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Effects of Silica Addition on the Chemical, Mechanical and Biological Properties of a New α-Tricalcium Phosphate/Tricalcium Silicate Cement

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The addition of tricalcium silicate (C_3S) to apatite cements results in an increase of bioactivity and improvement in the mechanical properties. However, adding large amounts raises the local pH at early stages, which retards the precipitation of hydroxyapatite and produces a loss of mechanical strength. The introduction of Pozzolanic materials in cement pastes could be an effective way to reduces basicity and enhance their mechanical resistance; thus, the effect of adding silica on the chemical, mechanical and biological properties of α-tricalcium phosphate/C_3S cement was studied. Adding silica produces a reduction in the early pH and a decrease in setting times; nevertheless, the presence of more calcium silicate hydrate (C-S-H) delays the growth of hydroxyapatite crystals and consequently, reduces early compressive strength. The new formulations show a good bioactivity, but higher cytotoxicity than traditional cements and additions higher than 2.5% of SiO_2 cause a lack of mechanical strength and an elevated degradability.

Keywords: *calcium phosphate cements, hydroxyapatite, tricalcium silicate, pozzolan*

1. Introduction

Calcium phosphate cements (CPCs) are a clinical alternative to traditional bioceramics because they are easy to handle and shape, mold themselves well to the contours of defective surfaces, and set in situ in the bone cavity to form a solid restoration[1]. Since their development in the mid-80's, CPCs have also attracted great interest due to their chemical similarity to the mineral phase of bone tissue and their good osteoconductivity[2].

One of the most important formulations is based on α-tricalcium phosphate [α-$Ca_3(PO_4)_2$; α-TCP], which sets in situ and forms a calcium-deficient hydroxyapatite [$Ca_9(HPO_4)(PO_4)_5(OH)$; CDHA] when hydrated[3]. However, it is not very strong under compression[4] and its mechanical strength is low when compared to that of cortical bone[5], limiting its application to areas subjected to low mechanical loads[6].

In view of the excellent bioresorbability of CDHA, researchers have focused their efforts on overcoming the mechanical weakness of calcium phosphate cements by using different fillers, fibers and reinforcing additives that lead to the formation of various multiphase composites, based on the idea that the filler in the matrix may eliminate crack propagation[7]. Nevertheless, the presence of fillers prevents bone ingrowths into pores and produces a denser cement with a slower resorption rate and hence a slower bone substitution[8]. Therefore, it is difficult to increase the strength of these cements without negatively affecting other properties.

Due to their spontaneous development of strength (spontaneous consolidation) towards water[9], the addition of Ca_3SiO_5 (C_3S) to the α-TCP-based cement could be an effective way to increase its mechanical strength[10] and improve the bioactivity and biocompatibility of traditional CPCs[11,12]. However, the pH increase during the initial stages delays setting times and prevents apatite formation in larger concentrations.

In the chemistry of ordinary Portland cement (OCP), synthetic colloidal silica (silica fume) is a highly reactive siliceous material which reacts with the calcium hydroxide formed in hydrated cement paste (pozzolan). Although the mechanisms by which silica fume operates are unclear, the presence of small amounts of silica can accelerate the hydration of C_3S by forming colloidal calcium silicate hydrate (C-S-H), thereby increasing the rate of early strength gain[13].

Thus the aim of this work was to study the effects of adding silica on the chemical, mechanical and biological properties of α-TCP/C_3S cement after ageing in simulated body fluid (SBF).

2. Experimental

2.1. Materials

To prepare the α-TCP/C_3S/SiO_2 cement, all chemicals of analytical grade were used.

α-TCP was prepared through solid state reaction, heating the appropriate mixture of γ-$Ca_2P_2O_7$ (Extra Pure, Dyne®) and $CaCO_3$ (Extra Pure, Nuclear) at 1300 °C for 5 hours followed by quenching in air[14]. After calcination, the product was wet milled for 4 hours in a polyethylene jar with alumina balls using an alcoholic medium (anhydrous ethanol, 99.5%, Cromoline) to an average particle size inferior to 10 μm.

Tricalcium silicate powders were synthesized by sol-gel route, using $Ca(NO_3)_2.4H_2O$ and $Si(OC_2H_5)_4$ (TEOS)[15]. Briefly, suitable amount of TEOS was added in 200 mL water under continuous stirring and then, required amount of $Ca(NO_3)_2.4H_2O$ was added to the solution. The solution was maintained at 60 °C until gelation occurred and the gel was dried at 120 °C. Repeated grindings and calcinations at 1400 °C were necessary to reach the product as monophasic as possible. In order to obtain powders with similar average particle size, the same milling treatment as in the case of α-TCP was used.

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2.2. Preparation of composite samples

Synthesized C_3S (7.11 μm) was mixed with α-TCP (10.71 μm) in powder ratios of 0, 5.0 and 10.0 mass % and precipitated silica WL180 (Auriquímica Ltda - Brazil) (surface area 133 $m^2.g^{-1}$ and mean diameter 19.54 μm) was added in a molar ratio SiO_2:C_3S=2. The liquid phase was a sodium phosphate buffer prepared from NaH_2PO_4 and $Na_2HPO_4.12H_2O$, and the liquid-to-powder ratio (L/P) was dependent of the content of C_3S added ranging from 0.4 to 0.44 $mL.g^{-1}$. Each powder sample was carefully weighed and mixed with the liquid phase in appropriate powder-to-liquid ratio, packed into silicon molds and aged at 36.5 °C with controlled humidity for 24 hours. The samples were identified as α-TCP, 5SiO_2 and 10SiO_2 being the number the quantity of C_3S added in mass percent.

2.3. Setting time measurement

Setting time of samples was measured according to ASTM C266-89 using a Gillmore Needles method[16]. Three specimens for each formulation were tested and standard deviation was used as a measure of the standard uncertainty. Initial setting time was determined as the end of moldability without serious damage to the cement structure and the final setting time as the time beyond which it is possible to touch the cement without causing serious damage.

2.4. In vitro tests

To assess in vitro bioactivity, the 24 hours-set pastes were soaking in simulated SBF at 36.5 °C SBF[17] for 14 days and afterward, gently rinsed with deionized water followed by ethanol dehydration and drying in atmospheric temperature.

The degradation behavior was characterized by monitoring changes in weight loss in SBF[18]. For degradability test, the disks were accurately weighed before and after immersion in SBF. The weight loss (WL) was calculated according to Equation 1 being W_0 the initial weight of the specimen and W_d the weight of the specimen dried after different degradation times (7, 14 and 21 days). Each measurement was performed three times and the average value was calculated.

$$WL\% = \frac{(W_0 - W_d)}{W_0 \times 100} \quad (1)$$

2.5. Cytotoxicity test for cements

The cell viability assay was performed by direct contact test according to the ISO 10993-5 (Biological evaluation of medical Part 5: Tests for in vitro cytotoxicity) using peripheral blood mononuclear cells (PBMCs) and the MTT assay.

The PBMCs were assembled cultured in a Dulbecco's modified Eagle's Medium (DMEM) (Sigma) with HEPES (Sigma) (free acid, 2.5-3.7 $g.L^{-1}$) supplemented with 10% fetal bovine serum (FBS) (Cultilab, Sao Paulo, Brazil), at 37 °C in a humidified atmosphere of 5% CO_2 for one 24 and 48 hours. The dissolution extracts were prepared adding the culture medium to cement discs previously incubated in SBF for 7 days, placed in a 24-well plates and sterilized by ethylene oxide. The cell suspension was adjusted to a density of 10^5 cells in 0.5 mL of HDMEM and was added to each well of a 24-well plate. As negative control, conventional α-TCP, also incubated in SBF for 7 days was used[19].

The number of viable cells was quantitatively assessed by MTT test. After incubating at 37 °C and 5% CO_2 for 24 and 48 hours, 150 μL of 3 $mg.mL^{-1}$ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added to extract/cell constructs and cultured for 3 hours at 37 °C. Then 100 μL of dimethyl sulfoxide (DMSO) was added to each well, and the product was colorimetrically assessed with a Model Multiskan EX Microplate Reader (Labsystems, USA). Absorbances were read at a wavelength of 540 nm.

Experimental values were analyzed via one-way ANOVA test follow by Tukey's Multiple Comparison Test.

2.6. Characterization techniques

Phase composition of the samples was determined by X-Ray Diffraction (XRD) in a PHILLIPS® diffractometer (X´Pert MPD) and Cu-target. Diffractograms were recorded employing Ni-filtered radiation (λ = 1.5406 Å) with a step size of 0.05° and a time/step ratio of 1 second.

Morphological variations before and after soaking in SBF were characterized by Scanning Electron Microscopy (SEM) using a JEOL microscope (JSM-6060) on gold-coated samples.

Compressive strength (CS) was measured in servohydraulic Universal Testing Machine (MTS 810) with a load measuring cell of 10 kN and a loading rate of 1 mm/min. The number of replicas was n = 10 and Student Multiple Comparison Test was used to compare mean values.

pH measures were carried out during soaking in SBF and lectures were made in an μPA-210 pHmeter at 36,5 °C.

3. Results and Discussion

Figure 1 shows the X-ray of silica powder used in different formulations. The results showed a mixture of different polymorphs in addition to amorphous form; even though that the only stable form under normal conditions is α-quartz and this is the form in which crystalline silicon dioxide is usually encountered. The phases found were: Cristobalite Low (JCPDS 76-0936); Tridymite M Low (JCPDS 76-0894); Quartz (JCPDS 79-1913); SiO_2 (JCPDS 16-0980). The characterization of raw materials α-TCP and C_3S was described in previous works. For α-TCP powder, the presence of approximately 18% β-Ca_3PO_4 (β-TCP), determined by quantitative analysis[19,20], was found in addition to α-TCP phase, due to the presence of Mg^{2+} in raw materials. In the case of C_3S, the most intense characteristic peaks of CaO due to the decomposition of Ca_3SiO_5 were observed[21].

The initial and final setting times of α-TCP and the different composites are shown in Figure 2. The setting times of α-TCP were higher than those reported in the literature for similar materials[22] due

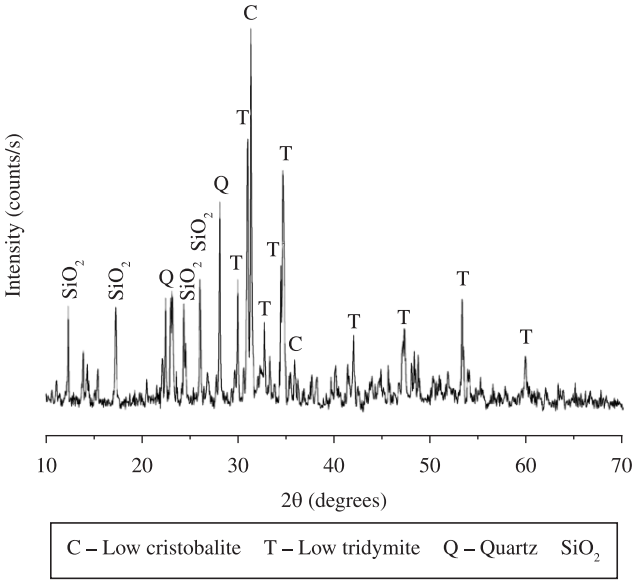

Figure 1. X-Ray diffraction patterns of SiO_2 powder.

to the presence of β-TCP in the starting powder that delays -TCP hydrolysis (Equation 2). When C_3S is added, a significant increase in setting times is observed in relation to traditional -TCP cement (more than 200 minutes for initial setting time and 400 minutes for final setting time) this being directly related to the content of C_3S added[12]. The presence of SiO_2 reduces the setting time of the α-TCP/C_3S compositions with a 5% weight content of C_3S ($5SiO_2$); however, the values obtained are still higher than those for α-TCP cements. Higher additions of SiO_2 increased the setting time and are not suitable for immediate clinical applications[23].

$$3\alpha - Ca_3(PO_4)_{2(s)} + H_2O = Ca_9(HPO_4)(PO_4)_5OH_{(s)} \quad (2)$$

When C_3S is added, the solubility of α-TCP decreases due to the formation of $Ca(OH)_2$ and C-S-H during Ca_3SiO_5 hydrolysis (Equation 3); and setting times increase consequently. The addition of silica reduces the alkalinity of the medium by reacting with the $CaOH_2$ formed (Equation 4), thus the setting times are reduced. Nevertheless, by increasing the amount of added C_3S and SiO_2 ($10SiO_2$), more dense calcium silicate gel (C-S-H) is formed on the

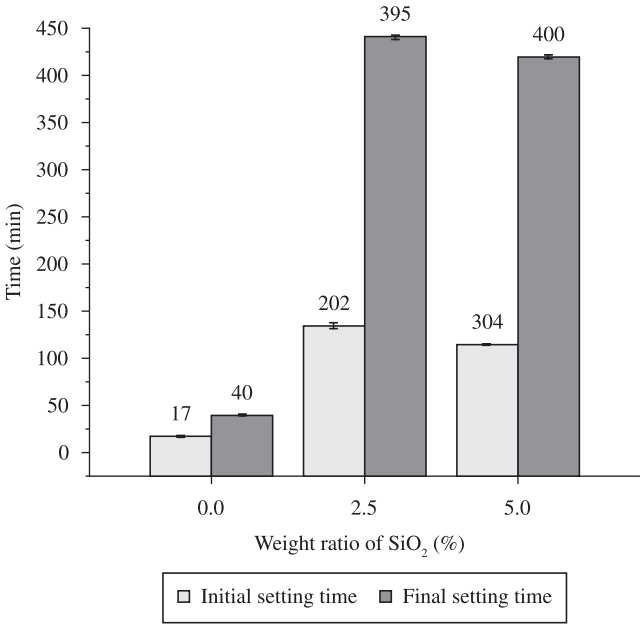

Figure 2. Initial and final setting time of the paste samples with different SiO_2 contents (L/P) = 0.4 mL.g^{-1}).

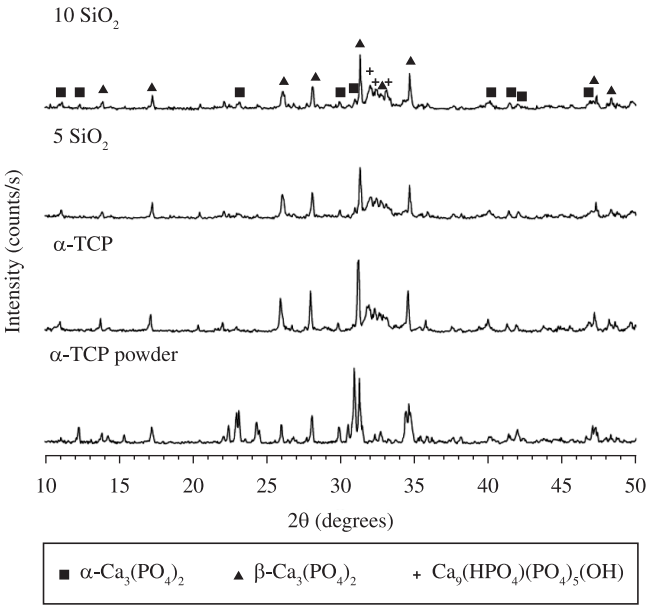

Figure 4. XRD patterns of cements after 7 days in SBF.

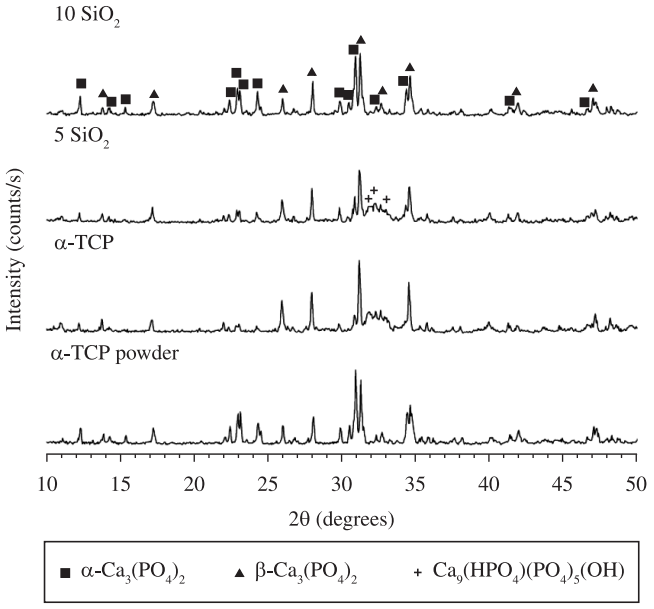

Figure 3. XRD patterns of cements 24 hours set.

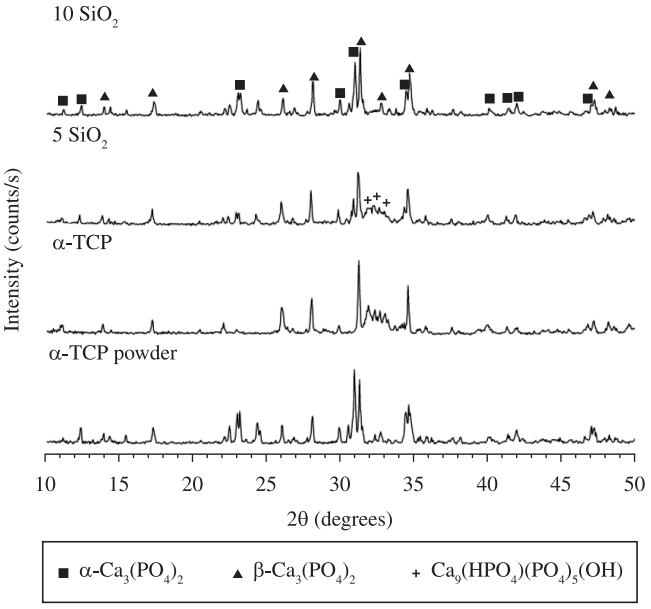

Figure 5. XRD patterns of cements after 14 days in SBF.

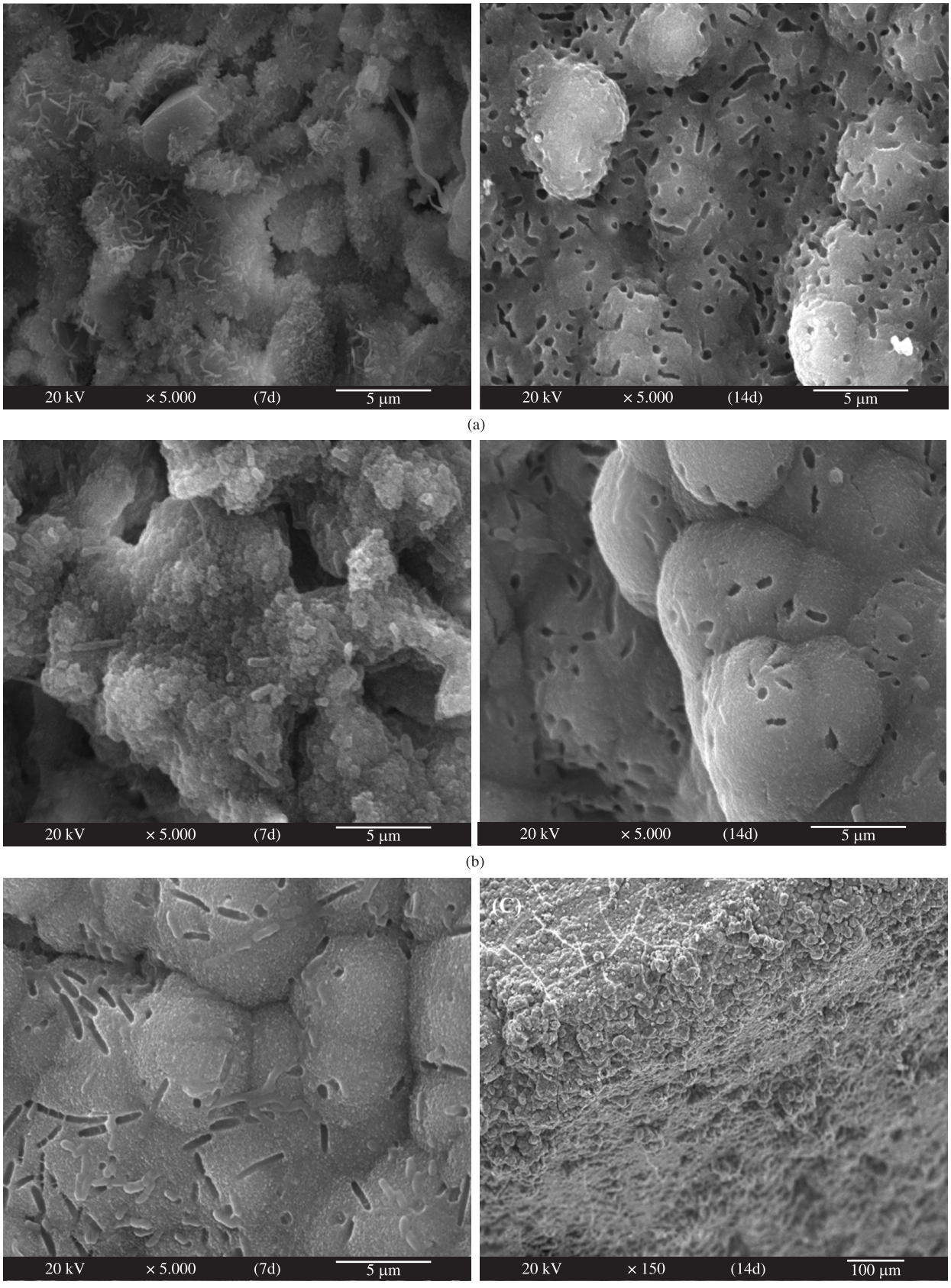

Figure 6. SEM micrographs of surface of a) α-TCP; b) $5SiO_2$; and c) $10SiO_2$ after soaking in SBF for 7 and 14d.

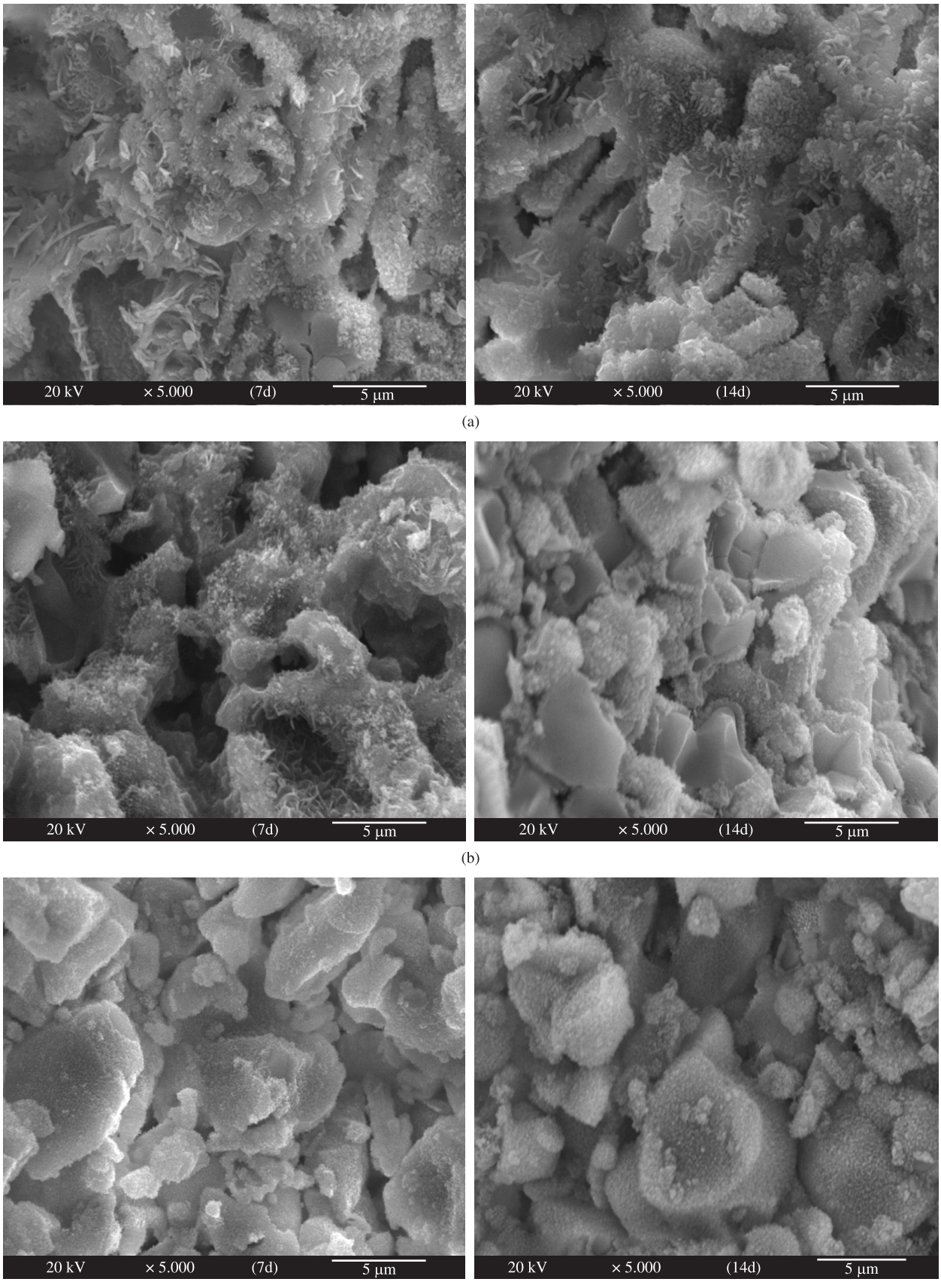

Figure 7. SEM micrographs of fracture surface of a) α-TCP; b) $5SiO_2$; and c) $10SiO_2$ after soaking in SBF for 7 and 14d.

surface of α-TCP particles preventing dissolution of the grains and precipitation of CDHA and increasing the setting times as a result.

$$Ca_3SiO_{5(s)} + 3H_2O = CaO.SiO_2.H_2O_{(gel)} + 2Ca(OH)_{2(s)} \quad (3)$$

$$Ca(OH)_{2(s)} + SiO_{2(s)} = CaO.SiO_2.H_2O_{(gel)} \quad (4)$$

The x-ray diffraction patterns of different composites before and after soaking in SBF are displayed in Figures 3-5. For α-TCP, mainly peaks of CDHA (JCPDS 46-0905) were observed in addition to β-TCP (JCPDS 09-0169) peaks at all soaking times. The β-TCP is considered an impurity in the starting powders as it not involved in the hydration reaction and remains unreacted; this is why their most intense peaks appear with great intensity in all diffraction patterns.

After 24 hours setting (Figure 3), in composites containing SiO_2, the X-ray diffraction peaks of CHDA appeared less intense, and unreacted -TCP (JCPDS 09-0348) were also detected as a result of the delay in the dissolution of the α-TCP particles. The greater the amount of C_3S/SiO_2 added, the greater the intensity of the unreacted α-TCP peaks and the lower the CDHA formed.

A few differences were observed in X-ray patterns of composites after 7 and 14 days of SBF soaking (Figures 4-5). The X-ray spectra of all formulations were very similar and the presence of CDHA, in addition to β-TCP and α-TCP, was observed in all cases. However, owing to the limitations of the technique, it was not possible to differentiate between the hydroxyapatite precipitated from the α-TCP and that obtained by precipitation in SBF, and observations of the surfaces and fracture surfaces by SEM were necessary to clarify this.

On the other hand, the presence of calcium-silicate-hydrate (C-S-H) was not detected, probably due to the low content on the sample and the amorphous character of these hydrates.

The observation by SEM is an effective way to estimate the bioactivity of materials as apatite grains and layers formed have differentiated features[24]; while several authors have considered the materials to be biocompatible judging from the presence or absence of a superficial layer of hydroxyapatite formed after immersion in simulated physiological solutions. Figure 6 shows the SEM images of α-TCP, $5SiO_2$ and $10SiO_2$ after soaking in SBF for 7 and 14 days. For α-TCP cement (Figure 6a) a network of entangled needle-like crystals or petal-like plates, typical for set α-TCP-based calcium phosphate cements, was observed after 7 days of immersion, whereas a layer of bone-like apatite, precipitated under physiological conditions, was detected within 14 days of SBF soaking. Although measures were taken to maintain aseptic conditions, bacterial contamination by Bacillis and Cocci colonies was observed on samples surface[25].

Adding small amounts of SiO_2 produces a bumpy and amorphous appearance after 7 days (Figure 6b) and a homogeneous layer of CDHA with globular shape morphology, typical of bioactive materials after 14 days[26] immersed in SBF. The higher the silicon content in the composite, the shorter the time required for the formation of the surface layer (Figure 6c). No typical features of type I C-S-H[27] were observed for silica additions maybe due to the large quantity of quartz present in the sample, since the SiO_2 modification used has a decisive influence on the processes of formation of calcium silicate hydrates[28].

Different microstructural features can be seen in the fracture surface of different composites (Figure 7). Since early stages, α-TCP shows the typical petal-like plates covering the biggest grains and the growth of these plates with time, at the expense of smaller grains, within the interstices of fracture surface (Figure 7a). With low contents of SiO_2 (Figure 7b) incipient precipitation of some CDHA crystals can be seen; crystals that grow with soaking time enclosing the larger α-TCP grains. For further additions of SiO_2 (Figure 7c), no significant differences with soaking time were found and the cross section appeared covered or speckled with small distinct features that could be identified as small crystals of dry C-S-H[29]. No needle-like crystals or petal-like plates were observed ascribed to the lack of time for CDHA growth to occur besides insufficient time and conditions for the material to react completely.

With the presence of C_3S and SiO_2, the $HSiO^{3-}$ ions are released during the hydration of the composite paste, acting as sites for nucleation of apatite crystals and hence accelerating the deposition of apatite on the surface[30].

Even though it was possible to reduce pH values with the addition of SiO_2 (Figure 8), the compressive strength did not improve. After addition of SiO_2, the values of compressive strength were inferior to 1MPa, even after 7 days of soaking in SBF (Figure 9), and only for $5SiO_2$, similar values to those of α-TCP cement were achieved after 14 days.

The increase in mechanical strength of the cement occurs as a result of the formation of the interlocking of hydroxyapatite crystals precipitated after α-TCP solubilization and in general, increases with immersion time in SBF[31]. However, since the particles of SiO_2 act as nucleation sites for C_3S hydration and can cause blockage of the pores, which densifies the hydrating gel structure, α-TCP dissolution and precipitation are delayed owing to the formation of a dense calcium silicate on the surface of α-TCP particles, hence causing the initial low compressive strength. Moreover, although less significant, the increase of porosity caused by the degradation of the materials in early stages, also contributes to the decrease observed. For $5SiO_2$, with the increment in soaking time, and the advancement of the hydration reaction and crystal growth of CDHA, the compressive strength augmented.

The implantation of materials into living tissue causes wound and foreign body reactions. To predict the possible harmful effects of materials on the surrounding host tissues, it is important to gain information on the degradability of the implanted materials[32]. The relationship between the weight loss rate of the composites and the immersion time in SBF (Figure 10) shows that the weight of α-TCP and $5SiO_2$ increased with time, whereas the weight of the composite cement decreased over time when the SiO_2 content in the composite was 5% ($10SiO_2$). Moreover, adding SiO_2 produces an increase in the degradation rates of composites; however, the degradation rate of $5SiO_2$ sample was lower than those of the composites containing only C_3S[12] and the behavior observed was very similar to those of traditional CPCs.

Degradability of some CPCs is very slow both in vivo and in vitro[33] and conventional CPCs usually often experience a mass gain after long soaking periods as a result of hydrolysis of α-TCP or due to the formation of apatite on the surface of the samples. Considering the fact that the degradability is primarily governed by the chemical composition, the reason for the higher degradation rate of the α-TCP/C_3S/SiO_2 composites could be the higher solubility of the C-S-H as compared with CDHA. In addition, the dissolution of C-S-H and/or other phases could be larger than the amount of apatite deposited on the cement surface and formed due to the hydrolysis of α-TCP, so the weight of samples decreased.

It is generally accepted that the in vitro cell-material interaction is a useful criterion in the evaluation of new biomaterials. Figure 11 shows the result of direct cytotoxicity test for composites against the PBMCs after incubation for 24 and 48 hours. Although differences were no statistical significant, composites containing SiO_2 seem to be more cytotoxic than traditional α-TCP. Furthermore, it was found that the viability of PBMCs showed a tendency towards a decrease in all compositions with aging probably due to the occurrence of

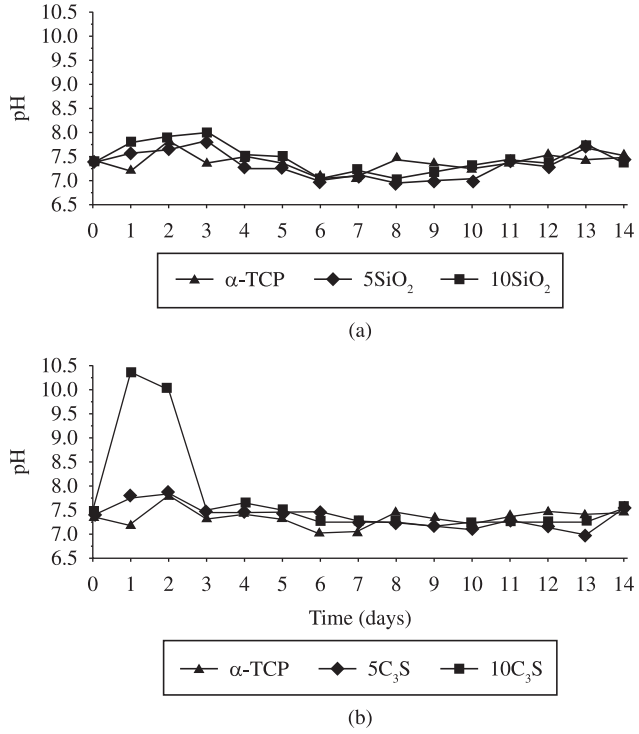

Figure 8. Changes in pH value of SBF soaked with a) α-TCP/C_3S; and b) α-TCP/C_3S/SiO_2.

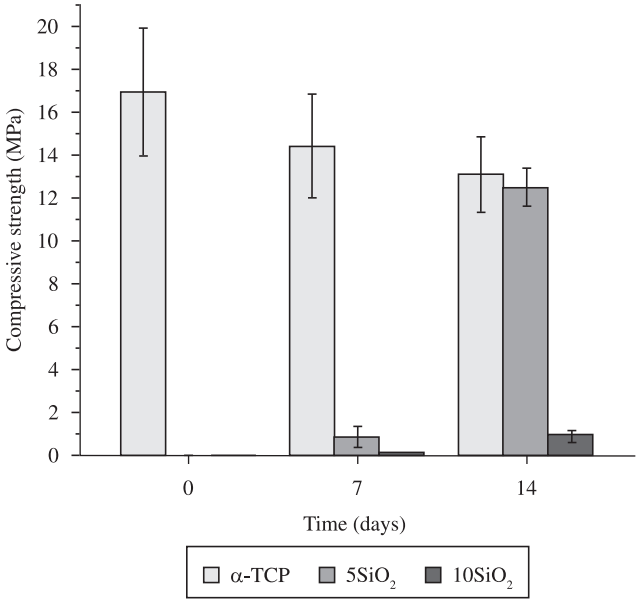

Figure 9. Compressive strength of the α-TCP α-TCP/C_3S/SiO_2 composites after soaking in SBF.

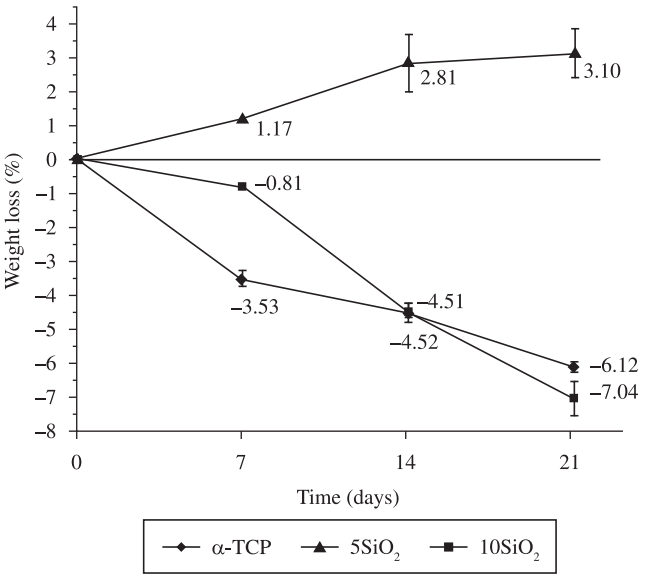

Figure 10. Weight loss of the α-TCP and α-TCP/C_3S/SiO_2 composites after soaking in SBF.

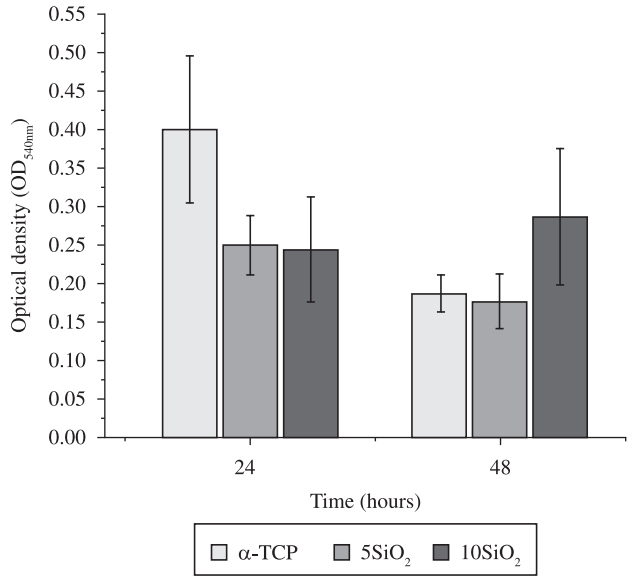

Figure 11. Cell viability of PBMCs on the different pastes after culturing for 24 and 48 hours.

some chemical transformations of the material in culture medium since after 7 days in aqueous media the pastes continues hydrating.

When only C_3S is added, compositions are less cytotoxic and more compatible than pure α-TCP-based cement as a result of the dissolution of silicate ions present in α-TCP/C_3S pastes that stimulate cell proliferation[34,35]. With the presence of SiO_2, more C-S-H is formed and more $HSiO^{3-}$ is released during hydration which can accelerate the hydroxyapatite deposition on the surface and enhance biocompatibility. However, contrary to our expectations, the results showed a fall in cell viability maybe due to the higher solubility of compositions and the poor cohesion of the cement in the presence of SiO_2 which may have masked the actual results.

4. Conclusions

The addition of SiO_2 reduces local pH during C_3S hydrolysis and accelerates the hydration of C_3S; however, it does not produce a noteworthy increase in compressive strength compared to conventional CPCs in the early stages as a result of the suppression of α-TCP dissolution. Furthermore, the presence of SiO_2 increases the

degradability of cements and does not improve the biocompatibility of materials by reducing the cytotoxicity. Nevertheless, the α-TCP/C_3S/SiO_2 composites possess excellent bioactivity, as indicated by the early formation of bone-like apatite in SBF, being attributed to the presence of C_3S and not of SiO_2.

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