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Silicate and borate glasses as composite fillers: a bioactivity and biocompatibility study

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Abstract Composites filled with a silicate glass (CSi) and a new borate glass (CB) were developed and compared in terms of their in vitro behaviour both in acellular and cellular media. Acellular tests were carried out in SBF and the composites were characterized by SEM-EDS, XRD and ICP. Biocompatibility studies were investigated by in vitro cell culture with MG-63 osteoblast-like and human bone marrow cells. The growth of spherical calcium phosphate aggregates was observed in acellular medium on all composite surfaces indicating that these materials became potentially bioactive. The biological assessment resulted in a dissimilar behavior of the composites. The CSi demonstrated an inductive effect on the proliferation of cells. The cells showed a normal morphology and high growth rate when compared to standard culture plates. Contrarily, inhibition of cell proliferation occurred in the CB probably due to its high degradation rate, leading to high B and Mg ionic concentration in the cell culture medium.

1 Introduction

Acrylic polymers have been extensively used in orthopaedic and dental applications as filling and fixing agents [1]. However, a long-term problem associated with this material is the formation of fibrous tissue at the bone-cement interface which may compromise the fixation of the prosthesis [2]. Thus, over the years, a lot of research effort has been put into optimization of the properties of these materials [1, 3, 4].

The main condition for a synthetic material to form a stable bond with the bone is the precipitation of an apatite layer on its surface, which is responsible for its bioactivity [5]. Several compositions of glasses may promote this behaviour and they can be used to improve the bone bonding capability of inert matrices like PMMA.

According to previous work, the formation of hydroxyapatite (HA) seems to be induced by functional groups existing on material surface, such as Si–OH [6]. So, it is accepted and well characterized that glasses like 45S5 (Bioglass®) form a silica-rich gel layer (Si–OH), through ion exchange reactions with the physiological medium followed by precipitation of calcium and phosphate ions, resulting in a bioactive material, which enhances bonding capability with bone and soft tissues [7–9].

Recent researches demonstrated that borate-based glasses derived from the 45S5 glass by fully or partially replacing the SiO_2 with B_2O_3, can be converted into hydroxyapatite when placed in dilute phosphate solution, being the conversion rate to HA more rapid for the glasses with higher B_2O_3 content [10, 11]. It is believed that the formation of apatite on B-based glasses follows a set of dissolution-precipitation reactions similar to those of a Si-based glass, but the silica gel layer is absent [10].

The biological performance of the B-based glasses has been addressed in previous studies. In vivo, particles of a boron-modified 45S5 glass containing 2 wt% of boron oxide, implanted in rat tibia bone marrow, promoted new bone formation and a significant increase of the thickness

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of osseointegrated tissue when compared with control 45S5 glass [12]. Also, in vitro, boron-modified 45S5 glass containing varying amounts of B_2O_3, tested as-prepared and partially converted to HA (by soaking in a K_2HPO_4), allowed the proliferation of osteoblast cells, although inhibition of cell growth was observed for the glasses with higher B_2O_3 content, especially for the as-prepared samples and tested in static conditions [13]. In this context, it seems useful to investigate new materials and expand the range of glass compositions available for use in biological applications and the B-based glasses might be a promising material for biological applications.

In terms of filler, innovative studies published by the present group showed that some glasses of the $3CaO{\cdot}P_2O_5$–MgO–SiO_2 system have the potential to be used as biomaterial [14] and the high MgO content in the composition of these glasses does not hinder their apatite forming ability [15, 16]. Therefore further investigation into its biological behaviour will be of great interest, since the effect of this filler on the proliferation of cells has not been disclosed.

In this context, the main purpose of the present work is to analyse the behaviour of PMMA-co-EHA composites filled with silicate (CSi) and new borate (CB) glasses, in acellular and in cellular media, regarding their capability for calcium phosphate formation and osteoblast cell proliferation and differentiation. The proposed borate glass composition was prepared by replacing all the silicate of the silica based glass by borate, thus becoming to our knowledge the only B-based glass with addition of MgO, for biomedical application, found in the literature. Bioactivity of the composites was assessed in SBF and cytocompatibility studies were performed firstly with the osteoblast-like MG63 cell line for a rapid screening assay and, afterwards, with human bone marrow cells to assess the performance of the material regarding osteoblastic proliferation and differentiation events.

2 Materials and methods

2.1 Preparation of the glasses

Two different glass compositions were used in the experiments. A silicate glass composed of (mol%) 38% CaO, 12.7% P_2O_5, 24.8% MgO, 24.5% SiO_2 and a borate glass, which consists of a similar composition where SiO_2 was entirely replaced by B_2O_3. The glasses were prepared through the classic melt-quenching method and the resultant glass frit was dry-milled (Retsch, RM100 Mortar Grinder Mill) to a powder with mean particle size of 10 μm, measured with a Coulter LS Particle Size Analyzer. The amorphous character of the glasses was confirmed by X-ray diffraction (XRD, Rigaku Geigerflex Dmax-C with CuKα radiation).

2.2 Preparation of the composites

PMMA/EHA/Glass composites in a ratio (wt%) of 25:25:50 respectively, were synthesized and compared according to the glass composition [16]. Methylmethacrylate (MMA) and ethylhexylacrylate (EHA) were obtained from Aldrich Chemical Company and Merck supplied benzoyl peroxide (BPO). All the reagents were used as received. The composites were prepared by free radical polymerization at 80°C for 24 h and no activating agent was used. The monomers were first mixed in a glass recipient, afterwards BPO (polymerization initiator) was dissolved in this liquid mixture and finally the glass was incorporated into the mix. It was poured into a Teflon mould where polymerization took place. Samples of $5 \times 5 \times 3$ mm^3 were prepared for the bioactivity and biocompatibility studies, and sterilized by 70% alcohol.

2.3 In vitro bioactivity

In order to evaluate the in vitro bioactivity and compare the degree of apatite formation on the composites, specimens were mounted vertically and soaked in simulated body fluid (SBF) at physiological conditions of temperature and pH, respectively, 37°C and 7.4. The SBF solution was prepared according to the formulation of Kokubo and Takadama [17], with ion concentrations nearly equal to those of human blood plasma (Table 1). This solution was previously filtered through a Milipore 0.22 μm system and it was used a constant specimen surface area to solution volume ratio of 0.1 cm^{-1}. The materials were soaked for periods of 1, 3, 7, 14 and 21 days. After immersion the samples were removed from the fluid and their crystallinity, morphology and surface modification were followed by X-ray diffraction (XRD) and scanning electron microscopy coupled with X-ray energy dispersive spectroscopy (SEM-EDS, Hitachi S-4100, Japan) at an acceleration voltage of 25 keV and beam current of 10 μA. The solution was characterized by inductively coupled plasma spectroscopy (ICP, Jobin Yvon, JY70 Plus) to measure ionic concentration and pH was evaluated at the different times.

Table 1 Ionic concentrations (mM) of SBF and human blood plasma

	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
Plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5

2.4 Biocompatibility studies

2.4.1 MG63 osteoblast-like cells

The MG63 cell line, originally derived from a human osteosarcoma, has shown numerous osteoblastic features, being largely used in biocompatibility tests [18]. These cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air, in α-Minimal Essential Medium (α-MEM) containing 10% fetal bovine serum, 50 μg/ml ascorbic acid, 50 μg/ml gentamicin and 2.5 μg/ml fungizone. For subculture, PBS (phosphate-buffered saline) was used to wash the cell monolayer twice, which was then incubated with trypsin—EDTA solution (0.05% trypsin, 0.25% EDTA) for 5 min at 37°C to detach the cells. Cells were resuspended in culture medium and cultured (2×10^4 cell cm^{-2}) for 7 days in standard polystyrene culture plates (control) and on the surface of the composites. The medium was changed every 2–3 days. Control cultures and seeded material samples were evaluated at days 1, 3, and 7 for cell viability/proliferation and observed by confocal laser scanning microscopy (CLSM; Leica SP2 AOBS).

2.4.2 Human bone marrow cells

Human bone marrow, obtained from orthopaedic surgery procedures (after patient informed consent), was cultured in the same experimental conditions as those used in the culture of MG63 cells. Primary cultures were maintained until near confluence (10–15 days) and, at this stage, adherent cells were enzymatically released (trypsin–EDTA solution). The cells were seeded at a density of 2×10^4 cell/cm^2 in control conditions (standard plastic culture plates) and on the surface of the composites. Control cultures and seeded material samples were cultured for 21 days in the presence of 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone, experimental conditions reported to allow the osteoblast differentiation in this culture system [19]. All the experiments were performed in the first subculture, since the sequential passage of bone marrow cells results in a progressive loss of the osteoblastic phenotype [20]. Control cultures and colonized composites were evaluated throughout the culture time for cell morphology, cell viability/proliferation, alkaline phosphatase (ALP) activity and ability to form calcium phosphate deposits, as follows.

2.4.3 Cell viability/proliferation

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a simple colorimetric method to measure cytotoxicity and viability/proliferation, first developed by Mosmann [21]. This method is based on the capacity of viable cells to metabolize tetrazolium salt by forming purple formazan crystals, which can be dissolved and quantified by measuring the absorbance of the solution at 600 nm. Cultures were incubated with 0.5 mg/ml of MTT in the last 4 h of the tested culture period; the medium was then decanted, formazan salts were dissolved with 200 μl of dimethylsulphoxide and the absorbance was measured in an ELISA reader. Results were compared in terms of macroscopic surface area and expressed as Acm^{-2}.

2.4.4 Alkaline phosphatase activity

Alkaline phosphatase is a glycoprotein that participates in processes leading to mineral formation in tissues like bone [22]. ALP activity was determined in cell lysates (obtained by treatment of the cultures with 0.1% triton in water) and assayed by the hydrolysis of p-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, and colorimetric determination of the product (p-nitrophenol) at $\lambda = 405$ nm: Hydrolysis was carried out for 30 min at 37°C. Results are expressed in nanomoles of p-nitrophenol produced per min per μg of protein (nmol min^{-1}/μg protein).

2.4.5 SEM and CLSM microscopy

The samples for SEM observation were fixed in 1.5% glutaraldehyde in 0.14 *M* sodium cacodylate buffer (pH 7.3), then dehydrated in graded alcohols, critical-point dried, sputter-coated with gold and analysed in a JEOL JSM 6301F scanning electron microscope equipped with a X-ray energy dispersive spectroscopy (EDS) microanalysis capability (voyager XRMA System, Noran Instruments).

For CLSM assessment, the samples were fixed in 3.7% paraformaldehyde (10 min). Cell cytoskeleton filamentous actin (F-actin) was visualized by treating the cells with Alexa Fluor®0 488 Phalloidin (1:20 dilution in PBS, 1 h) and counterstaining with propidium iodide (1 μg ml^{-1}, 10 min) for cell nuclei labelling. Labelled cultures were mounted in Vectashield® and examined with a Leica SP2 AOBS (Leica Microsystems) microscope.

2.5 Statistical analysis

Values are expressed as mean ± standard deviation (SD) of three replicates and were compared using the student's *t*-test, with a significance level of $p < 0.05$.

3 Results and discussion

3.1 In vitro bioactivity

Bioactivity of CSi and CB composites was assessed in SBF, which is widely employed as synthetic plasma, unlike

most of the reported studies regarding the bioactivity of the B-based glasses [10, 11, 13, 23], that used a 0.02 M K_2HPO_4 solution (with a phosphate ions concentration approximately 20 times that of the human blood plasma).

SEM images of CSi and CB composites after immersion in SBF for various periods are shown in Fig. 1. Similar results were obtained for the two composites, prepared with different glass compositions, clearly showing the precipitation of a surface layer on both.

As seen in Fig. 1 the initial samples (before immersion) were constituted by the glass particles dispersed in the polymeric matrix. This morphology changed with the soaking time and, after 3 days in SBF, the spherical precipitates began to grow and partially cover the surface of the composites. After 7 days, the composites were covered with a homogeneous layer of precipitates which consisted of numerous needle-like crystalline aggregates characteristic of hydroxyapatite [24]. With further increase in the soaking time, there was no significant change in the surface morphology, and a layer identified as calcium phosphate could still be observed after 14 and 21 days (not shown).

The surface chemical analysis was carried out by EDS in SEM and the patterns of the composite surface composition before and after soaking in SBF are depicted in Fig. 2. The obtained results for 0 days confirmed that the elements that constitute the composites before immersion are in agreement with the glass composition. The CB composite revealed the presence of Ca, P and Mg (B is not detectable by EDS) and for CSi, besides these same elements, Si was also identified. The surface of composites CB and CSi exhibited an increase in Ca and P concentration and a corresponding decrease of the Mg and Si signal (when it is

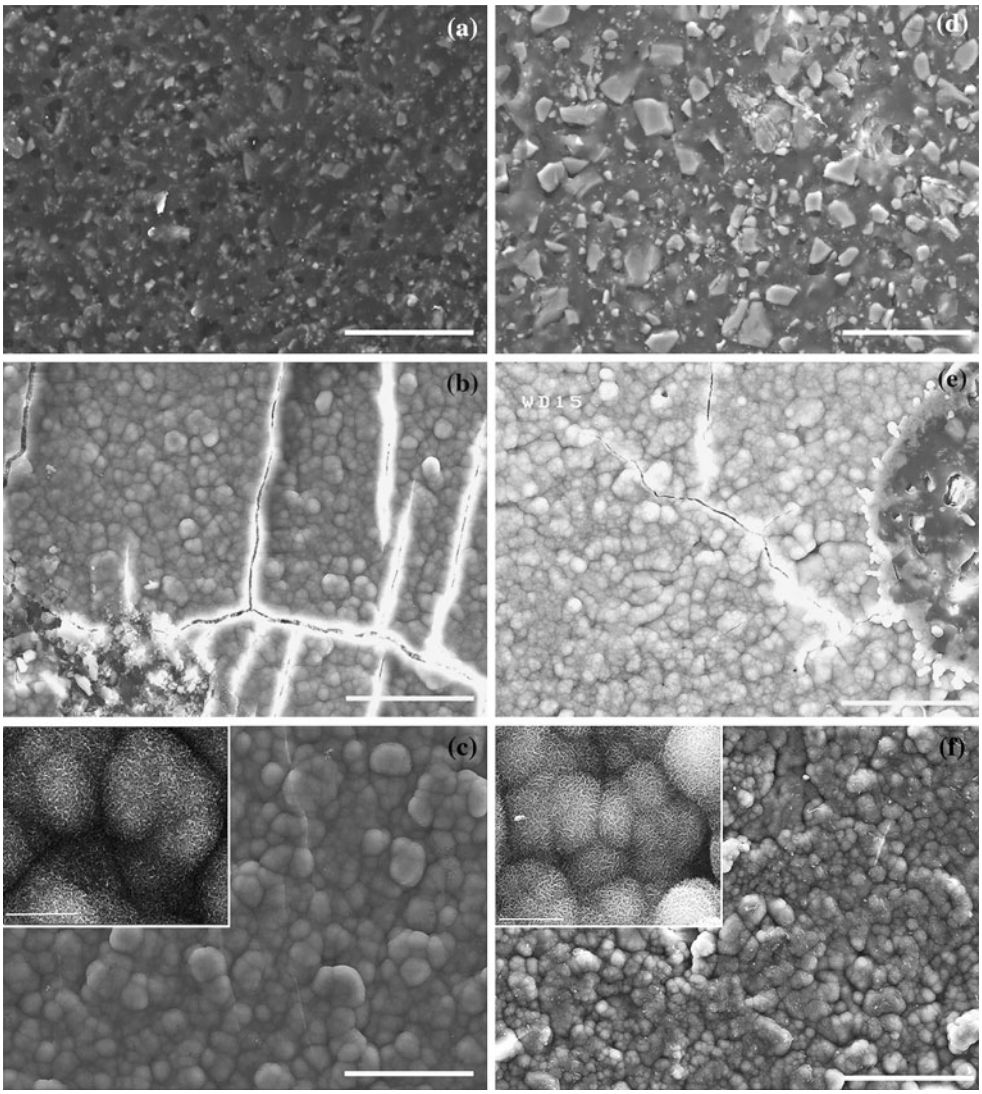

Fig. 1 SEM micrographs, for the CSi composite **a** before immersion, **b** after 3 days and **c** after 7 days in SBF. For CB, **d** before immersion, **e** after 3 days and **f** after 7 days. *Bars* 60 μm (*inset*: *bar* 6 μm)

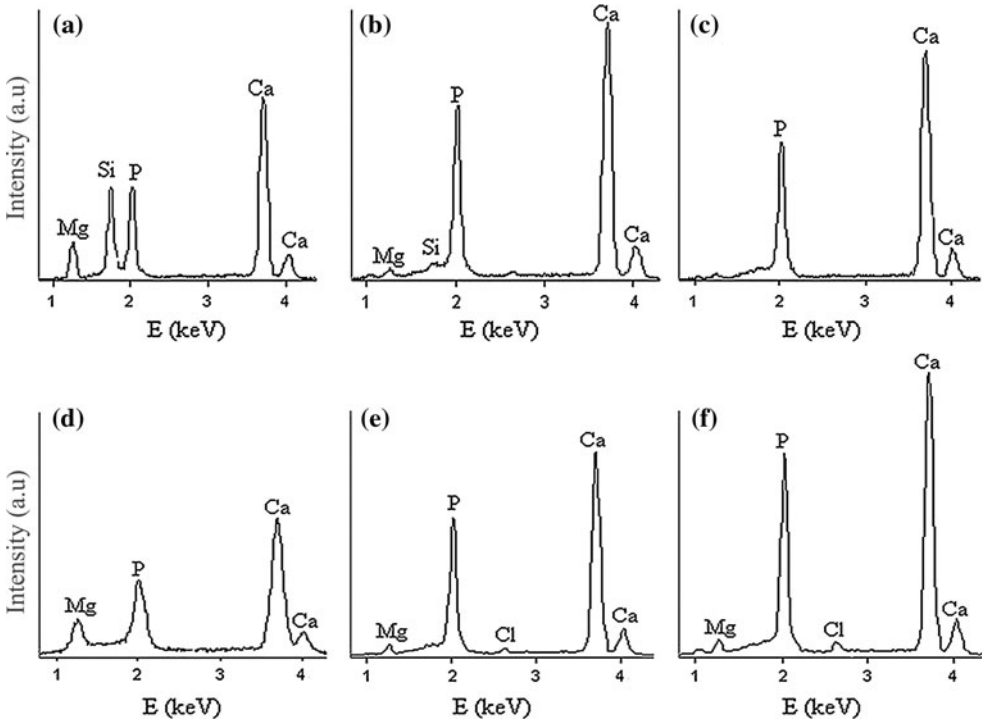

Fig. 2 EDS results, for CSi **a** before immersion, **b** after 3 days and **c** after 7 days. For CB **d** before immersion, **e** after 3 days and **f** after 7 days

present) after 3 days of immersion, evidencing a calcium phosphate formation. Furthermore, for the CB composite the presence of a small amount of Cl ions incorporated in the mineral phases was detected, probably coming from the SBF. The surface analysis of the composite CSi, for 7 days, only showed the occurrence of Ca and P, and the Si and Mg signal disappeared. For CB, after 7 days, the spectrum was similar to earlier time. The measured Ca/P molar ratio for this immersion time was 1.69 for CSi and 1.57 for CB.

XRD patterns of composites after the several soaking periods are illustrated in Fig. 3. The composites without immersion showed typical spectra of amorphous phase and absence of detectable crystalline phase. Peaks accusing crystallinity of the surface precipitates began to appear after 3 days of immersion and sharpened for the longest period. The results indicate that a calcium phosphate was deposited on the surface of CSi, being identified the peaks characteristic of hydroxyapatite at $2\theta = 26^\circ$, 32°, 50° and 53° attributed to reflections (0 0 2), (2 1 1), (2 1 3) and (0 0 4) respectively [25]. The intensity of these peaks increased with soaking time, due to the growth, on the composite surface, of an apatite layer of enhanced crystallinity with time. The CB spectrum revealed the presence of Mg-substituted tricalcium phosphate phase (whitlockite) together with the apatite phase in the newly formed layer. After 3 days, the peaks of diffraction for $2\theta = 28^\circ$, 31° and 35° were assigned to the reflection (2 1 4), (0 2 10) and (2 2 0) of whitlockite. The apatite phase was detected after 7 days immersion through the peaks at $2\theta = 26^\circ$, 32°, 53°. The precipitation of two phases (apatite and whitlockite) was also identified on the surface of sol–gel glasses containing Mg when exposed to SBF [26, 27]. When magnesium is incorporated into the atomic structure of HA, the central calcium atom is substituted by magnesium. Since the ionic radius of Mg^{2+} (0.69 Å) is considerably smaller than that of Ca^{2+} (0.99 Å), the placement of Mg into Ca position distorts the HA structure, resembling more to whitlockite [28]. Prolonged soaking time of the composite arose the apatite phase herewith whitlockite.

Variation of ions concentration and pH in SBF solution versus soaking time of the composites are presented in Fig. 4. For CSi composite, the release of Ca and P occurred during the first period of immersion due to the dissolution of the glass followed by their consumption for the growth of calcium phosphate layer, resulting in the observed decrease in the concentrations of these ions in the solution. The Mg and Si concentration slightly increased as a result of glass dissolution and ionic exchange with the solution. For CB composite, the release of Ca and P showed a similar profile to CSi indicating that these ions are required for the build up of the calcium phosphate layer on the composite. The Mg and B concentration in solution increased continuously up to 14 days, reaching values much higher than those regarding CSi. The less cohesive structure of borate glass compared to the silicate glass can be responsible for its higher degradation rate. Despite the high dissolution of the borate glass, the pH did not change significantly until the end of the test. The elevated Mg concentration in SBF for this composite can explain the formation of both apatite and whitlockite phases detected

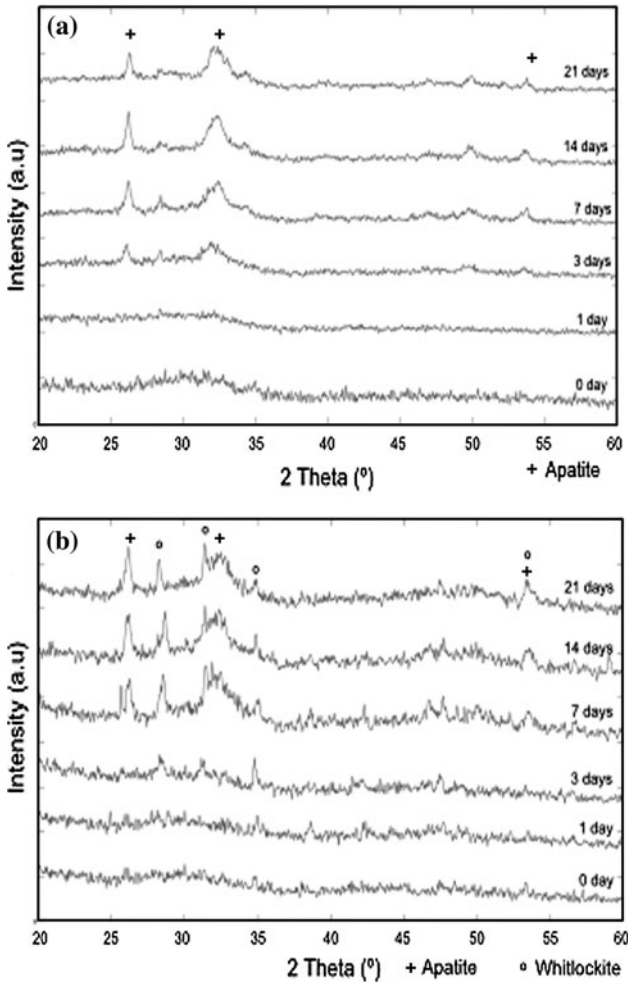

Fig. 3 DRX patterns of composite **a** CSi and **b** CB

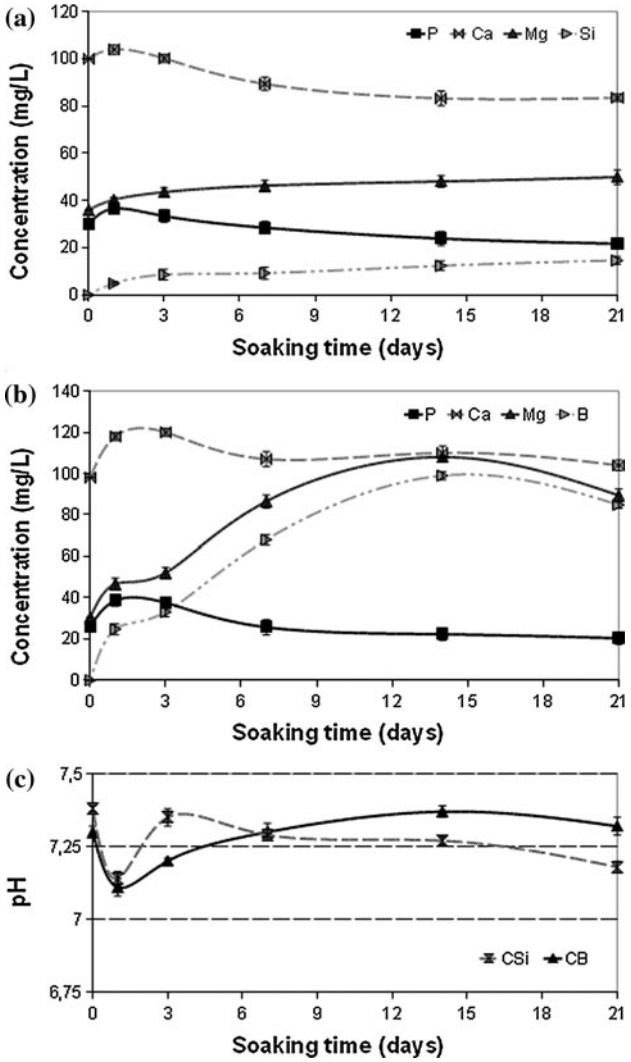

Fig. 4 Variation of ionic concentration in SBF due to immersion of **a** CSi and **b** CB, and **c** pH evolution with time

by XRD and consequently the presence of its signal in EDS for all soaking periods. For other bioactive materials it is reported that when the Mg/Ca molar ratio of the solution is higher than 0.05, a Mg-substituted TCP is formed [29].

3.2 Biological assessment

As-prepared CSi and CB composites were seeded with MG63 osteoblast-like cells for a preliminary and quick screening. Results are presented in Fig. 5. Regarding the MTT assay, control cells, seeded in standard polystyrene culture plates, presented a lag phase followed by an increase in the cell proliferation, especially from days 3 to 7. Comparatively, seeded CSi displayed significantly higher values at day 1, suggesting that a higher number of cells attached to the composite, and, afterwards, cells proliferated with a similar growth rate, resulting in higher MTT reduction values throughout the entire culture time. By contrast, seeded CB composite presented low MTT values at day 1, and, following, cell proliferation increased slowly during the culture time. CLSM observation of the cultures is in line with the MTT assay. At day 7, seeded CSi was completely covered by a continuous and thick well-organized cell layer, whereas CB showed only few and altered attached cells.

In addition, CSi and CB composites were seeded with human bone marrow cells and were cultured in conditions known to favour osteoblast differentiation [19]. Observation of the seeded materials by CLSM (Fig. 6) showed that, following cell plating, cells attach to the material surface within minutes. Over CSi, at 1 h, attached cells showed varying degrees of cytoplasm expansion, displaying an elongated morphology at 24 h with a well defined nucleus and F-actin cytoskeleton. Cells proliferated throughout the culture time and, at day 21, the material surface was covered with a continuous and well organized cell layer. The MTT assay, Fig. 7 confirmed this behaviour, i.e., an

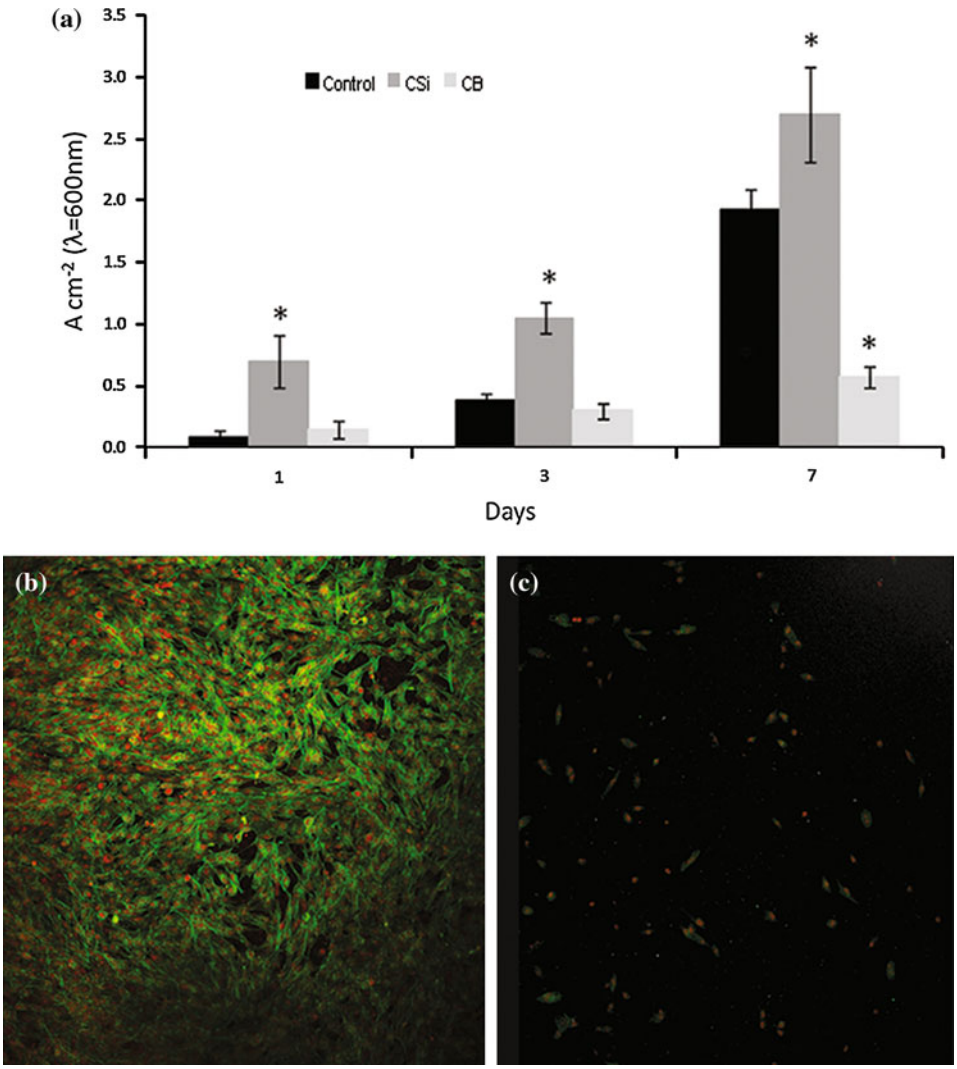

Fig. 5 Behaviour of MG63 osteoblast-like cells cultured up to 7 days over CSi and CB composites. **a** Cell viability/proliferation, estimated by MTT assay, (*) Significantly different from control culture. CLSM images at 7 days, on **b** CSi and **c** CB

increase in the cell viability/proliferation during the culture time. In addition, cells presented a high ALP activity, which increased significantly during the third week suggesting that the growing cells were engaged with an osteoblast differentiation process [20, 30]. Accordingly, SEM observation of colonized CSi composite at day 21 showed a thick cell layer with a fibrillar matrix and associated calcium phosphate mineral deposits, Fig. 8, a proof of the complete expression of the osteoblast phenotype [19]. By contrast, cells cultured over CB composite showed signs of deleterious effects regarding the cell adhesion process, reflected by a low number of attached cells at 1 h and impaired cytoplasm expansion at 24 h. Cell proliferation was also impaired with only small cell clusters scattered over the surface at day 21. ALP activity was also lower than that on CSi, and matrix mineralization did not occur. Results for CB composite are shown in Figs. 6, 7, 8.

As mentioned before, CSi and CB composites were seeded "as-prepared" and the differences regarding the biological performance might be related to the behaviour of the composites following cell culture. Exchange reactions between the material surface and the culture medium such as dissolution/precipitation reactions along with the simultaneous adsorption of biologically active molecules such as peptides and proteins creates a specific microenvironment that can positively or negatively influence cell adhesion and the subsequent proliferation and differentiation events [31–33]. Results reported in the previous section showed a different behaviour of CSi and CB following immersion in SBF, suggesting that differences in the chemical composition and dissolution of the glasses have an important impact on cell growth.

The improved biological performance of CSi compared with CB is related with its lower degradation rate, which is directly correlated to the chemical composition and structure features of the glass used as filler in percentages of 50 wt%. The structure and network connectivity strongly determine the solubility and bioactivity of the glass

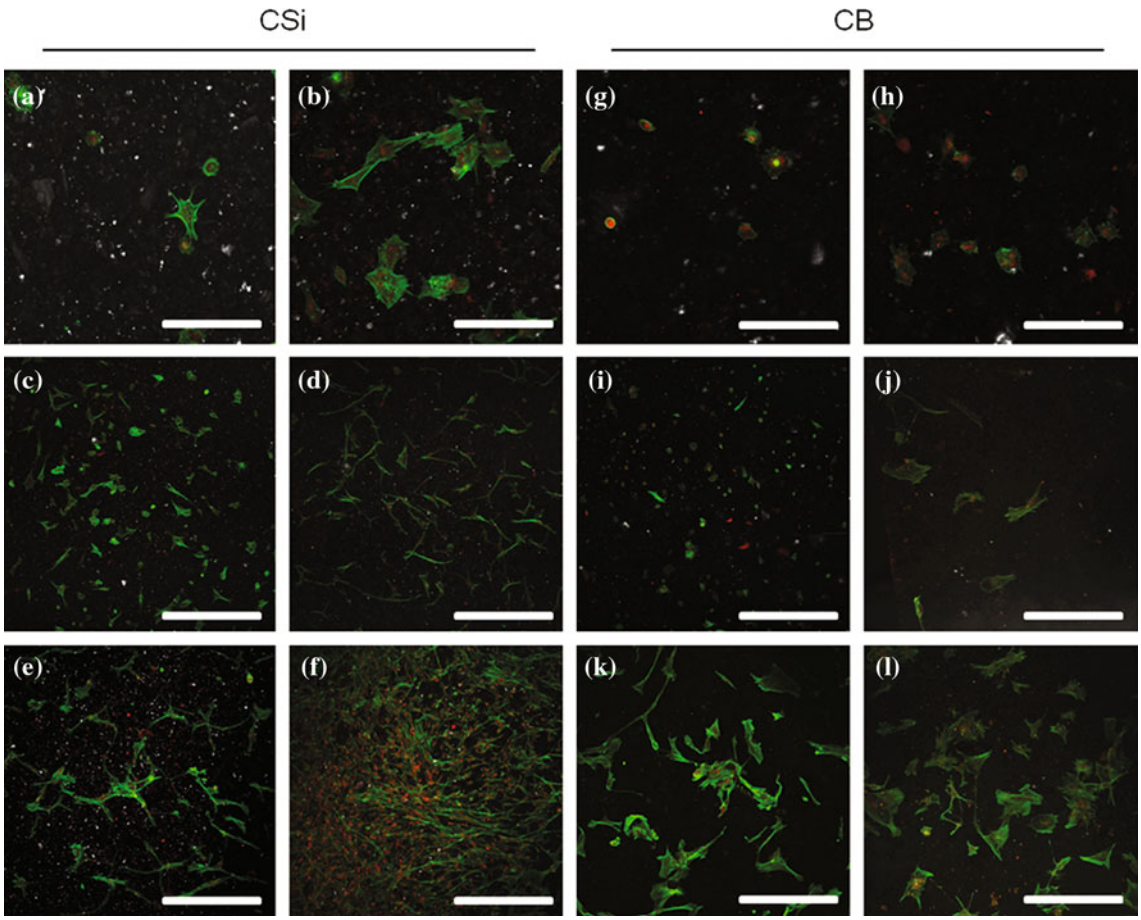

Fig. 6 CLSM observation, for CSi composites seeded with human bone marrow cells and cultured for **a** 1 h, **b**, **c** 24 h, **d** 7 days, **e** 14 days and **f** 21 days. For CB composite **g** 1 h, **h**, **i** 24 h, **j** 7 days, **k** 14 days and **l** 21 days. **a**, **b**, **g** and **h** *bar* 60 μm; **c**–**f** and **i**–**l** *bar* 500 μm

showing that the adhesion and proliferation of the cells on the composite filled with silicate glass is favoured. In addition, the positive effect of CSi on cell behaviour may also be related to the release of silicon, which plays an important role in physiological process during the growth and development of the bone, acting on the proliferation and differentiation of osteoblasts [34]. The levels of the released Si (20 ppm) are within a wide range, between 0.1 and 100 ppm, which leads to a dose dependent increase of human osteoblast-like cells' proliferation and differentiation, in short-term cultures [35], and within the variable physiological range in humans [36].

In the CB composite, the structure of the borate glass is less cohesive and therefore more soluble, resulting in two apparently competing effects: the formation of calcium phosphate layer improving biological performance, and the high release of ions into the cell culture medium causing a greater inhibition of cell proliferation. As suggested by the results found in SBF, B and Mg ions in the culture medium released from CB may attain levels high enough to cause cytotoxicity. Previous in vitro works demonstrated that borate glass resulted in a greater inhibition of cell proliferation under static culture conditions, if the boron concentration was above a certain threshold value [13, 23]. Although it has already been reported the whitlockite stimulation of cell proliferation and the synthesis and secretion of collagen [37], the ICP measurements showed an abnormal concentration of magnesium, which together with B may result in a negative effect on cell proliferation. Results provided in vitro evidence of poor biocompatibility for this composite. However, it is believed that, in vivo, the effects of ion release might be less severe than those seen in the cell cultures experiments performed under static conditions. The body fluids represent a more dynamic system in which the local chemical changes are attenuated by metabolic processes and by the continuous circulation, preventing the excessive level of ions at the interface cell/material. Moreover, in living body, upon the implantation of a material in the bone tissue, there is a continuous availability of osteoblast progenitor cells that can adhere to the material surface when the appropriate sets of conditions are met. These factors most probably will increase the performance of the CB composite.

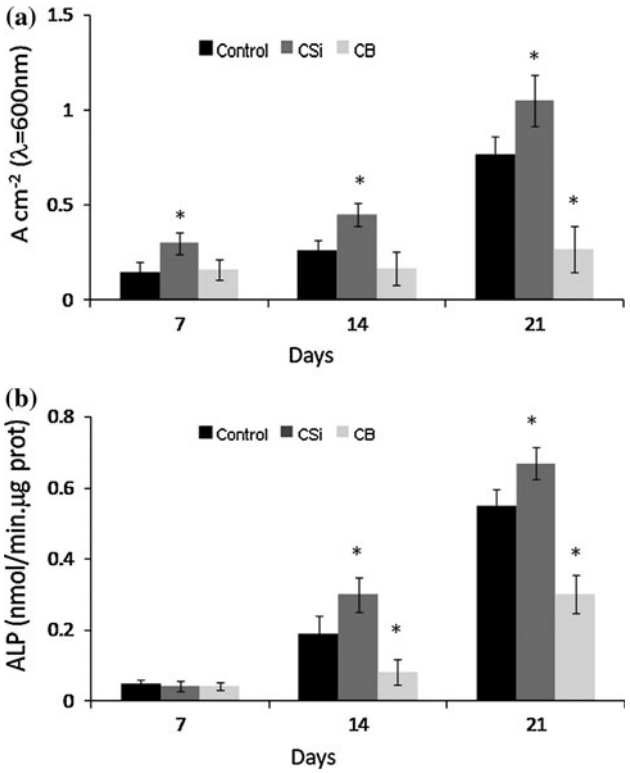

Fig. 7 Cell viability/proliferation by MTT assay (**a**), and alkaline phosphatase activity (**b**) of human bone marrow cells grown over CSi and CB for 21 days. (*) Significantly different from control culture

4 Conclusions

The investigated composites, CSi and CB, promoted the growth of spherical calcium phosphate aggregates after soaking in SBF, indicating that these materials are potentially bioactive. The key difference between their cell behaviour is the chemical nature and structural features of the glass fillers, responsible for the dissolution rate and chemical environment around the cells. The CSi composite demonstrated an inductive effect on the proliferation of MG63 and human bone marrow cells, and stimulated specific metabolic activities such as ALP activity and matrix mineralization suggesting that this composite may have a stimulatory effect on bone formation in vivo. The CB composite produced a certain inhibition of cell proliferation, probably due to the excessive presence of its ionic dissolution products in culture medium. The use of more dynamic cell culture conditions is expected to alleviate the observed deleterious effect on cells. Composites filled with lower percentage of borate glass may have better cellular behaviour, due to an expected decrease of ionic concentrations of the glass dissolution products. The comparative study of CSi and CB composites evidenced the high performance of CSi regarding bioactivity and biocompatibility and clearly indicated that, with respect to CB composite further and new experiments are required and a more in

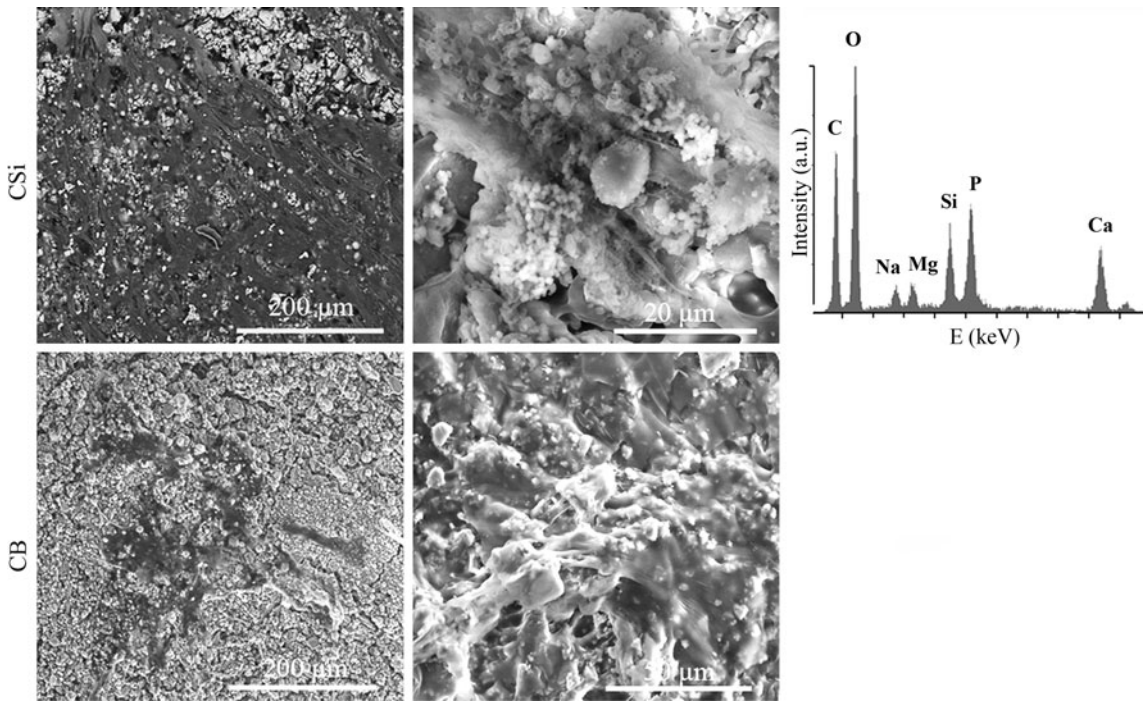

Fig. 8 SEM appearance of human bone marrow cells cultured for 21 days over CSi composite, *inset*: EDS spectrum of the mineralized structures, and CB composite

depth study should be made in order to assess its performance in biomedical applications.

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