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Article:

Fernandes, J., Gentile, P., Crawford, A. et al. (2017) Substituted Borosilicate Glasses with Improved Osteogenic Capacity for Bone Tissue Engineering. Tissue Engineering - Part A, 23 (23-24). pp. 1331-1342. ISSN: 1937-3341

https://doi.org/10.1089/ten.tea.2016.0386

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1 **Substituted Borosilicate Glasses with Improved Osteogenic Capacity for Bone**
2 **Tissue Engineering**

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25 **Abstract**

26 Borosilicate bioactive glasses (BBGs) have shown capacity to improve the new bone
27 formation when compared to silicate bioactive glasses. Herein, we assessed the capacity
28 of BBGs to induce the osteogenic differentiation of bone marrow mesenchymal stem
29 cells (BM-MSCs), as a function of their substituted divalent cations (Mg^{2+}, Ca^{2+}, Sr^{2+}).
30 To this purpose, we synthesized BBG particles by melt quench. The cell viability,
31 proliferation and morphology, (i.e. PrestoBlue®, PicroGreen®, and DAPI and Phalloidin
32 stainings, respectively) as well as protein expression (of ALP, osteopontin and
33 osteocalcin) of BM-MSCs in contact with the BBGs were evaluated for 21 days. We
34 observed an enhanced expression of bone-specific proteins (ALP, OP and OC) and high
35 mineralization of BM-MSCs under BBG-Mg and BBG–Sr conditioned osteogenic
36 media for concentrations of 20 and 50 mg/ml with low cytotoxic effects. Moreover,
37 BBG-Sr at a concentration of 50 mg/ml was able to increase the mineralization and
38 expression of bone-specific proteins even under basal media conditions. These results
39 indicated that the proposed BBGs improved the osteogenic differentiation of BM-
40 MSCs. Therefore, showing their potential as relevant biomaterials for bone tissue
41 regeneration, not only by bonding to bone tissue, but also by stimulating new bone
42 formation.

43

44 **Key words:** Borosilicate glasses, strontium, mineralization, osteogenic induction, BM-
45 MSCs

46

1. Introduction

The properties of bioactive glasses (BGs) support their key relevance of in clinical applications associated to bone tissue repair and regeneration.(1, 2) They are part of a tissue engineering-based strategy that can overcome the drawbacks of the traditionally used autologous bone grafts (e.g. lack of adequate amount and quality of bone, donor site morbidity). BGs are considered relevant for bone tissue repair since they: (i) promote osteointegration (forming a bone-like hydroxyapatite (HA) layer on their surface); (ii) are biocompatible; and (iii) their degradation shows positive biological effects after implantation.(2-5) There are however drawbacks associated with conventional BGs including *in vitro* cytotoxicity related to the release of Na^{+} ions, and interest in modified compositions has increased in recent years.(6)

Recently, borosilicate bioactive glasses (BBGs) have attracted interest in bone tissue engineering.(1, 7, 8) BBGs have shown capability to improve the new bone formation when compared to silicate-based BGs.(9, 10) They present controllable degradation rates and have a high compositional flexibility that potentially allows BBGs to be tailored with enhanced osteogenic and angiogenic properties, as well as with antibacterial capacity.(11, 12) On one hand, as shown by Huang *et al.*, a glass network composed of borosilicate's have more controllable conversion rates to HA.(13) This has also been demonstrated to occur *in vivo*.(14) As a matter of fact, the addition of borate to the glass network can also be beneficial for bone healing, as well as formation, and maintenance of new bone, while supporting cell osteogenic differentiation.(15) Frequently, it has been associated with the increase in bone resistance to fracture.(16, 17) On the other hand, by exploiting the compositional flexibility of BBGs, inorganic divalent cations, such as Mg^{2+}, Ca^{2+} and Sr^{2+} can be incorporated and play a key role in bone metabolism. For instance, Mg^{2+} increases bone formation rate as well as stimulates

72 bone cell adhesion increasing their stability.(18, 19) The Ca^{2+} is known to be essential
73 during the apatite formation process, being also favorable to osteoblast proliferation,
74 differentiation and the mineralization of the extracellular matrix (ECM).(20) Sr^{2+} also
75 has bone therapeutic potential. Different studies evidenced its beneficial effects on bone
76 cells and bone formation *in vivo,*(21, 22) being even used for the treatment of
77 osteoporosis.(23)

78 BGs are known to be osteoinductive materials, capable of stimulating the function and
79 osteogenic differentiation of bone and stem cells without any additional
80 supplementation. Findings from Fu *et al.* suggested that the borosilicate 13-93B1
81 scaffolds supported the proliferation and function of osteogenic Murine Osteocyte-like
82 Cell Line MLO-A5,(15) while Gentleman *et al.* demonstrated that Sr^{2+}-substituted
83 bioactive glasses stimulated osteoblast metabolic activity promoting cell proliferation
84 and ALP activity.(24) More significantly, Santocildes *et al.* demonstrated that Sr-
85 containing BGs appeared to be capable of promoting osteoblastic differentiation in a
86 proportion of bone marrow mesenchymal stem cells (BM-MSCs) that were in some way
87 pre-committed to this lineage.(25) Liang *et al.* showed that borate glasses support the
88 attachment and differentiation of human bone marrow derived mesenchymal stem cells
89 and human mesenchymal stem cell derived osteoblasts.(26)

90 The present study aims to fabricate three substituted BBGs to be used in bone tissue
91 repair and regeneration field. The incorporation of different divalent cations (i.e. Mg^{2+},
92 Ca^{2+}, Sr^{2+}) into the BBGs was tested due to the fact that the release of these cations
93 might induce specific effects on BM-MSCs, such as: in their proliferation,
94 differentiation and the mineralization of the ECM, as well as stimulating bone cells and
95 promoting bone formation.(11) BM-MSCs are a cell type of especial interest for bone
96 tissue research and therapy because of their ease and reproducibility of isolation as well

as their ability to differentiate into mesodermal lineage cells such as osteoblasts, osteoclasts and osteocytes.(27) Therefore, we aimed to investigate the effects of BBGs biological activity over viability, proliferation, and differentiation of BM-MSCs, providing novel biocompatible BBGs that promote osteointegration and cell differentiation for bone tissue engineering.

2. Experimental

2.1. Preparation of BBGs

The BBGs of general formula $0.05Na_2O \cdot x MgO \cdot y CaO \cdot (0.35\text{-}x\text{-}y)SrO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$ (molar ratio, where x, y = 0.35 or 0.00, and $x \neq y$) were synthesized by melt-quenching. The suitable amounts of, silica (SiO_2, Macherey-Nagel, Germany), boron oxide (B_2O_3, Alfa Aesar, Germany), sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, Australia), and magnesium oxide (MgO, Sigma-Aldrich, Portugal), or calcium carbonate ($CaCO_3$, Sigma-Aldrich, Portugal), or strontium carbonate ($SrCO_3$, Sigma-Aldrich, Portugal) were thoroughly mixed with the addition of ethanol in a porcelain pestle with the help of a mortar, vacuum dried overnight and transferred to a platinum crucible. After entirely dried, each batch was heated to 1450 ˚C in air for 1 h and subsequently the melt was quickly poured into cold water to form the glass frit. Afterwards, the as-quenched glasses were ground in an Agate mortar (RETSCH, Germany) and sieved to a particle size <63 μm. Before the *in vitro* tests BBG-Mg ($0.05Na_2O \cdot 0.35MgO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$), BBG-Ca ($0.05Na_2O \cdot 0.35CaO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$) or BBG-Sr ($0.05Na_2O \cdot 0.35SrO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$) were weighted, dried and sterilized at 160 ºC for at least 2 h.

2.2. Morphology and chemical composition of BBGs

The morphology of the synthesized BBGs was observed by scanning electron microscopy (SEM, model S360, Leica Cambridge, UK) equipped with energy dispersive X-ray spectroscopy (SEM/EDS link-eXL-II) for the determination of the surface chemical composition.

2.3. Isolation and expansion of mesenchymal stem cells

Bone marrow mesenchymal stem cells (BM-MSCs) were isolated from bone marrow of 4-5 week-old male Wistar rats according to the method established by Maniatopoulos *et al.* (28) and recently proposed by Santocildes *et al.*.(25) BM-MSCs were expanded in basal medium consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, UK), supplemented with 100 U/ml penicillin (Sigma,-Aldrich, UK) and 1 mg/ml streptomycin (Sigma-Aldrich, UK). Cells were cultured at 37 ºC in an atmosphere of 5% CO_2.

Prior to the *in vitro* studies, BM-MSCs, at passage 2, were harvested and seeded into 24 well plates, at a density of 2×10^4 cells per well. Cells were cultured in the presence of the BBGs at concentrations of 20 and 50 mg/ml, for 7, 14 and 21 days under static conditions. The BM-MSCs cultured in the absence of BBGs were used as negative control and in the presence of 45S5 bioglass® as positive control. The BBGs at the desired concentrations and 45S5 bioglass® were deposited on top of the cells, in cell culture inserts with porous membranes (0.4 µm ThinCerts™ Cell Culture Inserts; Greiner, Germany), as used in previous works (29). The ThinCerts™ were used as an inert platform to support the glass particles under cell culture conditions. All conditions were cultured in basal and osteogenic differentiation media (basal medium supplemented with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 10^{-8} M dexamethasone). Figure 1 presents a schematic of the experimental design.

144 **Figure 1.** Schematic of the experimental design.

145 2.4. Potential cytotoxic effect of BBGs dissolution on BM-MSCs

146 **Cell viability and proliferation (PrestoBlue® and PicoGreen® assays).** The
147 PrestoBlue® reagent (Fisher Scientific, UK) is a resazurin-based solution that is reduced
148 to resorufin by viable cells which can be detected fluorimetrically. The cell viability
149 assay was executed according to the manufacturer's instructions. In brief, the
150 PrestoBlue® reagent was added to a final concentration of 10% to the wells and the cells
151 were incubated for 1 h at 37 ^{o}C. Afterwards, 200 µl samples of the culture medium were
152 removed and placed in 96-well plates and the resorufin fluorescence was quantified
153 spectrophotometrically using a plate reader (Tecan Infinite M200). The fluorescence
154 was determined at an excitation wavelength of 560 nm and emission wavelength of 590
155 nm. The metabolic activity was presented in fluorescence values and compared with the
156 control (cell cultured in the absence of glass particles under basal medium conditions).

157 The PicoGreen® dsDNA reagent (Invitrogen, USA) is an ultrasensitive fluorescent
158 nucleic acid dye for quantification of double-stranded DNA (dsDNA) in solution. This
159 assay enables the measurement of cell proliferation. After each culturing period, the cell
160 monolayers were washed with PBS and then incubated at 37 ºC for 3 h followed by a
161 freezing step at -80 ºC for at least over night in ultra-pure water (1 ml) to ensure cell
162 lysis. The assay was performed according to the manufacturer's protocol. And the
163 fluorescence was determined at an excitation wavelength of 485 nm and emission
164 wavelength of 528 nm. The DNA concentration was presented in µg/ml and compared
165 with the control (cell cultured in the absence of glass particles under basal medium
166 conditions).

167 **Cell morphology and distribution**. After each culturing period the cell grown in tissue
168 culture coverslips were washed with PBS and fixed with 4% formalin solution (0.5 ml)
169 for 15 min at room temperature (RT). The cell layers were then washed with PBS,
170 containing 0.2% Triton X, for 2 min. After the fixation and permeation steps, the cell
171 monolayers were washed again with PBS and stained with 4,6-diamidino-2-phenyindole
172 dilactate (1:1000 DAPI, Sigma, UK) for 2 min at RT, and phalloidin-
173 tetramethylrhodamine B isothiocyanate (Sigma, UK) for 1 h at RT. Finally, the cells
174 were washed and observed using an Axioplan 2 imaging fluorescent microscope with a
175 digital camera QIC AM 12-bit (Zeiss, UK).

176 **2.5. Osteogenic capacity of BBGs on BM-MSCs**

177 **Alkaline phosphatase quantification**. The concentration of alkaline phosphatase
178 (ALP) was determined for all the culture time periods, using the lysates used for DNA
179 quantification. Briefly, the ALP quantity was assessed using the Alkaline Phosphatase,
180 Diethanolamine Detection kit (Sigma-Aldrich, UK) in which p-nitrophenyl phosphatase
181 (pNPP) solution is hydrolyzed by ALP to yellow free p-nitrophenol. In brief, a buffered
182 pNPP solution was prepared and equilibrated at 37 ºC. Afterwards, 2% (v/v) of sample
183 or control were added. Immediately after mixing the absorbance was read at 405 nm in a
184 plate reader (Tecan Infinite M200) for ≈ 5 min. An ALP standard solution was used as
185 control and buffer as blank. The units were calculated according to the following
186 equation: $\frac{(\Delta A_{405nm}/\min Test - \Delta A_{405nm}/\min Blank) \times df \times V_F}{18.5 \times V_E}$. Where df = dilution factor; V_F =
187 Volume of final solution; 18.5 = millimolar extinction coefficient of pNPP at 405 nm
188 and V_E = Volume of samples/ALP standard solution. ALP activity was calculated by
189 normalizing ALP concentration per DNA concentration for each condition and time
190 point.

191 **Alizarin red staining.** After 21 days culture, the cells grown in tissue culture coverslips
192 were fixed in 70% ice-cold methanol at -20 °C at least for 30 min. The cell layers were
193 then washed with PBS and dried overnight. Afterwards, cells were stained with alizarin
194 red solution for 10 min [342 mg of alizarin red, (Sigma-Aldrich, UK) in 25 ml of
195 distilled water and the pH was adjusted to 4.1 with 10% ammonium hydroxide (Sigma-
196 Aldrich, UK)] for 10 min. The coverslips were then, washed in distilled water,
197 dehydrated in acetone/xylene (Sigma-Aldrich, UK) solution and mounted using an
198 aqueous mountant. The stained constructs were observed under an optical microscope
199 (BX51, Olympus Corporation, UK) and images captured by a digital camera (DP70,
200 Olympus Corporation, UK). The BM-MSCs morphology and mineral deposition was

201 also observed using SEM (model S360, Leica Cambridge, England) equipped with
202 energy dispersive X-ray spectroscopy (SEM/EDS link-eXL-II) for the determination of
203 the surface chemical composition.

204 **Immunodetection of bone-specific proteins.** Osteopontin (OP) and osteocalcin (OC)
205 protein expression of BM-MSCs was assessed by immunoassay technique to evaluate
206 their osteoblastic differentiation. The procedures were executed according to the
207 manufacturer's instructions. The concentrations of OP and OC were determined for all
208 the culture time periods, using the lysates used for DNA quantification. The OP
209 quantitative determination was performed using Mouse/Rat Osteopontin Quantikine
210 ELISA Kit (R&D Systems, UK). In brief, 50 µl of assay diluent RD1W and 50 µl of
211 standard (2500 to 39 pg/ml), control and samples were added and the plate incubated for
212 2 h at RT. After 4 washing steps and perfectly dried, 100 µl of Mouse/Rat OP
213 Conjugated were added and incubated for 2 h at RT. The sandwich complex was
214 washed 4 times and allowed to react with 100 µl of substrate solution before adding 100
215 µl of stop solution. Finally, the optical density was determined at 450 nm and the
216 concentration of OP obtained from a standard curve plot. OC quantitative determination
217 was performed by the use of Rat Gla-Osteocalcin High Sensitive EIA kit (Takara
218 Clontech, Japan). In brief, 100 µl of samples and standard solution (16 to 0.25 ng/ml)
219 were incubated for 1 h at 37 ºC with the capture-antibody, rat osteocalcin C-terminus-
220 specific antibody. After OC capture and 3 washing steps, 100 µl of the enzyme-labelled
221 antibody (GlaOC4-30) specific to Gla-OC was incubated for 1 h at RT. The sandwich
222 complex was washed 4 times and allowed to react with 100 µl of substrate solution for
223 10-15 min. Finally, after adding the stop solution the optical density was determined at
224 450 nm and the concentration of OC obtained from a standard curve plot. OP and OC

225 content was calculated by normalizing OP or OC concentration per DNA concentration
226 for each condition and time point.

227 **2.6. Statistical analysis**

228 Results are expressed as mean ± standard deviation with n = 3 for each sample. Error
229 bars represent standard deviations. The data was analyzed by non-parametric statistics:
230 Kruskal-Wallis test ($p<0.0001$), followed by a Dunn's Multiple Comparison test. ***
231 $p<0.001$; ** $p<0.01$; * $p<0.05$ in respect to the control.

232 **3. Results and discussion**

233 **3.1 Morphology of BBGs and their chemical composition**

234 BBGs' frits were successfully obtained by melt quenching and ground in a controlled
235 manner. Figure 2a, 2b and 2c show the SEM/EDS analysis of BBGs, which exhibited an
236 angular shape with low sphericity and confirmed the successful incorporation of the
237 different modifier divalent cations (i.e. Mg^{2+}, Ca^{2+} and Sr^{2+}). The composition of BBGs
238 was confirmed in a prior work by the use of X-ray fluorescence and follows the general
239 formula $0.05Na_2O{\cdot}x MgO{\cdot}y CaO{\cdot}(0.35\text{-}x\text{-}y)SrO{\cdot}0.20B_2O_3{\cdot}0.40SiO_2$ (molar ratio, where x,
240 y = 0.35 or 0.00, and $x \neq y$). (30) Moreover, studies after immersion in SBF, showed that
241 the studied BBGs are bioactive due to the formation of bone-like apatite structures onto
242 their surface, and the constant release of ions to the reaction media over time. (29)

243

244 **Figure 2.** SEM/EDS micrographs of BBGs, a) BBG-Mg, b) BBG-Ca and c) BBG-Sr.
245 SEM images are shown as insets, displaying the morphology of the glass particles. The
246 specific modifier divalent cation is highlighted in yellow for each BBG.

247 **3.2 *In Vitro* biological evaluation**

248 Osteoblast differentiation can be divided into three stages: cell proliferation, ECM
249 synthesis and maturation, and ECM mineralization, each with a cellular characteristic
250 behavior.(31)

251 **3.2.1 Potential cytotoxic effect of BBGs leachables on BM-MSCs**

252 The effect of BBGs' concentration on cell viability and proliferation was studied on a
253 previous work (29, 30). It showed that a concentration between 20 and 50 mg/ml did not
254 significantly affect cell viability and proliferation. On the other hand, Romero *et al.* (32)
255 studied the osteogenic response of BM-MSCs to strontium-substituted bioactive glasses
256 (SrBG) and observed that 20 mg of Sr50BG promoted the osteoblastic differentiation of
257 BM-MSCs. Based on these results, we decided on the BBGs concentrations of 20 and
258 50 mg/ml.

259 Herein, we cultured BM-MSCs in basal and osteogenic differentiation media for 7, 14
260 and 21 days under static conditions, in the presence and absence of BBGs, in order to
261 evaluate their biological activity and capacity to maintain their structural integrity. The
262 cellular metabolism and proliferation was evaluated by quantifying the conversion of
263 resazurin-to resorufin by viable cells; the amount of double stranded DNA (live cells) in
264 the culture wells; and their morphology. The data was analyzed in comparison with the
265 BM-MSCs (without any BBGs, i.e. control experiment) to evaluate the impact of BBGs
266 in the cell behavior.

267 Fluorescence microscopy images showed the morphology of BM-MSCs in culture
268 containing BBGs or 45S5 bioglass® (Figure 3). The adhered BM-MSCs exhibited a
269 well-spread morphology, exhibiting cell-to-cell contacts in a comparable manner on the
270 BBGs conditioned cultures as in the control experiment. While BM-MSCs cultured with

271 45S5 bioglass® presented a round shape for the last time point (21 days), suggesting cell
272 death. At the same timepoint, especially for cultures under osteogenic differentiation
273 media (e.g. BBG-Sr), there was distinguishable well-spread polygonal shape cells,
274 suggesting osteoblast-like morphology.

275

276 **Figure 3.** BM-MSCs morphology observed by fluorescence microscopy, after 7, 14
277 and 21 days culture with BBGs either under basal or osteogenic culture medium. Each
278 sample was incubated at two different concentrations (20 and 50 mg/ml). Cells cultured
279 with basal and osteo medium were used as negative control and 45S5 bioglass®
280 incubated with medium was used as positive control. Nuclei stained blue by DAPI;
281 Actin stained green by Phalloidin.

282

283 **Figure 4.** Metabolic activity (PrestoBlue® assay) and proliferation (PicoGreen® assay)
284 of BM-MSCs cultured either in basal or osteogenic media in the presence of different
285 concentrations (20 and 50 mg/ml) of BBG-Mg (a, b), BBG-Ca (c, d) and BBG-Sr (e, f).
286 The 45S5 bioglass® (g, h) was used as control. Standard culture medium was used as
287 negative control. Results are expressed as mean ± standard deviation with n = 3 for each
288 bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test (p<0.0001),
289 followed by a Dunn's Multiple Comparison test. *** p<0.001; ** p<0.01; * p<0.05 in
290 respect to the control. The statistical significance is represented relative to the control of
291 BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21
292 days).

293

294 From the PrestoBlue® data (Figure 4a, 4c, 4e and 4g) it is possible to observe that the
295 metabolic activity of BM-MSCs increased over the 21 days of culture. In the first 7 days
296 of culture, the BM-MSCs under conditioned cultures presented reduced viability in
297 respect to the cells culture under basal condition (negative control), especially for BBG-
298 Ca culture conditions, which may be due to the initial burst release of ions to the media.
299 In contrast, from day 14, BM-MSC cultures under osteogenic media displayed a
300 reduction of metabolic activity with respect to the control (absence of glass particles and
301 cell cultured under basal medium). It is noteworthy that BM-MSC cultures at day 21
302 with 45S5 bioglass® under osteogenic media showed very low metabolic activity when
303 compared with the control experiment (osteogenic media), being consistent with the cell
304 death observed by fluorescent microscopy (Figure 3). Complementary to the viability
305 analysis, the cellular proliferation was also assessed by measuring the total cell DNA
306 (Figure 4b, 4d, 4f, 4h). The PicoGreen® data showed an increase on the number of BM-
307 MSCs over time. However, under osteogenic media, the cells presented lower
308 proliferation rates than cultures under basal media from day 14. Noteworthy, is the
309 prominent reduction of BM-MSCs for cultures with BBG-Mg (osteogenic and basal
310 media) and 45S5 bioglass® (osteogenic media) at day 21. To emphasize, BBG-Sr and
311 45S5 bioglass® under osteogenic media showed a significant reduction on the cell
312 proliferation at day 14 when compared with the cells cultured under basal media,
313 suggesting an alteration of biological behavior.(33) Regarding the effect of
314 concentration, there was no potential toxic effects with time except in the case of BM-
315 MSCs cultured in the presence of BBG-Mg (osteogenic and basal media) and 45S5
316 bioglass® (osteogenic media) at day 21, where a large reduction in cell number was
317 observed in relation to the control (basal media).

318 **3.2.2 Alkaline phosphate quantification**

319 It is commonly accepted that ALP is a key player in the process of osteogenesis, being
320 ALP known to be involved in early stages of normal and pathological calcification. (34-
321 36) In general, an increase of the ALP activity is correlated with osteogenesis,
322 increasing during the bone formation stage.(37) Not surprisingly, the ALP
323 quantification data (Figure 5) showed a significantly higher ALP activity when cells
324 were cultured under osteogenic media rather than basal media.(38) However, at day 21
325 the levels of ALP activity on BBG-Mg, -Sr and 45S5 bioglass® cultured under
326 osteogenic media are significantly higher than the cultures without glass addition (e.g.
327 at day 21, under osteogenic media, the addition of 20 mg of BBG-Sr (c) induced a
328 highly significant ($p<0.001$) enhanced ALP activity in relation to the cells cultured in
329 the absence of glass particles). BBG-Mg, -Sr and 45S5 bioglass® were not capable of
330 inducing the ALP protein expression alone, however, they were capable to increase the
331 ALP expression during the differentiation process of BM-MSCs into osteoblasts under
332 osteogenic media (for 21 days of cell culture). It is relevant to point out that previous
333 studies quantified the chemical species released from BBGs in solution, demonstrating a
334 higher concentration of Mg^{2+} and Sr^{2+} ions (2-fold) when compared with Ca^{2+} (from
335 BBG-Mg, BBGs-Sr and BBG-Ca, respectively). (29, 30) The presence of higher
336 concentration of specific ions (i.e. Mg^{2+} and Sr^{2+}) might facilitate cell differentiation. It
337 is also relevant to highlight the fact that along with the increase of ALP activity for the
338 case of BBG-Mg and -Sr glasses there was observed an increase of cell proliferation. In
339 contrast, the viability and proliferation data, presented a reduction of viable and live
340 cells for the BM-MSCs cultured in the presence of these BBG-Ca and 45S5 bioglass®.
341 Remarkably, BBG-Sr promoted the increase of ALP activity for both concentrations of
342 glass particles, i.e. 20 and 50 mg/ml.

343

344 **Figure 5.** ALP activity of BM-MSCs (cultured either in basal or osteogenic media in
345 the presence of different concentrations (0, 20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca
346 (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are expressed
347 as mean ± standard deviation with n = 3 for each bar. The data was analyzed by non-
348 parametric statistics: Kruskal-Wallis test (p<0.0001), followed by a Dunn's Multiple
349 Comparison test. *** p<0.001; ** p<0.01; * p<0.05 in respect to the control. The
350 statistical significance is represented relative to the control of BM-MSCs culture in
351 basal media for the respective day of culture (i.e. 7, 14 and 21 days).

352

353 ### 3.2.3 Matrix mineralization

354 Similar results were found at day 21 after alizarin red staining for mineral deposits
355 (Figure 6). In agreement with ALP activity data, cells cultured under osteogenic media
356 yielded more bone-like nodules, resulting from ECM mineralization.(35) The intense
357 red spots dispersed in the cell culture correspond to the mineralized nodules. Of
358 importance, and corroborating with ALP activity data, mineral deposits were more
359 evident with BBG-Mg, -Sr and 45S5 bioglass® (Figure 6a, 6c and 6d) and an increase of
360 red nodules with the increase of concentrations can be observed in the case of the cells
361 cultured in the presence of BBG-Sr (**Error! Reference source not found.**c, osteo 20
362 mg/ml and 50 mg/ml). Higher concentrations of BBG-Sr and 45S5 bioglass® show the
363 presence of red nodules even in cells cultured in basal medium. However, ALP activity
364 data, suggests that BBG-Sr and 45S5 bioglass® (Figure 6c and 6d basal for a
365 concentration of 50 mg/ml) are capable to induce ECM mineralization by themselves.
366 Therefore, the combination of ALP activity and mineralization results suggest the use of
367 BBG-Sr to promote osteogenesis.(39)

368

369 **Figure 6.** Alizarin red staining of BM-MSCs cultured during 21 days, either in basal or
370 osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of
371 BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control.

372

373 **Figure 7.** SEM micrographs of BM-MSCs in the presence of different concentrations
374 (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c) after 21 days of cell
375 culture either in basal or osteogenic media. Higher SEM magnifications are shown as
376 insets, highlighting the formed apatite-like structures. The 45S5 bioglass® was used as a
377 control.

378 In Figure 7 we presented SEM micrographs of BM-MSCs cultured for 21 days in the
379 presence of BBGs and 45S5 bioglass®. In the images it is possible to observe the
380 deposition of minerals over the dense layer of cells, when they were cultured in the
381 presence of BBG-Mg, -Sr and 45S5 bioglass® (Figure 7a, 7c and 7d). The presence of
382 these glass compositions in the culture medium promoted a mineralization typical to
383 occur while BM-MSCs differentiate into osteoblasts.(38) This mineralization is in
384 agreement with the alizarin red data where the mineral deposits were more evident in
385 the cells cultured in the presence of BBG-Mg, -Sr and 45S5 bioglass® (Figure 6a, 6c
386 and 6d). In these culture conditions, it is observed in the SEM/EDS images calcium
387 phosphate deposits over the dense cellular layer. Once more, higher concentrations of
388 BBG-Sr and 45S5 bioglass® under basal culture conditions promoted the deposition of a
389 higher amounts of calcium phosphate structures, suggesting that BBG-Sr and 45S5
390 bioglass® (Figure 7c and 7d basal for a concentration of 50 mg/ml) are capable of

391 inducing ECM mineralization by itself, which could be beneficial for bone regeneration
392 (39).

393 **3.2.4 Protein expression (OP and OC)**

394 Complementary to the reported biological data, the differentiation level of BM-MSC,
395 cultured in the presence (20 and 50 mg/ml) and absence of BBG-Mg, -Ca, -Sr and 45S5
396 bioglass® (either in basal or osteogenic media) was assessed by the quantification of the
397 expression level of two major bone-specific proteins, i.e. OP and OC. The relative
398 expression of these proteins was normalized in relation to the number of cells, i.e.
399 amount of dsDNA. It is well known that osteoblasts are differentiated cells that
400 mineralize the bone matrix. OP is a phosphoprotein synthesized by bone forming cells,
401 which present calcium-binding domains and is responsible for cell attachment,
402 proliferation, and ECM mineralization.(40) In the case of OC, it is a bone-specific
403 glycoprotein capable of binding to calcium, which promotes ECM calcification.(40) Not
404 surprisingly, the OP and OC quantification data (Figure 8 and Figure 9) showed a
405 significantly higher protein expression when BM-MCSs were cultured under osteogenic
406 media rather than basal media.(41) In the case of OP, as expected, a delay in the protein
407 synthesis is observed (Figure 8). At day 7 there was no significant difference of OP
408 expression in relation to the control (absence of glass particles and cell culture in basal
409 medium). However, at day 14 there is a high expression peak by BM-MSCs cultured in
410 osteogenic medium (in the presence of BBG-Mg, -Sr and 45S5 bioglass®), which
411 determines the decay of the matrix deposition phase and the beginning of the
412 mineralization phase. Moreover, BBG-Sr and 45S5 bioglass® continue to induce a
413 significant overexpression of OP over time (e.g. at day 21), supporting the
414 mineralization demonstrated by ALP and alizarin red analysis (**Error! Reference**
415 **source not found.Error! Reference source not found.**Figure 5 and 6, respectively). In

416 the OC case there was a high protein expression up to day 14, indicating bone ECM
417 maturation (Figure 9).(42) At day 7 there is a significant difference in OP expression in
418 relation to the control (cell culture in basal medium and in the absence of glass
419 particles). After day 7 there was a reduction of OC expression, consistent with matrix
420 mineralization. Noteworthy is the observation that BBG-Sr under basal medium induced
421 the BM-MSCs to exhibit a peak of OC expression at day 14. This data suggested that
422 the BBG-Sr glass particles (at a concentration of 50 mg/ml) induced the OC protein
423 expression, which is in agreement with the ALP and alizarin red data. Also, BBG-Sr
424 and 45S5 bioglass® prolonged the OC overexpression over the 21 days of culture. In
425 addition, the 45S5 bioglass® promoted a high deposition of OC at day 21 (Figure 4h)
426 compared with high BM-MSCs density in the case of BBG-Sr (Figure 4f). However, it
427 is important consider that in the case of the cultures in the presence of 45S5 bioglass® a
428 very low BM-MSCs cell density was observed, which might be related with the
429 cytotoxicity of 45S5 bioglass®.(43) Therefore, and overall our data suggests that the
430 BBG-Sr glass particles are able to induce the BM-MSCs to express higher levels of OP
431 and OC, while maintaining the BM-MSCs cell density.

432

433 **Figure 8.** OP protein content of BM-MSCs cultured either with basal or osteogenic
434 media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a),
435 BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are
436 expressed as mean ± standard deviation with n = 3 for each bar. The data was analyzed
437 by non-parametric statistics: Kruskal-Wallis test (p<0.0001), followed by a Dunn's
438 Multiple Comparison test. *** p<0.001; ** p<0.01; * p<0.05 in respect to the control. The
439 statistical significance is represented relative to the control of BM-MSCs culture in
440 basal media for the respective day of culture (i.e. 7, 14 and 21 days).

441

442

443 **Figure 9.** OC protein content of BM-MSCs cultured either with basal or osteogenic
444 media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a),
445 BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are
446 expressed as mean ± standard deviation with n = 3 for each bar. The data was analyzed
447 by non-parametric statistics: Kruskal-Wallis test (p<0.0001), followed by a Dunn's
448 Multiple Comparison test. *** p<0.001; ** p<0.01; * p<0.05 in respect to the control. The
449 statistical significance is represented relative to the control of BM-MSCs culture in
450 basal media for the respective day of culture (i.e. 7, 14 and 21 days).

451

452 Hence, combining the obtained biological data, i.e. viability, proliferation,
453 mineralization and protein expression analysis (ALP, OC and OP), we were able to
454 identify the BBG-Mg, -Sr (at concentrations of 20 to 50 mg/ml) as relevant promoters
455 of the osteogenic differentiation of BM-MSCs. Several authors reported that Mg^{2+} ions
456 significantly enhanced osteoblast adhesion by the altering cell-matrix interactions,
457 which modulate the function of integrins related with cell differentiation. (19, 44) Our
458 results suggest that BBG-Mg also promoted BM-MSCs differentiation, which might be
459 related with the presence of Mg^{2+} in the culture medium. Remarkably, BBG-Sr (at a
460 concentration of 50m/ml and 21 days of culture) presented the capacity to induce
461 osteogenic response in BM-MSCs in the absence of osteogenic medium. Other authors
462 reported Sr containing glasses to stimulate osteoblast metabolic activity, inhibiting
463 osteoclast differentiation, as well as promoting the increment of ALP activity. (24) For
464 instance, Hurtel-Lemaire *et al*. (45) have shown that Sr does induce osteoclast apoptosis

465 at concentrations higher than 9 mM. This is in accordance with our previous studies
466 (29) that demonstrated a concentration of Sr in the reaction media about to 20 mM after
467 3 days of culture. The ALP activity results showed the shift of BM-MSCs to a more
468 differentiated state, while the alizarin red analysis demonstrated that the cells in the
469 presence of BBG-Mg and BBG-Sr glass particles demonstrate intense and dispersed red
470 spots in the cell culture, corresponding to the mineralization promoted by the BM-
471 MSCs. Santocildes *et al.* (25) demonstrated that the dissolution of Sr-containing glasses
472 stimulated the upregulation of genes associated with the process of osteogenic
473 differentiation, such as *Bglap* (osteocalcin) and *Spp1* (osteopontin). In agreement with
474 this data, we also observed that BBGs-Mg an BBG-Sr might also influence the ECM
475 maturation and mineralization, through the promotion of the OP and OC protein
476 overexpression, which suggests that these glass compositions may be effective in
477 inducing and sustaining the osteoblastic phenotype. (46)

478 **4. Conclusion**

479 BBGs with different substituted divalent cations (Ca^{2+}, Sr^{2+} or Mg^{2+}) were successfully
480 synthesized by melt quenching. *In vitro* studies demonstrated that the studied BBGs
481 exhibit the capability to improve the osteogenic differentiation of BM-MSCs with no
482 deleterious effects over cell viability and proliferation. Specially, BBG-Mg and BBG-Sr
483 (at 20 and 50 mg/ml) provided favorable conditions for BM-MSCs to differentiate to
484 osteoblast-like cells and induce the formation of a high amount of mineralized nodules.
485 The phenotypic expression of two major bone-specific proteins, namely, OP and OC
486 confirmed the osteogenic potential of the BBGs.

487 Finally, the findings that the BBGs may promote *in vitro* cell differentiation into an
488 osteogenic lineage, support their potential application in regenerative medicine. Based
489 on these promising results we propose the incorporation of these BBGs into

490 biomaterials for bone regeneration. From our results, BBGs are able to enhance the
491 osteogenic potential of the implants and promote the osteogenic differentiation of BM-
492 MSCs. The proposed BBGs are relevant candidates for further *in vivo* evaluation.

493

494 **Acknowledgements**

495 The authors gratefully acknowledge financial support from Portuguese Foundation for
496 Science and Technology (Ph.D. grant BD/73162/2010), the European Union's Seventh
497 Framework Programme (FP7/2007–2013) under Grant No. REGPOT-CT2012-31633-
498 POLARIS. This work was also supported by the European Research Council grant
499 agreement ERC-2012-ADG-20120216-321266 for the project ComplexiTE and is
500 associated with the UK EPSRC Centre for Innovative Manufacturing of Medical
501 Devices-MeDe Innovation (EPSRC grant EP/K029592/1) where Fernandes was a
502 visiting researcher. The authors also gratefully acknowledge Mr Robert Burton
503 (Sheffield Hallam University) for his support and help with X-ray fluorescence analysis
504 and Prof. S. Armes and Dr. L. Fielding (Department of Chemistry, Sheffield University)
505 for their support with the ATR-FTIR analysis.

506 **Author Disclosure Statement**

507 No competing financial interests exist.

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