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PhD Thesis Summary

Contributions on the manufacturing of 3D constructs using biomimetic powders for biomedical applications

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Abstract

The continuous advances recorded in the biomaterials science have led to a near-complete understanding of processes involved in osseointegration.

In good congruence with this general direction, the main goal of the PhD thesis was (*i*) to refine a complex and reproducible synthesis technology for the manufacture of biomimetic calcium phosphate-based (CaP) ceramic materials (e.g., hydroxyapatite and tricalcium phosphate phases, in different proportions) and (*ii*) to develop, implement and multi-parametrically assess the performance of innovative porous products derived from marble and seashell precursors as potential bone graft substitutes (to be applied in the restorative and reconstructive orthopaedic surgery). The technology refined and advanced in this PhD thesis lies at the basis of the development of three types of products, whose mechanical and preliminary *in vitro* performances were further engineered by the addition of selected reinforcing components and porogen agents, respectively..

An important innovation is not only the adherence to the concept of nature-inspired biomedical solutions, but also the use of sustainable and cost-efficient resources for the elaboration of the 3D products. The multi-parametric physico-chemical, mechanical and in vitro biological (partially), employing advanced and complementary investigation/testing methods, successfully facilitated the selection of technology “algorithms” for the fabrication of 3D constructs with custom-made bio-ceramic composition, porosity degree (porosity on demand), internal porous networks, and mechanical features for a wide range of healthcare applications.

Keywords: biogenic CaPs; custom-made 3D constructs; induced porosity on demand

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Introduction

The natural bone structure can be assimilated to a complex nanocomposite consisting of mineral fractions (mainly a hydroxyapatite-like compound) interposed in an organic network. The development of an efficient regeneration solutions for the restoration of diseased, traumatized or damaged parts of the bone tissue to its initial anatomical, physiological and functional state, constitutes both a huge challenge and important goal in the biomedical field [1, 2]. This difficult task stems from bone limited capacity to repair itself when dealing with defects situated above the critical size (i.e., diameter anr/or length of 10-12 mm) [3]. The regeneration, restauration and regrowth of bones involves remineralisation and resorption stages.

The design and fabrication of an ideal bone graft substitute (scaffold) can be viewed as a step-by-step process: *(i)* fabrication of biocompatible, nontoxic, non-immunogenic and bioresorbable materials with a suitable degradation rate for cell/ tissue growth *in vitro* or *in vivo*; *(ii)* construction of a 3D framework structure for migration, specialization, proliferation and differentiation of the regenerative cells population; *(iii)* ensuring a porous internal structure with a network of interconnected pores/channels for cell growth and transport of nutrients inside the scaffold and in the native local environment; and *(iv)* improvement of optimal mechanical properties for each application [4, 5].

Despite the increased efforts made for the fabrication of polymer-ceramic composite materials as a potentially approach to mimic the bone structure and resistance, synthetic materials rely on a limited intrinsic toughening mechanism which leads to a reduced ability to undergo high mechanical deformations [6]. Therefore, the fabrication route proposed in this PhD thesis fosters the development of custom-made nature-inspired materials and 3D constructs, aiming to replicate the intricate bone architecture and functions, for a rapid and complete resolve of bone defects, irrespective of size. Importantly, no such products are yet available on the market, and thereby the progresses achieved in this PhD thesis could prove relevant for bone reconstruction/grafting interventions [7, 8].

Currently, bone defect restoration can be achieved by several methods, using a wide range of natural (allograft, autograft) or alloplastic (metallic, ceramic and polymeric biomaterials) materials [9]. Autografts and allografts are disadvantaged by the limited amount of tissue donor, their morbidity and the biological and immunological incompatibility risks [8]. From this perspective, synthetic materials are preferred as a reasonable, yet not integrated solution. In addition to these aspects, the socio-economic factor drastically tilts the balance in favour of new biomimetic products derived from natural (biogenic) resources [10, 11], that can more accurately replicate the mechanical and physiological requirements of the implantation bone site. Technically and economically, the necessity of these products is related to the extensive amount of material required for the large bone defects reparation.

In the view of developing, implementing and promoting a 3D biomodel with continuous spatial porosity and porosity gradient and increased mechanical features, the first step targeted the refinement of the manufacturing technology for the synthesis of biomimetic calcium phosphate materials (CaPs) based on biogenic resources with regenerative biological interest [12]. In this respect, the experimental results published in the **first doctoral article** exposed the bio-functionalization of marble and seashell resources as an eco-friendly, cost-efficient and sustainable approach for CaPs synthesis (hydroxyapatite, hydroxyapatite/brushite or hydroxyapatite/monetite nanomaterials) through thermal dissociation and subsequent treatment with acid with optimised composition (phosphoric acid in the 0-90% range additional to the stoichiometric amount) [13]. The *in vitro* assessment atestted their cytocompatibility and potential for stimulating the restoration/ conservation of physiological functions of injured areas [13].

In direct correlation, the second objective targeted the assessment of three fibres (*Luffa cylindrica,* hemp - *Cannabis sativa,* and wool) as possible natural templates for the manufacturing of 3D biotemplates with tuneable porosity. Preliminary results were presented in the **second doctoral article** regarding the successful incorporation of dried Luffa cylindrical fibres in the ceramic matrix, at various isostatic-pressing forces [14], without any binding materials or chemical treatments, as usually reported [15, 16]. The bioceramic materials were chosen to synthesise hydroxyapatite/brushite and hydroxyapatite from marble and seashells, respectively. The consolidation of green products and the generation of high level of porous architectures with pore and channel interconnectivity were attained by high temperature sintering by the sacrificial template (porogen) method. Such characteristic are desired for a proper cell in-growth and disposal of residues resulted from the cell activity [14]. This favourable hypothesis was further investigated during the **third doctoral article**, in terms of fibres thermal behaviour in two ambients (air and nitrogen) [17]. Topmost, based on the *in vitro* cytotoxicity evaluation of the resulting chars/combustion products, performed for the first time (to the best of our knowledge), the proposed route allowed to delineate the most adequate fibre to be further considered for the fabrication of patient-risk-free implants [17].

An advantageous compromise of the inter-dependent microporosity, macroporosity and mechanical features is also necessary to ensure a good *in vivo* functioning of the products. Therefore, the incorporation of natural fibres can induce an suitable internal porous structure, yet in the detriment of the products mechanical resistance. To warrant enhanced mechanical features, for both compact and porous products, the third objective targeted the possibility to incorporate graphene based materials, as reinforcing agent, directly into the ceramic matrix with no need for binding materials or chemically-routed methods, as reported in other studies [18, 19]. This also allowed for the clear delimitation of the optimal technological parameters for the proposed composite materials (mixtures with different mass ratios between: the ceramic powder/Luffa fibres/graphene nanoplatelets). The necessary mechanical properties were also reflected onto the 3D structures function of the cold isostatic pressing, as reported in ref. [20]. A controlled sintering program, performed in different ambients, applied to both types of developed products (compact and porous structure) was proposed in the **fourth doctoral article** [21]. This strategy aimed to reveal the influence of the sintering parameters on: *(i)* the ceramic materials conversion to different CaPs based on optimal hydroxyapatite, α or β-tricalcium phosphate phase ratio; *(ii)* the ability of Luffa fibres to generate proper architectures after complete reduction in the presence/absence of graphene nanoplatelets in the ceramic matrix; and *(iii)* the overall mechanical features function of the product type and the graphene amount.

In every research stage, from raw materials synthesis to the final biomimetic 3D models, a complex characterization protocol was conducted in order to ensure the fitted choice of simple or biphasic CaP ceramic materials, natural fibre (porogen agent) and the mass ratio of the incorporated reinforcing agent for precisely engineering their shape and properties. Morpho-compositional (SEM/EDS) and structural investigations (XRD, FT-IR spectroscopy) were performed for both source powdered materials and compact and porous products. The morphological aspects such as texture, roughness and topographic patterns stand as essential factors for the biological success of the ceramic materials [22]. Testing the *in vitro* behaviour of the synthesized materials is also imperative for the assessment of cell adhesion, survival and inflammatory responses [23].

Along with chemical composition, the surface-volume features dictate the cellular behaviour in terms of adhesion, differentiation, migration and proliferation (both *in vitro* and *in vivo*) and the degree of new bone formation [24, 25]. Therefore, the surface-volume features were outlined exhaustively by SEM mapping and roughness parameters assessment, contact angle and surface free energy, 3D reconstructions by nano-CT method (porosity degree, pore/channels interconnectivity, tortuosity, internal porous network) [14]. Due to the graphene nanoplatelets reinforcement, mechanical properties were subsequently surveyed in terms of micro-hardness, compressive strength and bending resistance for the forecasted applicability of the developed products.

The ultimate goal of the PhD thesis tackled the identification of the optimal technological flow necessary for biomimetic implants fabrication with tuneable features with respect to chemistry, internal porous architecture, and mechanical features (Figure 1). The delineated technology also responds to an ever-growing current requirement: custom-made/patient-personalized implants. A patent application was submitted for the proposed optimal manufacturing technology ().

The outcomes of the developed technology serve also for future original scientific advances of the scientific field: **(*1)*** the possibility of combining the ceramic biomaterials with natural antimicrobial elements (Ag) and antibiotics; **(*2)*** the manufacture of bulk products that can be customized intraoperatively by the surgeon, according to patient requirements; ***(3)*** enhanced mechanical properties provided through accurate processing of the ceramic powders; ***(4)*** the evaluation of the biomimetic products by *in vitro* standardized assays; ***(5)*** the use of bone implants as viable candidates for controlled drug, cytostatic and cell delivery medium; and **(*6)*** the favourable control of the ceramic biomaterials, natural fibre and graphene nanoplatelets ratios, can satisfy the most varied implant demands in orthopaedic surgery.

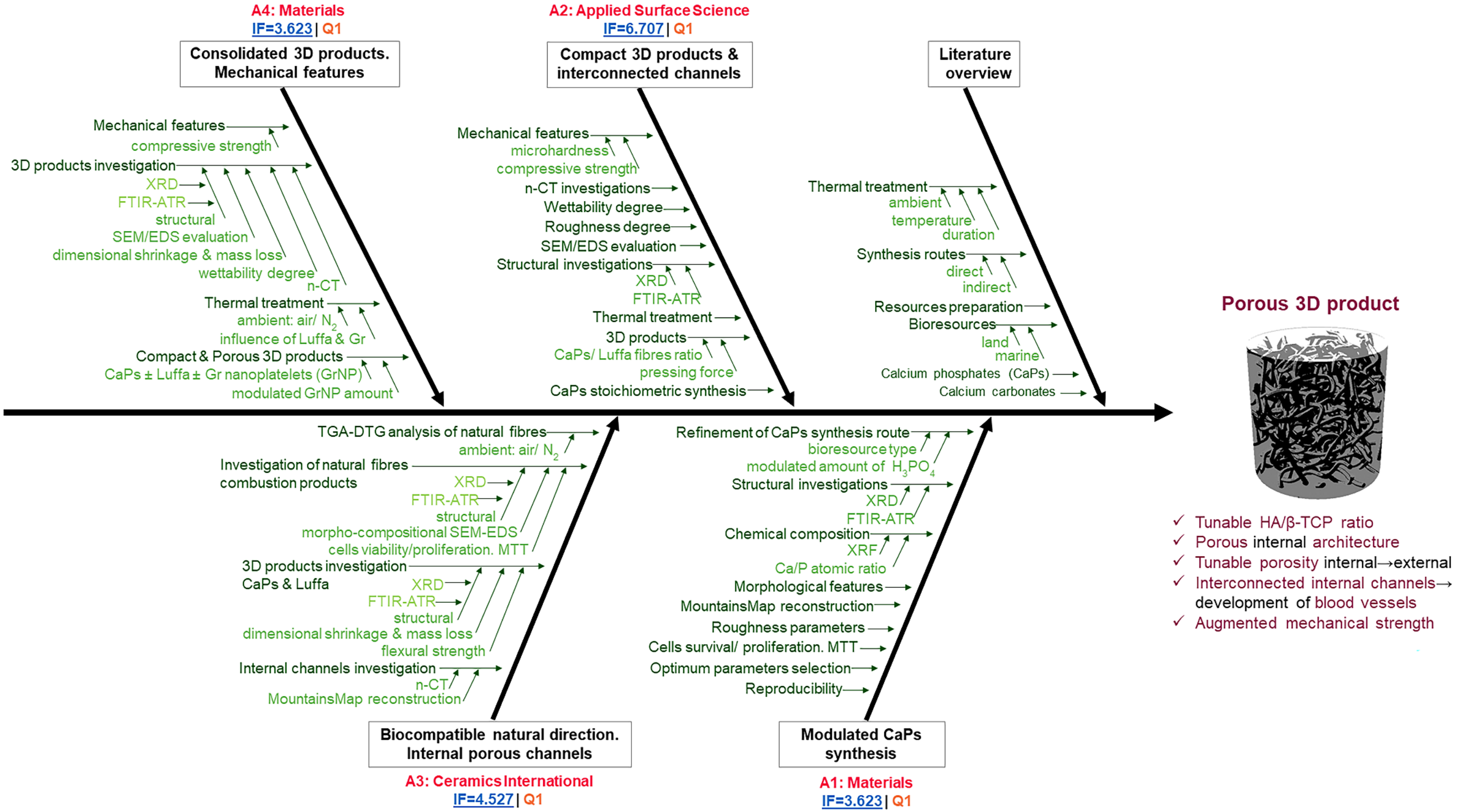

Figure 1. Schematic representation of the stages followed in the PhD thesis

Chapter 1 – Literature overview of the doctoral topic

1.1. Biogenic calcium carbonate resources used in biomedical applications

The vast majority of natural resources become useless after fulfilling their main purpose, turning into significant amounts of wastes daily, coming from different industrial areas and companies [26, 27]. Thus, a global challenge was created to recycle, transform and reintegrate such resources, with a significant harmful impact on the environment, into value-added materials and products, one of the targeted niches being that of bone regeneration [27, 28]. This increased the awareness among researchers, and some of the synthesis routes currently used to obtain calcium phosphate-based materials (e.g., hydrothermal, wet precipitation, sol-gel, solid reaction) were adapted for each type of natural resource or new ones have been developed [27-30]. A detailed exposition of them is offered in ref. [7, 31].

The alternative synthesis of a new generation of calcium phosphates was reported from many natural precursors (e.g., eggshells, seashells, fish bone, and bovine bone) [8, 10, 11, 28, 30, 32, 33]. In the second half of the 20th century, the *replamineform process* was developed, consisting of *replication of life forms*, applied in the first studies to preserve or duplicate the porous architecture of corals, and later other marine resources, in order to obtain products and materials based on hydroxyapatite for the medical field [34, 35]. Several aspects can be pursued in this regard, in terms of sustainable: i) economic, reduces the costs associated with the disposal of bio-wastes and the purchase of chemicals based on calcium and phosphorus, ii) processing, methods involve a small number of steps and therefore a short synthesis time, iii) environment, reduces the degree of pollution by emissions of toxic substances and/or gases and the energy consumption, and iv) human resources, reduces exposure to toxic chemicals and increases occupational safety and health [26-28, 36].

Therein, the first important aspect when designing a technology based on raw calcium carbonate ($CaCO_3$) materials consists in the possibility of acquiring the biogenic resources. The geographically widespread calcium carbonate is found in both land (marble quarries) and marine environment (biological systems/skeletons along coastlines – seashells, snails, corals) [12, 37]. In this respect, the availability of the resources was not an impediment, but a key element for the research studies developed throughout the PhD thesis.

From the range of natural resources based on calcium carbonate, studies reported the possibility of involving the same conversion path for two autochthonous precursors – dolomitic marble and seashells – thus setting the foundation for an adapted and fully reproducible method of calcium phosphate powder synthesis [12, 38]. The investigations carried out at the precursor level indicate the presence of three different polymorphic forms of calcium carbonate – calcite and dolomite for marble, calcite and aragonite for seashells [31, 38]. It is thus confirmed that the stratified morphology is given by the successive perpendicular arrangement of the calcite/dolomite and calcite/aragonite lamellae [38].

One of the advantages of the adapted wet precipitation method is the possibility to convert powdery materials into patient-custom-made implantable products [12, 17, 21].

1.2. Synthesis of calcium phosphates by wet precipitation method

The need to develop a synthesis route and a technology for obtaining bioceramic materials with potential applicability in the field of orthopaedic implantology has been widely disseminated in recent decades [34, 39]. In terms of natural resources conversion into biogenic calcium phosphates, at the beginning of this study, the challenges resided in the absence of complete, accurate and reproducible data regarding the synthesis parameters. A recent study [7] revealed that current experimental procedures limit the mechanical and biological features of naturally-derived materials, leading to poor quality products.

However, a series of answers were outlined in regard to the wet precipitation synthesis method, but through the involvement of various biogenic resources and consisting mainly of $CaCO_3$ as the precursor material [12, 13, 38]. Other synthesis methods based on the reaction between calcium hydroxide ($Ca(OH)_2$) and phosphoric acid (H_3PO_4) have been also developed for preparing hydroxyapatite powder. Most of them strongly depend of $Ca(OH)_2$ [40]/ calcium oxide (CaO) [16, 41] and different acids concentration. The synthesis route that underlies this study consists in the precipitation of calcium phosphates by improving the method developed in 1939 by Rathje [42].

Therefore, this study comes as a resolution for both the incomplete and inconsistent dedicated studies already published and the reconstructive orthopaedic surgery. It starts thereby through conceptual approaches adopted from some studies [43, 44] and applied to the selected precursors. Given the importance of increased accessibility to biogenic resources involved during the synthesis, studies conducted on this PhD thesis focused on two types of $CaCO_3$, one found in the terrestrial environment (dolomitic marble, Rușchița, Romania) and the other in the marine environment (*Mytilus galloprovincialis* seashells, Black Sea, Romania).

1.2.1. Thermal decomposition of marble and marine seashells

The need for clarification of experimental parameters begins from the preliminary stage of synthesis, the calcination/thermal decomposition of raw $CaCO_3$ materials. Research studies place the optimum dissociation temperature between 600 and 1200°C, depending on the polymorphic form of the involved $CaCO_3$ [45]. The influence of the content of additional elements, brought from the natural habitat of the resources (e.g., Mg, Na, Si) was also reported. This aspect becomes important considering the dolomitic form of calcium carbonate ($CaMg(CO_3)_2$) from the local marble [38]. Thus, the determination of the optimal temperature dissociation of both natural resources was further based on the endothermic chemical reaction: carbon dioxide (CO_2) is released and CaO is formed, according to the reaction 1 [7, 38, 46].

$$\mathrm{CaCo_3} \overset{t^{\circ}}{\rightarrow} \mathrm{CaO} + \mathrm{CO_2} \tag{1}$$

Due to the influence of the physical form of precursors upon the dissociation degree [47], the marble and seashells used in the synthesis process were cut into downsized pieces. In order to investigate and concretely determine the thermal dissociation parameters for both selected resources, TGA/DSC analyses were performed with a temperature range up to 1500°C and a heating rate of 10°C/min. [38]. Following the obtained results, both the succession of irreversible thermal transformations from the dolomite/aragonite polymorphic phase to the calcite phase, and later to the CaO type compound, for marble and shell, as well as the maximum dissociation temperature correlated with them, were confirmed (~900°C). However, in order to preserve the mass stability, it was established that at 1300°C a complete conversion of both types of precursors into CaO is ensured.

1.2.2. Key synthesis parameters refinement

The calcination temperature alongside the concentration of reagents, the solution pH and magnetic stirring, are the key parameters that govern the performance of the synthesis method for these materials. The reported reagents concentrations largely vary between 0.15-1.0 M [40]. These variabilities often lead to the synthesis of poor qualitative materials with a Ca/P molar ratio far more different than that of the biological apatite (impure or incomplete reacted hydroxyapatite) [48]. Another key factor that determines the degree of conversion of precursor materials into calcium phosphates, is the hydration of calcium oxide resulting from the applied heat treatment. Calcium oxide can be hydrated and converted into calcium hydroxide according to the reaction 2. This step has been reported as optional [16], but not in the case of this synthesis route. If the optional direction is followed, the results indicate an incomplete conversion of CaO into calcium phosphate, and the remaining amount becomes $Ca(OH)_2$ after hydration during the synthesis process [38].

$$\mathrm{CaO} + \mathrm{H_2O} \rightarrow \mathrm{Ca(OH)_2} \tag{2}$$

The further conversion of $Ca(OH)_2$ was achieved solely by the improved method. The improvements followed the concentration of phosphoric acid from the calculated value according to the chemical stoichiometry (reaction 3) and varied by gradually increasing it with 50% in the preliminary study [38], and with 10% in all the following others [12-14, 17, 20, 21]. The reaction between $Ca(OH)_2$ and H_3PO_4 is based on the following steps: the phosphate anions released following the dissolution of acid phosphates in water react with Ca^{2+} cations, released prolonged after the gradual dissolution of $Ca(OH)_2$ in water, and form a coordinated network named the *reaction product*, which under favorable conditions is converted into various calcium phosphates [49, 50]. The Ca^{2+} cations react with water molecules and form positively charged "aquasols" by hydrolysis. They in turn react with phosphate anions and form hydrophosphate salts. As the exothermic reaction takes place, the solution turns into a gel with weakly interconnected molecules. Gel thickening occurs gradually to the point where mixing becomes difficult to achieve. This is when the gel crystallizes around each grain of unreacted calcium oxide in the form of a crystalline lattice. The degree of crystallinity of the obtained products is dependent on the rapidity with which the dissolution of $Ca(OH)_2$ occurs. The importance of the magnetic stirring (established at a value of 700 rpm) and a constant temperature (25°C) present throughout the synthesis process were associated with an increased crystallization, a reduced tendency to cluster formation and the absence of $Ca(OH)_2$ traces [38]. Variation of these parameters is imperative since some reported synthesis methods were found unreliable [7, 51].

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_2O \tag{3}$$

The preliminary study was conducted starting with a lower amount of $Ca(OH)_2$ powder (2 g) and a H_3PO_4 acid with a higher concentration (95% solution). This set-up led to the development of a liquid sample with the doubling of the acid amount, in the case of powder derived from seashells, and therefore a maximum limit of the chemical reagent was established. In this respect, the synthesis process subsequently underwent a series of refining steps for the involved parameters, delieneating first the proportions of stoichiometric mixture for the $Ca(OH)_2$ powder and H_3PO_4, but of lower concentration (85% solution). Thus the following reported studies [12, 52] start from a mixture of 10 g of $Ca(OH)_2$ powder and 5.5 ml of H_3PO_4 added dropwise at an addition rate of 1 mL/min, in the presence of 200 ml of distilled water (amount that has been kept constant throughout the experiments). The possibility of modulating the amount of acid was reduced to an increment of 10%, without reaching twice its amount. The synthesized slurries from each precursor followed a wash-filter-aging process at room temperature (72 h). After removing the water excess, the complete elimination of the remaining water and acid was achieved by drying the ceramic powders using an autoclave (100°C for 2 h). This step is necessary for the molecular stability conservation [8].

As a result, it was established that the reaction between the resulted $Ca(OH)_2$ and H_3PO_4 works from stoichiometric calculated amounts up to 30% increased amount of H_3PO_4 for biphasic CaPs synthesis with variable HA/brushite or HA/monetite ratios. In order to verify the reproducibility of the synthesis route for this range of H_3PO_4 and to investigate the amount of reagent that favours the appearance of these types of calcium phosphates, the studies presented in this PhD thesis followed the maximum range of H_3PO_4 addition (0%=the stoichiometric amount, up to 90%=maximum accepted quantity) [13].

1.2.3. Calcium phosphates derived after precipitation

The most frequently mentioned calcium phosphate based biomaterial is the stoichiometric hydroxyapatite (HA) with the Ca/P molar ratio of 1.67 [53]. However, natural apatite found in the bone tissue is non-stoichiometric (Ca/P molar ratio over 1.67), with carbonate groups and traces of some other chemical elements incorporated in its structure [54].

According to the reported results, during the refining steps of the synthesis method, the concentration of HPO_4^-, $H_2PO_4^{2-}$ and PO_4^{3-} ions changes with the pH of the solution [49]. Hence, the obtaining of pure HA is favoured only under stoichiometric preparation conditions, and only by the conversion of $Ca(OH)_2$ powder derived from seashells [12, 13, 38, 52]. The general trend of transformations that occur at pH≤6.5 and ambient temperature is as follows: ACP (amorphous calcium phosphate)→brushite (DCPD)→OCP (octacalcium phosphate)→HA [55]. In the case of dolomitic marble type resources, due to the presence of

Mg in its composition, a biphasic composition of HA and a secondary phase of DCPD type occurred from the first acid concentration. A magnesium substrate (present from the beginning of the reaction) influences the crystallization of DCPD and HA as follows: Mg^{2+} ions formed after hydrolysis of $Mg(OH)_2$ take up more space in the crystal lattice of calcium phosphate and decreases its crystallinity [55, 56]. Studies also report the inhibitory effect of magnesium on the growth of HA crystals [57].

Brushite is the least stable of the moderately soluble calcium phosphates and occurs as a primary or by-product of precipitation from solutions of moderate or neutral acidity at temperatures up to 40°C [55, 56, 58]. Due to its metastable thermodynamic character, it can remain stable, dissolve, reprecipitate or disintegrate. Therefore it is considered as a precursor phase in the formation of HA [59]. Its dissolution or reprecipitation in the more stable thermodynamic phase (HA) occurs only in environments that are subsaturated in calcium and phosphate ions [60]. In particular, the presence or absence of HA nanocrystals, as a primary product formed, strongly influences the degree of formation of DCPD since nanocrystals can also serve as nuclei for its formation [56]. Recrystallization is usually influenced by factors such as: temperature, pH and the presence/absence of metal ions (e.g., Mg^{2+}) [55].

Presenting a biological importance [58, 61, 62], DCPD has been proposed as an intermediate in both bone mineralization and dissolving enamel in acids (tooth erosion) [63]. In medicine it is used as primary component for calcium orthophosphates cements based. In the human body, DCPD particles are found in non-collagenous organic matter, around nonmineralized collagen fibres, being considered the "reservoir" of calcium and phosphate ions needed for further mineralization. Due to its high solubility compared to HA (in physiological conditions), it has an ideal *in vivo* resorption rate, taking part in the bone remodeling process [55, 64]. Moreover, it has been shown that DCPD tends to convert to HA *in vivo* [65].

With the increase of the acid amount and implicitly by the induction of the non-stoichiometric preparation conditions, the precipitation of the DCPD phase is favoured to the detriment of HA phase, registering a progressive decrease of the phase proportion of up to 18% HA [38]. Moreover, once the threshold of 20% and 30% of additional H_3PO_4 has been reached, for the marble and seashell derived powder, respectively, the appearance of monetite (DCPA) also occurs. Monetite is the anhydrous and less soluble form of brushite (due to the absence of water inclusions), which can be crystallized from aqueous solutions, but at 80-100ºC [58, 62]. Although it has a higher thermodynamic stability at low pH, from a kinetic point of view, the formation of brushite occurs faster. The weight loss of the monetite appears due to its high solubility and occurs in favor of the emergence of DCPD.

Unlike brushite, the monetite is not found in the human body in any normal or pathological calcification [58]. However, studies report a very high solubility in aqueous media, at physiological pH, compared to other biocompatible phosphates [66].

This series of changes in the DCPD and DCPA phases can occur from the beginning of the transformation reaction, either by the initial precipitation of the DCPD and its conversion to DCPA after the autoclave drying step, or by the direct formation of the DCPA phase as a result of high acid content in the synthesis solution (increased acidity) [67]. Generally, because DCPD precipitation occurs exothermically, compared to the endothermic mechanism of DCPA formation, the tendency is to form brushite as a stable intermediate/final phase [68, 69]. Once the above-mentioned concentration threshold was exceeded, the HA content becomes undetectable, the final composition up to 90% H_3PO_4 being biphasic (DCPD/DCPA in different proportions).

The performed experimental testing considering the research hypothesis led to a completely reproducible, facile and eco-friendly synthesis technology, developed for the conversion of calcium carbonate-based resources (dolomitic marble and seashells) into calcium phosphates (CaPs) with modulated composition [12, 13, 70]. Overall, these results confirm the possibility of extending the natural precursors range for HA preparation and also bring forward an alternative synthesis for DCPD/DCPA based cements.

For subsequent implantation, the appropriate chemical and phase composition, as well as the biomechanical and biological features of the starting materials will be further assessed for the manufacturing of biomimetic products.

1.3. Manufacturing technology of calcium phosphates consolidated bioproducts

The development of scaffolds with applicability in the medical field has been targeted, predominantly, through two categories of methods: conventional methods (e.g., foamin gas, progen leaching) and additive manufacturing methods, which allow the replication of certain morphological properties compatible with human bone, but not the geometric ones in the micrometric field [71, 72].

Since the internal architecture of the products dictates the bone tissue regeneration degree after implantation, the goal is to find how the developed products meet the requirements for nutrient diffusion, mass transport, adhesion, proliferation and cell migration, along with specific mechanical requirements for each bone segment. Therefore, the current focus targets to find simplified and cost-efficient manufacturing methods, but which can restore the heterogeneous and complex structure of the bone and also ensure the contribution of mechanical strength for both non- and load-bearing applications [34].

In this PhD thesis, the main goal was to develop, implement and promote a 3D nature-inspired biomodel with continuous spatial porosity and porosity gradient as an entity capable of reproducing the geometry, morphology and resistance of the biological structure (Figure 2). By this approach, major challenges were addressed: the elimination of the toxic solvents involved in the process and the lack of mechanical properties adequate for load bearing applications. An important innovation is the use of strictly biogenic materials throughout the production of these products, starting with the raw calcium carbonate resources.

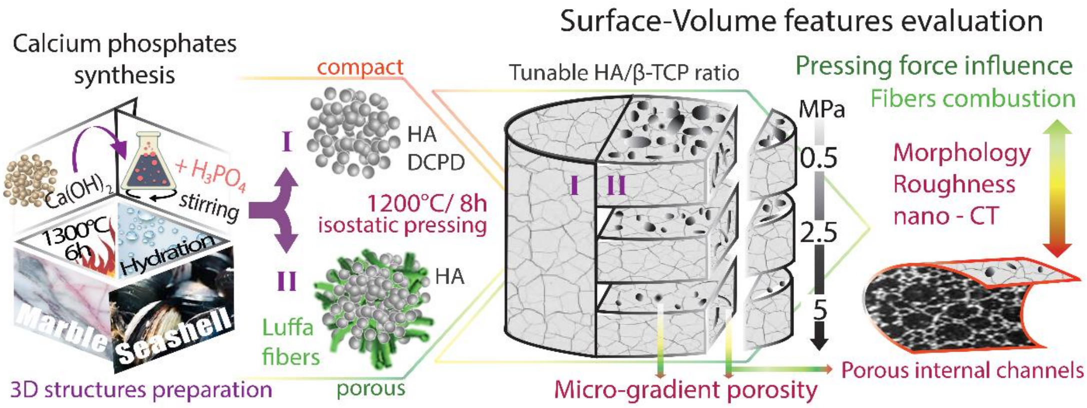

Figure 2. Schematic representation of the manufacturing technology followed within the PhD thesis [14]

This study comes as a paramount necessity to solve the absence of similar products on the customer market, the current high patient-risk orthopaedic surgical approaches and simplistic geometries, structural and mechanical features of 3D structures that cannot offer an optimal personalized bone compatibility for a specific clinical case. The reduced time required for biomimetic implants manufacturing (several days for a new ready-to-use product) can improve the life quality of a patient – reduce traumatic and medical recovery periods, reduce the overall costs and lead to a much faster return to current activities. In this respect, the synthesis and development of materials and manufacturing technologies based on biogenic resources, with regenerative biological interest, which can contribute to the restoration or conservation of physiological functions at the level of injured areas, constituted the first step.

1.3.1. Natural fibre-based porogen agents

The manufacturing of 3D porous products was envisioned by addressing the current limitations of the biogenic-derived materials development that have biological capabilities similar to those of the human bone. For the attained biomimetic products, the *in vitro* simulation methods as well as the *in vivo* results are dependent on the obtained architectural parameters: *i)* macroporosity, pore size and surface features that promote cell adhesion and proliferation, *ii)* high level of pore interconnectivity that allow in-growth of cells and disposal of residues resulted from the cell activity.

The creation and transition to porous 3D structures based on the developed ceramic materials [12, 13, 38], relied further on natural materials with energy efficiency and low cost. Strategies based on hierarchically porous systems were often exploited through different processing pathways in order to obtain porous materials. One of the possible directions to be investigated is the involvement of different types of natural fibres as porogen agents. Their redirection outside the core role offered in the textile and fashion industry, and their involvement in the development of various multi-purpose products, has led to their perception over time as *high performance fibres* [73].

This strategy is based on expanding the possibilities of obtaining new materials and products available at advantageous costs, while pursuing environmentally-friendly aspects. Thus, the terms such as renewable, recyclable, biodegradable and sustainable have become keywords and are highlighted in all studies dedicated to this field [74-76]. Some of these fibrous renewable materials come from agricultural activities undertaken for the economic development of certain geographical areas and industrial areas (e.g., building sector, automotive, plastic industry), and therefore have benefited from an increasing accessibility in recent years [76-78].

Several natural fibres (e.g. luffa cylindrica, hemp, wool) emerged as possible natural templates for the desired bioceramic products and biomedical applications and were involved in this proposed technology. Furthermore, opposed to their well-known reinfoircing direction, a less explored route was followed here, namely the *sacrificial template method*. The method involves the use of fibres as a sacrificial porogen agent, i.e. the incorporation into the desired ceramic matrix, with a certain mass ratio, and their degradation by heat. In the fibres incorporation area, the space occupied by them prior to the heat treatment will be transposed after sintering in a network of pores of variable sizes and interconnected channels in the form of an internal spatial arrangement, similar to that of natural bone [14, 17].

In this respect, a complete overview of the natural fibres features, including their thermal behaviour in both oxidative and reductive sintering atmosphere, was carefully assessed in ref. [17]. The compositional and morphological aspects of the raw fibres were investigated in several studies. Given that the applied thermal treatment is, generally. the last fundamental technological stage, the thermal stability is of outmost importance in order to establish their dissociation temperature, their predictable reduction (the sacrificial component) and the influence of the sintering ambient, as reported in ref. [14, 17, 21]. Next, the examination of the morpho-compositional, structural and cytotoxic properties of the resulting combustion products (also known as chars), which may influence the *in vitro* and *in vivo* biological behaviour of the 3D structures, was also pursued and revealed in ref. [17].

a. Fibres of vegetal origin

Natural fibres of plant origin (e.g., Luffa cylindrica, hemp, cotton, sisal, bamboo, jute, wood) benefit from a wide distribution, some being found predominantly in tropical areas (e.g., Luffa fibres are cultivated in Asia and Africa) [79, 80]. They are considered lignocellulosic nanocomposites composed of cellulose fibres arranged in a spiral in an amorphous matrix composed of compounds such as hemicellulose, pectin, lignin and wax [74, 81]. Fibrils are round, elongated three-dimensional reticular structures with hollow cross-sections, which are connected to each other by resinous materials [74, 79]. The whole assembly gives plant fibres properties such as mechanical strength (tensile and flexural) and considerably high hardness, but also a low resistance to high temperatures and environmental factors which influence their aging and degradation processes [82]. Therefore, their usefulness was initially limited to applications in the field of textile manufacturing (e.g., fashion products, mats, bath sponges, ropes, insole materials, filling materials) and less in the industrial area [74, 81].

The beneficial properties of Luffa were recognized and recorded more than 400 years ago [79]. The scientific advance has led to the involvement of Luffa fibres for the development of various industrial products for pharmaceutical engineering, biotechnology and environmental engineering fields [79, 83]. In the medical field, the use of very small pieces of Luffa sponge for certain sinus diseases or as a support for the immobilization of proteins and cells has been reported [74, 84].

From a morphological point of view, Luffa fibres are arranged randomly and multi-directionally in the form of fibro-vascular networks forming bundles, each inner column being composed of three layers arranged from outside to inside (outer, middle and inner layer) [15, 34, 79]. The diameter of the fibres varies in the range of 0.2–1 mm for each individual fibre or along the length of the same fibre, leading to a diversity of properties [74]. With a much longer use, over several civilizations, hemp fibres have been preferred due to their high strength and larger dimensions in length, for making ropes, paper and textile products [85, 86]. Hemp, and mainly industrial hemp (*Cannabis sativa*), is one of the most cultivated plants, generating materials in the form of fibres [77].

Generally, the outer layer of vegetable fibres is rich in lignin and/or pectin, which homogeneously surrounds the entire fibre, followed by the middle layer of cellulose and the inner layer of hemicellulose [74]. Cellulose is a natural semicrystalline polysaccharide composed of anhydroglucose units connected by glycosidic bonds and with a high degree of

polymerization [82, 87]. Hemicellulose is not a type of cellulose, but differs from it in terms of the number and type of sugar units, as well as the low degree of polymerization by an order of magnitude of 10–100 times, and lignin is a hydrocarbon polymer composed of aliphatic and aromatic compounds [82]. Also, hemicellulose and lignin polymers are completely amorphous [77]. Depending on the habitat conditions in which they grew (e.g., soil type, climate, species), the chemical composition of the fibres may differ – most studies report the presence of elements such as C, O, N, P, Si [74, 84, 87].
A detailed elementary and structural analysis of raw fibres can be found in ref. [83].

The hypothesis presented in the proposed technology is based on the fact that the thermal degradation of the cellulosic-based materials (all natural fibres) leads to a mass loss of 60 wt.% between 300–500°C and ends up to 800°C with the formation of a small carbonaceous residue [88]. Specifically, their thermal degradation process is based on a succession of processes, which, depending on the heat treatment conditions (e.g., direct contact with the working atmosphere, the existence / absence of physico-chemical stimuli of heat, oxygen concentration), lead to fibres combustion: dehydration, depolymerization, decomposition of compounds resulting from depolymerization, formation of intermediate non-volatile products and their decomposition [80, 81]. These processes are delimited in three stages of decomposition, each having an associated temperature range due to the influences brought by the natural environment of the fibres [89, 90]. The preliminary step comprises the fibres degradation by dehydration to about 105°C [81]. With the progressive increase of the temperature, the fibres ignite (the main burning stage) by the rapid depolymerization of the cellulose and the hemicellulose layers, simultaneously with the beginning of the degradation of the lignin and pectin layers [77, 80, 87]. The combustion process ends at about 400–450°C by completely breaking the bonds between the glycosylic units and the formation of combustion products (also known as *chars*) [80]. The remaining amount of lignin can continue its dissociation process sometimes even up to a temperature of 900°C [87]. It is considered that up to this temperature, and above it, the stage of decomposition, without burning, of all combustion products, formed by cracking and reforming phenomena, takes place, resulting in a carbonaceous material (char) [81, 91].

b. Fibres of animal origin

Keratin-type proteins are found in birds, mammals and reptiles in various forms of exoskeleton material as the main component (e.g., hair, feather, skin, horns) [92, 93]. These are some of the most widespread non-food proteins, cheap and renewable, with a figure of several million tons produced annually as a result of the activities carried out by multiple industrial areas [93-95].

In the last decade, the recovery and recycling of these natural resources types, mainly in the form of fibres, has aroused interest and procedures have been proposed for the extraction and purification of keratin from their composition [94, 96]. Further, keratin can be reintegrated into the development of new materials (e.g., films, sponges, gels, foams) for various applications such as pharmaceutical, cosmetic, biotechnological, medicinal, textile and non-textile [95, 97]. In the medical field it has been reported as advantageous for drug delivery, tissue reconstruction, or as an implantable material [97].

Keratin is characterized by high thermal stability, low solubility and a complex structural arrangement given by the crossed organization of the double sulphur bonds between the cysteine amino acids in the composition [92, 93, 96]. The level of sulphur and cysteine differs depending on the investigated area – for wool fibres it was found that the outer layer is rich in cysteine and S bonds, while the cortex is composed of microfibrils with low S content [93]. Therefore, the strength and stability of animal fibres are provided by a high sulphur content [92]. At structural level, the characteristic diffraction patterns of the fibres show the existence of either α-type or β-type keratin, with the exception of materials from birds or reptiles that can produce both types. Also special, are the wool fibres, which in their natural state have only α keratin helixes, but with their deformation, β-type structures appear also [92]. Both fibrillar structures are integrated into an amorphous matrix of interconnected polypeptides [98].

When exposed to high temperatures, wool fibres show a degradation behaviour from inside to outside, the process starting with the loss of adsorbed water up to a temperature of 120–190°C [93, 98]. Decomposition is continued in two successive stages, by alteration of the low-sulphur structures found in the form of α keratin, up to about 250°C [96]. Above 300°C and up to about 600–620°C, the gradual decomposition of β- type rich-sulfur structures also occurs [93, 94]. The process is thus, considered completed; the combustion product resulting from the volatilization of double keratin is composed of various compounds based on nitrogen and sulphur [93, 96].

c. Fibre-templated products

Some methods for obtaining natural fibre/collagen/polymer and hydroxyapatite composites were already patented [99]. However, they focus on the preservation of the porous aspect of the natural template [2, 6, 34] which fall short in terms of accessibility and regeneration of natural resources, predefined level of porosity, pore size and shape, morphology and mechanical behaviour of the final product.

Specifically, for the fabrication of products, pieces of defined sizes and shapes of Luffa are generally sectioned and used as skeletal material [16, 100, 101]. Their preparation procedure usually involves: washing and/or soaking in distilled water for a few minutes to a few hours [16, 100], immersion in sodium hydroxide solution [15, 74, 100] or exposure to various chemical (e.g., alkalis, silane-based agents, maceration) or physical (e.g., corona discharge) treatments [74]. When in contact with such substances, beneficial changes occur, both structurally and in the surface of natural fibres, for the improvement of mechanical properties or absorption abilities [74, 78]. The skeleton thus prepared, is further immersed / impregnated in / with binding slurry prepared with polymers such as starch [16], PVA [101, 102], cellulose and/or PLLA [15], wood chips [100] and calcium phosphates based-powder in different proportions. Then, the drying step follows (a few hours to a few days at ambient temperature) and/or sintering to remove the skeleton, at temperatures in the range of 600–1250°C, for 2 hours [16, 100-102]. Following the procedure, the skeleton architecture is the one that will dictate the internal arrangement of interconnected pores/channels of the resulting ceramic products. For industrial applications, which require the production of reinforced composite products with a high degree of adhesion between the fibres and the matrix material, the sintering step of the fibrous skeleton is eliminated [90, 103, 104].

Bone regeneration requires pore sizes between 100-150 μm; a proper vascularization and ossification requires pore diameter of at least 300 μm [105]. The minimum pore size to reach an appropriate porosity is considered 100 μm up to 1000 μm for bone regeneration *in vivo*, while *in vitro* osteogenesis requires lower magnitudes [106]. Moreover, the size of the interconnections varies in the range of 15–50 μm, which can ensure the vascularization of the structure and a larger surface area [5, 14]. Proper vascularization is essential for colonization of osteoblast cells and bone ingrowth [5]. This translates to the forecast of addressing multiple orthopaedic requirements related to micro/macro-porosity degree and interconnectivity [26].

Up to this stage, few research studies were published regarding the potential framework of porous structures based on biocompatible naturally-derived calcium phosphates and 14 wt.% of luffa fibres, through the *sacrificial template method*. The proposed procedure involves the use of a ceramic skeleton and the mixing of Luffa fibres without binding agents or solutions in order to generate a custom porosity level and model [14, 17, 21]. This can simulate the random distribution of pores and channels (tortuosity) and the percentage of porosity according to the affected bone region. Results revealed that the fibres combustion at 1200°C led to the successful formation of a porous structure with interconnected pores and channels and micro-gradient porosity formed inside the channels [14, 17]. Moreover, the total porosity (max. ≈20%) and mechanical features were influenced also by the pressing force. Increased pressing forces for 3D products development favour bone-like compressive strength values (20-65 MPa), but reduce the pores and channels dimensions and possibly the vascularization and bone ingrowth capability) [14].

1.3.2. Graphene-based reinforcement agents

Given the outlined compromise between the mechanical and architectural features of the 3D products, a toughening mechanism through graphene-based materials incorporation (max. 2 wt.%) was further pursued to sustain the biofunctionalization of the porous structures. In particular, due to the graphene reinforcement, mechanical properties were a target assessment in terms of micro-hardness, compressive strength and bending resistance [20, 21]. Their choice is generally motivated throughout the mechanical loads along natural bone and the forecasted applicability of the developed products.

Given the fragile nature and low wear and tensile strength of ceramic structures based on calcium phosphates (porous and compact), the need to strengthen them has become a research direction in itself [107-110]. With the appearance of a defect, the brittle fracture occurs rapidly from the surface of the structure inwards, having catastrophic effects from the early stages of implantation, which is why the use of such products for load-bearing applications of bone reconstruction has been restricted [111-114]. Therefore, research studies aim the combining and strengthening calcium phosphate-based materials with other materials with suitable characteristics for implantation [115]. In the last decade, some of the materials that have attracted the most attention from researchers are those based on graphene [116, 117].

The term graphene is dedicated to the unilayered arrangement of carbon atoms with a honeycomb structure, graphene being a first example of a two-dimensional crystal [116, 118, 119]. Starting from the number of successive layers of carbon atoms (between 2–10 layers), a series of derived materials were later developed, also called the *family of graphene-based materials*, with a maximum thickness of about 100 nm: graphene nanoplatelets (GNP), graphene nano-sheets (GNS), graphene nano-tubes (GNT), graphene nano-onions (GNO), graphene nano-ribbons (GNR), graphene oxide (GO) and reduced graphene oxide (rGO), the last two being obtained by chemical oxidation methods [18, 118]. Graphene can be synthesized by several physical and chemical processes, each leading to a different morphology of the material, hence the names presented above [18]. Due to their mechanical (Young modulus = 0.5–1 TPa, intrinsic strength ≈130 GPa), surface, electrical, thermal, chemical, optical and biological properties, graphene-based materials have also been introduced in biomedical applications (e.g., orthopaedics, drug delivery, biosensing, cancer therapy) as reinforcing component in order to improve the stability and mechanical strength of different types of products [107, 115, 120-123].

The incorporation of graphene-based materials as a secondary phase for the consolidation of ceramic products for bone regeneration leads to both increased mechanical strength and improved structural and biological properties, especially since once exposed to temperatures above 600°C graphene it oxidizes and is progressively altered [124, 125]. In order to induce the desired effects, a special attention must be made to the process of incorporation into the matrix material, and, as such, a number of factors have been identified that can significantly influence the final behaviour of the products: i) the interfacial bonding strength, ii) the amount of graphene, iii) the dispersion distribution method and degree, iv) the mechanical features of the matrix, v) the particle sizes of the powder matrix, vi) the efficiency of stress and load transfer from the matrix to graphene and vii) the route preparation of the reinforced products [19-21, 121, 126].

For composite products based on calcium phosphates and graphene, the reinforcement mechanisms aim at forming a crack propagation barrier through crack deflection, branching and bridging [19]. Graphene, in the form of nanoplatelets, has a higher specific surface area and a three-dimensional multi-layer arrangement of graphene sheets (between 10 and 30), being able to coat the ceramic particles and generate an increased contact surface with the matrix material [110, 127]. Also, the morphology, size and surface roughness of GNP play an important role for the transfer of mechanical load from the ceramic matrix, facilitating the increase of mechanical interlocking and barrier (e.g., strength, rigidity, hardness) and the improvement of the elastic modulus of products [127].

Studies report the successful reinforcement of HA and/or HA/β-TCP biphasic materials with GO, rGO, nano-platelets graphene type, by various methods such as in-situ synthesis, plasma sintering, electro-spinning or deposition, hot isostatic pressing (HIP), vacuum cold spraying and conventional mixing [114, 128-130]. Due to the functional groups that can be easily detached and reattached (forming hybrid materials), graphene oxide (GO) has been indicated as suitable for industrial applications that require the modification of the graphene surface [131]. The influence of oxygen functionalities on HA crystallization has also been demonstrated, as HA nanocrystals can grow on the surface of GO or rGO [132].

Composite products based on HA and graphene can be made in different forms (e.g., powder, bulk, scaffold, coating, film), each with an associated type of application (e.g., bone reconstruction, coating of metal implants) [19]. The process of preparing composite products has been reported in some studies starting to a first stage of surface preparation of graphene-based materials (mostly GO and rGO) in solution of isopropyl alcohol, calcium chloride and water (volume ratio 1:1) and continuous ultrasonic/magnetic stirring for several tens of minutes, after which the HA powder is added to the resulting solution; the final reinforced product results after the drying step for one day at 40°C or by lyophilisation [133, 134]. For GO and rGO type materials the preparation can be made in solutions, given their excellent dispersion capacity in aqueous media [113]. Due to these properties, GO can also be used to bind metal ions or nanoparticles [134].

The incorporation of GNP-type materials has been reported through both wet and dry routes. In the first case, the solutions are different from GO/rGO, mostly based on bromides, and the suspension created with the ceramic powder is further subjected to mixing stages by ball milling and hot pressing [135]. To reduce and even eliminate the risk of material contamination in this manner, recently published studies propose the strengthening of the ceramic matrix by mechanical incorporation and ultrasonic dispersing of GNP, regardless of the amount of involved agent [20, 21]. The products are also obtained by pressing, but at ambient temperature. During compaction and pressing, the preferential orientation of the GNP between the ceramic particles occurs, thus blocking the formation and propagation of cracks or the extension of their area [135].

The improvement of the mechanical functions was generally correlated with the mass ratio between the HA/graphene-based material matrix (GO, rGO, GNS, GNP) and the degree of materials homogenization, so that an amount of GO/rGO of 1 wt.% generally led to an increase in hardness by ≈30%, in elastic modulus by ≈50–70% and in fracture toughness by 80–100% [127, 128, 130]. Compared to a HA-only product, the breaking strength and compressive strength of composite products can even reach double values with the incorporation of up to 5 wt.% of graphene, falling within the range of values reported for natural trabecular bone [120, 126]. The improvement of the tribological properties of the products has been explained as the effect of the formation of an adhesive carbon-based film on certain areas of the particles/products surface at the time of wear [116].

In the case of GNP-reinforced products, the bending strength and fracture toughness increased by up to 55% and 75% respectively compared to the values obtained for products without reinforcing agent [135], and the compressive strength reached at least double values for 1 wt.% GNP [20, 21]. The most recently published studies also expose the possibility of strengthening porous structures developed through natural fibres (strengthening the ceramic matrix exposed by the two routes in Figure 2), and precisely delimit the optimal amount of graphene for both types of products (compact and porous) [20, 21].

1.3.3. Influence of the sintering parameters on the global features

According to the strategy proposed in this PhD thesis, three different products were developed, function of the addition and incorporation of two agents, Luffa fibres and graphene nanoplatelets as porogen and mechanical reinforcement, respectively (CaPs±Luffa±GNP) [13, 14, 17, 20, 21]. The consolidation of the finally pressed products was attained by high temperature sintering. Sintering is a crucial step for inducing the defining characteristics of products, acting through various mechanisms such as surface diffusion and grain boundaries, volume diffusion and evaporation-condensation, ultimately leading to the densification of the microstructure [136, 137].

The sintering process, depending on temperature and ambient environment, establishes the compositional, architectural and mechanical behaviour of ceramic structures [138-140]. This strategy allows the adjustment of the ceramic matrix to various HA/TCP phase composition after sintering, without any residual calcium oxide. Due to the modulated synthesis parameters and the subsequent heat treatment in controlled oxidative and reductive atmosphere, a very low risk of contamination with residues (traces of calcium oxide), arising from the reaction between $Ca(OH)_2$ (obtained by thermal transformation of natural calcium carbonate) and H_3PO_4, is ensured [12, 13].

At higher temperatures, the decomposition of HA leads to the partial formation of β-TCP and α-TCP [108, 127, 139]. It was previously demonstrated that at temperature of 1200°C, maintained for 8h in air atmosphere, the initial chemical composition of synthesised ceramic materials (HA and HA/DCPD) converts to biphasic calcium phosphates with modulated HA/β-TCP ratios (70-100%/30-0%) [12, 14]. Maintaining the temperature and sintering time, a reductive ambient, namely nitrogen, was also considered in order to evaluate the prospect of different phase compositions and natural templates degradation. Therefore, the results indicated an almost complete decomposition of HA and DCPD in β-TCP, α-TCP and $Ca_2P_2O_7$ phases, with different proportions depending on the presence and amount of graphene [21]. Also, the DCPD phase has been indicated as responsible for promoting HA decomposition into TCP-type phases [141], while its progressive decomposition may lead to the appearance of pyrophosphate phases ($Ca_2P_2O_7$) [142, 143].

Both TCP materials exhibit an increased solubility than that of the hydroxyapatite and are more rapidly absorbed *in vivo* [29, 144]. The reasoning behind the development of such materials is based on their compositional similarity with the mineral component of the natural bone, as well as well-known properties of biodegradability, bioactivity, osteoconductivity and osseointegration that sustains bone tissue remodelling [13, 52, 145]. Generally, biphasic materials govern the dissolution rate and regeneration of host tissue after implantation [145].

The natural templates dissociation temperature, predictable reduction and the influence of the sintering medium was also assessed in both oxidative and reductive atmospheres. It has been established that the air treatment of Luffa fibres occurs faster due to the pyrolysis process, compared to a reductive ambient, where the burning of the fibres is hindered and the degradation occurs by carbonization [17, 84].

In regard to the effect of the sintering ambient on the products developed by sacrificial template method, once the temperature of 1200°C is reached, the fibres decompose completely, regardless of the involved ambient [17, 21]. In this way, interconnected pores and channels, similar to the Haversian channels of the natural bone, are generated in their place, without remnant traces of combustion products on the inside, and a gradient of micro-porosity due to the volatile substances eliminated during the decomposition process [14, 17]. Research studies report that the shape/geometry (e.g., spherical, rectangular, trabecular-like), size (>100–200 μm), tortuosity and interconnectivity between pores/channels, are generally important factors for implantable porous structures in view of a proper cell adhesion, proliferation, colonization and development leading to the formation of blood vessels and new bone tissue [3, 146]. In this respect, the 3D products sintered in both ambients comply with the bone regeneration requirements.

The graphene-based reinforcing agent incorporated in the ceramic matrix is reduced during sintering at high temperatures (> 600 ° C) by oxidation processes [114, 147] and acts as an accelerator for the decomposition of HA and DCPD and generator of micro-porosity in both sintering ambients [20, 21]. In these studies, the possibility of combining graphene with a fibrous porogen agent of natural origin was also reported for the first time. Thus, in the case of porous products, graphene acts as an adjuvant from a compositional and mechanical point of view, independent of the sintering ambient. In respect to mechanical features, the more pronounced effects of the decomposition of the ceramic matrix and the formation of a much pronounced porosity gradient, from the surface to the volume of the products, induced a weakening of the structures directly dependent on the amount of incorporated graphene [21, 148].

Thus, depending on the type of required product, the sintering program followed and the amount of agents (porogen and reinforcing), the results delimit optimal intervals for the fabrication of custom-made implants.

1.3.4. Surface-volume 3D reconstruction of products

Due to the possibility of microstructural and topographic visualization, and the quantification of certain essential parameters (e.g., roughness, porosity and pore/channel interconnectivity), the methods of digital surface and volume, 3D reconstruction have been successfully involved in various applications for several research areas (e.g., material science, geoscience, food science, biology, additive manufacturing) [149, 150].

For the topographic reconstruction of the surfaces of interest (e.g., materials, cells, 3D products), microscopy images acquired at different magnifications with high resolution microscopes (e.g., atomic force, confocal or scanning electron) are needed [151-153]. Subsequent processing of these images with dedicated topographic reconstruction software was performed in most studies through the MountainsMap® software suite (Digital Surf, Besançon, France) [151-156]. The first step of the reconstruction process involves isolating the topographic layer and the intensity layer of the sample – analysis from the surface to the maximum depth detected by a colour code (each section of depth is rendered visually with a different colour from bright orange – peak surface and down to navy blue – valley depth). By combining the two images, the desired features (e.g., roughness parameters and profiles) can be highlighted and quantified by different methods (e.g., threshold, segmentation, circular detection) [152, 154, 156].

The X-ray CT technique was introduced into current practice in the second half of the 20th century with a resolution of only 300 μm, which later reached higher levels in the submicrometric and nanometric field [149, 157]. In order to render the internal architecture (porous structure) at a high level of performance, it was reported that the ideal methods of reconstruction and visualization should be non-destructive, non-invasive, three-dimensional, and should also allow repeated analysis of samples in their original state, without prior preparation and at normal working parameters (ambient temperature and atmospheric pressure) [149, 158].

The resolution of CT reconstruction images is dictated by several factors, such as the optical magnification of the system involved, the type and resolution of the detector, and the size of the X-ray beam [157]. In view of the physical principle, the entirely analysed sample is virtually reconstructed by rotating it around an axis while it is scanned by the X-ray beam, after which, through mathematical functions, individual 2D slices are created and analszed [26, 149, 157]. From a digital point of view, filtering functions are used to improve the contrast, and a threshold is necessary to isolate and to characterize the required details in each image-slice, especially those related to the shape, size, distribution and interconnectivity of the pores (voids) [149, 157, 159].

The parameters related to the internal network of pores and/or channels dictate the way in which the transport of nutrients, the cell invasion and their survival and/or the formation of blood vessels, and implicitly the degree of newborn bone tissue formation, are ensured after implantation [160]. Thus, compared to classical methods of determining the porosity, which possess a number of disadvantages, the use of non-destructive techniques can constantly monitor the architecture of the samples during the technological flow and can lead to improved product characteristics for optimal compatibility with the bone reconstruction area [26, 150, 161].

Regarding the porous 3D products developed through the involvement of natural Luffa fibres, the nano-CT technique has been involved from the beginning of the experimental study to highlight the feasibility of the fabrication route [14]. Even more so, the ability of these fibres to generate interconnected internal pores and channels, randomly distributed inside the products, by complete decomposition, without traces of combustion products, has been outlined and demonstrated [14, 17, 21]. Thus, it was also observed the way that the fibres structure transforms under the influence of the high temperature from compact into tubular sections with walls in the form of a porous matrix [17]. These tubular sections isolated by 3D reconstruction at the product level are identified with the formed channels, which preserve the tortuous appearance according to the way the fibres were integrated in the ceramic matrix [17, 21]. Moreover, given the elimination process of volatile substances during the thermal degradation of fibres – reported as responsible for the porous microstructure of the fibres after sintering – the internal surface of the channels was also investigated [14]. It has been demonstrated, by digital processing of SEM micrographs, that the porosity properties are transferred from the fibres to the ceramic matrix during heat treatment, the channels presenting an internal microporous texture and a considerable degree of roughness, considered favourable for and easier anchoring of cells and a faster formation of new-bone tissue [17, 162].

1.4. In vitro characterization and testing of the obtained products for biomedical applications

In order to perform a complete investigation, the materials and products based on CaPs synthesized and processed according to the developed procedure, were evaluated for their *in vitro* biological behaviour by several methods. Thus, powders derived from marble and seashells at all H_3PO_4 concentrations were studied for cell viability and proliferation (LIVE/DEAD and MTT assay) on pre-osteoblast cell lines, in extraction media [13]. The results confirmed their ability to sustain and facilitate cell survival and proliferation, their morphology and density remaining unchanged by the amount of acid/ type of CaPs synthesized (HA, DCPD, DCPA), for both incubation periods (1-3 days). Therefore, all developed materials have suitable biocompatibility properties for bone regeneration.

With the establishment of processing routes for powdery materials, green and sintered products were also subjected to a complex cell testing, using the same range of pre-osteoblast cells [12, 52]. Toxicity tests by LDH enzyme release analysis did not indicate any plasma membrane alteration even at long incubation periods (4 days). Moreover, cell viability and proliferation tests showed an increased cell-affinity of ceramic products derived from marble and seashells compared to the reference sample (commercial HA). Regarding the cell adhesion and morphology, fluorescence microscopy images confirmed the ability of actin and vinculin filaments to develop, mature and spread on all investigated surfaces.

The development of porous products based on natural porogens, must take into account the effect of heat treatment applied not only on the ceramic matrix, but also on fibres, given that after this stage they decompose and the resulting products may induce cytotoxic effects. Therefore, the *in vitro* cytotoxicity evaluation of pure combustion products, a new approach in scaffold characterization, is mandatory to establish which templates are appropriate for further *in vivo* consideration and are also patient-risk-free. In this respect, char powders were investigated for the first time [17]. Only in the case of Luffa fibres studies indicated a viability and proliferation of osteoblast cells greater than 95% for both incubation periods, so there lies a very low probability of cytotoxic effects and risks associated with the patient if traces of combustion product remain on the surface of the internal channels.

Another key aspect for the success of bone regeneration *in vivo* is the investigation of the biological behaviour of the mechanical reinforcement component [35]. Thus, dedicated studies for graphene-based materials have reported that the biological response and viability of cells are primarily dependent on their size and morphology [18, 163]. Dimensions of nanometric order have led to a decrease in cell viability by up to 50% in the case of GNS type materials. However, the materials support the growth and proliferation of fibroblast and osteoblast cells depending on the incorporated amount [130]. In terms of the well-known antibacterial effect of graphene, it has been shown that, depending on the structure of bacteria, exposure time and graphene concentration, it inhibits the growth of Escherichia coli, Staphylococcus aureus and Bacillus subtilis bacteria and destroys their cell membrane a few hours after initiating direct contact [118, 163, 164].

materials

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Naturally-Derived Biphasic Calcium Phosphates through Increased Phosphorus-Based Reagent Amounts for Biomedical Applications

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Abstract: Calcium carbonate from marble and seashells is an eco-friendly, sustainable, and largely available bioresource for producing natural bone-like calcium phosphates (CaPs). Based on three main objectives, this research targeted the: (i) adaptation of an indirect synthesis route by modulating the amount of phosphorus used in the chemical reaction, (ii) comprehensive structural, morphological, and surface characterization, and (iii) biocompatibility assessment of the synthesized powdered samples. The morphological characterization was performed on digitally processed scanning electron microscopy (SEM) images. The complementary 3D image augmentation of SEM results also allowed the quantification of roughness parameters. The results revealed that both morphology and roughness were modulated through the induced variation of the synthesis parameters. Structural investigation of the samples was performed by Fourier transform infrared spectroscopy and X-ray diffraction. Depending on the phosphorus amount from the chemical reaction, the structural studies revealed the formation of biphasic CaPs based on hydroxyapatite/brushite or brushite/monetite. The in vitro assessment of the powdered samples demonstrated their capacity to support MC3T3-E1 pre-osteoblast viability and proliferation at comparable levels to the negative cytotoxicity control and the reference material (commercial hydroxyapatite). Therefore, these samples hold great promise for biomedical applications.

Keywords: dolomitic marble; seashell; $CaCO_3$ derived-calcium phosphates; modulated synthesis set-up; SEM; image analysis; pre-osteoblasts

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Full length article

Internal and external surface features of newly developed porous ceramics with random interconnected 3D channels by a fibrous sacrificial porogen method

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ABSTRACT

The bone remodeling research field has shifted focus towards sustainable, eco-friendly and reproducible manufacturing technologies of 3D structures. It is now accepted that a suitable internal architecture and an active interface between the 3D structure and host bone-tissue constitute the two most critical traits for a successful bone tissue engineering application.

A completely reproducible synthesis set-up was recently developed for calcium phosphate (CaP) bioceramics preparation from natural highly available marble and seashells. The influence of the pressing force in the fabrication process of porous 3D scaffolds derived from such CaPs by a sacrificial porogen method using natural fibers is here investigated. The fiber-ceramic based-products underwent thermal processing, followed by surface and volume features characterization. After fibers` thermal removal, interconnected 3D channels were obtained, which could allow a suitable in vivo irrigation and implant-associated negative side-effects prevention. This method provides the prospect of tunable HA/β-TCP content in the case of both precursors' derived-scaffolds.

The morphological results revealed the internal and external pores dimensions, modulated through different pressing forces that led to a controlled total porosity, evidenced by computed tomography techniques. Further, the wettability and mechanical features supported the advance of the novel porous-ceramic-structure designs as reliable bone reconstruction alternatives.

1. Introduction

Incrementally, in the past two decades, it has come to attention the possibility to integrate/adapt plentiful natural resources for bone tissue engineering applications. This comes as a response to the (*i*) higher frequency and (*ii*) intricacy of orthopedic problems, correlated with (*i*) global population growth, tendency to pro-active lifestyles and increasing life expectancy, and (*ii*) large defects generated by trauma, pathological conditions or tumor resection [1,2]. Given the complexity of the defects that fall outside the bone ability to self-repair, bone tissue engineering is often seen as an interdisciplinary strategy meant to reduce and overcome the need for autologous bone transplantation, which is the gold standard for osseous reconstruction [3,4]. For successful surgical intervention, the substitute structure needs to harmoniously combine key bone-mimicking requirements such as chemical composition, 3D structure, mechanical features and biological behavior [4–6].

In this regard, scaffolding methods from conventional techniques to additive layer manufacturing have drawn special attention and occupy important places amid the fabrication technologies of 3D structures [7]. However, none of them is flawless, especially when it comes to bone-like ceramic materials processing. Nowadays, the prominent materials for bone reconstruction are the calcium phosphates (CaP), among which hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) and β-tricalcium phosphate (β-TCP, rhombohedral $Ca_3(PO_4)_2$) are the most important due to their adequate chemistry, mechanical features and capacity to promote

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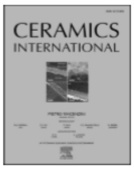

Comprehensive analysis of compatible natural fibre as sacrificial porogen template for tailored ceramic 3D bioproducts destined for hard tissue reconstruction

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ABSTRACT

For successful bone remodelling, the implantable 3D structures require suitable internal architectures which can be achieved by the use of fibres as natural templates. The ability of fibres to generate complex configurations for 3D bioceramic products was preliminary reported by sacrificial fibrous-porogen method. This study aims to demonstrate the safe-prospect of repurposing natural-fibres (i.e. luffa, hemp, wool) for embedment into a calcium phosphate (CaPs) matrix prepared through a completely reproducible route, and the beneficial influence of fibres upon structural, topographic and mechanical features of CaPs-products, since a complete assessment of the fibres-combustion-products resulted after thermal treatment was not yet disclosed. The complex investigation program based on i) thermo-gravimetric (TGA-DTG), ii) structural (XRD, FTIR-ATR), iii) morpho-compositional (SEM/EDS) and, most importantly, iv) biological cytotoxicity assays of fibres-derived chars, clearly indicated that luffa-fibres are the safest (>95% cell-survival) to be considered for bioceramic porous-orthopaedic-implants. Further, as exposed by nano-CT, the high temperature pyrolysis of luffa-fibres led to 3D interconnected channels inside the products, which allows a suitable vascularization and osteointegration. The topographic reconstruction of channels-inside-surface revealed a secondary 3D network of micro-pores. Along with the mechanical features, the novel bioceramic porous structures stand as reliable bone-repair alternatives.

1. Introduction

Concerning the direct impact on the bone tissue engineering field, the strategy of employing natural porous resources (e.g. marine sponges, algae, coral, wood) as reinforcement for the bioceramic products by replica method or direct foaming [1–3] may come as a response to current high frequency age-related pathologies, the limited capacity of bone to repair itself in critical situations (e.g. large defects generated by trauma, chronic diseases or tumour resection) and to the well-known demerits of the bone repair gold standard (i.e. insufficient donor sites, disease transmission and morbidity) [4,5]. Therefore, the target is to reduce the need of autologous grafts harvesting by developing valid tissue-engineering alternatives.

The last century historical events, the economic downside and the global increase of energy consumption motivated the current generation of scientists to comprehensively explore the future of "high-performance fibres (HPF)", apart from their natural destination in the fashion industry [6,7].

Biofibres, either as plant-derived (e.g. hemp, cotton or luffa sponge-gourds), mineral (e.g. graphite) or animal-keratin-based (e.g. hairs,

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Article

Fiber-Templated 3D Calcium-Phosphate Scaffolds for Biomedical Applications: The Role of the Thermal Treatment Ambient on Physico-Chemical Properties

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Abstract: A successful bone-graft-controlled healing entails the development of novel products with tunable compositional and architectural features and mechanical performances and is, thereby, able to accommodate fast bone in-growth and remodeling. To this effect, graphene nanoplatelets and *Luffa*-fibers were chosen as mechanical reinforcement phase and sacrificial template, respectively, and incorporated into a hydroxyapatite and brushite matrix derived by marble conversion with the help of a reproducible technology. The bio-products, framed by a one-stage-addition polymer-free fabrication route, were thoroughly physico-chemically investigated (by XRD, FTIR spectroscopy, SEM, and nano-computed tomography analysis, as well as surface energy measurements and mechanical performance assessments) after sintering in air or nitrogen ambient. The experiments exposed that the coupling of a nitrogen ambient with the graphene admixing triggers, in both compact and porous samples, important structural (i.e., decomposition of β-$Ca_3(PO_4)_2$ into α-$Ca_3(PO_4)_2$ and α-$Ca_2P_2O_7$) and morphological modifications. Certain restrictions and benefits were outlined with respect to the spatial porosity and global mechanical features of the derived bone scaffolds. Specifically, in nitrogen ambient, the graphene amount should be set to a maximum 0.25 wt.% in the case of compact products, while for the porous ones, significantly augmented compressive strengths were revealed at all graphene amounts. The sintering ambient or the graphene addition did not interfere with the *Luffa* ability to generate 3D-channels-arrays at high temperatures. It can be concluded that both *Luffa* and graphene agents act as adjuvants under nitrogen ambient, and that their incorporation-ratio can be modulated to favorably fit certain foreseeable biomedical applications.

Keywords: marble; graphene; *Luffa*; sintering ambient; reinforced bio-products; biomedical; applications

1. Introduction

Incipient discoveries in the realm of ceramic materials for bone healing applications can be dated back to over half a century ago [1,2]. However, it was not until the beginning of the 1980s that the medical community started using and marketing calcium phosphates

Chapter 3 – General conclusions

Globally, in the field of dentistry and orthopedics, a high number of cases involving the implantation of bone replacement products are annually reported. The subject of the PhD thesis was chosen for reasons related to aspects in close regard to bone defects that can be more difficult to solve using the currently available products. The experimental research aimed to develop a new, complete, reproducible, cheap and sustainable manufacturing technology of 3D bone restoration and substitution/reconstruction products. The products that can be obtained using this technology are made from ceramic biomaterials derived from natural resources, with a high biological performance and have internal (composition, structure, porosity), external (surface properties) and adapted mechanics characteristics, depending on certain patient needs.

It is generally considered that an ideal material for bone regeneration must harmoniously combine compositional, structural, geometric/architectural and mechanical characteristics to ensure the *in vivo* success. Based on these considerations, the experimental design was structured according to the scheme presented on Figure 1. The results obtained using this logical sequence were published in specialized journals and presented at conferences (for each step or stage of 3D material/product development there is at least one published scientific article).

Following an environmental-friendly direction, the study started from the premise of possible synthesis of biomimetic calcium phosphate ceramics from natural precursors such as marble and local seashells (calcium carbonate) as advantageous alternatives for the synthesis of bone replacement materials. Therefore, the chosen synthesis route was wet precipitation, with the addition of the necessary chemical reagent (phosphoric acid). Starting from the data presented in the literature, it was possible to refine the synthesis route, by using a quantity of phosphoric acid up to the maximum proposed level, in addition to the calculated stoichiometric amount. Thus, biomimetic calcium phosphates were obtained by the separate conversion of the two natural resources of calcium carbonate (Article 1). In this first stage, the main objective was to find/design a reproducible route, with well-established working parameters, on the basis of which it is possible to synthesize and finally to obtain a wide range of powders with modulated phase composition: HA→HA/DCPD→HA/DCPA→β-/α-TCP, in different proportions.

Taking into account the destination of the materials and their derived products, advanced studies were needed for the surface and the biological properties investigation. Digital topographic reconstructions made using MountainsMap software highlighted microporous textures for the entire range of synthesized materials, regardless of the natural precursor or the amount of acid involved. The biocompatibility and non-cytotoxicity properties of the powdered materials were demonstrated by viability, cytotoxicity and cell proliferation tests and microscopic fluorescence analysis, highlighting levels comparable to those of a commercial hydroxyapatite material.

For the development of prototypes for porous 3D products using synthesized bioceramic materials, the chosen solution implied the incorporation of natural fibres, of vegetable (Luffa and hemp) and animal origin (wool) (Articles 2 and 3). The first stage in the development process of porous structures, correlated with their necessary compliance with the biocompatibility criteria, involved the characterization of combustion products of Luffa, hemp and wool fibres that were chosen as potential porogen agents. By heat treatments (up to 1200°C) the complete decomposition was observed only in the case of Luffa fibres, in oxidative ambient environment (air). Research continued with the evaluation of cytotoxicity, demonstrating the cytocompatibility of all combustion products. The highest rates of cell viability and proliferation have been identified for combustion products resulting from Luffa fibres.

The obtained prototype products showed internal channels formed by the thermal disintegration of the *Luffa cylindrica* porogen agent type. *In vivo*, they could allow for the irrigation of the massive ceramic products for bone substitution with an adequate blood flow and body fluids. Thus, using bioceramic materials synthesized under stoichiometric conditions (hydroxyapatite from seashells and biphasic calcium phosphate HA/DCPD from marble) (i) compact products and (ii) porous structures with internal interconnected channels by the one-step addition of Luffa natural fibres were successfully developed. No binder materials or chemical treatments previously applied to the fibres were used; the final sintering was performed in air, at high temperatures (1200°C).

In the case of bone substitution products, some of the most important clinical aspects are related to surface and mechanical properties. In this respect, these were studied for both types of developed products, in order to obtain adequate surface-volume characteristics related to a key parameter, the pressing force. In the case of ceramic products obtained by the sacrificial template method, the pressure with which the mixtures of bioceramic powders and fibres are compressed induces the size (diameter) of the channels and the mechanical characteristics. Researches made in this regard has led to the conclusion that the use of high value forces can improve the mechanical behaviour and properties, but drastically changes the appearance of the internal 3D network of micro-pores and channels, by greatly reducing the size of their diameters and implicitly the possibilities of vascularization and formation of new bone tissue. This leads to the first compromise needed to ensure the optimal porosity and mechanical strength characteristics for adequate osseointegration.

In order to obtain superior mechanical characteristics, a further research stage was implemented, based on the continuation of the one-step development process of compact and porous products, by reinforcing them with graphene nanoplatelets. Their homogeneous dispersion in the bioceramic powder was achieved, regardless of the amount used. The consolidation and the defining particularities for the mechanical behavior of the finished products (compact and porous) were studied according to the applied heat treatment. A key factor is the gaseous atmosphere inside the heat treatment furnace (Article 4). Thus, the modulation of the sintering environment in the presence of the reinforcing agent leads to its disappearance and to the appearance of a favourable compositional variability of monophasic, biphasic and tri-phasic calcium phosphates, each combination presenting mechanical characteristics in accordance with the requirements of bone regeneration. In addition, the conversion of the ceramic matrix into a different range of calcium phosphates offers the possibility of establishing a programmable resorption of bioceramic bone substituents and, consequently, a faster bone regeneration, after the implantation of the respective products.

The favourable consequences of the addition of graphene can be traced further by the mechanical behaviour, by generating an additional micro-porosity degree at the level of the compact ceramic component. Correlated with the ability of Luffa fibres to generate internal channels with a distribution similar to the spatial distribution of blood vessels in the natural bone, products obtained using this technology have the optimal characteristics necessary for bone substitutes used in dentistry and orthopaedics. Modification of the compositional range also induces important morphological changes for the overall mechanical strength of the products, by expanding the size of the grains and pores in the ceramic matrix.

In the long run, it is expected that the developed manufacturing technology will lead to the emergence of superior medical products, primarily by expanding the range of possible orthopaedic applications. Depending on the particular medical case, products with different compositional, morphological, architectural and mechanical characteristics can be used for each patient. The technology allows the modulation of each parameter, by the favorable choice of the natural precursor, the optimal ratio of porogen/reinforcing agent and up to the selection of the sintering ambient, in order to obtain the beneficial effects of custom-made products (bone regenerator sites).

Chapter 4 – Original scientific contributions to the field

The experimental studies presented in this PhD thesis involved a series of original contributions that aimed to develop an integrated technological route for obtaining bioceramic materials based on calcium phosphates of biogenic origin. It has been possible to obtain new materials for bone replacement, with direct applicability as dental and orthopaedic implants for the reconstruction of bone defects with variable dimensions and characteristics. The proposed technology can be seen as a three-stage development process.

The first stage was based on the refinement of the synthesis route of some bioceramic materials of biogenic origin. Sustainable natural resources based on calcium carbonate, such as marble and local marine shells, were used to obtain the targeted materials in an easy and cost-efficient way: single-phase and/or biphasic calcium phosphates. In this respect, it was established that a first key factor involved in the correct and reproducible synthesis of bioceramic materials is the amount of chemical reagent required for the conversion of natural resources. The performed physico-chemical characterizations outlined the dynamism of the chemical reactions, highlighting *three compositional ranges* with reproducible results and the precipitation of HA under stoichiometric conditions, only for seashell-type resources.

◉ An important contribution in the PhD thesis field is represented by the fact that two types of compositions were identified for the first time by wet precipitation synthesis from marble and seashell resources. The first is the biphasic HA/DCPD mixture obtained up to 10% and 10-30% phosphoric acid additions. The second involves the possibility of obtaining the biphasic HA/DCPA mixture, stable at ranges of higher concentrations, up to a maximum of 90%, for both types of derived materials. In order to determine the optimal range (between 0-30%) of the chemical reagent, in accordance with the requirements for bone substitution and reconstruction products, complex studies of digital rendering of the topography were performed, using the MountainsMap software. Thus, the microporous character of the materials was highlighted, independent of the natural precursor or the amount of acid used, but with a different degree of roughness. All synthesized materials were characterized *in vitro* as to determine their biological performance.

◉ Another element of novelty highlighted in this thesis is the establishment of a one-step manufacturing protocol of "green" ceramic products with controlled spatial porosity and internal architecture and with interconnected channels, similar to that of natural bone. Achieving this goal involved the use of porous agents from the range of natural fibres with the possibility of decomposition and reduction at high temperatures, a method called *fibrous sacrificial template method*. The proposed process contributes to the elimination of the intermediate stages of chemical treatment of fibres, the need of binders (reported to ensure the connection between the porogen agent and the matrix material) and implicitly the risks associated to them even after sintering at high temperatures (e.g., toxic residual residues), and to the solving of the vascularization problem of osteo-reconstruction products.

- For the first time, it was possible to parameterize the technological process, by establishing the optimal conditions for compression and sintering of mixtures of ceramic material and natural fibres. The combustion products resulted from the thermal decomposition of natural fibres in two sintering environments (air/nitrogen) were thoroughly and comparatively investigated. Heat treatments made on three types of natural fibres (e.g., Luffa cylindrica, hemp, wool) highlighted the possibility of complete thermal degradation only of Luffa fibres and especially in air sintering ambient, at temperatures higher than 1000°C.

- Another important feature for the targeted field is represented by the exposure for the first time of the biocompatible character of the products resulting from the thermal decomposition of the fibres, by performing *in vitro* cytotoxicity tests. Among other aspects, the type of natural fibre that generates a patient-risk-free combustion product (>95% cell viability and proliferation) and capable of generating channels with dimensions comparable to those in bone tissue has been identified.

- Another important aspect is the identification of the result of the thermal decomposition process of the natural fibres itself, which is accompanied by the evacuation of volatile compounds. This process leads to the formation of a porous matrix inside the channels, thus appearing a micro-porosity gradient identified from the surface of the porous samples and to their entire volume. The presence of Luffa fibres during heat treatment leads to the preservation of the biphasic calcium phosphate formed HA/β-TCP, with increased crystallite size and different proportions depending on the natural precursor (70-100%/30-0%), without the alteration of the network parameters.

Maintaining the porous configuration of the products and at the same time improving their mechanical characteristics required the development of a compatible way of consolidation by strengthening the bioceramic matrix. It was decided to use a reinforcing material with high distribution and incorporation capacity in the ceramic matrix of calcium phosphate, and superior densification of products induced at high temperature, following the sintering process in two different ambients (oxidative vs. reductive/ air vs. nitrogen).

- An important finding consists in demonstrating the influence of the graphene nanoplatelet reinforcing agent on the compositional and architectural characteristics, in order to obtain compact products, as well as its compatibility with Luffa fibres. These aspects were analyzed for all quantities of embedded graphene, clearly delimiting the appropriate combination ratio of the two agents for porous products, a unique aspect in the literature.

- The effect of the heat treatment environment on the obtained products was established. For both sintering environments (air and nitrogen), the concomitant presence of graphene and Luffa is a compositional and structural influencing factor. The oxidative medium favours an accentuated decomposition of the ceramic matrix until the extinction of HA or HA/DCPD and the integral formation of the tricalcium phosphate β-TCP, while the reductive environment leads to a decomposition of the initial compounds in variable proportions of β-TCP, α-TCP and $Ca_2P_2O_7$ with the increment of the graphene amount.

⦿ The optimal graphene amount that can be used has been determined, depending on the type of desired product and the sintering environment. Thus, for compact products, in the case of nitrogen sintering, the maximum quantity is 0.25 wt.%, while for the porous ones the restrictions are imposed by air sintering, up to a maximum of 0.50 wt.%. Each combination of calcium phosphates has different mechanical properties and resorbability degrees, suitable for several types of bone regeneration applications.

Consequently, the research led to the development of a completely new technology for the fabrication of compact and porous 3D products (composite assemblies) with modular properties, able to satisfy a wide range of requirements for further implementation in bone substitute load- and non-load bearing applications. The products that can be obtained in this way can be custom-made function of the specificity of the bone defect and the range of properties required at the patient level.

The importance of the original contributions highlighted in the field of materials engineering and presented in this PhD thesis was supported and recognized in 5 international conferences and by publishing the results in 4 scientific articles, Clarivate Analytics ranked, having an impact factor in the 3.623 – 6.707 range.

Based on the developed manufacturing technology proposed in the PhD thesis, a national patent application was filed (**A-00258**) on May17th 2021: „*Procedeu de obținere a unui produs pentru reconstrucția defectelor osoase, pe bază de hidroxiapatită și fosfat de calciu bifazic biogen*” by F. Miculescu, A.C. Mocanu, G. Stan, I.V. Antoniac, M.C. Costoiu, Ș.I. Voicu, M. Miculescu, I.M. Mateș, A. Semenescu.

The results obtained in this PhD thesis will be complemented in the future with further experimental insights:

↠ preclinical testing, complex *in vitro* assays in cell cultures and *in vivo* tests on animal model, and validation of the manufacturing technology and the characterization program for materials and products derived from marble and seashells;

↠ reproducibility studies of the completed synthesis route following the influence of the raw resources natural habitat on the final properties of the materials and products (applicable for seashells);

↠ development of reference/control materials, with well-defined synthesis and processing parameters, with known ratios between the different types of constituent calcium phosphates, and which can be subsequently used in the analysis of the quality of other bone type substitutes.

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