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In vitro degradation of calcium phosphates: effect of multiscale porosity, textural properties and composition

A. Díez-Escudero^{1,2}, M. Espanol^{1,2}, S. Beats^{1,2}, M.P. Ginebra^{1,2,3}

¹ Biomaterials, Biomechanics and Tissue Engineering Group, Department of Materials Science and Metallurgical Engineering, Universitat Politècnica de Catalunya (UPC), Av. Eduard Maristany 10-14, 08019 Barcelona, Spain.

²Barcelona Research Center in Multiscale Science and Engineering, Universitat Politècnica de Catalunya, Av. Eduard Maristany 10-14, 08019 Barcelona, Spain

³Institute for Bioengineering of Catalonia, C/ BaldiriReixac 10-12, 08028 Barcelona, Spain

*Corresponding author

Maria Pau Ginebra Molins

Biomaterials, Biomechanics and Tissue Engineering Department

Department of Materials Science and Metallurgical Engineering

Universitat Politècnica de Catalunya

Avinguda Eduard Maristany 10-14, 08019, Barcelona, Spain

Tel: +34 934017706

Email: maria.pau.ginebra@upc.edu

Abstract

The capacity of calcium phosphates to be replaced by bone is tightly linked to their resorbability. However, the relative importance of some textural parameters on their degradation behaviour is still unclear. The present study aims to quantify the effect of composition, specific surface area (SSA), and porosity at various length scales (nano-, micro- and macroporosity) on the in vitro degradation of different calcium phosphates. Degradation studies were performed in an acidic medium to mimic the osteoclastic environment. Small degradations were found in samples with interconnected nano- and micropores with sizes below 3 μm although they were highly porous (35-65 %), with maximum weight loss of 8 wt%. Biomimetic calcium deficient hydroxyapatite, with high SSA and low crystallinity, presented the highest degradation rates exceeding even the more soluble β -TCP. A dependence of degradation on SSA was indisputable when porosity and pore sizes were increased. The introduction of additional macroporosity with pore interconnections above 20 μm significantly impacted degradation, more markedly in the substrates with high SSA ($>15 \text{ m}^2/\text{g}$), whereas in sintered substrates with low SSA ($<1 \text{ m}^2/\text{g}$) it resulted just in a linear increase of degradation. Up to 30% of degradation was registered in biomimetic substrates, compared to 15% in β -TCP or 8% in sintered hydroxyapatite. The incorporation of carbonate in calcium deficient hydroxyapatite did not increase its degradation rate. Overall, the study highlights the importance of textural properties, which can modulate or even outweigh the effect of other features such as the solubility of the compounds.

Keywords: calcium phosphates, porosity, textural properties, degradation

1. INTRODUCTION

Bone has a remarkable capacity for self-repair. However, this self-healing capacity is not sufficient to bridge critical sized bone defects and moreover it can be impaired in some pathological situations. In these cases, the use of bone grafts, either natural (autografts, allografts or xenografts) or synthetic, is crucial to restore bone function [1]. An ideal bone graft should provide initial strength at the implantation site while actively supporting bone remodelling. Therefore, it is important to have a tight synchronisation between graft resorption and new bone deposition to allow a gradual replacement of the bone graft by newly formed bone [2]. Although bone autografts remain the gold standard for such applications, site morbidity and volume availability are major concerns that limit their use [3]. The need for synthetic bone grafts is clear, but their choice and design remain still complex due to many considerations such as material composition, architecture, mechanical stability, degradation products, etc. that can potentially affect the remodelling process [4].

Among synthetic bone grafts [5-7], calcium phosphates (CaP) are very interesting for remodelling purposes as they possess a close resemblance to the mineral phase of natural bone consisting of ~70 wt% of nanocrystalline hydroxyapatite (HA) [8]. Owing to the close compositional resemblance of CaP to bone mineral, they can induce a biological response similar to that taking place during bone remodelling. Indeed, CaP can potentially be resorbed by osteoclastic cells and can also support bone formation under the action of osteoblastic cells [9]. However, despite the potential of CaP, there is presently no material matching the remodelling rates of natural bone. The bottleneck for most CaP formulations, including hydroxyapatite-based formulations, is their poor resorption rate, especially for sintered HA, which is one of the most commonly used materials [10]. *In vivo* studies have proved that even after 9 months of implantation

sintered HA remained at the site with hardly any sign of resorption [11,12]. To circumvent this problem, HA has been combined with more soluble phases such as β -tricalcium phosphate (β -TCP) [13–16]. Alternatively, more inherently soluble phases such as brushite/monetite have also demonstrated higher resorbability [17,18]. In addition, doping the crystal structure of HA with e.g. carbonate ions was found to enhance its resorption and to promote osteoclastic activity *in vitro* [19–24].

However, not only composition plays a role in degradation rates. The modification of crystallinity, grain size, specific surface area [25,26], or the porosity content [27–29] are relevant physicochemical features which have been shown to help tailor resorption rates, hence improving the *in vivo* performance of such implants [30]. Despite the various works proving increased degradation in materials with lower crystallinity, high specific surface area, decreased grain density, and a higher degree of porosity, the extent to which each parameter influences resorption remains unclear. The complexity of isolating the contribution of each of these parameters lies in the close interrelation between these factors, as changing one can potentially affect the other/s.

One key aspect in the evaluation of the *in vitro* degradation of biomaterials is the selection of the degrading medium. Although degradation of CaP has been assessed in different solutions [31–35], the simulation of the resorption process by osteoclasts requires working in solutions at acidic pH. This is because resorption by mature osteoclast proceeds through the development of a tight ring-like zone of adhesion (i.e. the sealing zone) that delimits the resorbing area onto which osteoclasts generate an acid milieu that can reach values below pH 3, resulting in the dissolution of the underlying mineral [36]. In this regards, studies performed at low pH can be used as predictors of osteoclastic degradation [37–40].

The aim of this study was to provide quantitative information on the relative importance of CaP composition, specific surface area, and porosity in an *in vitro* model of CaP

degradation. We took advantage of the possibility offered by the fabrication of hydroxyapatite scaffolds using biomimetic routes, i.e., by low temperature dissolution – precipitation reactions, to systematically introduce controlled levels of porosity at the nano, micro, and macroscale, as well as to modify the specific surface area [41–43]. Thus, the porosity at the nano-microscale and the specific surface area were modified by varying the L/P ratio and the particle size of the starting powder, respectively [41,42]. In turn, macroporosity was introduced through a foaming process [43]. Moreover, the effect of doping with carbonate ions on the degradation behavior was analyzed. Finally, the degradation behavior of the biomimetic scaffolds was compared to that of sintered materials, namely β -TCP and sintered HA, obtained after thermal treatment of the biomimetic scaffolds, which, in addition to the change in composition, exhibited different textural parameters. To facilitate comparison between materials and to discard any effect due to the use of different reagents (e.g. purity, presence of ionic traces of foreign ions, etc.), all materials were prepared from the same Ca and P sources.

2. MATERIALS AND METHODS

2.1. Preparation of biomimetic calcium deficient hydroxyapatite

α -Tricalcium phosphate (α -TCP) was used for the preparation of biomimetic calcium deficient hydroxyapatite (CDHA) through a cementitious reaction. Briefly, α -TCP was obtained by heating calcium hydrogen phosphate (CaHPO_4 , Sigma-Aldrich, St. Louis, USA) and calcium carbonate (CaCO_3 , Sigma-Aldrich, St. Louis, USA) at a 2:1 molar ratio at 1400°C for 15h and then quenching in air. Subsequently, the particles obtained were milled as described elsewhere [41] to obtain two different α -TCP powder sizes, coarse (C: 5.2 μm median size) and fine (F: 2.8 μm median size). α -TCP, with 2 wt% of precipitated hydroxyapatite (PHA, Merck KGaA, Darmstadt, Germany), was mixed with a liquid phase consisting of an aqueous solution of 2.5 wt% disodium hydrogen phosphate (Na_2HPO_4 , Merck, Darmstadt, Germany) which acted as a reaction

accelerant. Both phases were mixed in a mortar for 1 min and the resulting paste was transferred into 15x2mm² PTFE disc moulds where the discs were left to set in water at 37°C for 7 days. CDHA samples with different liquid to powder ratios (L/P) were prepared, ranging from 0.35 to 0.65 mL/g.

2.2. Preparation of biomimetic carbonate-doped hydroxyapatite

Carbonated calcium deficient hydroxyapatite (C-CDHA) was obtained by performing the setting of the cement in a saturated sodium bicarbonate solution (NaHCO₃, Sigma-Aldrich, St. Louis, USA) instead of water, for 17 days at 37°C. Samples were immersed in the carbonated setting medium once cohesion was achieved, i.e., approximately after 5h of preparation. In these specimens, water was used as the liquid phase of the cements instead of a disodium hydrogen phosphate solution.

2.3. Preparation of biomimetic foams

To prepare the macroporous scaffolds, the powder phase consisting of 98wt% α -TCP and 2 wt% of precipitated hydroxyapatite (PHA) was mixed with an aqueous solution of 1 wt% Polysorbate 80 (Sigma-Aldrich, St. Louis, USA) as foaming agent [43]. Both phases were foamed with a domestic food mixer for 30 s at 7000 rpm and then transferred to 6x12 mm PTFE cylindrical moulds where they were left to set in water at 37°C for 10 days. The L/P ratios for the foams were adjusted to 0.55 and 0.65mL/g for coarse and fine α -TCP powders respectively, as these proportions produced scaffolds with similar macroporosities for the two powders.

2.4. Preparation of sintered hydroxyapatite and beta-tricalcium phosphate

Sintering of CDHA and C-CDHA at 1100°C for 9 h resulted in beta-tricalcium phosphate (β -TCP) and sintered hydroxyapatite (SHA). This protocol was applied both to non-foamed and foamed cements. The non-foamed specimens corresponding to β -TCP and SHA were obtained from coarse particles since no differences were observed

regarding their physicochemical features after sintering. Figure 1 summarizes the various synthesis procedures and the nomenclature assigned to the different materials.

2.5. Physicochemical Characterization

2.5.1. X-ray Diffraction (XRD)

Phase characterization of the samples was performed by X-ray diffraction using a D8 Advance diffractometer (Bruker) equipped with a Cu K α anode, operated at 40kV, and 40mA. Data were collected in 0.02° steps over the 2 θ range of 10°-80° with a counting time of 2s per step. Prior to analysis, the samples were finely ground into powders. The experimental patterns were compared to those of hydroxyapatite (JCPDS 09-0432), α -TCP (JCPDS 09-0348) and β -TCP (JCPDS 09-0169). Phase quantification was performed using analysis software (DIFFRAC.EVA software, Bruker).

2.5.2. FTIR spectroscopy

Samples were analyzed by Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) using a Nicolet 6700FTIR equipped with a He/Ne beam, Csl beam splitter and DTGS-Csl detector. 64 scans were acquired with a resolution of 4 cm⁻¹ in the range of 4000 to 575 cm⁻¹ with a Germanium crystal.

2.5.3. Carbonate quantification

Elemental quantification of total carbon content was performed by bulk combustion using a Thermal combustion element analyzer, Thermo EA 1108(TC/EA), working in standard conditions (Helium flow at 120 mL/min, combustion furnace at 1000°C, chromatographic column oven at 60°C, oxygen loop 10 mL at 100 kPa). The amount of carbonate was calculated according to the following equation:

$$\text{Percentage of Carbonate} = \frac{MW_{CO_3^{2-}}}{MW_C} \cdot \%_C \quad (\text{Eq.1})$$

where, $MW_{CO_3^{2-}}$ is the molecular weight of carbonate ion, MW_C is the molecular weight of carbon, and $\%_C$ is the percentage of carbon given by the measurement. Prior to

analysis, samples were crushed using an agate mortar, and the powders were dried at 120°C overnight.

2.5.4. Morphological analysis

Scanning electron microscopy (Zeiss Neon40dual beam FIB/SEM) was used to study the microstructure of the samples. All micrographs were taken from the cross-section of samples. Prior to imaging, samples were coated with carbon to enhance conductivity.

2.5.5. Specific Surface Area (SSA)

The specific surface area was determined by nitrogen adsorption using the BET (Brunauer-Emmett-Teller) method (ASAP 2020, Micromeritics). Prior to measurement, samples were outgassed in vacuum conditions (10 μ mHg) at a holding temperature of 100°C for 2 hours.

2.5.6. Porosity

The open porosity and pore entrance size distributions were obtained by mercury intrusion porosimetry (MIP, Autopore IV Micromeritics). All samples were dried at 100°C for 2 hours prior to measurement. Mercury intrusion-extrusion curves were recorded from 30 to 30000 psia.

2.5.7. Skeletal density and compression tests

The skeletal density of samples was measured by helium picnometry (AccuPyc 133, Micromeritics) for the different compositions CDHA_C, CDHA_F, C-CDHA_C, β -TCP and HA. The compression strength of the different compositions and L/P ratios were measured using a Universal Testing Machine (Instron 8511) at a cross-head speed of 1 mm/min until fracture. Ten cylindrical samples (6 mm diameter by 12 mm high) were analyzed for each composition and L/P. Compressive strength values are shown in the supplementary information (Appendices 1).

2.6. Accelerated degradation study

An accelerated degradation study was performed by immersing the samples in an acidic solution consisting of 0.01 M hydrochloric acid (HCl, Panreac AppliChem) and 0.14 M sodium chloride (NaCl, Panreac AppliChem) at 37°C, based on previous studies [36,44]. Prior to degradation, samples were dried at 120°C overnight until a constant weight was achieved. Since the solution was not buffered, to prevent any change in the solution pH that would alter the degradation behavior, the samples were immersed in a large volume of acidic medium (15 mL) to keep the pH constant. The solutions were placed in sterile polypropylene tubes (50 mL) over 8 h. Every hour, samples were transferred to new vials with fresh medium. After degradation, samples were rinsed thrice with distilled water and dried overnight at 120°C until constant weight. The weight loss percentage ($n=3$) was obtained by calculating the difference in weight according to the equation:

$$\text{Percentage of weight loss} = \frac{m_0 - m_f}{m_0} \cdot 100 [\%] \quad (\text{Eq.2})$$

where, m_0 is the initial wet sample mass and m_f is the final mass after drying. Supernatants at each time point were collected and pH values were measured using a pHmeter (MultiMeter MM 41).

2.7. Statistical analyses

The degradation experiments were conducted with three samples. The results are presented as the average and standard deviation. Statistical significance with a level of $p < 0.05$ was evaluated for dense and foamed samples. One-way ANOVA was used for dense samples since they presented a normal distribution. The analyses of foamed versus non-foamed specimens were carried out using the non-parametric Mann Whitney test since no normal distribution was found. Compressive strength is presented as mean and standard deviation. All statistics were performed using SPSS software (IBM).

3. RESULTS

3.1. Physicochemical Characterization

Figure 2A shows the X-ray diffraction results for the different samples. The XRD profile of the biomimetic samples differed significantly from the sintered ones in terms of peak sharpness as a consequence of the low crystallinity of biomimetic CDHA, and was more pronounced in CDHA_F than in CDHA_C. The three CDHA samples contained traces of unreacted α -tricalcium phosphate (3 wt%). It is noteworthy that the addition of carbonate altered the CDHA conversion kinetics, and 17 days were required for conversion of C-CDHA samples into similar levels as those of CDHA coarse and fine. Similarly, the absence of accelerant in the liquid phase, as is the case of foamed specimens required 10 days of setting. This was confirmed by the similar levels of unreacted α -TCP. Upon sintering CDHA at 1100°C, pure β -TCP was obtained, whilst sintering of C-CDHA resulted into stoichiometric HA.

The ATR-FTIR spectra of the different materials studied are shown in Figure 2B. Similar to what was observed by XRD, sintered samples showed better resolved bands than biomimetic materials. This was particularly evident when comparing CDHA with SHA. Typical phosphate (PO_4^{3-}) bands in HA appear at 570, 600, 960, 1030 and 1090 cm^{-1} , corresponding to vibrational modes ν_4 , ν_1 , ν_3 respectively [45]-[47]. The stoichiometric high temperature SHA showed well defined and sharper bands than CDHA for the phosphate groups. The hydroxyl band, at 630 cm^{-1} appeared also sharper in SHA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) than in CDHA, consistent with the non-stoichiometric nature of the apatites obtained by hydrolysis of α -TCP (i.e., $\text{Ca}_9(\text{PO}_4)_5(\text{HPO}_4)(\text{OH})$) [47]. The presence of HPO_4^{2-} in CDHA was proved by an additional band appearing at 870 cm^{-1} . Typical carbonate bands (CO_3^{2-}) at 1414 and 1471 cm^{-1} , and at 871 cm^{-1} were visible in C-CDHA, which are representative of ν_2 vibrational mode accounting for a B-type carbonated apatite (i.e. phosphate substitution) [48], [49]. The β -TCP spectrum showed typical phosphate bands at 551, 604, 945, 970, 1024, 1042, 1080 and 1118 cm^{-1} [46], [50]. The skeletal density of the biomimetic compositions slightly differed.

CDHA_C and CDHA_F resulted in 2.70 ± 0.02 g/cm³ and 2.67 ± 0.02 g/cm³ respectively, while the value for C-CDHA_C decreased to 2.54 ± 0.03 g/cm³ consistent with the incorporation of carbonate in the crystal structure. As expected, sintered β -TCP and HA compositions, showed the highest values of density with 3.08 ± 0.02 and 3.18 ± 0.04 g/cm³ respectively.

TC/EA analyses of carbonate-containing samples are summarized in Table 1. The carbonate levels were similar for all samples regardless of their porosity, ranging from 11 to 13 %wt. Control samples of CDHA_C and CDHA_F were included and proved no carbonation occurring from ambient carbon dioxide or from precursor chemicals.

Table 1. Carbonate content of the different carbonated CDHA samples.

	L/P ratio	Carbonate level
	[mL/g]	[%wt]
C-CDHA_C	0.35	12.25 ± 0.78
	0.45	13.30 ± 1.27
	0.55	12.33 ± 0.25
	0.65	13.23 ± 0.04
Foam-C-CDHA_C	0.55	11.33 ± 0.39
Foam-C-CDHA_F	0.65	12.58 ± 0.32
CDHA_{C/F}	0.55	0.05 ± 0.07

Scanning electron micrographs of the different compositions are shown in Figure 3. The different biomimetic substrates showed microstructures consisting of aggregates of nano-micrometric crystals. For CDHA_C, the aggregates consisted of plate-like crystals. CDHA_F instead consisted of needle-like crystals due to the smaller particle size of α -TCP used for its synthesis. The introduction of carbonate resulted in a plate-like morphology of the crystals, both for the C-CDHA_C and C-CDHA_F samples, which showed a very similar microstructure. The high temperature CaPs, β -TCP and SHA, showed the typical polyhedral grain structure of sintered samples.

Micrographs taken at a lower magnification of the CDHA and β -TCP non-foamed samples obtained at different L/P ratios and micrographs of the foamed samples revealed significant differences according to the processing methods of the materials (Figure 4). The increase in the L/P ratio led to more open structures, consistent with an increase in the separation between the starting α -TCP particles, due to the higher amount of liquid. Foaming resulted in the formation of larger macropores, with pore interconnections, i.e., openings between adjacent macropores.

The specific surface area and total open porosity values for all materials are shown in Figure 5. Figure 5A displays the values for non-foamed materials. Materials synthesized at high temperature showed low SSA values ($<1 \text{ m}^2/\text{g}$) whilst low temperature biomimetic substrates showed higher SSA values. Specifically, CDHA_F with needle-like structure possessed the higher SSA values ($40 \text{ m}^2/\text{g}$) whereas CDHA_C, consisting of bigger plate-like crystals, showed lower values ($15 \text{ m}^2/\text{g}$). Carbonated samples (C-CDHA_C) showed values between those of CDHA_C and CDHA_F ($27 \text{ m}^2/\text{g}$). Carbonated CDHA fine (C-CDHA_F) was not analysed since porosity, microstructure and SSA was equal to the coarse counterpart. The L/P ratio did not have a significant effect on SSA values. Oppositely, L/P ratio had a great influence on porosity values. The increase in L/P ratio resulted in higher percentages of open porosity, significantly evident after the foaming process, as assessed by MIP.

Interestingly, in the non-foamed compositions, the increase of L/P ratio not only resulted in an increase in the total porosity, but also shifted the pore size distributions to larger sizes, as illustrated in Figure 6. All biomimetic samples (CDHA_C, CDHA_F and C-CDHA_C) showed bimodal pore populations. Larger pores account for the pores in between aggregates (micrometric range), and the smaller pores relate to the distance between crystals (nanometric range). In contrast, sintered β -TCP samples exhibited a large peak centered around $1 \text{ }\mu\text{m}$, and an almost total disappearance of the

nanoporosity, as a consequence of nanocrystal coalescence during the sintering process.

The effect of the foaming process on the pore size distribution is illustrated for biomimetic and sintered foams in Figures 6E and F, respectively. Biomimetic foams (Figure 6E) maintained the trend regarding nano-microporosities, with a superimposed macroporosity up to 100 μ m. Upon sintering, the macroporosity as well as the microporosity around 1 μ m were maintained, whereas no nanoporosity was observed as a result of the aforementioned coalescence of the nanocrystals (Figure 6F).

3.2. Accelerated degradation study

The results of the accelerated degradation study are illustrated in Figure 7. Figure 7A depicts the degradation of the different non-foamed materials depending on their L/P ratio while Figure 7B shows the degradation of the foamed samples (non-foamed counterparts are included as control samples). Overall, when the liquid to powder ratio increased, the degradation increased for all samples but to varying degrees depending on their composition, microstructure, and textural properties. For a given L/P, CDHA_F experienced a larger weight loss than the similar CDHA_C, and the degradation was more sensitive to the increase in L/P than the coarse counterpart. Surprisingly, the carbonated apatite (C-CDHA) exhibited the lowest degradation values of all samples, whereas β -TCP, despite being the most soluble phase, showed degradation values between CDHA and C-CDHA.

The incorporation of macroporosity in the foamed samples resulted in remarkable increases in degradation, compared to their non-foamed analogues (see Figure 7B). However, the extent of the increase was strongly dependent on the substrate. Thus, biomimetic CDHA showed the highest increase (three- and four-fold weight loss for the foamed CDHA_C and CDHA_F compared to their non-foamed counterparts). In the carbonated samples, C-CDHA, the impact of the macroporosity was smaller resulting in

a three-fold weight loss increase irrespective of the α -TCP particle size. In the sintered β -TCP, the impact of foaming in the degradation was again dependent on the size of the starting α -TCP, being two or three-fold for β -TCP_C and β -TCP_F respectively. SHA foams showed the lowest degradation among all foamed materials, yielding similar resorption as non-foamed materials. Figure 7C shows the pH values measured during the degradation experiment. Steady values in pH were observed for all samples throughout the experiment, irrespective of sample composition and porosity.

Figure 8 combines the degradation results obtained for each material as a function of porosity. Despite that non-foamed formulations exhibited a linear increase with L/P (differences in slope were observed depending on sample type), the introduction of interconnected macropores in the foamed formulations led to marked changes in the degradation behavior. Degradation increased exponentially (exponential growth function) on the biomimetic samples with correlation coefficients (R^2) of 0.986, 0.799, and 0.855 for coarse, fine, and carbonated CDHA respectively, but remained linear for the sintered β -TCP ($R^2 = 0.938$).

The samples were analyzed after degradation, to investigate possible changes in composition and morphology. The results for representative samples of each composition at a fixed L/P (0.55 mL/g) are shown in Figure 9. XRD (Figure 9A) proved that no additional phases were re-precipitated. SEM images (Figure 9B) taken of the surface of the materials clearly demonstrated the degradation of the pristine plate and needle like crystals in the biomimetic CDHA samples, whilst images of β -TCP exhibited a less-obvious degradation only visible at the grain boundaries. The microstructure of the non-foamed samples cross-sections remained intact indicating that the acid was not penetrating the sample bulk.

4. DISCUSSION

The present study analyses the degradation behavior of different calcium phosphate biomaterials. The simple *in vitro* model used here does not capture all the complex mechanisms occurring during the degradation process of a biomaterial once implanted in the body. Therefore, the results obtained cannot directly be translated to material performance *in vivo*. However, this model facilitates comparison of the sensitivity to acidic degradation of different synthetic calcium phosphate ceramics, and, most importantly, it makes easier analyzing the relevance of some textural properties, i.e. specific surface area and porosity, during the acidic degradation of synthetic calcium phosphates.

The use of cementitious reactions in the formulation of biomimetic samples was vital to control the level of nano- and microporosity of the samples [40]. Indeed, the hydrolysis of α -TCP at body temperature results in the precipitation of an entangled network of crystal aggregates responsible for the presence of nanoporosity in the samples (Figures 4 and 6). Increasing the L/P ratio during sample preparation gradually introduced an additional level of porosity within the micron range, due to an increase in the distance between crystal aggregates [40] (Figures 4 and 6). Furthermore, the foaming process allowed introducing an interconnected network of macropores in the material (Figures 4 and 6). This provided a platform to systematically assess the role of the multiscale level of porosity in the degradation behaviour of calcium phosphates. On the other hand, the change in the starting α -TCP powder size from fine (F) to coarse (C) led to a change in the morphology of the nanocrystals from needles to plates - regardless of the L/P ratio- with a consequent change in SSA. The SSA was controlled by the surface area of the crystals which was readily available due to porous nature of cements (crystals precipitate forming an open entangled network) and was not affected by the L/P ratio. Modification of the cements composition was achieved either by incorporation of carbonate ions during precipitation or by a sintering process.

The degradation behavior of the different materials can be analyzed in two separate blocs: the degradation of the non-foamed samples, i.e. micro/nanoporous samples, versus the degradation of the foamed formulations, i.e., macroporous samples. Although all samples were inherently porous, non-foamed specimens possessed a maximum pore size below 3 μm , whilst foamed formulations presented a superimposed macroporosity, typically above 10 μm .

Regarding the non-foamed CDHA materials, despite the similar porosity values of biomimetic coarse and fine samples at a fixed L/P ratio, fine samples yielded higher resorption (Figure 7A), thus revealing the effect of SSA (the only exception was for L/P 0.35). Thus, for a given porosity, CDHA_F degraded more than CDHA_C. However, the difference in weight loss was small, and depended on the porosity and pore size of the specimens. Changing the L/P from 0.35 to 0.65 mL/g led to a linear increase in the degradation of biomimetic C and F samples. This was due not only to the increase in total porosity with L/P, but also to the displacement of pore sizes to larger values, due to the increase in interaggregate spaces [40,41]. This led to the appearance of an interconnected pore network around 1-2 μm (Figures 6A and B) through which the acidic media could improve penetration even if this was limited to the nearby surface. As shown in Figure 8, the increase of degradation with microporosity was more pronounced for the CDHA_F than for CDHA_C (from 5 wt% to 6.5 wt% and 8 wt% respectively), leading to a three times larger slope (0.13 vs. 0.04). This can be associated to the higher SSA and is in agreement with the trend observed by Kuo *et al.* using calcium sulphate based materials [51].

However, for the less porous formulations: CHDA_C and CDHA_F at L/P of 0.35, a similar degradation of around 5wt% was observed even if CDHA_F doubled the SSA of CHDA_C. The lower packing efficiency of coarse samples results in interaggregate spaces slightly larger than those observed for fine samples (pores centered at 50 nm and 30

nm in Figure 6A and B, respectively) allowing more liquid penetration and the subsequent degradation of the exposed volume.

It is worth mentioning that in calcium phosphate cements the L/P ratio and the particle size of the starting powder, which determine the SSA and micro-/nanoporosity (Figs. 5 and 6), are usually adjusted in order to optimize the mechanical and rheological properties, but their effect on the degradation rate is often overlooked. The results obtained show that they also influence the sensitivity to acidic degradation, and therefore, this should be taken into account in the design of the cement formulation.

The setting of the α -TCP cement in a carbonate solution produced a carbonate-doped calcium deficient apatite. Interestingly, despite the comparable values in SSA between carbonated doped HA (C-CDHA_C) and the non-carbonated substrates (CDHA_C, CDHA_F), the degradation behaviour was significantly lower for the carbonated samples ($p < 0.05$). This was unexpected, since the incorporation of carbonate ions in the hydroxyapatite lattice is known to markedly disturb the crystal lattice [52] increasing its solubility [53–55]. Quantitatively, C-CDHA_C resorption was reduced by a 30-50 % compared to CDHA_F, regardless of the L/P ratio. Figures 7C and 9 showed that the carbonate ions released during degradation did not increase the pH of the degrading media, which could have altered the degradation behaviour. Additionally, the ions released did not cause re-precipitation of secondary phases either. Moreover, although in the present work high percentages of carbonate were obtained; those values were comparable to other reported works [56]. Most importantly, the position of the carbonate bands in the FTIR indicated the incorporation of carbonate ions in the phosphate positions as shown in Figure 2B. Typical bands of CO_3^{2-} at 871, 1414 and 1471 cm^{-1} [21,48,49] gave evidence of a preferred B-type substitution.

The reason for the unexpected decrease in degradation can be linked to the fact that in the present study, the degradation behavior of the carbonated samples was compared

to calcium deficient hydroxyapatite instead of stoichiometric hydroxyapatite, as most authors do. The former, i.e. $\text{Ca}_9(\text{PO}_4)_5(\text{HPO}_4)(\text{OH})$, is inherently more soluble than stoichiometric HA, i.e. $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ [57], with solubility products of 85.1 ($-\log K_{\text{PS}}$) and 116.8 ($-\log K_{\text{PS}}$) respectively [11], due to its distorted lattice and consequently lower crystallinity. The results obtained suggest that the incorporation of carbonate ions in this kind of pre-distorted lattice does not have the same effect than in stoichiometric HA; rather than increasing the solubility the opposite happens.

Figure 7A also displays the results for the degradation behavior of β -TCP samples at the different L/P. It is interesting to note that, in spite of having a much higher solubility, with a solubility product of 28.9 ($-\log K_{\text{PS}}$) [11],[58] and larger pore sizes (Fig. 6), its degradation was lower than that of CDHA, which can be explained by the much smaller SSA. Similar to what was already observed for the biomimetic samples, the increase in L/P led to a gradual increase of the degradation of sintered β -TCP samples from 4% to 6% for L/P 0.35 to 0.65, respectively, with a slope similar to that of CDHA_{F} .

The second part of the study aimed to discern the effect of macroporosity on the degradation rates of biomimetic and sintered samples. Since not all L/P could be foamed, only one L/P per material was selected to assess the effect of macroporosity on degradation, that being the one that allowed obtaining similar values of macroporosity in the different materials (Fig. 6B). For biomimetic CDHA samples, foaming resulted in an increase of the extent of degradation to levels between a 20-30 wt% (Fig. 7B). The carbonated samples showed a smaller increase, in agreement with the decreased degradation observed in the non-foamed specimens. For the sintered samples, foaming led to a maximum of 15 % of mass loss. The degradation values for β -TCP obtained upon sintering CDHA_{C} were rather low if compared to β -TCP obtained from CDHA_{F} . This can be explained by the low porosity of the former formulation (Figure 5B). For comparison purposes, Figure 7B also included the degradation of a stoichiometric HA foam. As expected, SHA presented the lowest degradation among all

foams comparable to the non-foamed materials (5-7 wt%) due to its low solubility and low SSA.

The benefits of having an open porous structure in the degradation of CaP was also observed by Schaefer *et al.* using 2D dense disks and 3D porous scaffolds consisting of sintered HA and β -TCP [14]. Figure 8 combines the degradation behavior obtained for the non-foamed and foamed materials as a function of porosity. The graph for each composition shows that for the biomimetic substrates, foaming results in an exponential increase in degradation, which can be explained by the large available area that becomes exposed to the acidic medium, and therefore is susceptible to degradation. The relevance of the SSA, in this respect, was highlighted by the observation that the introduction of similar levels of macroporosity resulted in a significantly higher increase in degradation for the fine than for the coarse CDHA foams (29 % vs 19 % weight loss), corresponding to a SSA of 30 and 14 m²/g respectively. Thus, similar levels of added macroporosity represented a 3-fold increase in degradation for the coarse CDHA, whereas for the fine CDHA the increase was 4-fold.

Interestingly, for the sintered β -TCP, the impact of the macroporosity introduced by foaming on the degradation was much lower, as it only resulted in a linear increase in degradation. On one hand, this can be explained by its low SSA, and on the other, by the larger size of the micropores already present in the substrate which may help to improve penetration of the acidic solution within the material, even in absence of macropores, therefore reducing the impact of the macroporosity. In spite of having a high SSA, an intermediate behavior was found for the carbonated hydroxyapatite samples that did not experience such a large increase in degradation upon foaming as did the CDHA samples, probably due to the low solubility already found in the non-foamed samples.

One important aspect to check was whether the degradation of the calcium phosphates generated any by-products due to the acidic environment during the experiment. Indeed, in previous works, where degradable polymers like polylactic acid or derivatives were incorporated [59,60] to increase the degradation of CaP, the precipitation of brushite was reported due to the acidic environment caused by the degradation of these polymeric phases [61]. As demonstrated by the XRD patterns (see Figure 9A), the composition of all samples remained unaltered after degradation, while SEM images evidenced the microstructural changes post-degradation (Figure 9B). However, whereas the microstructure of the CDHA surface appeared clearly degraded due to the acidic environment, the dissolution on sintered β -TCP was noticeable mainly at the grain boundaries, which induces the formation of cracks and irregularities on the grain surfaces and was also highlighted by Koerten *et al.* [37]. Despite the visible effects of degradation, the penetration degree of the acidic medium was limited to a few micrometers from the surface (e.g. up to 10 μm for a L/P=0.55) for the non-foamed samples. In the case of the foamed samples the presence of interconnected macropores allowed the penetration of the acidic medium throughout all material.

Overall, this study demonstrates the importance of textural properties, which can modulate or even outweigh the effect of other intrinsic properties such as the solubility of the compounds. The tuning of porosity and SSA at a multiscale level (nano, micro and macroporosity) is a powerful tool to control the degradation of CaPs, which can even exceed the effect of the intrinsic solubility of the material.

5. CONCLUSIONS

The *in vitro* degradation behavior of different CaPs was compared in terms of composition, SSA, and multiscale porosity (nano, micro, and macroporosity) using an isotonic acidic solution of pH 2 to emulate the acidic osteoclastic environment during bone remodeling. The SSA played an important role in the degradation as shown by

the comparison between biomimetic samples with different SSA and a range of porosities at different length scales. In this respect, it is important to stress the advantage offered by the biomimetic processing of CaP, which allows tuning the SSA in a much higher range than the high temperature sintering methods. However, the effect of SSA on degradation was dependent on the porosity and pore size, which conditioned the extent of acid penetration within the samples. The combination of SSA and porosity (nano-, micro- and macro) resulted in an exponential increase in the degradation for high SSA materials but to a linear increase in sintered materials with low SSA. Varying these parameters allowed tuning degradation from 3 wt% up to 30 wt%. Without the foaming step, degradation could not go beyond the 8 wt%. In contrast to what happens with stoichiometric hydroxyapatite, doping with carbonate did not result in an increase of the chemical degradation of biomimetic CDHA, indicating that carbonate did not have the same effect, probably due to the lower crystallinity and the already distorted network of CDHA compared to stoichiometric hydroxyapatite.

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FIGURE LEGENDS

Figure 1. Sketch summarizing the various CaP synthesis procedures and sample nomenclature.

C = carbonated; F = foam; CDHA = calcium deficient hydroxyapatite; SHA = sintered hydroxyapatite; β -TCP = beta tricalcium phosphate. C and F subscripts denote the size of starting α -TCP. *In this work, the degradation of sintered hydroxyapatite (SHA_C and SHA_F) was only analysed in foamed samples. **The effect of microporosity on the degradation of non-foamed β -TCP was assessed using the coarse samples (β -TCP_C)

Figure 2. XRD patterns (A) and ATR-FTIR spectra (B) of all materials studied, prepared with a L/P ratio of 0.55 mL/g.

Figure 3. Scanning electron micrographs showing the microstructures of the different samples prepared with L/P ratio of 0.55 mL/g (scale bar: 500nm).

Figure 4. Scanning electron micrographs at low magnification of the non-foamed CDHAc and CDHAF and β -TCPc samples obtained with different L/P ratios (0.35 and 0.65 mL/g), as well as of the foamed counterparts. The scale bar in the two first columns corresponds to 5 μ m, and in the third column (foams) to 100 μ m.

Figure 5. Specific surface area (SSA) and percentage of open porosity values for biomimetic and sintered non-foamed compositions (A) and for the corresponding foamed formulations (B). Note that for the foams two L/P were studied, 0.55 and 0.65 mL/g, corresponding to coarse and fine formulations, respectively. The error bars for the SSA are associated to the measurement and were provided by the instrument.

Figure 6. Pore entrance size distributions of the different samples, as determined by MIP.

Figure 7. Accelerated *in vitro* degradation for biomimetic and sintered samples (N=3). A) Non-foamed samples groups identified by the same superscripts are not statistically different ($P > 0.05$). Letters indicate differences between different L/P within the same formulation; numbers identify differences between compositions for the same L/P ($P < 0.05$); B) Degradation for foamed samples and the corresponding non-foamed counterparts. According to subscripts, coarse samples have a L/P of 0.55 mL/g and fine analogues 0.65 mL/g. * denotes statistically significant differences ($p < 0.05$) between samples, dense and foamed, respectively, C) pH values for L/P ratio 0.35 and 0.65 mL/g.

Figure 8. Degradation of the different samples as a function of the open porosity. C-CDHA_c and β-TCP include the foamed fine specimens.

Figure 9. XRD and SEM images for different compositions with L/P of 0.55 mL/g before (solid line) and after degradation (dashed line). SEM images of the surface of the specimens for the biomimetic CDHA (coarse and fine) and β -TCP, before and after degradation (scale bar: 1 μ m).

Statement of Significance

The physicochemical features of calcium phosphates are crucial to tune biological events like resorption during bone remodeling. Understanding *in vitro* resorption can help to predict the *in vivo* behavior. Besides chemical composition, other parameters such as porosity and specific surface area have a strong influence on resorption. The complexity of isolating the contribution of each parameter lies in the close interrelation between them. In this work, a multiscale study was proposed to discern the extent to which each parameter influences degradation in a variety of calcium phosphates, using an acidic medium to resemble the osteoclastic environment. The results emphasize the importance of textural properties, which can modulate or even outweigh the effect of the intrinsic solubility of the compounds.