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Material characterization and in vivo behavior of silicon substituted α-tricalcium phosphate cement

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Abstract

The possibility and biological effects of substituting silicon in α-tricalcium phosphate by way of solid state reaction have been evaluated. α-TCP powders with varying substitution amounts (1 and 5 mol% Ca_2SiO_4) were synthesized reacting mixtures of $CaCO_3$, $Ca_2P_2O_7$, and SiO_2, at a rate of 4 °C/min to 1100 °C, left to dwell for two hours then heated to 1325 °C at 4 °C/min and left to dwell for a period of four hours. The powders were then rapidly quenched in air. Si incorporation could be verified by XRD analysis, indicating an increase of the lattice volume with increasing Si content from 4284.1 (8) to 4334 (1) Å^3 for pure α-TCP and α-Si5%TCP, respectively. The hydrolysis of milled α-SiTCP powders was monitored by isothermal calorimetry and the compressive strength of set cements was tested. The results showed changes in speed and amount of heat released during reactivity tests and a decrease in mechanical strength (60, 50, and 5 MPa) with increasing Si content. *In vitro* **bioactivity of the set cements after soaking in simulated body fluid for 4 weeks was also tested. The formation of a bone-like apatite layer on the surface of the set cements could be observed and was thickest for 1%Si (20 µm). These results were in good agreement with the** *In vivo* **studies performed, which showed strong evidence that the cement containing 1% silicon doped α-TCP enhanced mesenchymal cell differentiation and increased osteoblast activity compared with α-TCP.**

Key words: **silicon-substituted α-tricalcium phosphate (α-TCP); calcium deficient hydroxyapatite (CDHA); reactivity; bone formation; bioactivity.**

Introduction

The development of new bone fillers and novel methods of bone fixation has been driven by the need to produce more efficient medical clinics and less invasive surgical procedures. Bone grafting is an effective and efficient way of filling bone defects. However it is accompanied by donor site pain in the case of autografts, or possible infection in the case of xenografts and allografts. In this context, the development of new synthetic substitutes gains special relevance. Calcium phosphates have been used by the biomedical and medical communities for several years [1]. Moreover, the ability of certain phosphate compounds to undergo a hydrolysis reaction resulting in the formation of an apatite, as well as being mouldable and injectable during the reaction period, have been properties attracting clinicians and have favored the development of calcium phosphate cements (CPC). These materials were first developed by Brown and Chow in the 70's–80's studying tetracalcium and dicalcium phosphate blends [2, 3] and continuing into the 1990's with systematic studies of other orthophosphate blends [4]. The resulting reaction products of the setting reaction of CPC's, mainly hydroxyapatites, have proven to be osteocompatible, and are used in certain applications for bone void filling, as well as bone augmentation for fixation of fractures.

Strong interest in tricalcium phosphate (α-TCP) grew later, due to the reactive nature as well as the single-phase composition needed to produce an apatitic cement product [5]. The hydration process

is a mildly exothermic two-step reaction involving dissolution of α-TCP and precipitation of calcium deficient hydroxyapatite (CDHA) following the reaction [6,7]:

$$3Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9(HPO_4)(PO_4)_5OH \quad \text{(Eq. 1)}$$

Due to a deeper understanding of these materials regarding amorphicity and powder compaction, the versatility of phosphate cements may improve. Among convenient application methods currently being investigated is injection. Higher mechanical strengths will allow a wider range of clinical applications [8-11].

Although hydroxyapatite is the main mineral component of bone, various trace elements such as fluorine, aluminum, boron, cadmium, chromium, silicon and strontium [12-14] have an important role in bone growth and metabolism. Of these, silicon has been shown to be an important element when discussing bone formation and osteoblastic activity [15, 16]. Soluble silica has formerly been shown to play an essential role in the cross-linking of collagen and proteoglycans during bone growth [17]. In addition, it is well known that the presence of Si plays a significant role in the bioactivity of several glasses and ceramics [18-20]. In apatite materials, the incorporation of silicon has been found to increase the dissolution rate of HA, both *in vitro* and *in vivo*, enhancing the bone remodeling process [21-27]. Several studies have reported that a solid solution is formed in the system $Ca_3(PO_4)_2$ – Ca_2SiO_4 for a Ca_2SiO_4 content below approximately eight molar percent [28], stabilizing the alpha form of the TCP.

The aim of this study was to produce silicon substituted α-TCP by solid-state reaction that could be used as an injectable CPC that would hydrate forming an Si-substituted CDHA. The effects of Si on the reactivity of the α-TCP cements, the mechanical strength and the in *vitro* bioactivity were investigated. Upon preliminary results the study was followed by an *in vivo* investigation of whether the silica containing α-TCP cement would increase bone formation and integration compared to an α-TCP control in a rabbit bone harvest chamber model.

Experimental

Materials preparation

Silica doped α-TCP was prepared by mixing and heat-treating mixtures of calcium pyrophosphate (produced by sintering $CaHPO_4$, Sigma C-7263, at 1000ºC for 16 hours), amorphous fumed silica (Merk 13126) and calcium carbonate (Sigma C-4830). The mixtures were calculated in order to obtain a solid solution of $(Ca_3(PO_4)_2)(1\text{-}x)$-$(Ca_2SiO_4)(x)$, which gave the equation:

$$(1+x)CaCO_3 + (1-x)Ca_2P_2O_7 + xSiO_2 \rightarrow Ca_{(3-x)}(PO_4)_{2(1-x)}(SiO_4)_x + (1+x)CO_2 \quad \text{(Eq. 2)}$$

for x = 0, 0.01 and 0.05.

Reagents were mixed and heated in a platinum crucible at a rate of 4°C per minute to 1100°C, left to dwell for two hours then heated to 1325°C at $4°C(min)^{-1}$, and left to dwell for a period of four hours. The materials were then removed from the oven and quenched rapidly in air. Once cooled the products were uniformly milled to produce a fine powder (Retsch100 ball mill with agate mortar and mortar balls). Materials are respectively termed α-TCP, α-Si1%TCP and α-Si5%TCP, according to the values of x = 0, 0.01 and 0.05.

Cement pastes with the different TCP solid solutions were prepared, mixing the powders with liquid at a liquid to powder ratio of 0.34 $ml(g)^{-1}$, at 37.5ºC. The liquid was a 2.5-wt% aqueous solution of Na_2HPO_4 (Merck, 1.06586), to accelerate the setting reaction.

Materials analysis

X-ray diffraction analysis of the silica doped α-TCP powders and the fully set cements was performed using a Guinier focusing camera equipped with an imaging plate (Huber Imaging Plate Guinier Camera 670). Data were collected for 32000 seconds (≈ 9 h) using $CuK_{\alpha 1}$ radiation from 2θ = 8 to 100º, with a 2θ-step of 0.005º. The lattice constants of the materials were determined by Rietveld refinements using the Winpow program [29], a local Windows version of the Rietveld analysis program LHPM 1 by Hill and Howard [30] and the refined crystal structures of α-TCP, β-TCP, and hydroxyapatite reported in the literature [31-33] as reference.

The specific surface area of the α-TCP powders was measured by BET nitrogen gas absorption analysis (Micrometrics model ASAP 2400). Samples were degassed overnight before measurement.

Assessment of the reactivity of Si-substituted α-TCP

Isothermal calorimetry analysis was performed with a TAM isothermal calorimeter at 37.5°C. One gram of material was hydrated with Na_2HPO_4 solution at a liquid to powder ratio of 0.34 ml(g)$^{-1}$. Data from the calorimeter was sampled at 1 Hz with PICO software (www.pico.com) until the reaction was complete. The electrical potential was measured and converted into W(mol)$^{-1}$ and then integrated, resulting in a total heat of reaction in kJ(mol)$^{-1}$ apatite formed.

Assessment of the mechanical strength

Cement pastes, prepared as described previously, were thoroughly mixed by hand for one minute then placed in Teflon moulds to set. The moulds were immediately immersed in Ringer's solution at 37.5°C. Samples were then removed at periodic time intervals for use as test specimens. After setting times of 1, 3, 6, 12, 24, 48, 72, 96, and 168 hours, eight specimens of each cement (diameter = 4 mm; height = 8 mm) were removed from the Teflon moulds and compression tested in a biaxial Instron 8511 load frame with MTS TestStar II controller. Testing was performed at room temperature with a crosshead displacement of 1 mm(min)$^{-1}$. Samples were loaded until failure. After testing, the specimens were put in acetone and saved for further characterisation.

Assessment of the in vitro bioactivity of Si-HA

The set cements were removed from Teflon moulds, dried and immersed in a simulated body fluid (SBF) [34], with ion concentrations nearly equal to those of human blood plasma, for 4 weeks at physiological temperature (37.5°C) and analyzed by means of scanning electron microscopy to observe apatite deposition on the surface of the materials as well as crystal morphology.

The cylindrical specimens of the set cements soaked in SBF for 4 weeks were dried and cleaved to analyze the thickness of the new apatite layer as well as the chemical composition and morphology of the bulk apatite and the new apatite layer. Micrographs were taken from at least 6 different locations on each sample. The thickness was measured with GATAN Digitalmicrograph software at 6 different locations in each micrograph. X-ray energy dispersive spectrometry (XEDS) was performed on carbon coated samples at an accelerating voltage of 20 kV, using a JEOL JSM 840A equipped with an Oxford Instrument three-window XEDS detector mounted at a high-angle position. The samples were then recoated by gold sputtering for further imaging at 10kV.

Assessment of the in vivo bioactivity of Si-HA

Six adult, lop-eared rabbits were used (weight 4.0 to 5.5 kg). Bone harvest chambers were implanted bilaterally in the proximal tibiae [35]. The chamber contains a core with a 1 × 1 × 5 mm groove. The groove with holes was in direct contact with bone and allows tissue ingrowth. The chamber can be used for repeated harvesting. In the study, α-TCP and -Si1%TCP were injected in a mold with 1 mm diameter to obtain a standard amount of material. The material was placed into the center of the channel in the chamber. One chamber received α-TCP as control and the other chamber received α-Si1%TCP, randomly. Six paired samples from each time period were harvested at 1, 2 and 3 weeks from the same population of six rabbits.

The harvested tissue was then evaluated by fixing in 2.5% buffered glutaraldehyde, dehydrated with ethanol, embedded in Technovit, cut into 6 µm sections, and stained with H&E, Goldner and TRAP. Osteoclasts were counted in the initial ingrown tissue at one week and along the circumference of materials at two and three weeks. Bone formation in the chamber and new bone grown onto the circumference of the materials were measured by an imaging system (analySIS, Soft Imaging System, Münster Germany) connected to a microscope (Olympus BX50, Olympus Optical co. Ltd., Japan). The percentage of new bone and rate of new bone formation on the materials were calculated by dividing the area with new bone growth by the total area or new bone bordering the materials. Student's paired t-test was used for the data analysis.

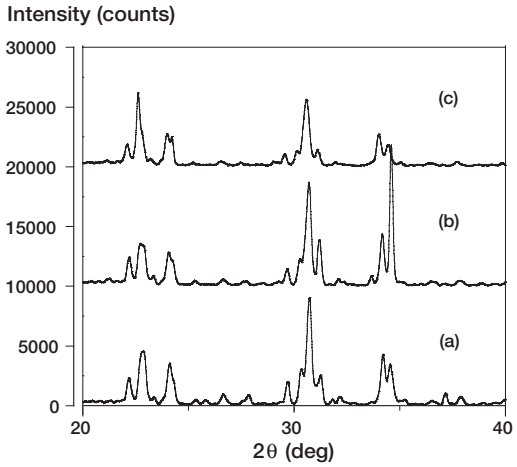

Figure 1: X-ray diffraction patterns of (a) pure calcium α-TCP, (b) α-Si1%TCP and (c) α-Si5%TCP.

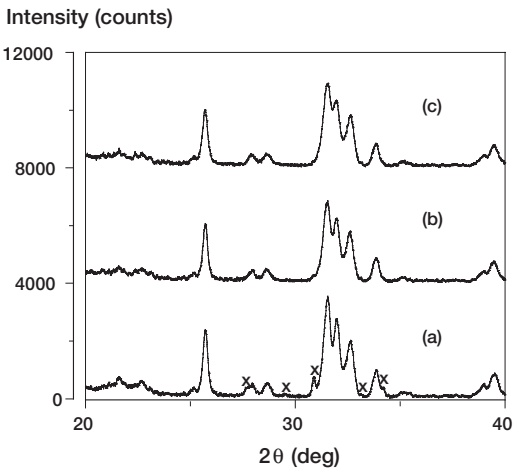

Figure 2: X-ray diffraction patterns of (a) pure calcium CDHA, (b) Si1%CDHA and (c) Si5%CDHA. The crosses indicate the presence of β-TCP, remaining from the TCP

Results

Characterization of the α-TCP powders and the matured cements

The examination of the X-ray diffraction patterns of the TCP powders prepared with varying Si amounts revealed the presence of α-TCP (space group $P2_1/a$) as major phase with traces of β-TCP (space group R3c) formed during the quenching (Fig. 1). Rietveld analysis of the powder data revealed a systematic increase of the lattice volume with increasing silicon content, with refined values of 4284.1(8), 4291(1), and 4334(1) $Å^3$ respectively for pure α-TCP, α-Si1%TCP and α-Si5%TCP, indicating the incorporation of silicon in the crystal structure. The analysis of the fully hydrated cements revealed the presence of an apatitic phase (space group $P6_3/m$) with traces of β-TCP (space group R3c) (see Fig. 2). Rietveld refinements of the lattice parameters indicated a slight increase of the lattice volume from 528.26(8) $Å^3$ for pure CDHA to 528.77(7) $Å^3$ for Si5%CDHA, with a lower volume of 526.51(7) for Si1%CDHA. Though these variations were very small compared to the volume change in the α-TCP structure and could be the result of a partial Si incorporation, if any at all. The most probable explanation for this would be the formation of a crystalline or amorphous silicate, as a separate phase. No such silicate phase was however detected by XRD. Sintering at 1000°C for 2 hours did not give more evidence of the presence of silicate phases or silica.

The specific surface areas obtained in the BET analysis of pure calcium α-TCP, α-Si1%TCP and α-Si5%TCP were similar, respectively 1.8, 1.6, 1.7 $m^2(g)^{-1}$. Through observing that the Langmuir equation resulted in a linear distribution, it was concluded that the surface contained little, if no, micro pores between 0.1 and 3 nm.

The examination with SEM of the set cement showed the same features for all the samples (see Fig. 3); a network of entangled plate-like apatite crystals grown from the α-TCP grains. Typical Hadley grains could be observed, showing an empty thick shell of hydration products inside which a α-TCP grain had fully reacted.

Reactivity of the α-SiTCP powders

The isothermal calorimetry measurements (Fig. 4) showed a similar evolution of the heat of reaction for all materials, with a quick primary reaction followed by a slower secondary reaction. However the α-SiTCP cements presented a faster initial reaction than pure α-TCP and a slower secondary reaction, no more heat being evolved after 36 and 55 hours, for α- Si1%TCP and α-Si5%TCP, compared to about 24 hours for pure α-TCP. The total heat evolved by the reaction of hydration was increased with increasing Si content, respectively 128, 152 and 165 kJ per mol CDHA. This could be the result of strain introduced in the matrix upon Si-doping.

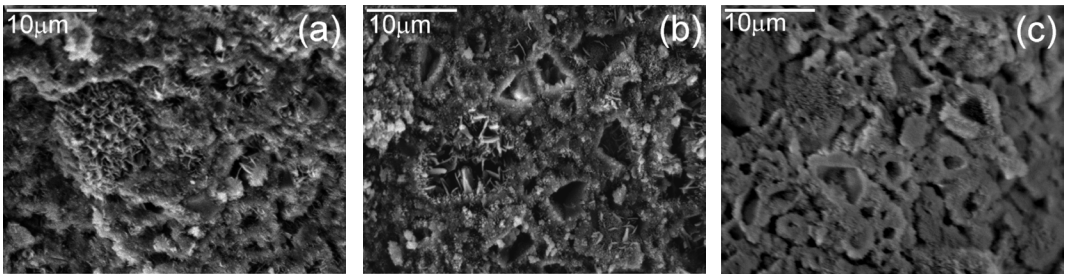

Figure 3: Scanning electron micrographs of the bulk of the fully hydrated Si-cements: (a) pure α-TCP, (b) α- Si1%TCP, and (c) α-Si5%TCP.

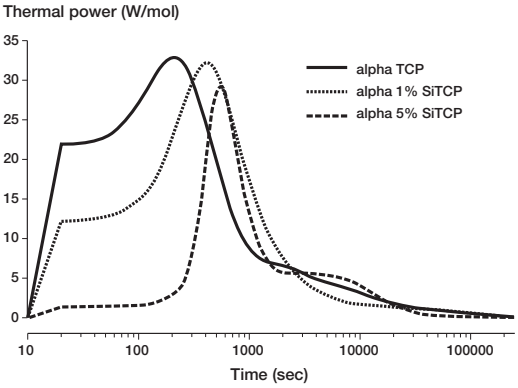

Figure 4: Isothermal calorimetry curves showing the rates of heat evolution during hydrolysis of the α-TCP powders.

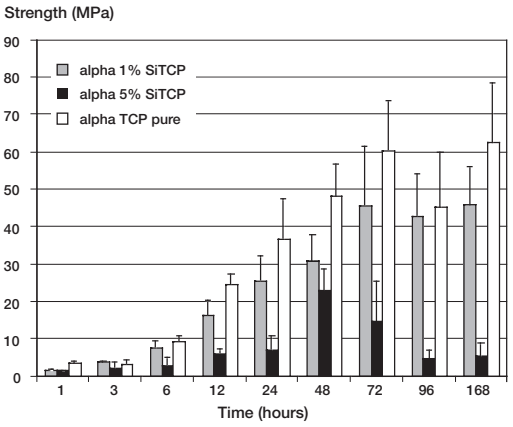

Figure 5: Compression strength development of the hydrating cement pastes.

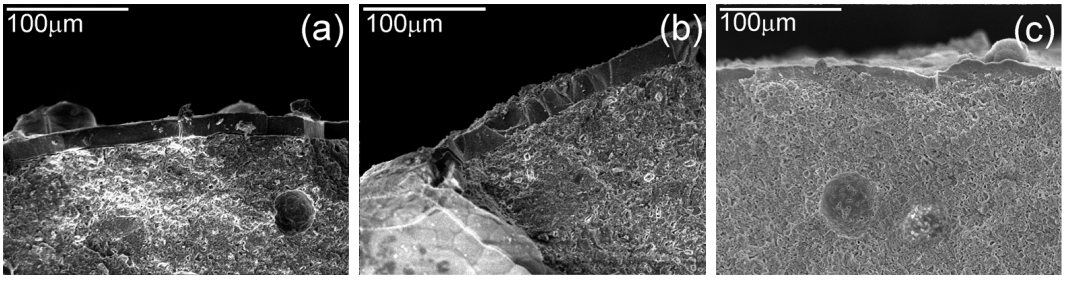

Figure 6: Scanning electron micrographs of the fully hydrated Si-cements, soaked in SBF for 4 weeks. (a) α-TCP, (b) α-Si1%TCP, and (c) α-Si5%TCP.

Compressive strength

Pure α-TCP paste resulted in a mean average strength after 72 hours of 60 MPa, even though the material was not compacted. α-Si1%TCP shows a reduction in compression strength of about 10 MPa on average compared to undoped α-TCP (Fig. 5). -Si5%TCP exhibits a significant reduction in compressive strength.

Bioactivity in vitro

SEM examination of the fully set cements soaked in SBF for 4 weeks at 37°C showed the formation of a thin and dense bone-like apatite layer on their surface (Fig. 6). The newly formed apatite layer consisted of nodules of crystal platelets grown together, forming a compact layer. The thickness of the precipitated apatite layers was thickest for the Si1% sample, respectively 16 ± 3, 20 ± 3, and 10 ± 4 μm for α-TCP, α-Si1%TCP and α-Si5%TCP.

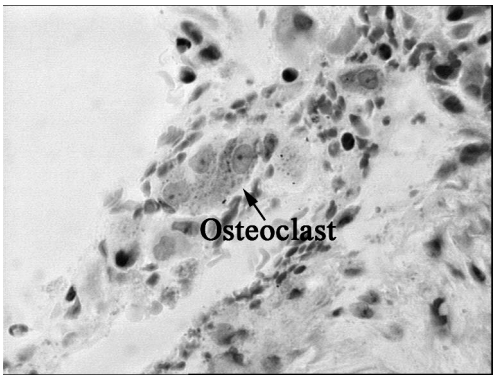

Figure 7: Osteoclast presence on the α- Si1%TCP (40x).

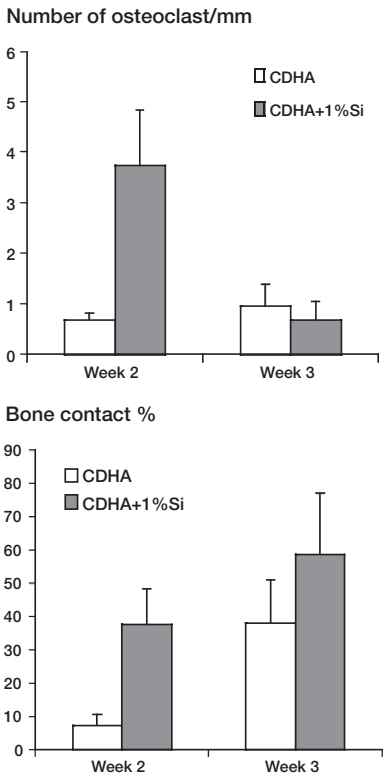

Figure 8: a. Number of osteoclasts on the material interface (Mean±SE). b. Bone contact % on the material.

Bioactivity in vivo

The initial ingrown tissue in the chamber consisted of blood cells and mesenchymal cells as well as osteoclasts and osteoblasts at week one. Close contact between osteoclasts and the materials was seen (Fig. 7). The number of osteoclasts in the initial ingrown tissue samples was 5.7±4.8 in CDHA, compared with 15.7±6.8 in Si1%CDHA. This was 2.8 times more in the tissue with Si1%CDHA than CDHA alone ($p<0.05$).

At week two there were signs of new trabecular bone formed in the chamber, towards the center of the channel. The number of osteoclasts in contact with the materials was 5.4 times more in the Si1%CDHA compared to α-TCP alone ($p<0.05$, Fig. 8a, 8b). Furthermore, osteoblasts with new bone trabecular or osteoid were observed on the Si1%CDHA, however less on CDHA alone ($p=0.05$, Fig. 9a, 9b). The number of osteoclasts was reduced in both materials, in week three (Fig. 8a). New trabeculae were more mature and in close contact to the materials in the case of Si1%CDHA. There was no significant difference in the bone formation, in the chamber at two and three weeks. There was a tendency to increase bone contact with Si1%CDHA.

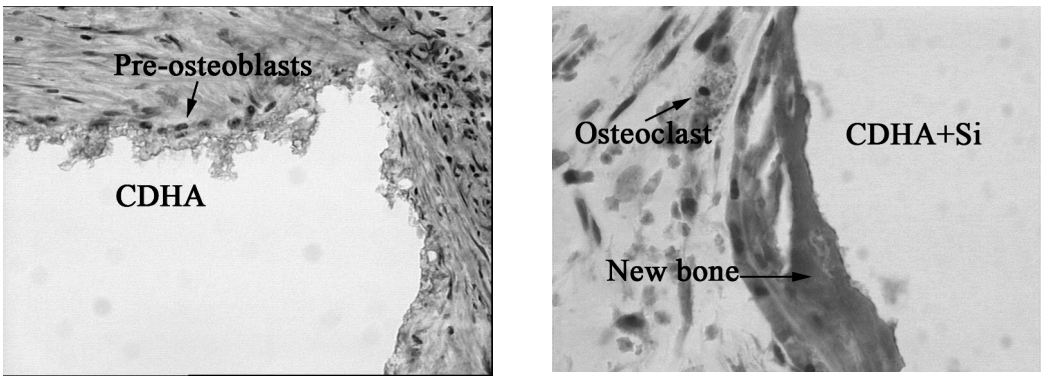

Figure 9: Images from 2 weeks specimens. a. The interface between tissue and α-TCP with pre-osteoblasts laid on the material. b. Osteoblasts with newly formed bone on the α- Si1%TCP (40x).

Discussion

XRD analysis of the silicon substituted α-TCP powders revealed a significant change of the lattice volume with increasing Si content. From these results the Si-incorporation, at least partial, could be verified. The mechanism of Si-substitution in the α-TCP structure is however uncertain. According to the phase diagram two phosphate groups are substituted by one silicate group, in order to maintain the electroneutrality of the structure, anionic and cationic vacancies are introduced. As mentioned previously, the α-TCP solid solution would correspond to the stoichiometry $Ca_{3-x}(PO_4)_{2(1-x)}(SiO_4)_x$ and the introduction of 1%Si would correspond to the formula $Ca_{2.99}(PO_4)_{1.98}(SiO_4)_{0.01}$ and 5%Si to $Ca_{2.95}(PO_4)_{1.9}(SiO_4)_{0.05}$. These results are in agreement with other studies where the formation of a silicon containing α-TCP is reported, although the substitution of one phosphate by one silicate group and therefore the introduction of excess cations is proposed [36-38]. The hydrolysis of pure α-TCP (Ca/P = 1.5) results in a non-stoichiometric apatite, often called calcium-deficient hydroxyapatite (CDHA) [7], with the same Ca/P = 1.5. In this respect, the hydration of the Si-substituted α-TCPs prepared in this study, with Ca/(P+Si) = 1.502 and 1.513, should be expected to form Si-containing apatites. However from the XRD analysis of the hydration products, the very low lattice volume variations did not permit to conclusion of the Si-incorporation. If not incorporated in the apatite crystals, silicate ions present in solution might precipitate to form a silica gel or other crystalline or amorphous calcium silicate. A study by Barnes et al. [39] on the hydration in the system Ca_2SiO_4-$Ca_3(PO_4)_2$, actually revealed that hydration of phosphate rich TCP forms hydroxyapatite, as a major phase, coexisting with a calcium silicate hydrate, both having variable compositions. In an attempt to substitute silicon in the apatite structure, Balas et al. [23] also observed the formation of C-S-H gel, at a critical Si content. However the small amounts of such phases could not be detected here, neither by XRD nor by XEDS.

Nevertheless, the incorporation of small silicon amounts in the cement matrix had noticeable effects on the properties of the biocements, and more particularly on the mechanical strength. Addition of Si decreased the mechanical strength from 60 MPa measured in the undoped material. However, the 1% doped material had a strength of 50 MPa, which is significantly stronger than many sulphate based materials currently used as bone fillers. The decrease in strength for 5%Si was very significant and might be explained by the deposition of a silica gel on the initially precipitated apatite crystals, limiting their growth but also their entanglement. This could also explain the slight decrease in reactivity observed by calorimetry, increasing amounts of silicon inhibiting the transformation of α-TCP to CDHA. A similar phenomenon was observed by Tanizawa et al. [36] who reported an inhibiting effect of the silicate ions on the transformation of dicalcium phosphate di-hydrate (DCPD) into apatite. Another possible explanation of the decrease of the compressive strength could be the early dissolution of the cement, after 48 hours of immersion in Ringer's solution.

In vitro bioactivity was observed with all the set cements, the most bioactive being the Si1%CDHA, exhibiting on its surface an apatite layer of about 20 μm thick. This activity limit is lower than in previously reported studies for ceramic Si-HA, since Balas et al. [23] reported a maximum activity for the Si-substituting molar fraction 0.05 and Porter et al. could observe an increase from 0.027 to 0.053 [26]. However it should be noted that the bone-like apatite deposition depends also on the surface characteristics of the Si-HA material. All the cement samples studied here were completely covered by bone-like apatite material after 4 weeks.

The *in vivo* study of silica-doped α-TCP resulted in observation of significantly enhanced bioactivity. It enhanced differentiation of mesenchymal cells to osteoclasts in the early period, and also increased osteoblast activity resulting in osteoid and mineralized bone on the material. This study supports with recent studies that incorporation of silicon affects the surfaces structure, surface charge, and biological activity of α-TCP in vitro [20, 27, 40, 41]. A possible explanation is that the early osteoclasts may play a role of promoting the dissolution of α-TCP, which may have stimulatory effect on osteoblast differentiation and proliferation as well as the bone mineralization process.

Conclusion

The initial aim was to incorporate Si into α-TCP and this has been done successfully. It is difficult to determine the degree of Si incorporation into CDHA after hydration. However, the incorporation of 1%Si in the cement reactant increased bioactivity and developped good mechanical strength (50 MPa), whereas higher amounts of Si had limited effect on bioactivity and significantly lowered strength. α-Si1%TCP showed significant and positive results of increased osteoclastic and osteoblastic activity and bone integration when placed in a bone chamber model of rabbits.

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