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RESEARCH

Synthesis and cytotoxicity analysis of porous β-TCP/starch bioceramics

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Abstract

The production of porous ceramics for biomedical applications is widely available in the Ceramics industry. In bioceramic applications, interconnected pores are pertinent to increase osteoconductivity and cell proliferation. However, an increase in pore size and the pore amount decrease the mechanical properties. For this reason, pore properties must be precisely controlled. In this study, the effect of a natural pore-forming agent, corn starch addition, and sintering conditions on mechanical properties and biocompatibility was investigated. During mixing, four different starch amounts (1, 3, 5, and 10 wt%) were added to pure beta-tricalcium phosphate (β-TCP) ceramic powders and pressed. Pressed pellets were sintered at 1000, 1100, 1200, and 1300 °C. A scanning electron microscope (SEM) is used to investigate microstructure, texture, pore size, and cell adhesion. The mechanical properties of the β-TCP ceramic parts were further characterized by measuring the density and compressive strength. Cytotoxicity tests were carried out with MTT assays. The optimum mechanical properties were obtained at 1100 °C sintered biocomposites. Although starch starts to burn around 410 °C and analytical results show no presence of starch after the sintering process, biocomposites initially containing 10% starch showed improved cell proliferation. However, a reduction of 59% in compressive strength and a 16% reduction in the density were also recorded. It was observed that 10 wt% starch addition increases cell proliferation by 10% in sintered β-TCP samples. Starch powder additions can be used to increase the cell viability of the material by facilitating the creation of pores, as a low-cost pore-forming agent for porous bone graft and non-load-bearing material in both orthopaedics and maxillofacial applications.

Keywords β-Tricalcium phosphate · Porous bioceramic · α-Tricalcium phosphate · Starch · Biocompatibility

Introduction

Porous bioceramics with good mechanical properties and biocompatibility are widely sought in orthopaedic and maxillofacial applications. The bones and skeletal systems in vertebrates have many essential physiological duties such as protecting vital organs; supporting functional movements with appropriate muscle attachments; producing red and white blood cells; and storing calcium, phosphate, and other necessary ions needed by the body.

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All these tasks are of great importance for maintaining vital functions, and problems in the bone structure can lead to serious health problems. Bones generally consist of water (9 wt%), collagen (20 wt%), calcium phosphate (CaP) (69 wt%), and other minerals and organic material in small quantities [1]. The majority of bone is made of calcium phosphate, namely carbonate hydroxyapatite. On the other hand, synthetic hydroxyapatite and other calcium phosphate compounds produced for bone graft applications adequately show high biocompatibility and bioactivity with the natural bone structure in medical applications. Bone tissue has excellent regenerative properties that can repair its structure and function on its own under minor fractures and defects. However, if the size of the defect is rather big above a critical size, depending on its location, it might not be possible for bone tissue to fully regenerate. Under such circumstances, to aid the bone in its regeneration function, bone grafting is applied. The most crucial function of bone grafting is to support the defect site and increase osteogenic activity and accelerate healing. The desired properties of bone grafts are to be compatible with the tissue to which the graft is applied, to be structurally durable and to provide support and stability to the tissues, to be non-toxic and non-carcinogenic and easily obtainable, and to be osteoinductive and/or osteoconductive [2–4]. Calcium phosphate bioceramics are bioactive and osteoconductive that make the material suitable for bone repair applications. Although there are different types of calcium phosphate materials, some are more remarkable. Each comes with a set of advantages and disadvantages. For example, calcium sulphate can be absorbed rapidly in the body before adequate bone formation and bone might collapse. On the other hand, depending on its size and position within the body, hydroxyapatite (HA) might take a long time to be absorbed. β-Tricalcium phosphate (TCP) has good dissolution and absorption properties, and like hydroxyapatite, it can supply needed calcium and phosphate ions to aid the bone regeneration process [5]. TCP has three polymorphs, under sintering temperatures of 1125 °C β-TCP, above 1125 °C α-TCP, and lastly above 1400 °C α′-TCP forms. While β-TCP is absorbed rapidly, α-TCP can take longer dissolution periods or even may not be completely absorbed. Although β-TCP supports bone formation by having excellent osteoconductive properties, the volume of newly formed bone is always lower than the graft filling volume [6]. To prevent this problem, it is used together with biogenic additives like growth factors such as osteogenin or collagen that support bone formation and might increase the osteoinductive properties of the material. In addition, HA can be mixed with β-TCP to form "biphasic calcium phosphate" to take advantage of the good properties of both materials [7]. There are various methods to increase the porosity of CaP ceramics. Some of these methods are costly and require complex laboratory studies. At the same time, some of the porosity-producing or porosity-foaming agents used might also have toxic effects, which can add to the cost of manufacturing to remove these impurities [8]. Hence, during the last few decades, interests in non-toxic and natural pore-forming or pore-foaming agents have been increasing. Naturally obtained starches can be used to overcome this problem. Starch is a natural, inexpensive, biodegradable, and renewable polymer formed by the chain formation of glucose monomers with glycosidic bonds. Starch is frequently used in food, chemistry, textile, and pharmaceutical industries. The primary starch sources in the world are corn, potato, and cassava plants [9]. Starch granules are separated from plant seeds and roots by industrial processes such as grinding, screening, and washing. Starch has been used as a burnable pore-forming agent to produce porous ceramics in the ceramic processing industry in recent years. It has been suggested that the contamination of alkali and other metal element ions with ceramic material is negligible due to the low ash content compared to other biological pore-forming agents [10]. Previous work by Lyckfeldt and Ferreira, in 1998 [11], focuses on producing porous ceramics by starch additions. They focused on different quantities of starch, ranging from 10 to 50% which was mixed with β-TCP and other calcium phosphates. They reported the successful production of low-density ceramics with interconnected pores. In another work, Mastalska-Popławska et al. in 2019 [8] used starch in ceramic production where starch can be a polymerizing agent in the sol-gel solution and they further suggested that it can be used as a rheological modifier or even strengthening agent in porous ceramics. It was proposed that due to the low-dimensional changes occurring during the consolidation process, starch can help to control the porosity of the bioceramics, which leads to better mechanical properties of the final product. They further added that because of gelation at low temperatures, it can be used as an eco-friendly cross-linking agent for the synthesis of biocomposites. In a relatively new study by Taherimehr et al. in 2021 [12], starch-based nanocomposites containing 3, 5, and 10 wt% of β-TCP nanoparticles were produced using a co-rotating twin-screw extruder. Nanocomposites were characterized by physicochemical methods, their degradability, and swelling degree. To investigate the dispersion of β-TCP nanoparticles in the composite and biomineralization of the nanocomposites after incubation in SBF, scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX) were performed. Evaluation of mechanical properties of nanocomposites demonstrated that an increase in β-TCP content enhanced mechanical properties. In another earlier work by Nakahira et al. in 2004 [13], scaffolds composed of monolithic β-TCP and β-TCP/α-TCP composites were fabricated by a low-pressure pressing processing method. The pore introduction was controlled by using starch powder. The aqueous slurry of the mixture of β-TCP and starch was prepared and cast into a slip-casting mould. The heterogeneity of the starch in aqueous slip-casting was solved by the addition of calcium phosphate gel into the slurry of the β-TCP/starch mixture. Sintering of green bodies was carried out by a pressureless sintering technique. It was suggested that the porosity of β-TCP and β-TCP/α-TCP composites could be controlled by the addition of starch at different fractions and different sintering temperatures. All these previous investigations used starch with different calcium phosphates and different production methods. Although in previous studies the characterization methods were similar to our knowledge, no cytotoxicity or cell culture studies were carried out. The uniqueness of this work lies in this cell culture studies. In this current work, we investigated the addition of different percentages of starch to produce porous β-TCP bioceramic. We utilized low quantities (1–10 wt%) and relatively dry use of starch, without gel formation. This allows the production of dense β-TCP bioceramics with and without interconnected pores, which can be beneficial in both engineering and medical applications.

Material and methods

Materials

Corn starch was purchased from Sigma-Aldrich (Yasin Teknik Company, Turkey). The β-TCP powder was purchased from Merck, Inc., Germany. The human osteoblast cell line was obtained from the American Type Culture Collection (ATCC) for cell viability assays.

Preparation of β-TCP/starch blends

β-TCP/starch blends were prepared by hand mixing. The corn starch powders were added to the β-TCP powders at a ratio of 1, 3, 5, and 10 wt%. Homogenization of the blends was achieved by 2 h of mixing in a planetary ball milling machine (RETSCH-S100, Germany). Granulation of powder blends was carried out by spraying 40 ml purified water per 100 g of the powder during hand mixing. Cylindrical pellets with a diameter of 11 mm and thickness of 11 mm from granules were produced using hardened steel dies according to British Standard no. 7253 [14]. Additionally, 2-mm-thick pellets were fabricated to observe cell-material interactions during SEM analysis. All pellets were sintered in an electric furnace (Nabertherm, Germany) by holding 4 h at different temperatures (1000, 1100, 1200, and 1300 °C). Both the heating and cooling cycles were kept for 4 h to attain target temperatures.

Characterization of sintered β-TCP

Mechanical property analysis compromised of compression tests of sintered pellets was conducted using a universal compression test machine (Devotrans Inc., Turkey). Five samples were compression-tested for each sample group, size and diameter data obtained from samples were put into the programme, pellets were compressed with 5-mm/min displacement, and the average of five samples were calculated. Density test results were obtained and averaged from mass/volume measurements of 7 samples for each sample group, the average values and standard deviation values obtained from each sample group were calculated using Excel. X-Ray diffraction analyses were performed using CuKα radiation 2θ range of 10–80° with a step size of 0.02° on each sintered sample group and unsintered β-TCP to characterize phases. Rietveld refinement was applied to XRD analysis. Fourier transform infrared spectroscopy (FTIR) was performed to determine the chemical compounds and makeup of sintered samples (FTIR, JASCO FT/IR-4700, Japan). The FTIR spectra were applied in the wavenumber range 450–4000 cm^{-1}. SEM (ZEISS MA/EVO 10, Germany) was used to investigate the microstructure, morphology, and cell attachment on the sample in cell culture studies.

MTT cytotoxicity assay

The human osteoblast cell line (from the ATCC) was used for all cell experiments in growth medium (Dulbecco's Modified Eagle's Medium (DMEM)) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. All samples were placed into 96-well plates, followed by ultraviolet (UV) light sterilization for 2 h. The cell suspension with 1×10^4 cells per well was cultured on the samples and cell plate (2D) and then incubated at 37 °C and 5% CO_2 for 72 h. MTT (3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide) protocol was applied to detect cell viability in our material. After incubation, the cell medium was removed; then, 0.2 mL of 0.5 mg/mL MTT in PBS was added to each well, followed by incubation for 4 h at 37 °C with 5% CO_2. The supernatant was removed gently, followed by the addition of dimethylsulfoxide (DMSO). Then, each sample was transferred to a 96-well plate to measure the absorbance at 590-nm (reference 660 nm) wavelength using a microplate reader. The cells were fixed on the pellets with 2.5% glutaraldehyde solution and purified by passing through a series of ethanol solutions (30, 50, 70, 80, and 95%) to see the cell morphology on ceramic pellets. The dried pellets were coated with a thin layer of gold (90 s) and examined at 10 kV using the SEM.

Results and discussions

Materials for bone tissue engineering applications should be non-toxic and promote cell growth in the applied area. Accordingly, to assess cell-material interactions and to determine sintered material’s ability to be used in bone graft applications, the effects of starch additions on pore formation and densities for bioceramics sintered at 1100 and 1300 °C were investigated.

Table 1 shows the samples’ compressive strength and classical density values prepared with different starch additions and at different sintering temperatures. It was observed that for each specific sintering temperature, the density and compressive strength of the samples decreased accordingly in comparison to the fully dense TCP sample with the addition of starch, as can be seen from Table 1. This is in good agreement with other researchers [15, 16]. This study observed that the density of all samples increased significantly in sintering up to 1100 °C. In other studies, it is stated that the sintering of calcium phosphates is completed above 1050 °C. With the increase in density, the best compressive strength results were obtained from samples sintered at 1100 °C. Increasing the sintering temperature further decreased the strength. This decrease is attributed to the microstructure (excessive grain growth) and most likely due to the phase transitional changes (β → α) occurring above 1100 °C. Table 2 gives phase transformation percentages of TCP from β to α. When the sintering temperature is above 1200 °C, nearly 30% α-TCP was obtained. As Yetmez et al. [17] obtained a similar result in their study on biphasic calcium phosphate (BCP) ceramic, extensive grain growth in samples sintered at 1200–1300 °C and β-TCP to α-TCP and phase transformation between 1180 and 1430 °C formed intergranular and intragranular cracks between the phases, thus increasing fragility and lowering mechanical properties of the material. Fig. 1 shows electron micrographs of pure β-TCP samples sintered at different temperatures with 3000× and 15,000× magnification. As seen from the micrograph in Fig. 1a, sintering at 1000 °C is insufficient to densify the β-TCP particles. When the temperature is raised to 1100 °C, sintering and grain growth happen. Sintering above 1100 °C did not improve the density. A fully dense, close pore structure could be easily observed with excessive grain growth from Fig. 1c and d. Fig. 2 shows the pore size distribution of the samples in SEM images. Pores range in size from 6.7 to 13.6 µm. It is postulated that starch starts to burn and forms pores around 410 °C; no significant pore size difference was detected as a function of sintering temperatures and starch additions in SEM images. Fig. 3 gives XRD patterns of the samples to help to understand the effect of starch addition and the effect of sintering temperatures on the β-TCP crystal structure. Fig. 3a compares the X-ray diffraction analysis of β-TCP ceramics unsintered, sintered at 1100 and 1300 °C. Fig. 3b shows XRD patterns of pure β-TCP and 10 wt% starch-added pellets sintered at 1100 °C. As seen in Fig. 3b and d, there is no effect of starch addition on the crystal structure of the β-TCP. Unlike starch addition, sintering temperature changed crystal structure significantly, as seen in Fig. 3a. These changes could be investigated as qualitative

Table 1 Classic density and compressive strength data of β-TCP/starch samples sintered at varying temperatures (1000–1300 °C)

Properties	Sintering temperature (°C)	Percentages of starch (wt%)				
		0	1	3	5	10
Density (g/cm^3)	**1000**	2.37 ± 0.05	2.34 ± 0.03	2.25 ± 0.07	2.2 ± 0.1	2.04 ± 0.09
	1100	2.75 ± 0.07	2.69 ± 0.04	2.64 ± 0.1	2.54 ± 0.11	2.31 ± 0.07
	1200	2.81 ± 0.06	2.74 ± 0.06	2.68 ± 0.06	2.59 ± 0.09	2.38 ± 0.03
	1300	2.73 ± 0.03	2.75 ± 0.06	2.69 ± 0.05	2.59 ± 0.03	2.39 ± 0.06
Compression strength (MPa)	**1000**	79.8 ± 20.1	76.8 ± 27.8	66 ± 15.5	61.5 ± 3.5	24.6 ± 6.4
	1100	89.3 ± 2.6	83.2 ± 9	74.3 ± 8.4	67.8 ± 6.1	36.8 ± 6.6
	1200	85.8 ± 20.1	81.4 ± 9.9	72.5 ± 15	66.5 ± 14.7	34 ± 8.4
	1300	55 ± 2.3	54.3 ± 2.3	63.3 ± 9.8	58 ± 19.7	33.2 ± 7.2

Table 2 Quantitative analysis of the crystalline phases formed during the sintering process

	Phase rate (%)	JPDS code	1000 °C	1100 °C	1200 °C	1300 °C
Phase analysis of pure β-TCP samples	Tricalcium bis(phosphate(V))-beta	98-007-6896	97.9	99.2	64.6	68.1
	Tricalcium bis(phosphate(V))-alpha	98-001-2638			34.4	31.3
	Hydroxylapatite	98-001-2638	2.1	0.8	1	0.6

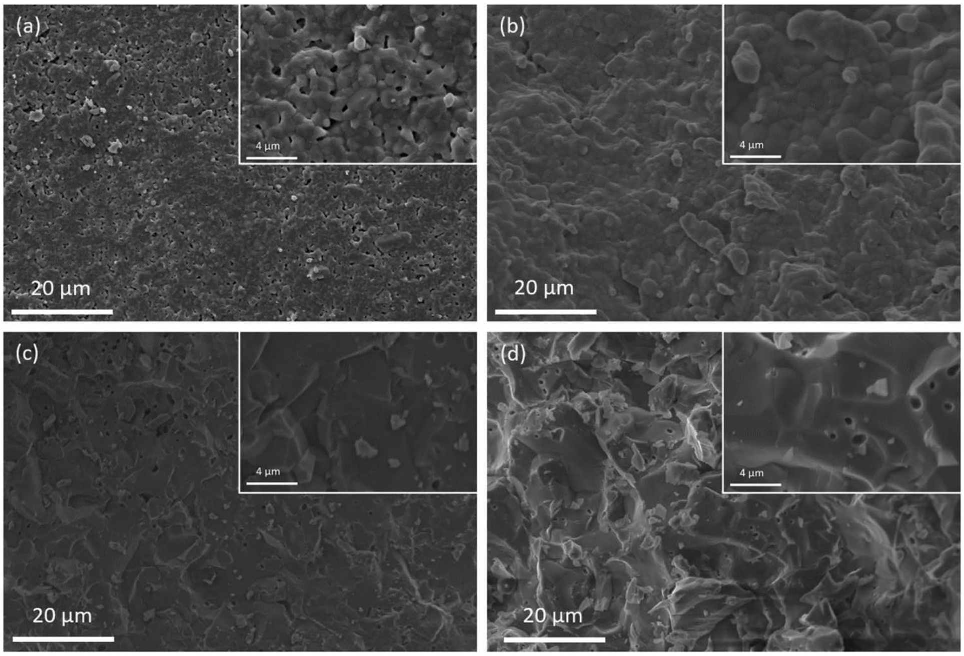


Fig. 1 SEM micrographs of pure β-TCP samples sintered at 1000 °C (**a**), 1100 °C (**b**), 1200 °C (**c**), and 1300 °C (**d**) with low 3000× and high 15,000× magnification

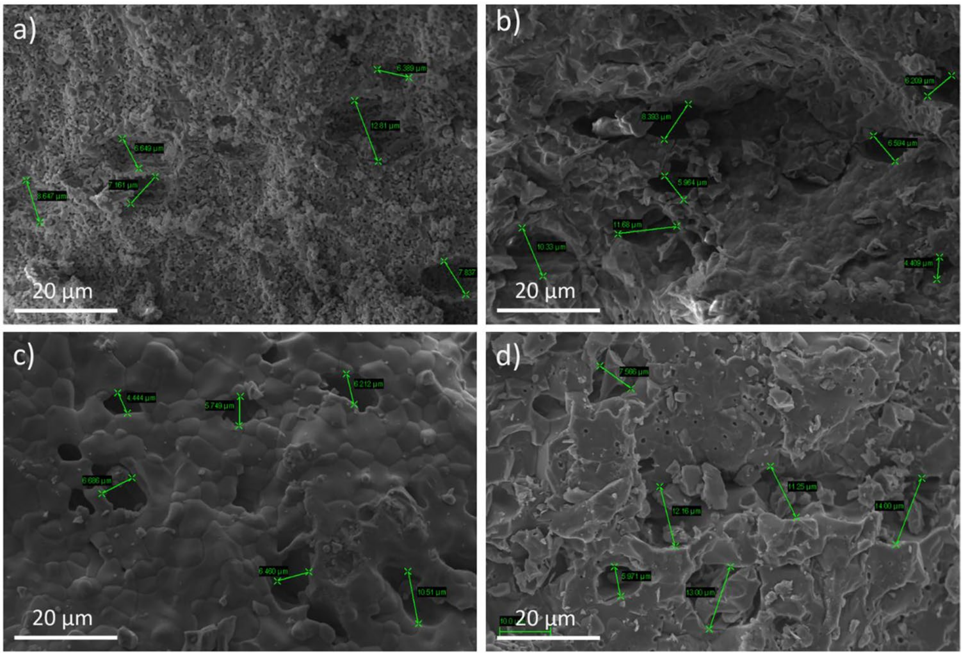


Fig. 2 SEM micrographs of 3 wt% starch-added samples sintered at varying temperatures between 1000 °C (**a**), 1100 °C (**b**), 1200 °C (**c**), and 1300 °C (**d**)

values from Table 2. The hydroxyapatite (HA) phase as sintering product was up to 2.1% at all sintering temperatures examined, but the number of HA decreases during the conversion to TCP phases as the sintering temperature increases. It is well-known that β-TCP can dissociate and the formation of HAp is possible at much lower temperatures [18]. Quantitative phase analysis shows 64.6% and 68.1% tricalcium bis(phosphate(V))-beta (JPDS code: 98-007-6896), and approximately 34.4% and 31.3 α-TCP (JPDS code: 98-001-2638) phases are encountered in the structure after sintering at 1200 and 1300 °C, respectively. As stated in similar studies, β-TCP begins to transform into the α-TCP phase at temperatures above 1125 °C [19–22]. This phenomenon was observed in all samples as a result of quantitative analysis. These results are consistent with the α-TCP and β-TCP peaks in the literature [23–25]. In Fig. 4, FTIR spectra show changes in the surfaces of TCP ceramics due to the phase transformation with varying sintering temperatures.

Unsintered β-TCP FTIR spectrum, Fig. 4a shows the characteristic PO_4^{3-} vibration bands of the β-TCP at 1022 cm^{-1} (v3), 962 cm^{-1} (v1), 599 cm^{-1} (v4), and 559 cm^{-1} (v4). PO_4^{3-} ions have v1 (symmetric stretching), v2 (symmetric bending), v3 (asymmetric stretching), and v4 (asymmetric bending) vibration modes [26, 27]. As a result of sintering, peak intensities of 1022-cm^{-1} (v3) and 559-cm^{-1} (v4) vibration modes decreased and shifted to 1130 cm^{-1} and 660 cm^{-1}, while the peak intensity of 962 cm^{-1} (v1), 599-cm^{-1} (v4) vibration modes increased and shifted to 1062 cm^{-1} (v1) and 719 cm^{-1} (v4). FTIR spectra agreed with XRD patterns on no meaningful effect of starch after sintering.

SEM micrographs of cell-material interactions are shown in Fig. 5. On day 7, cells cultured on 10% starch-added samples sintered at 1100 °C had appeared to attach the material well and cells started to extend locally and connect by filopodia. As seen in the SEM images, osteoblasts adhered and spread better on the surfaces of the samples sintered at 1100 °C. On the other hand, less adhesion and less cell viability are observed in the sample when sintered at 1300 °C due to the high crystalline structure of the surface. It is well-known fact that surface topography influences cell spreading, growth, and proliferation. SEM imaging shows sintered β-TCP/starch material is suitable for human osteoblasts, as cells

Fig. 3 X-ray diffraction analysis of unsintered β-TCP (**a**) and pure β-TCP powders sintered at 1100 (**b**)–1300 °C (**c**) temperatures and 10 wt% starch added 1100 °C (**d**)

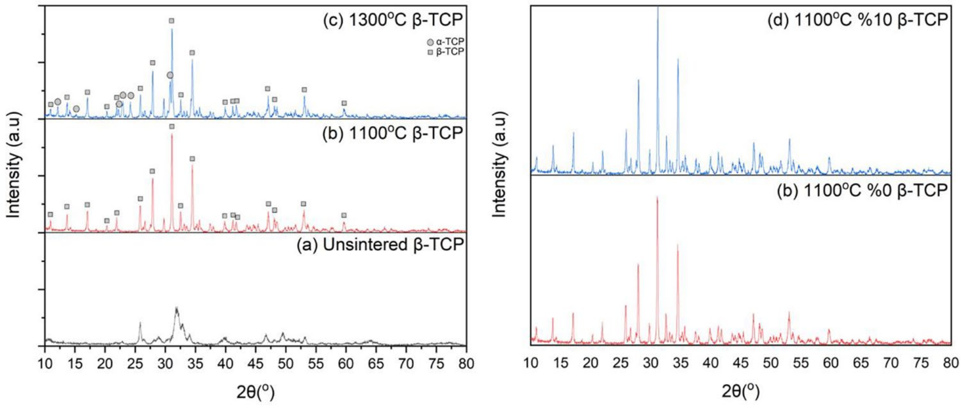


Fig. 4 FTIR spectra of unsintered β-TCP (**a**), pure β-TCP samples sintered at temperatures of 1100 °C (**b**), 1300 °C (**c**), and 10 wt% starch-added samples sintered at 1100 °C (**d**)

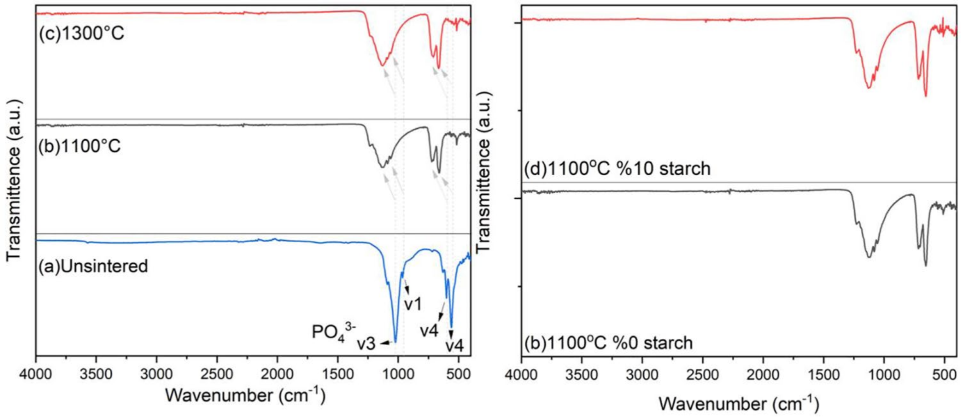
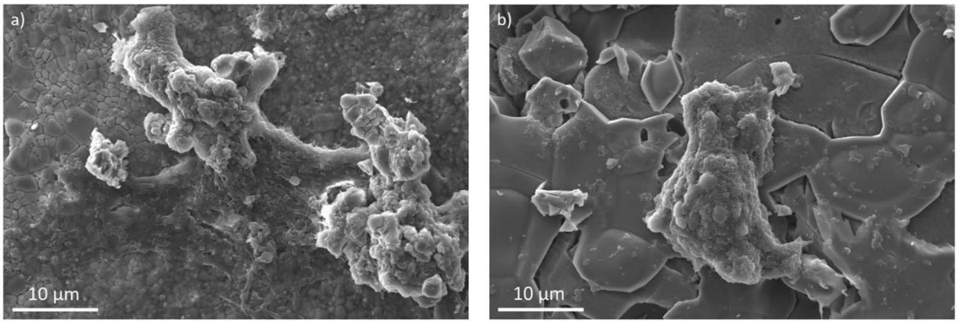


Fig. 5 SEM micrographs of cell-material interaction between osteoblasts and β-TCP pellets sintered at 1100 °C (**a**) and 1300 °C (**b**)



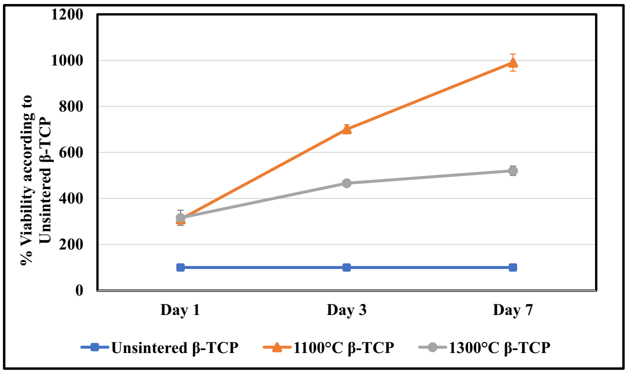


Fig. 6 Cell viability of β-TCP samples sintered at 1100 °C and 1300 °C, according to unsintered β-TCP

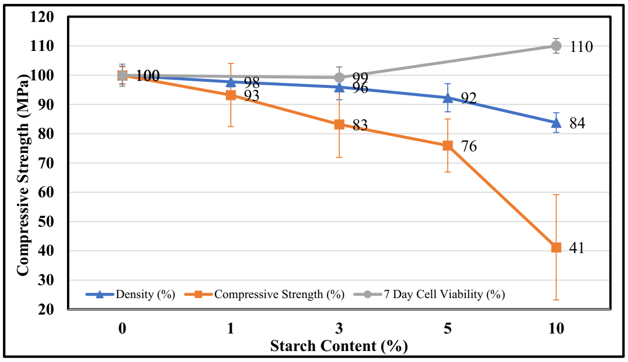


Fig. 7 The effect of starch additive on mechanical properties and cell viability of sintered β-TCP at 1100 °C

cultured on β-TCP/starch samples changed their morphologies by attaching and spreading, thus forming an excellent cell-material interaction. Fig. 6 shows "cell viability" results of β-TCP samples sintered at 1100 and 1300 °C compared to unsintered β-TCP. Sintered samples showed much better cell viability results compared to unsintered β-TCP. On days 3 and 7, 1100 °C samples were given distinctively better cell viability results with increased cell viability up to 197% compared to samples sintered at 1300 °C on day 7. These results indicate that the phase transformation of β-TCP to α-TCP occurring at temperatures higher than 1150 °C and increased crystallinity and hence changed surface topography can negatively affect cell viability. Fig. 7 shows the effect of starch additive on mechanical properties and cell viability in percentage. With 10 wt% starch addition, cell viability has increased by 10%. However, the density decreased by 16% and the compressive strength decreased by 59%. Although adding starch increases the biocompatibility of β-TCP, the resulting high mechanical strength loss may prevent the material from being used in load-bearing applications. An additional 10% cell proliferation may be beneficial in powdered bone graft applications that do not require high mechanical properties such as non-load-bearing applications. Additionally, even 10 wt% starch additive is insufficient to form interconnected pores that may benefit cell proliferation. The correct pore size is essential for tissue engineering applications. Small pores compared to the cell size may prevent cells from migrating towards the material centre and transporting nutrients, while large pores with their large surface area do not support cell adhesion. However, large pores around 100 to 500 μ can help with vascularization. Even though small pores do not directly support cell proliferation, they can support cells by increasing their attachment, which may be the reason why starch supplementation increased cell proliferation in our study [28]. Future studies could investigate the effects of different starch types to form varying sizes of pores on cell proliferation.

Conclusions

In this study, the mechanical properties and biocompatibility of porous β-TCP/starch composites were investigated. Starch is used by burning off during the sintering process to increase pore size and distribution, thus increasing biocompatibility. Analytical results show that after sintering, there is no presence of starch. Even though obtained pore size is not ideal for increasing cell proliferation, increased porosity may be beneficial by increasing cell attachment. The sintering temperature of 1100 °C was determined as the optimum temperature for β-TCP/starch samples for high mechanical properties and biocompatibility. It was observed that 10 wt% starch addition increases cell proliferation by 10% in sintered β-TCP samples. Although starch addition lowered the density by 16% and compressive strength by 59%, starch is still a low-cost alternative to increase the biocompatibility of the material in powder applications that do not require high mechanical properties. For further studies, it is advised that different types and sizes of starch can be used to increase the pore size. By forming interconnected pores, material biocompatibility can be further improved. Additionally, the effect of grain size on the powdered material and biodegradation tests can be beneficial to determine material properties even further.

Declarations

Competing interests The authors declare no competing interests.

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