Research Article

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# Bioglass and bioceramic composites processed by Spark Plasma Sintering (SPS): biological evaluation Versus SBF test

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**Abstract:** The biocompatibility of hydroxyapatite (HA), a lab-made bioglass (BGCaMIX) with high crystallization temperature and different HA/BGCaMIX composites, produced by Spark Plasma Sintering (SPS), was tested with respect to murine osteocytes both by direct and indirect tests, in order to also investigate possible cytotoxic effects of the samples' extracts. Previous investigations demonstrated that the samples' bioactivity, evaluated in a simulated body fluid solution (SBF), increased with the increasing amount of BGCaMIX in the sample itself. Although none of the samples were cytotoxic, the findings of the biological evaluation did not confirm those arising from the SBF assay. In particular, the results of direct tests did not show an enhanced "biological performance" of materials with higher glass content. This finding may be due to the high release of ions and particulate from the glass phase. On the contrary, the performance of the BGCaMIX alone is better for the indirect tests, based on filtered samples' extracts. This work further demonstrates that, when considering bioglasses and HA/bioglass composites, the results of the SBF assays should be interpreted with great care, making sure that the results arising from direct contact tests are integrated with those arising from the indirect ones.

**Keywords:** Bioactive glasses, Spark Plasma Sintering, Cytotoxicity Tests

## 1 Introduction

A significant challenge in clinical practice is the treatment of osseous defects, which are the result of bone loss caused by benign or malignant neoplasms, traumatic events, severe osteoporosis and infections. Autograft and allograft reconstructions, where bone tissue is taken from the patient itself or from donors, respectively, are among the strategies commonly used for bone regeneration. Both procedures have their inherent shortages, associated for example to immune responses and disease transmission risks for allografts, donor site morbidity, pain and limited tissue availability for autografts [1–3]. During the last four decades, a variety of synthetic alternatives to be employed as bone graft substitutes has been developed, with the aim of minimizing these complications. Specific calcium phosphates, and in particular hydroxyapatite (HA), are commonly used as bone substitute materials, in place of autografts and allografts, due to their biocompatibility, osteoconductivity (*i.e.* they promote bone apposition to their surface) and low cost [4, 5]. Several investigations also demonstrated the osteoinductive ability of HA: this material in fact appears to promote new bone formation [6, 7]. Despite HA is commonly used as bone grafts – in form of granules, powders, porous or dense bodies – and as coatings for metal implants with the aim to increase the biomimetic response of the prosthesis [8], this material is thermodynamically stable at body temperature and physiological pH: its reactivity *in vivo* is typically low and so

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HA is resorbed very slowly [9, 10]. This fact represents a major clinical drawback, since bone grafts should degrade at the same rate as the new tissue forms, thereby resulting in complete substitution by functional bone tissue. The realization of bulk HA products, which typically involves pressureless or classical pressure-assisted sintering methods to consolidate the starting powders, requires high temperature conditions (between 1150°C and 1350°C [11]), with possible HA decomposition [12] and, correspondingly, negative effects on the biological and mechanical performance of the final materials.

The production of HA-based composites with bioactive glasses as second phase is an intriguing alternative to go beyond these limitations. In fact bioactive glasses, and in particular the 45S5 Bioglass® (45S5) – the first developed and most used bioactive glass [13] – bond to bone more rapidly than HA. 45S5 is osteoinductive, seems to induce neo-vascularization and it exerts an antibacterial effect on a range of oral bacteria [14, 15]. For these reasons, the fabrication of HA/bioactive glass composites could lead to innovative systems with tunable bioactivity, tailored to specific clinical applications. In fact, by modifying the volume fractions of the constituents, it is possible to control the biological performance of the final system. At the same time, bioactive glasses can be used to incorporate important ions from a biological point of view (*i.e.* strontium, magnesium, silicon, etc. [16]) within the HA lattice, in order to mimic the composition of the biological apatite, *i.e.* the mineral phase of bone.

In the same way as dense HA, HA/bioglass composites are usually fabricated by means of pressureless or conventional hot-pressing sintering techniques. Unfortunately, such methods require high temperatures (up to 1200°C–1300°C, depending both on the HA to glass ratio and the glass composition [12]), which exceeds the crystallization temperature of the glass. For example, 45S5 starts to crystallize at about 600°C and it has a peak crystallization temperature between 650 °C and 690°C, depending on the heating rate and particle size [17–19]. Such high temperature treatments not only induce the crystallization of the bioglass in the composite, with possible negative effects on the resulting bioactivity [20], but may also cause HA decomposition and reactions between the ceramic and the glassy phase. For these reasons, the production of HA/bioglass composites requires (1) novel bioactive glass compositions with high bioactivity and lower tendency to crystallize than 45S5; and (2) the exploitation of more efficient densification techniques to obtain fully dense ceramic products under milder sintering conditions, as an alternative to classical sintering. In particular, the latter ones could be useful also to sinter HA powders alone.

In this context, in recent years a novel CaO-rich, $K_2O$ containing bioglass (BGCaMIX) was specifically designed to be employed whenever a thermal treatment is required, thanks to its low tendency to crystallize. temperature (880°C [21]) and sinterability than 45S5: while the consolidation process of BGCaMIX powders by pressureless sintering can be completed at about 800°C, in 45S5 such thermal treatment is typically performed between 950°C–1100°C [22, 23]. The feasibility of BGCaMIX powders to produce HA-based composites with different HA/bioglass ratio has been recently investigated [24]. Unfortunately, even though it was possible to fabricate such composites by means of pressureless sintering, the samples with lower bioglass amounts were characterized by some residual porosity after the heat treatment, which negatively affected their mechanical properties.

Recently, HA/BGCaMIX composites with lower porosity, higher compactness and hardness than the counterparts obtained by conventional sintering techniques were successfully produced by means of Spark Plasma Sintering (SPS) [25]. SPS is an efficient and powerful consolidation method with respect to conventional ones, in terms of both temperature and processing time [26]. SPS principle is based on the presence of a pulsed electric current flowing across a conductive die containing the powders to consolidate, so that they are rapidly heated by Joule effect, while a compressive load is simultaneously applied. Due to the relatively milder conditions generally required to achieve high density levels with respect to conventional consolidation methods, several beneficial effects, such as avoiding or limiting decomposition and crystallization phenomena as well as retaining grain growth, are associated with the use of SPS. Such features have been evidenced in recent studies, where the latter technology was successfully exploited for the consolidation of 45S5 powders [19, 27, 28]. In particular, the capability of the SPS method to avoid or limit devitrification phenomena even in glass systems with relatively low crystallization temperature was clearly confirmed.

Beside the production of HA/BGCaMIX composites, SPS has been used to consolidate BGCaMIX powders [19] as well as three different commercially available HA powders [29]. The bioactivity of the produced samples has been successfully tested *in vitro* using a simulated body fluid solution (SBF), thus demonstrating that the relative amount of hydroxycarbonate apatite which formed on the samples' surface during the immersion in SBF increased when the amount of BGCaMIX is augmented [25].

In this work, which is an ideal follow up from our previous publications [19, 25, 29], the biocompatibility of a set of pure HA, pure BGCaMIX and HA/BGCaMIX com-

**Table 1:** Experimental conditions adopted to produce bulk BGCaMIX, HA, and HA/BGCaMIX composites by SPS.

| System | Powder amount (g) | Dwell temperature, $T_D$ (°C) | Dwell time, $t_D$ | Mechanical pressure, P (MPa) |
|---|---|---|---|---|
| BGCaMIX | 1.40 | 730 | 2 min | 16 |
| 80BG_20HA | 1.49 | 800 | 3 s | 30 |
| 50BG_50HA | 1.53 | 1000 | 3 s | 30 |
| 30BG_70HA | 1.56 | 1150 | 3 s | 30 |
| HA | 1.6 | 1200 | 5 min | 30 |

posites (with different HA/BGCaMIX proportions, *i.e.* from 20 wt.% to 70 wt.% HA) produced by SPS was tested with murine long bone osteocytes (MLO-Y4) by means of a multi-parametric approach. Tetrazolium salt XTT and Neutral Red (NR) uptake assays were used to investigate cell viability, while Bromodeoxyuridine (BrdU) assay was employed to evaluate the effects of the produced materials on cell proliferation. Possible cytotoxic effects of the samples' extracts were also considered. In particular, the results of the biological investigation have been compared with the findings arising from the SBF tests discussed in our previous works [25].

# 2 Materials and Methods

## 2.1 Glass and Composites preparation

The BGCaMIX glass (composition: 47.3 mol% $SiO_2$, 45.6 mol% CaO, 2.3 mol% $K_2O$, 2.3 mol% $Na_2O$, and 2.6 mol% $P_2O_5$) was prepared by a melt-quenching route as previously reported [21, 30, 31]. Briefly, the commercial raw powder reagents – all reagent grade – ($SiO_2$, $Na_2CO_3$, $CaCO_3$, $K_2CO_3$, $Ca_3(PO_4)_2$ by Carlo Erba Reagenti, Rodano-Milano, Italy) were mixed in a laboratory shaker for 2 h and then melted at 1450°C for 1 h. The molten BGCaMIX was then quenched in water to obtain a frit which was dried at 110°C for 12 hours. Subsequently, the frit was ground for 40 min in dry conditions to produce a powder (grain size < 67 µm).

Proper amounts of commercial HA powders (CAPTAL® Hydroxylapatite, Plasma Biotal Ltd, UK) were mixed for 6 hours with BGCaMIX powders in order to prepare the following set of composites [25]:

- 80 wt.% BGCaMIX and 20 wt.% HA powders ("80BG_20HA");
- 50 wt.% BGCaMIX and 50 wt.% HA powders ("50BG_50HA");
- 30 wt.% BGCaMIX and 70 wt.% HA powders ("30BG_70HA").

## 2.2 Spark Plasma Sintering of glass, HA and composite powders

The SPS 515S apparatus (Fuji Electronic Industrial Co., Ltd., Japan) was used for the consolidation of BGCaMIX, 80BG_20HA, 50BG_50HA, 30BG_70HA, and HA powders. Details on the SPS equipment and the experimental procedure can be found elsewhere [19, 25, 29]. In particular, the amount of powders used for the obtainment of the various dense specimens is reported in Table 1 along with the adopted SPS conditions. It should be noted that the latter ones have been defined based on the systematic investigation conducted in previous works on the effect of the SPS parameters on powder densification [19, 25, 29]. Five series of sintered disks with 14.7 mm diameter and about 3 mm thickness were correspondingly obtained.

The relative density ($\rho$) of each polished specimen was measured by Archimedes method using distilled water. Weighting of the specimen was carried out taking advantage of a Ohaus Explorer Pro (Ohaus Corporation, NJ, USA) analytical balance (± 0.0005 g precision). The theoretical densities of the composite systems, *i.e.*, 2.915 (80BG_20HA), 3.015 (50BG_50HA) and 3.074 g/cm$^3$(30BG_70HA), were determined using a rule of mixture [32] and by considering the values of 3.16 and 2.86 g/cm$^3$ for HA and BGCaMIX, respectively. In this regard, it should be noted that the amount of powder used for the preparation of about 3 mm thick nearly full dense products was varied as reported in Table 1, according to the relative content of the two constituents in each series of bioceramics taken into account.

## 2.3 Biocompatibility tests

In this work MLO-Y4 (murine long bone osteocyte-like immortalized cell line) cells were selected to investigate the ability of the prepared samples (BGCaMIX, HA, 80BG_20HA, 50BG_50HA and 30BG_70HA) to support cell growth. The cytotoxicity of the materials was evaluated both through indirect contact – with the aim to test the possible negative effects of the materials' eluates – and direct contact, where the cells are seeded onto the sample and subsequently incubated under proper conditions. Cell survival, viability and proliferation were studied by means of Neutral Red (NR) uptake, tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) and Bromodeoxyuridine (BrdU) assays.

### 2.3.1 Cell culturing and preparation of materials' extracts

MLO-Y4 cells were cultured as indicated by the supplier in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Karlsruhe, Germany), supplemented with 10% foetal bovine serum (Invitrogen), sodium pyruvate 1 mM (Invitrogen), D-Glutamine 2 mM and 100 μg/ml penstreptomycin (Invitrogen). The cells were seeded in 6 well plates with the materials for the direct contact tests and the NR assay (see next paragraphs) was performed. The plates were incubated at 37± 1 °C in 5% ± 1% $CO_2$ humidified air (90% ± 5% humidity) and subcultured every about 72 h to maintain the cell populations at less than 70% confluent.

The materials' eluates for the indirect contact tests were obtained by treating them in centrifuge tubes (6 $cm^2$/ml area) containing DMEM. DMEM only and DMEM with 0.45% of phenol solution were used as negative (CTRL-) and positive (CTRL+) controls, respectively. The vials were maintained at 37°C for 5 days and every eluate was filtered by means of a 0.22 μm filter, according to ISO 10993–5 [33] and ISO 10993–12 [34] standard procedures. pH measurements were periodically carried on to control the physiological conditions of the culture medium.

### 2.3.2 NR test

The NR cytotoxicity (Neutral Red solution N6264 Sigma, Germany) assay is a widely used procedure to investigate the cell survival/viability. It is based on the ability of healthy viable cells to incorporate and bind neutral red, a supravital dye. MLOY4 cells were cultured in direct contact with the materials for 24 h and 72 h at 37°C ± 1°C, 90% ± 5% humidity, and 5.0% ± 1% CO2/air. After the removal of the samples and the culture medium, 250 μL of NR solution (Neutral Red solution N6264 Sigma, Germany) were added to all wells including the CTRL+ and the CTRL- and left to incubate for 3 h. Subsequently, the NR solution was removed, the cells were rinsed with 250 μL D-PBS and 100 μL NR Desorb (ETOH/acetic acid) solution, which is used to extract the NR from the cells, was added to all wells. After incubation (20 min), the amount of dye which has been incorporated by the cells was measured employing a spectrophotometer (Multiscan RC by Thermolab system, Finland) at 540 nm. CTRL+ and the CTRL- were used as references.

### 2.3.3 Morphological evaluations

The cells morphology after 24 h and 72 h direct/indirect contact with the materials was observed using optical microscopy (Nikon TMF, Japan).

### 2.3.4 XTT test

The XTT test was used to evaluate the cell viability after exposure to the samples' eluates. Such colorimetric assay is widely used to study the mechanisms of cell activation and damage via cellular metabolic activity. XTT protocol evaluates the cell viability based on the activity of mitochondrial enzymes in metabolic active cells, that reduce the yellow tetrazolium salt XTT to a water-soluble orange coloured product, whose amount is proportional to the number of living cells. Such newly synthesized orange formazan dye can be quantified by measuring absorbance at wavelength of 490 nm [35, 36]. Cells were grown in 96 well culture plates and incubated with the materials' eluates for 24 h and 72 h. Subsequently, the incubation XTT labelling solution (Cell Proliferation Kit II (XTT) Roche diagnostics, USA) was added (final concentration 0.3 mg/ml) and left to incubate for 4 h. The amount of the orange formazan dye generated from XTT was then measured by spectrophotometry (Multiscan RC by Thermolab system, Finland), using CTRL+ and the CTRL- as references.

### 2.3.5 Bromodeoxyuridine (BrdU) test

BrdU cell proliferation test is a commonly used assay to quantify the incorporation of 5-bromo-2-deoxyuridine in replicating DNA of proliferating cells, with the aim to eval-

uate cell proliferation. Cells were grown in 96 well culture plates and exposed to the materials' extracts for 24 h. Subsequently, 50 µl/well of BrdU labelling solution (Cell Proliferation ELISA, BrdU, Roche) was added and left to incubate. The incubation time (90 min) is long enough as BrdU is incorporated in place of thymidine into the newly synthesized DNA of the cycling cells. Subsequently the labelling culture medium was removed and FixDenat solution (Cell Proliferation ELISA, BrdU, Roche) was added, with the aim to fix cells and denature DNA in one step; such solution was then removed and the samples were further incubated with an antibody conjugated to peroxidase (anti-BrdU-POD), which is able to bind to the BrdU incorporated into the newly synthesized cellular DNA; in this way, it is possible to detect the immune complexes through the subsequent substrate reaction. The amount of the reaction product can be quantified by measuring absorbance at wavelength of 370 nm by means of a spectrophotometer (Multiscan RC by Thermolab system, Finland). The signal intensity is correlated to the amount of the newly synthesized DNA, and therefore to the number of proliferating cells.

#### 2.3.6 Statistical analyses

One-way variance analysis (ANOVA) was used to statistically treat the obtained results, which are expressed as the mean ± standard deviation; t-test analysis by a two populations comparison was employed to establish differences among groups. Statistical significance was considered at a probability $p < 0.05$.

## 3 Results and Discussion

The microstructure and mechanical behavior of the five series of sintered products, obtained under the SPS conditions summarized in Table 1, have been discussed in previous works [19, 25, 29], together with a systematic investigation of their phase composition and in vitro bioactivity in SBF. The main findings can be summarized as follows:

1. The obtained samples are characterized by higher compactness and density with respect to the counterparts obtained with classical sintering methods (density levels higher than 96% of their theoretical values). In particular, it was possible to produce for the first time by SPS a set of HA/BGCaMIX composites with high HA content (≥ 50 wt.%) with a density close to the theoretical value.

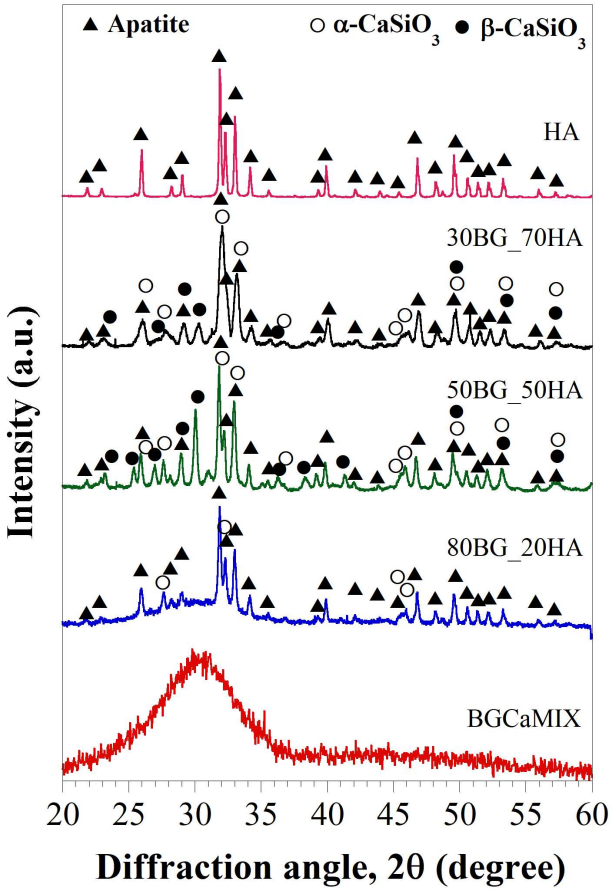


**Figure 1:** XRD patterns of bulk BG, HA, and BG-HA composite samples produced by SPS. A detailed description of the XRD analysis performed on the samples discussed in the present work has been previously reported in [19, 25, 29].

2. Thanks to both the low tendency to crystallize of BGCaMIX and the milder sintering conditions required by SPS, it was possible to reduce the crystallization of the glassy phase in the samples, with positive effects on their bioactivity. The XRD spectra acquired on the samples are compared in Figure 1. It is seen that the bioglass specimens maintained their original amorphous character after sintering. This holds also partially true when considering the composite system richer in the bioglass constituent. Indeed, the initial occurrence of the glass crystallization during SPS was evidenced by the presence of minor amounts of $\alpha$-$CaSiO_3$, as detected by the XRD analysis. Moreover, the content of $CaSiO_3$ in both the $\alpha$- and $\beta$- configurations was found to increase as the holding temperature was progressively augmented to achieve the nearly full densification in the other two HA-richer composites systems. Finally, the

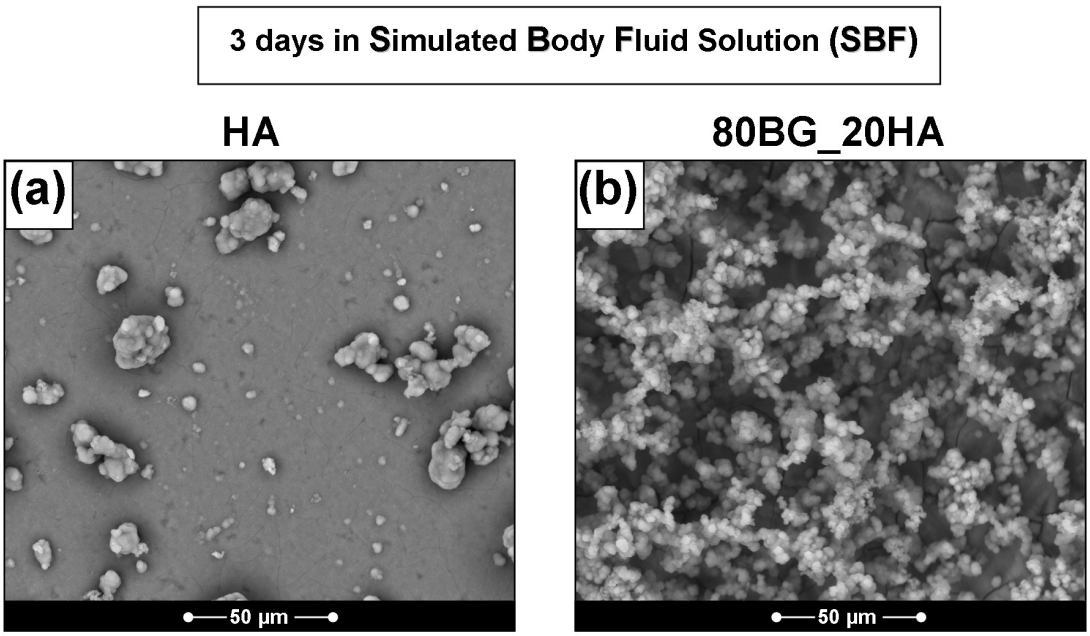


**Figure 2:** Scanning Electron Microscope images of the HA and the 80BG_20HA surfaces after immersion in SBF for 3 days. A detailed description of the SBF tests performed on the samples discussed in the present work has been previously reported in [19, 25, 29].

glass-free HA product only consisted of apatite with no evidence of secondary phases.

3. The samples' bioactivity in SBF increased when the amount of BGCaMIX in the sample is augmented.
4. Finally, the biocompatibility of BGCaMIX and of HA/BGCaMIX composites with different HA to glass ratio, produced by conventional sintering, has been successfully tested with respect to murine osteocytes and/or fibroblasts [24, 37].

SBF assay [38] aims to qualitatively evaluate the bone-bonding ability of a material (typically a bioactive glass, a bioceramic or a bioactive coating) by examining the ability of hydroxyapatite to form on its surface once in contact with SBF, which is a solution with ions concentration similar to that of human blood plasma. As an example, Figure 2 shows the surface of the HA and the 80BG_20HA samples (images observed by Scanning Electron Microscopy) after soaking in SBF for 3 days; for a detailed discussion of this point, see [25]. It is possible to observe that the relative content of the hydroxyapatite precipitates, which should reflects the samples' bioactivity and their bone-bonding ability, increased with higher BGCaMIX content in the sample. In this context the present contribution aims not only to test the biocompatibility towards cells of bioceramic samples obtained by SPS, which is a technique that only recently has been employed for the production of bioglass and HA/bioglass composites, but also to further demonstrate that SBF assays should be considered as preliminary screening tests, since SBF is not able to mimic the complexity of a real physiological environment, which contains cells, proteins, specific trace elements, etc [39]. Cytotoxicity tests, based on in vitro cell culture assays, are crucial in order to confirm the findings from the bioactivity evaluation with SBF, which has been reported in the literature to lead both to false negative and false positive results [40–42]. In this context, probably the most striking case is that of $\beta$-Tricalcium Phosphate: despite its high bone-bonding ability in vivo, it does not always induce the formation of hydroxyapatite in SBF [39]. Moreover, *in vitro* cell culture assays provide an estimate of cell viability, differentiation, proliferation and they can be employed to determine if a material itself has potential to be cytotoxic (*i.e.* through the so-called direct contact tests) or if a material has potential to release agents or degradation products that may be cytotoxic (the so-called indirect contact tests). In this work, the biological performance of HA, BGCaMIX and HA/BGCaMIX composites produced by SPS was studied by means of both direct and indirect contact approaches. Since the materials here proposed have been developed to be used in bone repair and regeneration, the samples were tested with respect to MLO-Y4, which is an osteocite-like immortalized cell line isolated from murine long bones [43, 44].

Figure 3 shows the results of the viability test with NR uptake, a colorimetric assay which is commonly used to

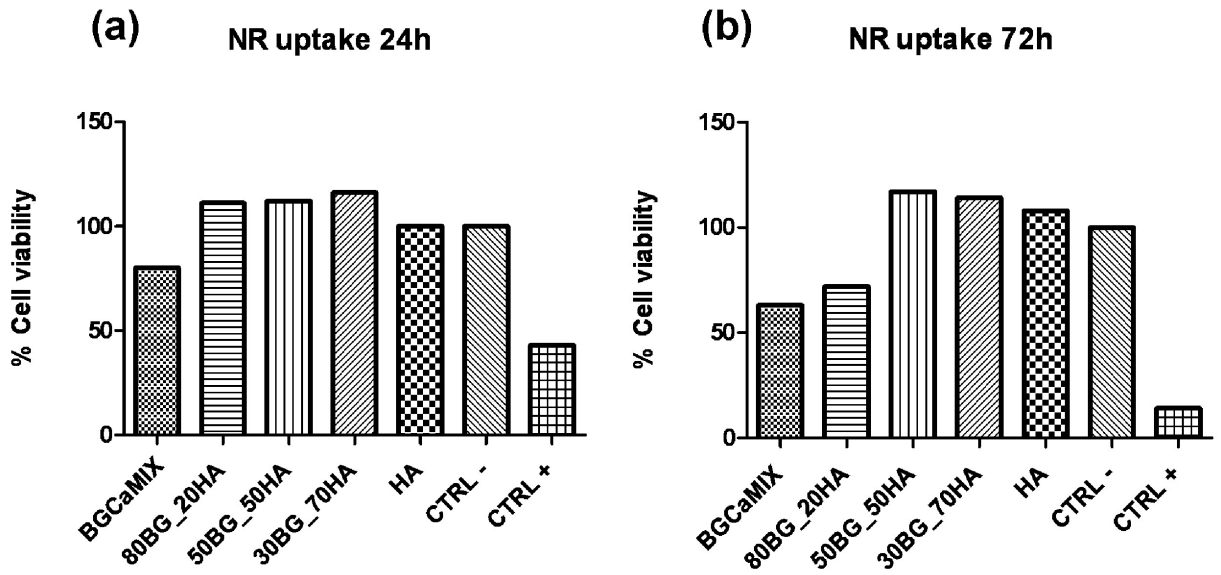


**Figure 3:** NR uptake after 24 h (a) and 72 h (b) in the different samples.

investigate the toxic effects of chemicals and biomaterials on a variety of cell lines from different origin [45, 46]. As it shows, healthy cells are able to incorporate the supravital dye neutral red, which is accumulated within their lysosomes. Therefore, a potential loss of integrity and function of the cell (for example, alterations of the cell or the lysosomal membrane), due to exposure to cytotoxic xenobiotics, is expected to result in a decreased uptake and binding of NR [47]. In this way, it is possible to distinguish between injured (or dead) and viable cells. NR uptake test has been adopted as a recommended assay for qualitative evaluation of biomaterials' safety by several agencies for testing and normalization [48]. The NR uptake and the cell cultures have been here evaluated after 24 h (Figure 3(a)) and 72 h (Figure 3(b)) of exposure to the materials. It should be noted that, observing the results of the SBF tests performed on the materials [25] and Figure 2), an enhanced biological performance of the samples with higher BGCaMIX content – characterized by higher formation of hydroxyapatite on their surface – could be expected. On the contrary, the results obtained in this work indicated that, after 24 h (Figure 3(a)), the exposure to BGCaMIX induces a slightly lower cell viability, although the decreasing in the lysosomal activity is not so significant to be considered a cytotoxic effect. Such effect is more pronounced after 72 h (Figure 3(b)), where the samples with higher BGCaMIX content (*i.e.* BGCaMIX itself and 80BG_20HA) determine a lower cell viability. However, also in this case the produced materials remained not cytotoxic. This fact was only detectable only by considering the results from the NR uptake assay (see the next paragraphs), which is a direct contact test, and it could be ascribed to the high release of ions and particulate from the glass phase. It is known that bioactive glasses have a typically higher reactivity than apatite. On the other hand, the literature also reports that the presence of particles and crystals in the culture medium may affect the accuracy of the absorbance readings employed to detect the dye neutral red [49–51]. Therefore, it is crucial to integrate such test with the results from additional cytotoxicity assays, in particular based on an indirect approach. In such an indirect test it is typically evaluated if a solution, which contains the extractable compounds of a given material, has potential to cause changes cells' morphology and/or lysis. The NR uptake after 24 h and 72 h of direct contact to 50BG_50HA, 30BG_70HA and HA was analogous or even higher than CTRL- (Figure 3), therefore also these materials did not show a cytotoxic response.

A morphological evaluation of MLO-Y4 cells after 24 h indirect contact with the samples is reported in Figure 4. Despite the BGCaMIX content is different in the discussed samples, no relevant effects were here observable in the cells' behaviour. In fact, MLO-Y4 grew very well in all the materials' extracts and showed a morphology similar to that of the cells in the CTRL-: no significant cell morphology changes were observed, including lysed or rounded cells. Figure 5(a, b) shows the results of the viability test performed by means of XTT colorimetric assay [52, 53]. After 24 h and 72 h of culture in the samples' eluates, the mitochondrial activity of the cells is higher than CTRL- for all the materials, including the samples with higher BGCaMIX

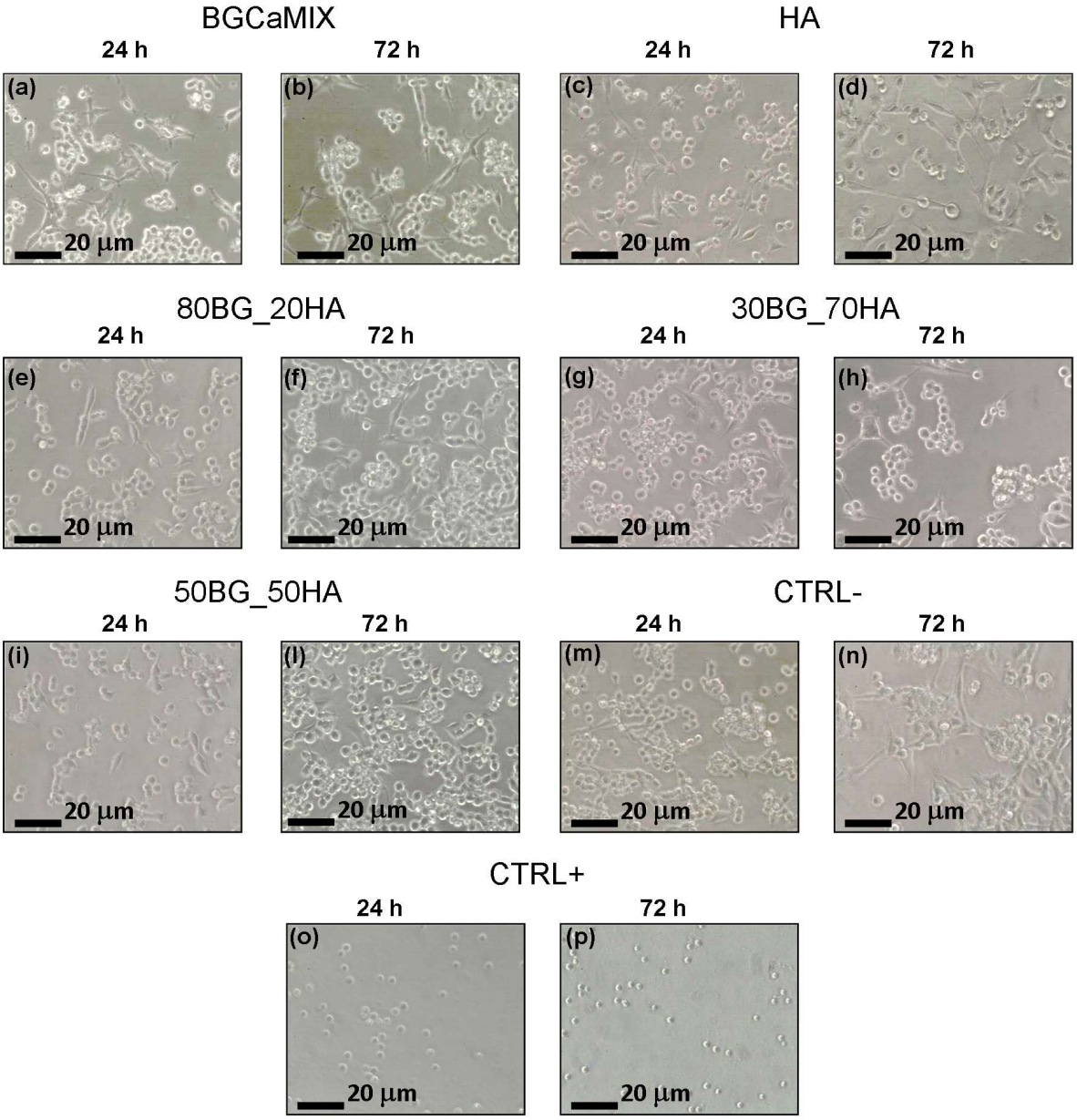


**Figure 4:** Morphological evaluation of MLO-Y4 cells after 24 h indirect contact with the samples using optical microscopy (Nikon TMF, Japan).

content, thus indicating that MLO-Y4 grew and proliferated normally. However, also in this case the pronounced *in vitro* bioactivity in SBF of BGCaMIX and 80BG_20HA ([25] and Figure 2) did not result in an enhanced biological performance with respect to pure HA. Finally, Bromodeoxyuridine test (BrdU) was used to evaluate the proliferation of MLO-Y4 cells cultured in extracts from the developed materials. BrdU is incorporated into the newly synthesized DNA in place of thymidine, therefore it acts as a marker of the cycling cells, which can be detected by immunohistochemistry [54]. According to the results reported in Figure 5(c), the produced materials did not affect negatively the cells. Here it is possible to observe that BGCaMIX, 80BG_20HA and 50BG_50HA samples preferably stimulate the cell proliferation. While this fact has been previously observed and discussed in several works (see, for example, [16, 55]) dealing with the "gold" standard Bioglass® 45S5 in powder or bulk form, analogous investigations carried out using sintered bioglasses and, in particular, bioglass-based composites sintered by SPS are lacking in the literature. Based on these findings, SPS seems to be a promising technique to produce sintered bio-

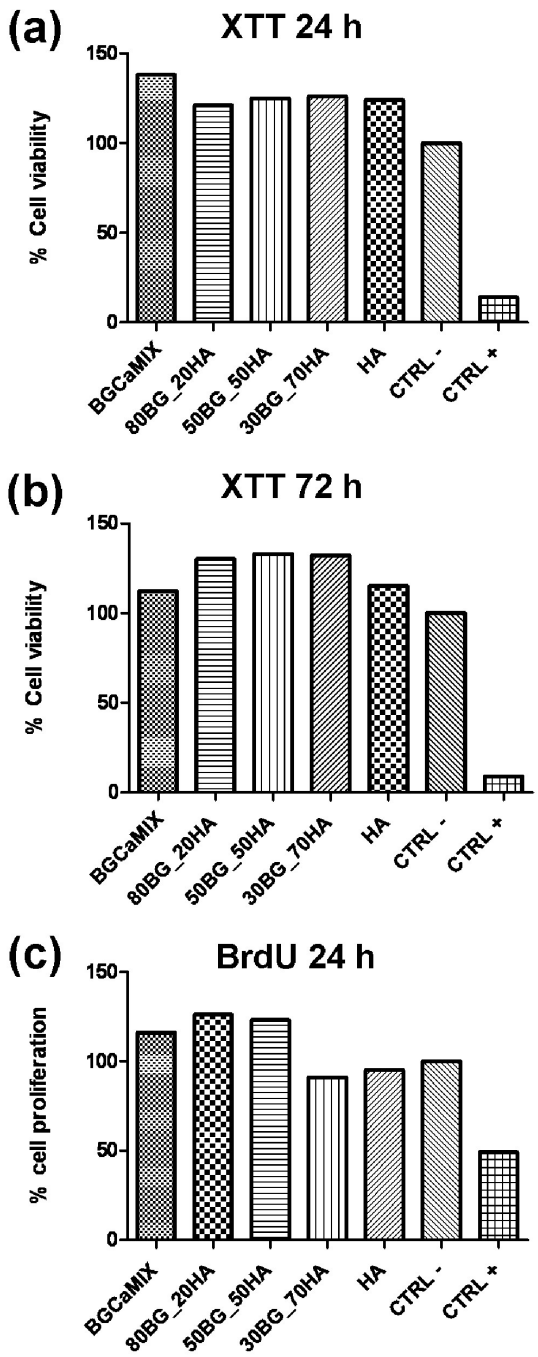


**Figure 5:** XTT test of MLO-Y4 cells cultured in eluate from the samples after 24 h (a) and 72 h (b); BrdU test after 24 h (c).

glasses and bioglass-based composites without negatively affecting the biocompatibility of the final system.

# 4 Conclusions

Recent investigations discussed the feasibility of bioglasses, HA and HA/bioglass composites processed by SPS. In particular, a novel lab made CaO-rich, $K_2O$ containing glass, named BGCaMIX was employed. BGCaMIX was specifically designed to be employed whenever a thermal treatment is required, thanks to its lower tendency to crystallize with respect to the widely used 45S5. According to SBF tests, the *in vitro* bioactivity of the samples, which should reflect the bone bonding ability of the material, is progressively improved with the increasing amount of BGCaMIX in the sample itself. Here, for the first time, the biocompatibility of a set of pure HA, pure BGCaMIX and HA/BGCaMIX composites produced by SPS was evaluated with respect to murine long bone osteocytes by means of both direct (NR uptake) and indirect test (XTT and BrdU). It should be noted that, although previous works [24, 37] confirmed the biocompatibility of both BGCaMIX and HA/BGCaMIX composites produced by conventional sintering, analogous investigations regarding bioglasses and bioglass-based composites sintered by SPS are lacking in the literature.

Despite none of the samples here discussed was cytotoxic, the findings of the biological evaluation did not confirm the ones arising from the SBF assay. In particular, the results of direct tests did not show an enhanced "biological performance" in the samples with increasing glass content. This fact may be ascribed to the high release of ions and particulate from the glass phase, which could affect the cell viability, although the literature also reports that the presence of particulate in the culture medium may affect the accuracy of the absorbance readings employed in the colorimetric assay. The performance of the BGCaMIX alone is, in general, better during the indirect tests: in fact, each eluate was filtered by means of a 0.22 µm filter according to ISO standard procedures [35, 36]. In particular, the samples with the higher glass content (BGCaMIX, 80BG_20HA and 50BG_50HA) slightly stimulate the cell proliferation. It is possible to conclude that SPS is a promising technique to produce sintered bioglasses and bioglass-based composites without negatively affecting the biocompatibility of the final system. Moreover, the present work demonstrates once more that the results of the SBF assays should be interpreted with great care, making sure that the results arising from direct contact tests are integrated with those arising from the indirect ones.

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