Feasibility of alumina and alumina-silica nanoparticles to fabricate strengthened betatricalcium phosphate scaffold with improved biological responses

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Abstract

Betatricalcium phosphate (β -TCP), nano-alumina– and nano-alumina/nano-silica-added β -TCP scaffolds were prepared by the foam replication method followed by sintering at 1200–1300 °C. The rheological properties of β -TCP and the nano-additive-containing β -TCP slurries were investigated using a computerized rheometer. The phase composition, mechanical strength, microstructure and rat-calvarium-derived osteoblast responses of the scaffolds were also studied. The results showed that nano-alumina decreased viscosity of β -TCP slurry and receded its thixotropy whereas a reverse result was obtained for the nano-silica additive. The phase composition of the scaffolds was whitlockite regardless of sintering temperature. The nano-alumina-added β -TCP scaffolds were 2–4 times stronger than pure β -TCP and their compressive strength values were in the range of 0.1–0.5 MPa, depending on the additive content and temperature. However, when nano-alumina and nano-silica were simultaneously used, the compressive strength was dramatically improved to 5 MPa for temperature 1250 °C and 15 MPa for temperature 1300 °C. Adding nano-alumina and nano-alumina/nano-silica to pure β -TCP resulted in a more compacted microstructure with reduced grain size. All scaffolds exhibited adequate cell attachment, but the proliferation and alkaline phosphatase activity of the cells increased when nano-alumina and nano-silica were simultaneously used. Overall, tissue engineering scaffolds with interconnected macropores and improved mechanical and biological properties can be fabricated using a small quantity of nano-alumina and nano-silica additives.

Keywords: tricalcium phosphate, scaffold, tissue engineering, nano-alumina, nan-silica, mechanical strength

1. Introduction

Calcium phosphate bioceramics are used in dense, granular, and porous forms. Among these forms, porous calcium phosphate ceramics are more efficient in substituting and regenerating damaged bones. Over the past two decades, porous calcium phosphate ceramics have been widely used to repair or reconstruct the damaged parts of the human body [1]. The proper porosity and bioactivity allow the ingrowth of bone tissue to achieve a full integration with the living bone tissues. In addition to bioactivity, the size, shape and interconnectivity of macropores are important factors which influence on osteoconductivity of a porous material [2]. Klawitter and Hulbert indicated that a minimum pore size of 45–100 μm is necessary for bone ingrowth into a porous bioceramic material, while the pore size for optimal fibrovascular tissue ingrowth is 100–150 μm [3]. Also, it is well agreed that the pores should be interconnective for successful surgical operations [2]. Interconnectivity is essential, because constrictions between the pores or dead-end pockets limit vascular support toward growing tissue [4].

Different methods, including foam replication, gas foaming, oil dispersion, 3D printing and porogen removal technique have been developed for manufacturing macroporous materials [5-7]. It has been proved that replication of a porous structure will produce interconnected, mono-sized holes with controlled pore size [8].

Among porous bioceramics, β -tricalcium phosphate (β -TCP) is vastly used in hard tissue regeneration because of its remarkable biocompatibility and its closed chemical similarity to biological apatite presented in human bones. On the other hand, β -TCP is resorbed *in-vivo* and replaced by the newly formed bone. This property imparts a significant advantage onto β -TCP compared to other biomedical materials, which are not resorbed (e.g. hydroxyapatite, HA, and calcium phosphate cements) and replaced with the newly formed bone tissues.

Therefore, β -TCP has been widely used as a scaffold in bone tissue engineering and plastic surgery [9].

However, besides the necessity of biocompatibility and 3D porous structure, the scaffolds should exhibit proper mechanical strength to maintain the shape against the stress during the surgical procedure and recovery. Unfortunately, the inherently low mechanical strength of all calcium phosphate ceramics (such as β -TCP and HA) limits their applications for load-bearing bones. Furthermore, the mechanical strength of the scaffolds is usually poor due to the high content of pores and the presence of large interconnective channels. The limitation of scaffold strengths is one of the major challenges in the scaffold fabrication. Similarly, the poor mechanical properties of β -TCP scaffolds have severely hindered their clinical applications. For many years, several efforts have been made to improve the mechanical strength of calcium phosphate bioceramics and scaffolds [2,9]. Adding Al_2O_3 to HA is an effective route for improving the low fracture strength of the matrix, because Al_2O_3 produces a matrix with high crack resistance through crack deflection and bridging mechanisms [10].

Chiba et al. showed that HA/ Al_2O_3 composite has a higher fracture toughness than HA monolithic material, and the fracture toughness increases linearly with Al_2O_3 content [10]. The results were also confirmed by Bouslama et al. who used alumina as a promising material for the reinforcement of calcium phosphate. In his work, the mechanical properties of a β -TCP–Fluorapatite composite was much improved by adding 2.5 wt.% of micro-sized alumina additive [11]. The same results were also reported by Youn-Ki Jun [2] and Saki [12].

Using nanosized ceramic additives for strengthening bone substitutes is highly regarded in recent years. For instance, Kaili et al. reported that the mechanical properties of β -TCP scaffolds improved when nano-sized powder was used as raw material [9]. Previously nanosized silicon carbide, titania and silica were employed to reinforce calcium phosphate bone cements [13].

The aim of the present study was to fabricate bioceramic scaffolds with improved mechanical properties using β -TCP as the main matrix and nano-alumina and nano-silica as reinforcing agents. The rheological behavior of the prepared aqueous suspensions used in the foam replication process was also evaluated. The effect of sintering temperature on physical and mechanical characteristics of the scaffolds was also studied. In addition to physical and mechanical properties, the effects of these additives on the scaffold cell responses were investigated.

2. Materials and methods

2.1. Preparation and characterization of starting materials

Nano-alumina ($n\text{-Al}_2\text{O}_3$), with a particle size of 20 nm was purchased from the Merck, Germany. Nano-silica ($n\text{-SiO}_2$), with an average particle size of 60 nm was also purchased from Shiv Kripa, India. The morphologies of $n\text{-Al}_2\text{O}_3$ and $n\text{-SiO}_2$ particles were observed by transmission electron microscopy (TEM). For this purpose, the powder was ultrasonically dispersed in ethanol to form a diluted suspension and then, a few droplets were dropped on a carbon coated–copper grid. The morphology of the particles was observed by a TEM instrument (GM200 PEG Philips) at an accelerating voltage of 200 kV. The specific surface area of the nano-particles was determined by N_2 adsorption technique using the Brunauer-Emmett-Teller technique.

To synthesize β -TCP, calcium carbonate (CaCO_3 , Merck 2069) and dicalcium phosphate, DCP, (CaHPO_4 , Merck 2144) powders were used as starting materials. β -TCP powder was synthesized at 1000°C through the reaction of CaCO_3 and CaHPO_4 according to the following equation [14]:

The phase composition and particle size of the obtained powder were analyzed by an automated X-ray diffractometer (XRD, Philips PW3710) and a laser particle size analyzer device (Fritsch Particle Size analysette 22), respectively.

2.2. The slurry preparation and rheological evaluations

Various slurries consisting of β -TCP, n-Al₂O₃, n-SiO₂, distilled water and other additives were prepared according to Table 1. The starting materials were mixed and homogenized together in a ball mill for 12 h.

The viscosity and thixotropy of slurries were measured at 25 °C using a rate-controlled rotational rheometer (Anton Paar MC-R300) with a coaxial cylinder sensor system. The outer diameter of the cylinder was 2.17 cm and its inner diameter was 1.7 cm. The slurry of each composition was poured into the rheometer for rheological characterization and the test was started two minutes after mixing by the program shown in Figure 1.

2.3. Fabrication of macroporous scaffolds

Rectangular-shape templates were prepared from the polyurethane (Pu) foams having 60 pores per inch (ppi) [1]. After stirring the slurry, the Pu sponges were immersed in it and then squeezed by passing the filled sponge through parallel rollers in which the space between the rolls was half of the sponge thickness. The scaffolds were dried at room temperature for 24 h, and then, at 100 °C for 12 h. The elimination of the polyurethane foams was carried out by a mild thermal treatment at a slow heating rate of 1 °C/min up to 600 °C. The thermal treatment was then continued at a faster rate, 10 °C/min, up to final sintering temperature. It should be noted that the samples were sintered at different temperatures: 1200, 1250 and 1300 °C for 5 h.

2.4. Characterization of the prepared scaffolds

The bulk density of scaffold (D_b), the volume of the macropores, i.e. the large pores produced by the foam replication process (P_{macro}), the volume of the micropores (P_{micro}) and total porosity (P_t) of the sintered samples were recorded using the following equations:

$$D_b = M/V \quad (2)$$

$$V = \text{length} \times \text{width} \times \text{thickness} \quad (3)$$

$$P_t = (1 - D_b/D_{\beta-TCP}) \times 100 \quad (4)$$

$$P_{macro} = (1 - D_b/D_{buf}) \times 100 \quad (5)$$

$$P_{micro} = P_t - P_{macro} \quad (6)$$

where M is the mass and V is the volume of samples, $D_{\beta-TCP}$ theoretical density of β -TCP, D_{buf} is the bulk density of unfoamed β -TCP (the sintered β -TCP bodies which were produced from the slurries, but no replication process were performed on them).

The compressive strength of the rectangular specimens with final dimensions of $10 \times 10 \times 5 \text{ mm}^3$ were recorded using a universal testing machine (STM 20, Santam Ltd) at a crosshead speed of 0.5 mm/min.

The phase composition of the samples was analyzed using XRD (Philips PW3710) diffractometer with Cu-K α radiation ($1.540600 \text{ \AA}$), operating voltage of 40 kV and current intensity of 10 mA. The XRD diagrams were recorded at $10^\circ \leq 2\theta \leq 50^\circ$ with a scan speed of $2^\circ/\text{min}$, a step size of 0.02° and a step time of 1 s.

The morphology of the synthesized samples was evaluated using a scanning electron microscope (SEM). The samples were coated with a thin layer of gold by sputtering (EMITECH K450X, England) and then observed by Streoscan S 360, from Cambridge Ltd.

To evaluate proliferation and attachment of osteoblastic cells on top of the samples, the cells were derived from newborn rat calvarium bone and cultured in Dulbecco–modified Eagle medium (DMEM; Gibco-BRL, Life Technologies, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS; Dainippon Pharmaceutical, Osaka, Japan) and 100 g/mL

1 penicillin–streptomycin (Gibco-BRL, Life Technologies) in 5% CO₂ and 95% air atmosphere
2 at 37°C for 1 week. The medium was changed every 2 days. The confluent cells were
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4 dissociated with trypsin and sub-cultured to 3 passages which were used for the tests.

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6 The porous cuboids (5×5×5 mm³) were sterilized using 70% ethanol and the osteoblastic cells
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8 were seeded on the tops of them at 2× 10⁴ cells/sample. The specimen/cell constructs were
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10 placed into 24-wells culture plates and left undisturbed in an incubator for 3 h to allow the
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12 cells to attach to them and then an additional 3 ml of culture medium was added into each
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14 well. The cell/specimen constructs were cultured in a humidified incubator at 37 °C with 95%
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16 air and 5% CO₂ for 1,7, and 14 days. The medium was changed every 3 days.

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18 The proliferation of the osteoblastic cells on specimens was determined using MTT (3-{4,5-
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20 dimethylthiazol-2yl}-2,5-diphenyl-2H-tetrazolium bromide) assay. For this purpose, at the
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22 end of each evaluating period, the medium was removed and 2 ml of MTT solution was
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24 added to each well. Following incubation at 37 °C for 4 h in a fully humidified atmosphere of
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26 5% CO₂ in air, the MTT was taken up by active cells and reduced in the mitochondria to
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28 insoluble purple formazan granules. Subsequently, the medium was discarded and the
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30 precipitated formazan was dissolved in dimethylsulfoxide, DMSO, (150 mL/well), and
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32 optical density of the solution was read using a microplate spectrophotometer (BIO-TEK Elx
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34 800, Highland park, USA) at a wavelength of 570 nm.

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36 The morphologies of the cells attached onto the surfaces of the specimens were also
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38 evaluated. The cells were cultured on top of the scaffolds as described above. After 7 days,
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40 the culture medium was removed, the cultured specimens were rinsed with phosphate
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42 buffered saline (PBS) twice and then, the cells were fixed with 500 mL/well of 3%
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44 glutaraldehyde solution (diluted from 50% glutaraldehyde solution (Electron Microscopy
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46 Science, USA) with PBS). After 30 min, they were rinsed again and kept in PBS at 4 °C.

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48 Then, the specimens were fixed with 1% Osmium tetroxide (Polyscience, Warminster, PA,
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USA) and dehydrated in ethanol solutions of varying concentration (30, 50, 70, 90, and 100%), for about 20 min at each concentration. Finally, the specimens were dried in air, coated with gold and analyzed by the previously described SEM instrument.

To evaluate the amount of alkaline phosphatase activity of the loaded cells, the alkaline phosphatase kit was employed. Briefly, after washing the cells with 400 μ l of PBS, p-nitrophenyl phosphate was spilled on them and kept in the dark for 30 minutes. Finally, the amount of the produced color indicating alkaline phosphatase activity of the cells was measured at wavelength of 405 nm. The test was carried out in triplicate and the results were standardized using the known concentrations of total protein.

For the statistical analyses, the data were processed using Microsoft Excel 2007 software. The results were produced as mean $\pm$ standard deviation of at least four experiments. The statistical significance between mean values was determined by a one-way analysis of variance, and significance in differences of the mean values was evaluated by Tukey's post hoc test (SPSS v10.0, Chicago, IL, USA). The $p \leq 0.05$ was considered significant.

3. Results and discussion

Figure 2 shows the TEM image of nano-alumina and nano-silica powders. For nano-alumina (Fig. 2a), aggregated particles with a particle size of about 20 nm are observed, which have produced larger particles with a size range of 100-200 nm. The TEM image of n-SiO₂ particles is also shown in Fig. 2b. Spherical nano-particles with a particle size lesser than 50 nm are seen in this picture. The n-Al₂O₃ and n-SiO₂ particles have a specific surface area of 80 and 120 m²/g, respectively.

In this work, tripolyphosphate (TPP) and carboxymethyl cellulose (CMC) was added to the suspensions as dispersing agent and binder, respectively. CMC can improve the cohesiveness of the suspension and advance green strength of unfired scaffolds. TPP is a water reducing agent that modifies fluidity and viscosity of slips.

Figure 3 shows shear stress–shear rate and viscosity–shear rate curves of pure β -TCP, 0.5 T-A, 5 T-A and T-A-S slurries. All slurries have a shear thinning behavior, i.e. the viscosity decreases with increasing shear rate. The viscosities of β -TCP and alumina-containing slurries at the beginning of the test are in the range of 3-5 Pa.s. By adding colloidal silica to the slurries, the viscosity of the first shear rate point (0.1 s^{-1}) increases.

The appearance of hysteresis loops in shear stress-shear rate curves of pure β -TCP and T-A-S slurries indicates that these suspensions have positive thixotropy, with a bigger loop area (and hence higher magnitude of thixotropy) for the latter. However, the thixotropic behavior disappeared by adding nano-alumina to β -TCP slurry. Actually, it was observed that nano-alumina acts as a dispersant in the β -TCP suspension. Nano-alumina adsorption on the β -TCP particle can affect forces between the surfaces, in particular van der Waals attractions and hence reduce the particle interactions and thixotropy. This phenomenon has been also observed in other suspensions containing nano-alumina particles [15]. A thixotropic slurry can be suitable for coating polymeric templates, because of its improved adhesiveness on polymer walls. This would even result in increasing the green strength of scaffolds. In this study, n-SiO₂ particles were used as a colloidal system. It is a dispersion of nanosized particles of silica with hydroxylated surface i.e. OH⁻ ions. When calcium phosphate particles are added, they are surrounded by colloidal silica particles followed by coagulation of the surrounding layers through the gelling mechanism. It would result in condensation of Si (OH)⁻ groups on the silica surfaces to form Si–O–Si bonds, resulting in increased viscosity and developed thixotropy. The presence of Ca ions released from the β -TCP particles into the slurry T-A-S can also accelerate the coagulation rate as described elsewhere [16].

Figure 4 shows the XRD spectra of β -TCP and n-Al₂O₃–incorporated scaffolds at temperatures 1200-1300 °C. Figure 5 presents these data for sample T-A-S. In both Figs, all peaks belong to β -TCP, whitlockite, with rhombohedral crystallographic system (ICDD 009-

0169). The XRD patterns of the samples sintered at 1250 and 1300°C also determine whitlockite as the only found phase. However, adding n-Al₂O₃ and n-SiO₂ would result in an obvious right-shift in the position of β-TCP peaks, suggesting the substitution of some Al and/or Si cations into the whitlockite crystal lattice. Overall, the results indicate that β-TCP phase is stable, even at 1300 °C and no phase transformation occurs, even in the presence of n-Al₂O₃. It is also noticeable that the XRD patterns of the additive phases cannot be detected (if they exist), because XRD is not sensitive to the concentrations lower than 5 wt.%. Thus, from the XRD patterns, it is found that no new detectable phase is produced at 1200–1300 °C and only ion substitutions in the β-TCP crystal lattice is suggestible.

Figure 6 shows the compressive strength of the samples after sintering at 1200-1300 °C. The mechanical strength of all samples increases with increasing the temperature. For temperature 1200 °C, the samples pure β-TCP and T-A-S exhibit poor mechanical strength and the maximum compressive strength value belongs to scaffold 2.5 T-A. By increasing the temperature from 1200 °C to 1250 °C, only the compressive strength of T-A-S increases significantly and reaches to ~ 4.5 MPa. Further increase of compressive strength is observed at 1300 °C, but the value of T-A-S (which is ~ 16 MPa) is much higher than that of others. At this temperature, pure β-TCP has a compressive strength of ~ 0.07 MPa and n- Al₂O₃–added scaffolds show values in the range of 0.2–0.5 MPa.

Alumina is a hard and rigid biomaterial with a greater resistance to fractures compared to HA and other calcium phosphates. It also exhibits extremely high stability within the human tissues. Therefore, Al₂O₃ powder has been added to calcium phosphate to obtain a high fracture toughness [4,10]. However, nano-particulates of alumina with high specific surface area and high surface energy could result better mechanical properties, though the best mechanical strength is obtained when n- Al₂O₃ and n-SiO₂ are simultaneously used.

The results of mechanical properties were in agreement with those of Bouslama et al. [11] who improved rupture strength of tricalcium phosphate-flourapatite composites using alumina micro-particles. In the present study, the compressive strength of the samples fabricated by mixing micro-sized β -TCP and nano-sized alumina and silica reached to ~ 16 MPa, which was almost 45% higher than the Lin's scaffolds fabricated using nano-sized β -TCP. It should be taken into account that the macropore size of the scaffolds produced in this study was in the same range with those of Lin et al. [9], however, in foam replication method, it is difficult to manufacture scaffolds with completely similar pore walls, though interconnecting macropores with a variable pore wall thickness (depending on the slurry concentration and the content of the material adsorbed onto the template walls) can be produced. It is clear that the thickness of the walls has a considerable effect on mechanical strength. On the other hand, for the same formulations, scaffolds with different compressive strengths will be obtained by using different scaffold forming methods.

The mechanical strength is inversely proportional to the porosity. The micro, macro and total porosity values of the samples are given in Table 2. In 1200 °C, the porosity values of all samples are nearly equal and the differences are not statistically significant. The total pore volume is about 80 %. At 1250 °C, decrease in the pore content of β -TCP is not statistically significant ($p > 0.05$), however; the microporosity values of nano-additive-containing scaffolds decrease significantly and are much lower than that of pure β -TCP. Compared to temperature 1200 °C, a 10 % decrease in micoporosity values of the nano-alumina-added scaffolds is observed at 1300 °C, meanwhile the scaffold T-A-S has the least total porosity value. It is noticeable that the variation in microporosity values mutates the total porosity values, whereas no significant changes in macroporosity of the scaffolds are observed by varying the sintering temperature. The reduction in micropore volume is due to the sintering

phenomenon and filling the pores with a viscous flow. The results agree with the SEM images and describe why the mechanical strength of 5 T-A is higher than 2.5 T-A at 1300 °C.

Figure 7 shows typical SEM images of β -TCP and 2.5 T-A. Spherical interconnected macropores with a pore diameter range of 200-500 μm are observed in this image. The other formulations exhibited the same macrostructure and hence, the images of them are not shown here.

In Figure 8, the microstructures of various samples (β -TCP, n-Al₂O₃-added β -TCP and n-SiO₂-added β -TCP) sintered at 1200 and 1300 °C are shown. At temperature 1200°C, pure β -TCP has a highly microporous structure (pore size less than 10 μm) in which the grains are elongated and locally joined together. The growth in grain size and decrease in micropore size and content of β -TCP is observed at temperature 1300 °C. From the pictures, the microstructure of nano-alumina- and nano-alumina/nano-silica-added β -TCP scaffolds are more compacted than that of pure β -TCP. Moreover, it seems that these additives limit the grain growth of β -TCP, especially when they are used simultaneously. Increasing in sintering temperature would result in grain growth and decrease in porosity. At 1300 °C the microstructure of 5 T-A is denser than 0.5 T-A. In sample T-A-S, the grain boundaries are nearly disappeared and it seems that a monolithic phase surrounds the grains. Thus, a more compacted microstructure is produced in T-A-S, though some pores with a size range of 1-10 μm are still observed. The exclusion of grain boundaries is probably due to the presence of a viscous flow mechanism that fills the grain margins. An interesting point seen in the micrograph of T-A-S is the presence of regions having rod-like morphology (arrows). Figure 9 that shows a higher magnification of T-A-S microstructure, discloses these rods among highly connected particles with a thickness of about 100-300 nm. The authors suggest this morphology, which can be related to the formation of an intermediate phase is responsible for the elevated mechanical strength, though it could not be detected by the XRD analysis.

Figure 10 shows the cell numbers and alkaline phosphatase activity of viable cells attached onto the scaffold surfaces containing different amounts of additives. For all samples, a gradual increase in cell numbers is observed by elapsing the culture time (Fig. 10a). However, the MTT test confirms the better proliferation of osteoblasts over specimen T-A-S compared to others, because the numbers of viable cells proliferated on T-A-S are significantly higher than that of pure β -TCP and other alumina-added scaffolds. It is notable that the presence of Al_2O_3 does not affect the proliferation rate of the osteoblasts.

Figure 10b shows the results of alkaline phosphatase activity test for samples β -TCP, 0.5T-A, 5 T-A and T-A-S. The alkaline phosphatase activity increases for all samples from day 1 to day 7 and then, decreases at day 14. At day 7, the cells produce a significantly higher amount of ALP on T-A-S compared to other samples ($P < 0.05$). The amount of ALP is inhibited after 14 days, although the average value of T-A-S is still higher than that of others. The restriction on the production of ALP is probably due to the confluence of the attached cells.

Figure 11 shows the morphology of the cells seeded on the scaffolds for 7 days. The difference in the cell morphology of the samples is less pronounced and for all samples, confluent, sheetlike cells with well spread cytoplasmic membrane are observed. The cells either have attached on to the scaffold surfaces or bridge upon the large pores. The results reflect adequate cell supporting capability of the scaffolds. The osteoblasts used in this study had been derived from Rat-calvarium which are broadly used for studying in-vitro biocompatibility and biological responses of bone implants [17].

In MTT assay, some enzymatic reactions reduce MTT reagent to a formazan product. Therefore the absorbance of formazan imitates the level of cell metabolism. This procedure is known as cell viability in culture medium.

The better proliferation and alkaline phosphatase activity of osteoblastic cells on scaffold T-A-S can be related to the presence of Si ions in its chemical composition. These ions can be

liberated from the porous material into the culture medium. It has been proved that Si ions play an important role in bone cell responses. Based on many reports, Si ions enhance the proliferation of osteoblastic cells and increase the synthesis of collagen [18, 19]. Figure 12 shows some high resolution SEM images taken from the cell-free parts of specimens β -TCP, 5 T-A and T-A-S kept in culture medium for 7 days. The up and bottom rows are related to the different magnifications. For β -TCP, no significant changes are observed in comparison with the images shown in Fig. 8, except that more micropores are observed here, probably due to the dissolution of calcium phosphate phase in the culture medium. In specimen 5 T-A, a porous microstructure comprising particles with roughened surfaces is observed. The particle surfaces coarsen due to the dissolution of β -TCP constituent of the scaffold and the presence of remaining nano-alumina particles. Moreover, it can be related to the initiation of calcium phosphate precipitation, which occurs during soaking the bioactive materials in physiologic media such as simulated body fluid, Ringer's solution and various cell culture solutions [16, 20]. In T-A-S, the microstructure is completely different than what was shown in Fig.8. In this specimen, the surface of the cultured scaffold consists of many balls comprising entangled nanosized needles. Based on the author's experience and regarding many papers, this morphology relates to the precipitates of biomimetic calcium phosphate [19, 21, 22]. Adding nano-silica to β -TCP scaffold provides many Si-OH groups on the material surface during the soaking procedure. These groups are capable sites for calcium phosphate precipitation. Thus, one can conclude that adding n-SiO₂ to nano-alumina-added β -TCP scaffold improves bioactivity and bone bonding ability, though more in-vivo studies are essential to support this statement.

4. Conclusions

The following conclusions can be pointed out from the results of this study:

- Incorporation of n-Al₂O₃ to β-TCP slurry decreases the level of thixotropy and viscosity, whereas n-SiO₂ has an adverse influence on the rheology of the suspension.
- Adding 0.5-5 % Al₂O₃ and 2.5 % SiO₂ to β-TCP **doesn't produce** detectable phase in its composition at sintering temperatures 1200-1300 °C, however; some ionic substitutions may occur.
- Adding n-Al₂O₃ to β-TCP improves the mechanical strength through producing a more compacted microstructure with limited grain growth.
- The simultaneous use of n-SiO₂ and n-Al₂O₃ in β-TCP scaffolds increases the mechanical strength drastically through reducing porosity, disappearing the grain boundaries and producing a monolithic microstructure.
- The rat calvarium-derived osteoblasts **exhibit a** higher proliferation rate on the scaffold containing both n-SiO₂ and n-Al₂O₃ additives; however all scaffolds exhibit adequate cell attachment.
- **Adding n-SiO₂ to nano-alumina-containing β-TCP scaffold causes precipitation of a falke-like phase during the cell culture period.**

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Tables

Table 1. Formulation of various slurries used for the preparation of the scaffolds (in wt.%)

Code	Distilled water	β -TCP	n-Al ₂ O ₃	n-SiO ₂	Sodium carboxymethyl cellulose	Sodium tripolyphosphate
β - TCP	35	65	0	0	2.6	0.84
0.5 T-A	35	65	0.5	0	2.6	0.84
2.5 T-A	35	65	2.5	0	2.6	0.84
5 T-A	35	65	5	0	2.6	0.84
T-A-S	35	65	2.5	2.5	2.6	0.84

Table 2. The pore volume of the scaffolds sintered at different temperatures

Temp. (°C)	Porosity (%)	β -TCP	0.5 T-A	2.5 T-A	5 T-A	T-A-S
1200	P_{micro}	47 ± 3	41 ± 5	42 ± 5	40 ± 4	45 ± 2
	P_{macro}	34 ± 2	38 ± 2	38 ± 1	39 ± 4	33 ± 4
	P_t	81 ± 3	79 ± 3	80 ± 6	79 ± 5	78 ± 2
1250	P_{micro}	45 ± 6	39 ± 4	37 ± 2	39 ± 3	38 ± 3
	P_{macro}	32 ± 2	35 ± 1	33 ± 5	36 ± 5	35 ± 6
	P_t	77 ± 3	74 ± 6	70 ± 8	75 ± 4	73 ± 5
1300	P_{micro}	39 ± 5	29 ± 3	30 ± 4	27 ± 4	21 ± 3
	P_{macro}	35 ± 5	32 ± 3	32 ± 1	36 ± 2	32 ± 5
	P_t	74 ± 2	61 ± 3	62 ± 4	63 ± 3	53 ± 3

Figure captions

Figure 1. The program used for the rheological test of β -TCP and nano-particulate-added β -TCP slurries.

Figure 2. The transmission electron micrographs of nano-alumina(a) and nano-silica (b)

Figure 3. The shear stress and viscosity curves of various slurries as a function of shear rate.

Figure 4. The XRD patterns of β -TCP and n- Al_2O_3 -incorporated scaffolds after sintering at 1200 °C, 1250 °C and 1300 °C.

Figure 5. The XRD patterns of the scaffolds containing both n- SiO_2 and n- Al_2O_3 (T-A-S) after sintering at 1200, 1250 and 1300 °C.

Figure 6. The compressive strength of β -TCP and nano-particulate-added β -TCP scaffolds after sintering at 1200 °C, 1250 °C and 1300°C.

Figure 7. Typical SEM images of β -TCP and 2.5 T-A at a low magnification.

Figure 8. The microstructures of β -TCP and other nano-particulate-added scaffolds after sintering at different temperatures.

Figure 9. The microstructure of specimen T-A-S sintered at 1300 °C observed at a magnification of $\times 10000$.

Figure 10. The cell numbers (a) and alkaline phosphatase activity (b) of viable osteoblasts attached onto the various scaffolds (*: $p < 0.05$).

Figure 11. The morphology of osteoblastic cells attached onto the surfaces of 7 days-cultured scaffolds.

Figure 12. The high-resolution SEM images of the cell-free areas of the scaffolds cultured for 7 days.

Figure 1

Figure 2

Figure 3

Figure 4

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Figure 12