similar peaks (**Figure 7**). The first peaks, corresponding to ν_4 symmetric bending of $(\text{PO}_4)^{3-}$ (563 cm^{-1} , 600 cm^{-1}) are more well defined for the bovine bone-derived material (bovine bone-derived ceramic) in comparison with marble and seashell-derived ones (marble-derived ceramic and

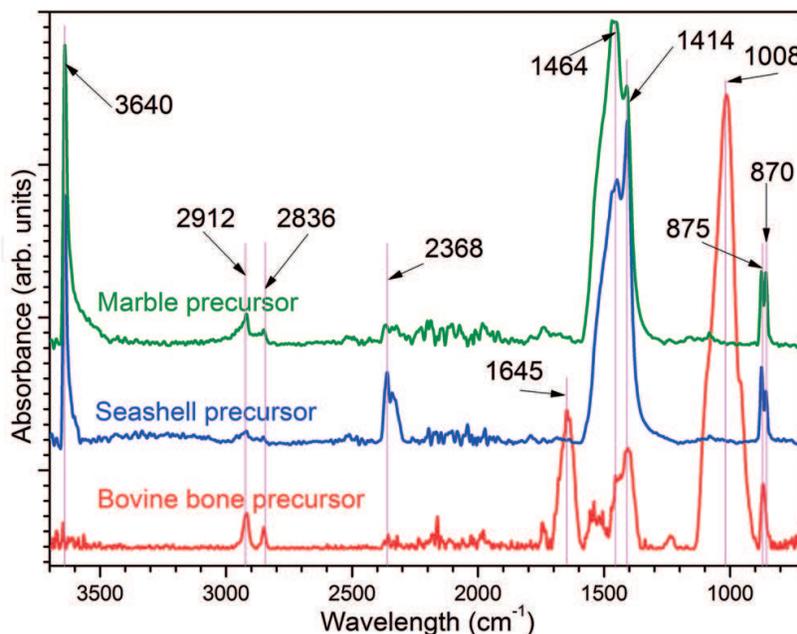


Figure 6. IR spectra of marble, seashell, and bovine bone precursors used for biocompatible calcium phosphate preparation.

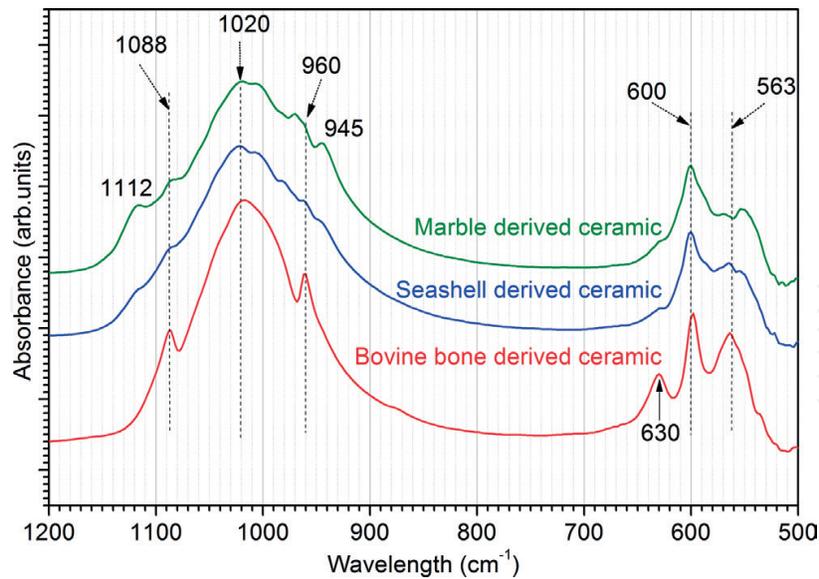


Figure 7. IR spectra of final bioceramic products obtained after indirect chemical synthesis and thermal treatment of marble and seashell precursors (marble-derived ceramic and seashell-derived ceramic, respectively) and thermal treatment at 1000°C of cortical bovine bone (bovine bone-derived ceramic).

seashell-derived ceramic, respectively). Bovine bone-derived ceramic samples also exhibit a peak corresponding to vibration of structural $(OH)^-$ groups (630 cm^{-1}), suggesting a higher water content in this samples. Peaks characteristic to phosphate groups were identified in the $900\text{--}1150\text{ cm}^{-1}$ region for all the three types of bioceramics. The IR spectra of bovine bone-derived ceramic includes a well-defined peak at 1020 cm^{-1} with two shoulders at 960 and 1088 cm^{-1} , which resembles well to the characteristic spectra of HA [44]. These peaks are assigned to ν_1 symmetric stretching of $(PO_4)^{3-}$ (960 cm^{-1}) and ν_3 asymmetric stretching of $(PO_4)^{3-}$ ($1020, 1088\text{ cm}^{-1}$). In good agreement with the XRD results (Figure 5), the marble-derived bioceramic spectra includes two additional peaks at 945 and 1112 cm^{-1} , corresponding to β -TCP [45], while the seashell-derived bioceramic (seashell-derived ceramic) exhibits a single peak of lower intensity at 1020 cm^{-1} , suggesting a lower crystallinity degree of the material with no significant differentiation between the HA and β -TCP peaks.

5. Conclusions and future perspectives

This study proved once more that in the quest of finding an excellent bone substituent, calcium phosphates raised a new level of knowledge due to the generous marine and land bioresources that can be converted. In this context, several drawbacks of current alloplastic methods can be forecasted and minimally invasive surgery shall be needed.

An insightful investigation was carried out in terms of three possible natural precursors—marble, seashell and bovine bone—for biogenic HA synthesis. For the first two, an improved and fully parameterized chemical method was proposed; marble itself serving as an innovative alternative. This led to significant morphological, compositional and structural variations

between final stage products. The precursors' structural examination revealed, as it was expected, three polymorphic calcium carbonate forms (calcite, dolomite and aragonite) and a typical bone-like phase composition. Thus, the intermediary thermal treatment affects the initial compact microstructure either by cracking and aeration (marble and seashell) or by transforming the haversian canals and lacunae into size distinctive pores (bovine bone). Further, post-synthesis heat treatment processing constituted a key objective for marble- and seashell-derived powders, which allowed for biphasic powdery calcium phosphates development. Contrary, in case of bone-derived products, there were no structural or compositional events, the final product consisting of pure crystalline HA.

In terms of naturally derived calcium phosphates, future perspectives are mainly correlated to [1] product manufacturing through both the conventional and additive (SFF) methods, [2] controlled porosity for an optimal vascularization and osseointegration and [3] complete standardization for industrial fabrication. In this respect, further thorough research is required.

Acknowledgements

This work was supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS – UEFISCDI, project number PN-III-P2-2.1-PED-2016-0892.

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