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**The Design and Properties of Novel Substituted Borosilicate Bioactive Glasses
and Their Glass-Ceramic Derivatives**

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1 **Abstract**

2 Three novel borosilicate bioactive glasses (BBGs) of general formula of $0.05\text{Na}_2\text{O}\cdot 0.35x$
3 $\cdot 0.20\text{B}_2\text{O}_3\cdot 0.40\text{SiO}_2$ (molar ratio, where $x = \text{MgO}$ or CaO or SrO) were prepared and used to
4 investigate the effect of crystallisation on their properties including cytotoxicity. The three post-
5 melt compositions were determined using X-ray fluorescence spectroscopy and crystallisation
6 events were studied using differential thermal analysis and x-ray diffraction. This information was
7 used to determine heat treatments to prepare glass-ceramics by controlled crystallisation. X-ray
8 diffraction analysis and Fourier transform infrared spectroscopy showed that, after higher heat
9 treatment temperatures (800-900 °C), borosilicate bioactive glass-ceramics (BBGCs) contained
10 mainly borate and silicate crystalline phases. Specifically, BBG-Mg, BBG-Ca and BBG-Sr glass-
11 ceramics detected the presence of magnesium silicate- $\text{Mg}_2(\text{SiO}_3)_2$ and magnesium borate- $\text{Mg}_2\text{B}_2\text{O}_5$;
12 wollastonite- $2\text{M}\cdot\text{CaSiO}_3$ and calcium borate- $\text{Ca}(\text{BO}_2)_2$; and strontium silicate- SrSiO_3 and strontium
13 borate- $\text{Sr}_2\text{B}_2\text{O}_5$, respectively. *In vitro* cytotoxicity tests were performed using the mouse fibroblast
14 cell line (L929). Glass and glass ceramic at concentrations lower than 50 mg/ml did not exhibit any
15 level of cytotoxicity when compared with the control. However, quantitative evaluation indicated
16 that greater cell growth occurred in the presence of materials with crystalline phases. Control of
17 BBGs crystallisation may therefore be used to influence the biocompatibility of these glass-ceramic
18 systems.

19 **Keywords:** Bioactive glasses, borosilicate, crystallisation, glass-ceramic and cytotoxicity.

1 1. Introduction

2 Bioactive glasses (BGs) and bioactive glass-ceramics (BGCs) have been evaluated for a wide range
3 of clinical applications related to bone tissue repair and regeneration ¹. BGs and BGCs are widely
4 reported to form a bone-like hydroxyapatite (HA) layer on their surface when placed in a simulated
5 biological environment ². This has also been demonstrated to occur *in vivo* ³. Formation of a bone-
6 like HA layer is a fundamental requirement for the establishment of a strong interfacial bond
7 between implants and bone ⁴. Since Hench *et al.* ² proposed the CaO: SiO₂: Na₂O: P₂O₅ system in
8 1969, 45S5 bioglass (or Bioglass[®]) has become the gold standard for this type of material.
9 However, slow degradation rate is a major disadvantage of the silicate based BG, which makes it
10 difficult to match the degradation rate of the BG scaffolds with the rate of new tissue formation ^{5,6}.
11 Moreover, some studies with silicate glasses report slow conversion rate to a bone-like HA and
12 often this conversion remains incomplete. Therefore unconverted glass containing SiO₂ could
13 remain in human body long after its implantation, raising uncertainty regarding the long-term
14 effects of SiO₂ *in vivo* ^{7,8}.

15 The addition of boron oxide to the glass network is one approach that has the potential to overcome
16 the limitations identified above by modifying dissolution rates as well as other properties including
17 tissue bonding ⁹. The incorporation of boron in a silicate glass matrix involves lower melting
18 temperatures, with an increased bio-degradation and increased conversion to HA ^{8, 10, 11}.
19 Furthermore, boron is reported to be beneficial for bone healing and its controlled release stimulates
20 bone formation and maintenance. Frequently, it has been associated with the increase in bone
21 resistance to fractures ¹²⁻¹⁴. Thereby, borosilicate bioactive glasses (BBGs) composition may be
22 used to achieve a more controlled release of specific component ions in order to trigger a range of
23 biological responses ¹⁵. Control of the surface reactions and therefore of the biodegradation and
24 bioactivity of implanted materials can be achieved by varying the chemical nature and/or

1 concentration of the BBGs constituents. For instance, Ciceo Lucacel *et al.* ¹⁶ have used the calcium
2 BBGs doped with silver (Ag^+) to better control Ag^+ antibacterial properties. Xu *et al.* ⁶ have also
3 proposed a sol–gel-derived calcium BBGs system for reinforcing glass-ceramic porous biomaterials
4 and accurately control their biodegradability. In another study by Wang *et al.* ¹⁷, evaluating the
5 osteogenic properties of BBGs doped with Zinc (Zn^{2+}) these BBGs significantly enhanced bone
6 regeneration in bone defects when implanted into the rats *in vivo*. Baiano *et al.* ¹⁸ used bioactive
7 glass-based trabecular coating for the development of a novel prosthetic acetabular cup to have an
8 improved *in vivo* interfacial bond with bone.

9 While studies have been demonstrating the ability of BBGs to better control degradation, thus
10 increasing the beneficial properties of ions release, there are still concerns regarding toxicity when
11 those glasses are implanted ^{19, 20}. There are several metal ions that at higher concentrations are
12 extremely toxic. In particular, boron release has been associated with cell growth inhibition for
13 concentrations greater than 2.5 mM and also causes cytotoxicity due to release of $(\text{BO}_3)^{3-}$ into the
14 medium ^{12, 19, 20}. Several cellular studies investigated the cytotoxicity of BGs and reported inhibition
15 of cell viability and proliferation for high doses of ions in culture ^{21, 22}. For instance, Santocildes-
16 Romero *et al.* ²³ have showed an increase of the cytotoxic effect of BGs dissolution by increasing
17 the amount of glass powders used and the level of strontium substitution added to the glass
18 composition. Moreover, specific component ions released in a controlled rate may stimulate
19 differently cells. Metallic ions such as magnesium (Mg^{2+}), calcium (Ca^{2+}) or strontium (Sr^{2+}) can
20 stimulate bone cell proliferation, differentiation and extracellular matrix mineralisation, as well as
21 increase the production rate of HA and bone ^{21, 22, 24-26}. For instance, Mg^{2+} is commonly related with
22 cells adhesion and stability ²⁴, while Ca^{2+} ²⁵ and Sr^{2+} ²⁶ are generally associated to apatite formation
23 process and cells differentiation and mineralisation. Moreover, Sr^{2+} has been successfully studied
24 for the treatment of osteoporosis²¹.

25 Various studies have been reported concerning the use of heat treatments in order to induce
26 crystallisation in BGs to promote changes in their physico-chemical properties ²⁷⁻³¹. For instance,

1 Rao *et al.* ³² have observed improvements in the dielectric constant and less dielectric loss of
2 sodium borosilicate glasses (SiO_2 – B_2O_3 – Na_2O system) with increase in the duration of the heat
3 treatment. Daguano *et al.* ²⁷ developed and characterised BGs and BGCs from the CaO - P_2O_5 - SiO_2 -
4 MgO system, using different degrees of crystallinity. They showed that partial crystallisation
5 improved mechanical properties by phase transformation, which modified the microstructure of the
6 base glassy material. Therefore, after studying the thermal profile of BGs, appropriated heat
7 treatment cycle with a controlled temperature increase could be applied to promote the re-
8 arrangement of the glass structures generating a well-ordered and crystalline structure. The
9 properties of the formed glass-ceramics are mostly influenced by the characteristics of the finely
10 dispersed crystalline and the residual glassy phases, which can be controlled by the composition of
11 the base glass. However, few authors have reported that the formation of new crystalline phases
12 provoked the modification of toxic effects induced by the glasses in cells ^{27, 30}. Specially, Hurrell-
13 Gillingham *et al.* ³⁰ investigated the effects of devitrification of glass-ionomer cements (GICs) from
14 SiO_2 - Al_2O_3 - P_2O_5 - CaO - CaF_2 system on glass-ceramic formation and *in vitro* biocompatibility. They
15 demonstrated that crystallisation might be used to improve the *in vitro* biocompatibility of GICs.
16 Also Freeman *et al.* ³³ studied the response to implantation of apatite glass-ceramics. They
17 demonstrated that crystallisation significantly improved the bone tissue response.

18 It seems likely that devitrification of glasses represents a route to modify and study their properties
19 including biocompatibility. However, few attempts to modify the biocompatibility of a medical
20 grade glasses using this approach have been reported ^{30, 33}. This study aimed to firstly synthesise
21 and characterise the thermal and chemical properties of three novel substituted BBGs. The
22 incorporation of different ions (i.e. Mg^{2+} , Ca^{2+} and Sr^{2+}) in BBGs lies on the fact that specific
23 effects can be used to achieve different human cell behaviour by the release of ionic dissolution
24 products. Secondly, we aimed to investigate the effect of BBGs crystallisation on their cytotoxic
25 effects, providing a method for the improvement of bioactive borosilicate glass-ceramics cellular
26 properties.

1 **2. Materials and methods**

2 **2.1. Materials**

3 All chemical compounds used for melt-quenched synthesis were reagent grade: di-boron trioxide
4 (Alfa Aesar, Germany), calcium carbonate (Sigma-Aldrich, Portugal), sodium bicarbonate (Sigma-
5 Aldrich, Australia), silica gel 60M (Macherey-Nagel, Germany), magnesium oxide (Sigma-Aldrich,
6 Portugal) and strontium carbonate (Sigma-Aldrich, Australia).

7 **2.2. Glass synthesis and preparation**

8 The novel BBGs of general formula $0.05\text{Na}_2\text{O} \cdot x\text{MgO} \cdot y\text{CaO} \cdot (0.35-x-y)\text{SrO} \cdot 0.20\text{B}_2\text{O}_3 \cdot$
9 0.40SiO_2 (molar ratio, where $x, y = 0.35$ or 0.00 , and $x \neq y$) were synthesised by melt quenching.
10 The appropriate amounts of SiO_2 , B_2O_3 , NaHCO_3 , and CaCO_3 or MgO or SrCO_3 were accurately
11 mixed with ethanol (Sigma, Portugal) in a porcelain pestle and mortar, fully dried overnight and
12 transfer to a platinum crucible. The batch was heated to $1450\text{ }^\circ\text{C}$ in air for 1 hour and subsequently
13 the melt was quickly poured in a water bath at $4\text{ }^\circ\text{C}$ to form a glass frit. The glass frit was ground
14 into an Agate mortar (RETSCH, Germany) to obtain microparticles and then, sieved for a size
15 smaller than $63\text{ }\mu\text{m}$.

16 **2.3. Characterisation**

17 **2.3.1. X-ray fluorescence (XRF)**

18 The X-ray fluorescence spectroscopy was used to confirm the glass composition (Philips PW2400
19 X-ray fluorescence spectrometer). Samples were prepared by fusing powdered glass with a known
20 quantity of boron and a flux producing a glass-like bead. After which, they were irradiated with
21 high-energy primary X-ray photons. All the samples were run in triplicate and the percentage (w/w)
22 of the oxides present were determined. Afterwards, molar percentage was calculated for all the
23 samples.

2.3.2. *Differential thermal analysis (DTA) and heat treatment*

The mid point of glass transition (T_g), and crystallisation (T_c) temperatures were determined by differential thermal analysis (Perkin-Elmer DTA7 running Pyris thermal analysis software in Unix) at a heating rate of $10\text{ }^\circ\text{C min}^{-1}$ from 50 to $1300\text{ }^\circ\text{C}$.

In accordance with DTA data, crystallised glass was prepared for each sample. Briefly, fast-quenched particles of the glass samples were heat-treated with a heating rate of $10\text{ }^\circ\text{C min}^{-1}$ and held for 120 min at each temperature (T_g , T_{c1} , T_{c2} and T_{c3}) before cooling to room temperature.

2.3.3. *X-ray diffraction analysis (XRD) and attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy*

The glasses were analysed before and after each heat treatment by X-ray diffraction analysis (XRD) and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). A STOE STADI P X-ray diffractometer was used to identify the crystalline phases. The glass samples were placed in aluminium holders and analysed by Cu radiation of wavelength 1.5406 \AA at 40 kV and 40 mA with a step size of 0.2° in a range of 2θ values from 10° to 80° at a scanning speed of 10 s/step. The crystalline patterns were identified by the use of the cards listed in the Joint Commission on Powder Diffraction Standards (JCPDS). The ATR-FTIR spectra were obtained using a Perkin-Elmer GX instrument in the range of $4000\text{--}550\text{ cm}^{-1}$ (resolution 4 cm^{-1}) for the identification of the chemical bonds present in the glass or glass-ceramic structures.

2.4. *In vitro cytotoxicity*

The *in vitro* cytotoxicity study was designed following the international guidelines ISO 10993-5:2009³⁴, using immortalised mouse lung fibroblast-like cell line (L929, Rockville, MD) in indirect contact with glass powders for 3 days. The samples were sterilised in an oven (Gallenkamp Hot Box, England) at $160\text{ }^\circ\text{C}$ for 120 min. After sterilisation, the glasses were added to Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, UK) solution at a concentration of 0.2 g/mL and incubated

1 for 24 h at 37 °C. After incubation, the glass-conditioned medium was removed and filtered through
2 a 0.2 µm membrane.

3 The L929 cells were expanded in DMEM supplemented with 10 % (v/v) fetal calf serum (Sigma-
4 Aldrich, UK), penicillin (100 U/mL, Sigma-Aldrich, UK) and streptomycin (100 µg/mL, Sigma-
5 Aldrich, UK), and non-essential amino acids (NEAA, Sigma-Aldrich, UK). The L929 cells were
6 cultured at 37 °C in an atmosphere of 5 % CO₂. Confluent L929 cells at passages between 15 and
7 19 were harvested and seeded into 48-well culture plates (growth area 0.95 cm²) at a density of
8 2×10⁴ cells/well. After a 24 h culture period the culture media was discarded and replaced with 0.5
9 cm³ of serially diluted, glass-conditioned medium (at a final concentrations of 100, 50, 25, 10, and 5
10 %). L929 cells cultured in the absence of glass particles, was used as negative control and 45S5
11 bioglass- conditioned medium as positive control. After 1 and 3 days time of culturing, the culture
12 medium was removed and the viability and DNA content of the cultures were analysed using
13 PrestoBlue[®] and Picogreen[®] assays respectively.

14 **Cell viability assessment:** The PrestoBlue[®] reagent (Fisher Scientific, UK) is a resazurin-based
15 non-fluorescent solution that is reduced to fluorescent resorufin by viable cells. The assay was
16 performed according to the manufacturer's instructions. In brief, the PrestoBlue[®] reagent was added
17 to a final concentration of 10% to the cell cultures and the cultures incubated for 1 h at 37°C. 200 µl
18 samples of the culture medium were removed and placed in 96-well plates and the resorufin
19 fluorescence quantified spectrophotometrically using a plate reader (Tecan Infinite M200). The
20 fluorescence was determined at an excitation wavelength of 560 nm and emission wavelength of
21 590 nm. The cell viabilities are normalised as % of the metabolic activity of the control cell
22 cultures.

23 **DNA measurement:** The PicoGreen[®] dsDNA reagent (Invitrogen, USA) is an ultrasensitive
24 fluorescent nucleic acid dye for quantification of double-stranded DNA (dsDNA) in solution. This
25 assay enables measurement of cell proliferation. After each culturing period, the cell monolayers

1 were washed with PBS and then incubated at 37 °C for 3 h followed by freezing step at -80 °C for at
2 least over night in ultra-pure water (1 mL) to ensure cell lysis. The assay was performed according
3 to the manufacturer's protocol. And the fluorescence was determined at an excitation wavelength
4 of 485 nm and emission wavelength of 528 nm. The DNA values were normalised to those of the
5 control cultures

6 **Morphological evaluation of cell cultures:** After each culturing period the cell monolayers were
7 washed with PBS and fixed with 4 % formalin solution (0.5 mL) for 15 min at room temperature
8 (RT). The cell layers were then washed with PBS, containing 0.2 % Triton X, for 2 min. After the
9 fixation and permeation steps, the cell monolayers were washed again with PBS and stained with
10 4,6-diamidino-2-phenylindole dilactate (1:1000 DAPI, Sigma, UK) for 2 min at RT, and phalloidin-
11 tetramethylrhodamine B isothiocyanate (Sigma, UK) for 1 h at RT. Finally, the cells were washed
12 and observed using an Axioplan 2 imaging fluorescent microscope with a digital camera QIC AM
13 12-bit (Zeiss, UK).

14 ***2.5. Statistical analysis***

15 Experiments were run at least in triplicate for each sample. All data were expressed as mean \pm
16 standard deviation (SD). Statistical analysis was determined by using Graphpad Prim[®] software,
17 version 6.0. The normality of the data distribution was monitored by Shapiro-Wilk test ($p < 0.05$).
18 Some of the datasets were not considered to have a normal distribution, requiring a non-parametric
19 statistical evaluation. In this context, Kruskal-Wallis test ($p < 0.05$) was applied to all dataset,
20 followed by a Dunn's Multiple Comparison test.

1 3. Results and Discussion

2 3.1. Glass characterisation

3 3.1.1. X-ray fluorescence

4 The X-ray fluorescence data, presented in Table 1, confirmed that the experimental compositions of
5 bioactive borosilicate glasses obtained by melt-quench method were close to the expected
6 theoretical composition, indicating that the glass network structure was formed as aimed. However,
7 the slight differences between theoretical and XRF data may have been caused by the high volatility
8 of alkali-borate compounds during melting process ³⁵.

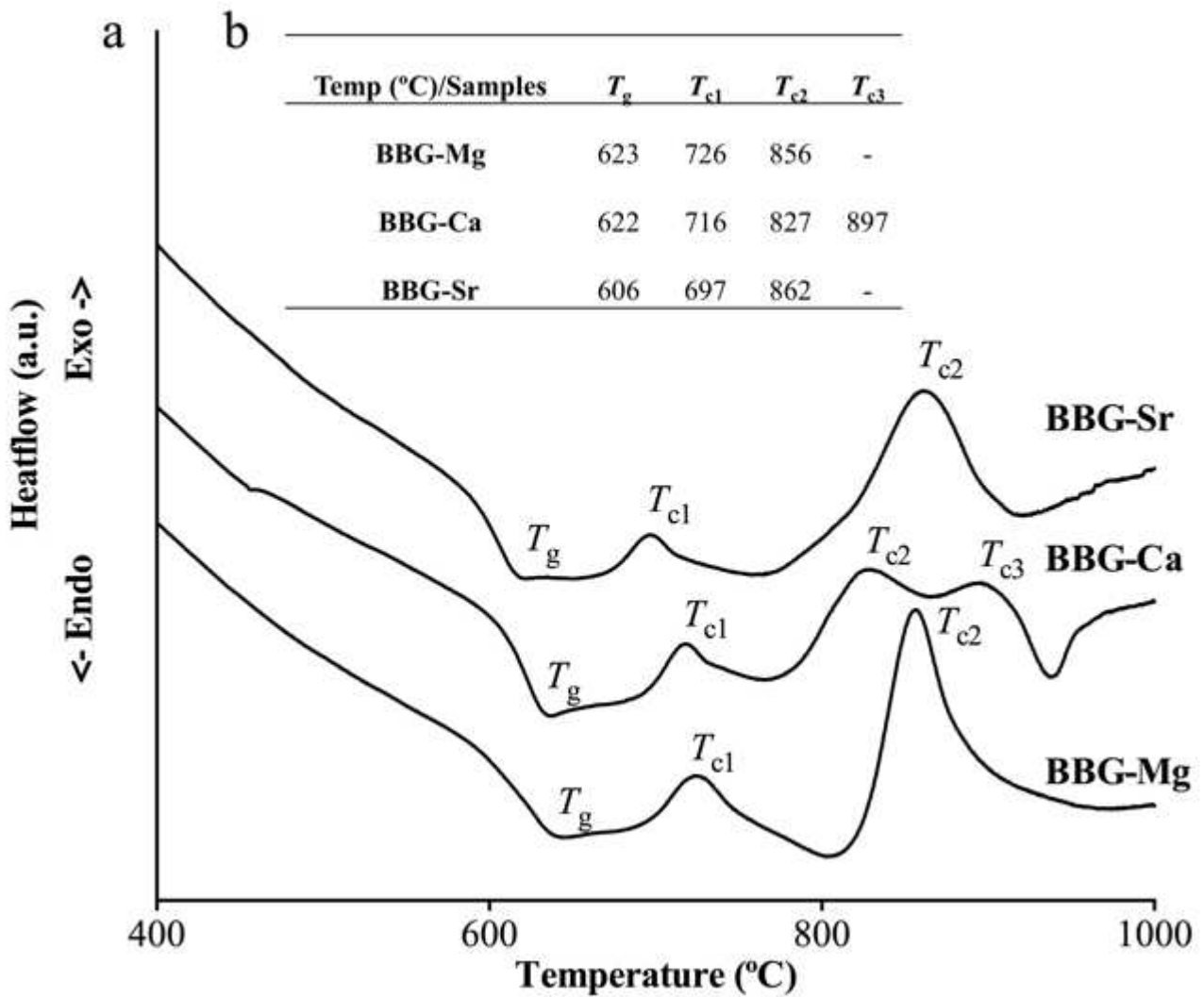
9 **Table 1** - X-ray fluorescence spectrometry estimation of elemental concentration (mol %) of BBGs.
10 BBG-Mg, -Ca and -Sr glasses (* varies with the specific ion for each glass). All analysis were
11 performed in triplicate and standard deviation < 0.05.

Samples / composition	Si (mol %)	B (mol %)	Na (mol %)	Ions* (mol %)
BBG-Mg	37.6 (40)	34.8 (40)	10.4 (10)	39.7 (35)
BBG-Ca	36.9 (40)	43.8 (40)	10.4 (10)	36.0 (35)
BBG-Sr	37.5 (40)	41.4 (40)	10.4 (10)	36.7 (35)

12 3.1.2. Differential thermal analysis

13 The DTA was used to study the phase transformation temperatures of the BBGs in order to
14 determine the heat treatment schedule. DTA patterns from all BBGs are presented in Figure 1a. For
15 BBG-Mg and BBG-Sr along with the mid point of glass transition temperature (T_g - endothermic
16 peak), two crystallisation temperatures (T_c - exothermic peaks) were found; while BBG-Ca glasses
17 presented a T_g and one exothermic (T_{c1}) peak followed by a doublet exothermic peak (T_{c2} and T_{c3}).

1 The T_g and T_c used for the different glass compositions are listed in Figure 1b. The BBGs revealed
 2 crystallisation temperatures within the range of temperatures of 700 to 900 °C with two phases
 3 frequently related with borate and silicate crystal phases ¹⁵. The endothermic peaks are related to
 4 the molecular re-arrangement in glass structure preceding and during glass crystallisation, a process
 5 known for crystal nucleation and growth ^{15, 29}. In order to induce crystal nucleus formation in the
 6 glass structure, BBGs were kept for 2 hours at T_g and subsequently for 2 hours at each T_c . Each
 7 well-defined exothermic peak may result in the formation of a different crystalline phases ²⁸. For
 8 each BBGs different heat treatment schedules were applied according to the endo- and exothermic
 9 peak temperatures reported in Figure 1b.



10

11 **Figure 1** - (a) DTA patterns of BBG-Mg, BBG-Ca and BBG-Sr glasses. T_g – mid point of glass
 12 transition temperature and T_c – crystallization temperature. (b) The mid point of glass transition

1 temperature and crystallization temperatures for the BBGs (BBG-Mg, -Ca and -Sr glasses)
2 determined by DTA analysis (T_g – mid point of glass transition temperature and T_c – crystallization
3 temperature).

4 *3.1.3. X-ray diffraction and attenuated total reflection Fourier transform infrared*

5 The XRD analysis was performed in order to study the crystallinity of the glasses before and after
6 heat treatments and the respective phases formed (Table 2). Figure 2a, 2b and 2c shows the phase
7 evolution XRD patterns of BBG-Mg, -Ca and -Sr glasses, respectively, from amorphous to
8 crystalline phase after the different heat treatment schedules. Heat treatment schedule at T_g were
9 performed and exhibit no significant difference in respect with the as quenched XRD patterns.

10 The XRD patterns of BBG-Mg glass after the first crystallisation heat treatment (BBG-Mg_ T_{c1})
11 showed a predominantly amorphous phase with weak diffraction peaks, which can be assigned to
12 silicon oxide (SiO₂, JCPDS Card No. 11-252). After T_{c2} heat treatment (BBG-Mg_ T_{c2}) two
13 predominant crystalline phases of magnesium silicate (Mg₂(SiO₃)₂, JCPDS Card No. 86-433) and
14 magnesium borate (Mg₂B₂O₅, JCPDS Card No. 73-2232) were observed.

15 In the case of XRD patterns of BBG-Ca glass after the first crystallisation heat treatment (BBG-
16 Ca_ T_{c1}) it was observed a predominantly amorphous phase with some weak diffraction peaks,
17 which can be attributed either to calcium silicate (CaSiO₃, JCPDS Card No. 76-186) and/or calcium
18 borate (Ca(BO₂)₂, JCPDS Card No. 31-155), indicating that after T_{c1} crystallisation started to occur.
19 After the second heat treatment (BBG-Ca_ T_{c2}) there is the formation of two predominant crystalline
20 phases, calcium silicate, commonly referred as wollastonite-2M and calcium borate. After the T_{c3}
21 heat treatment (BBG-Ca_ T_{c3}) the diffraction patterns are comparable to the previous heat treatment
22 patterns (T_{c2}), which indicates that no detectable amount of different crystalline phase is forming.

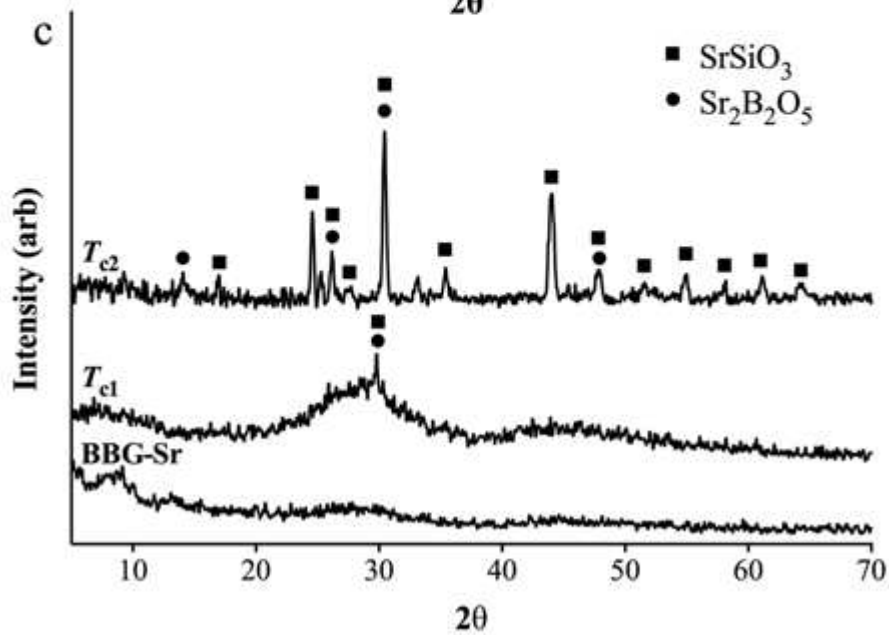
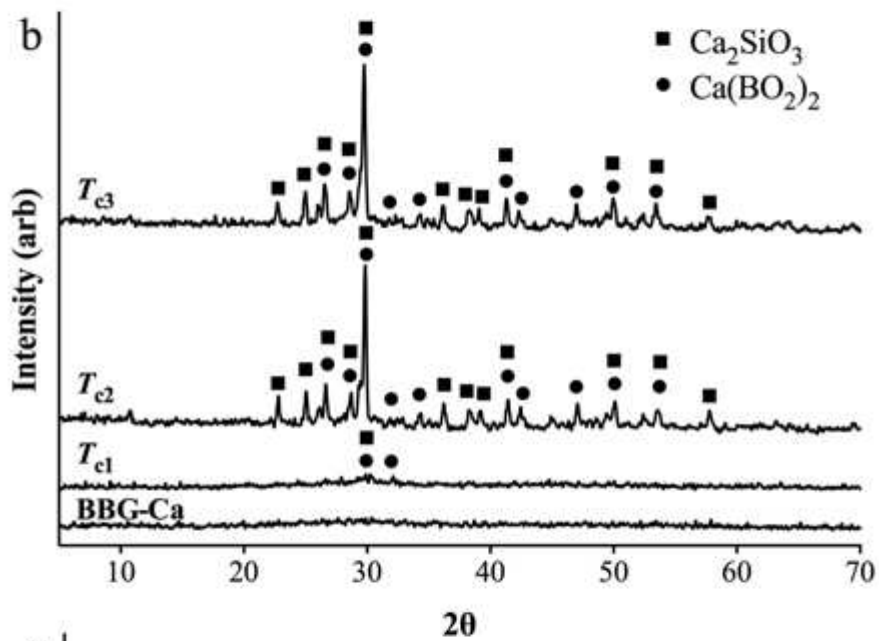
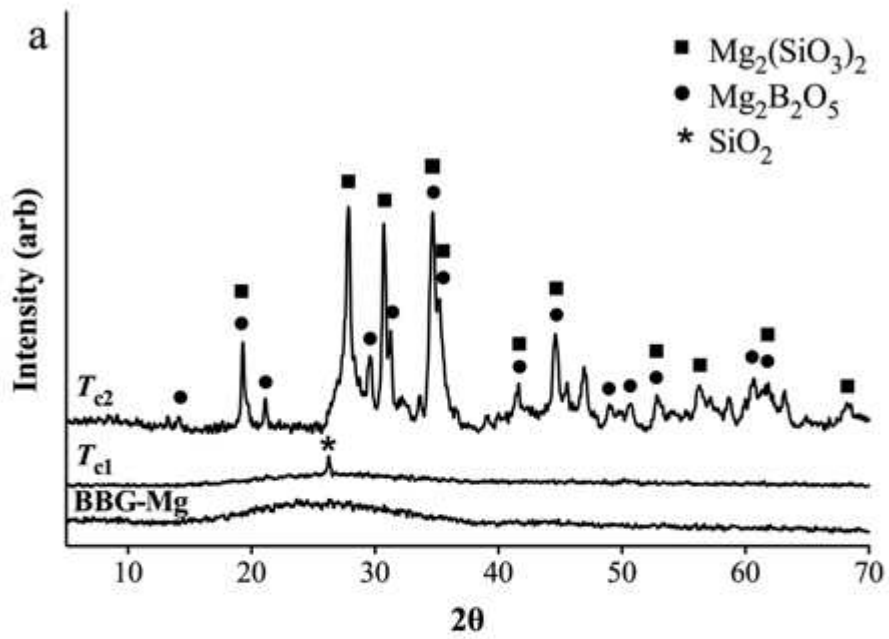
23 For the BBG-Sr glass, after the first crystallisation heat treatment (BBG-Sr_ T_{c1}) it was observed a
24 predominant amorphous phase of the glass and a weak diffraction peak, in which the weak peaks

1 can be attributed either to strontium silicate (SrSiO_3 , JCPDS Card No. 87-474) and/or strontium
 2 borate ($\text{Sr}_2\text{B}_2\text{O}_5$, JCPDS Card No. 19-1268), suggesting that the formation of crystalline phases
 3 starts after the first crystallisation heat treatment. After the second heat treatment (BBG-Sr_ T_{c2})
 4 there were formed two crystalline phases of strontium silicate and strontium borate.

5 Finally, XRD data showed that for all BBGs after heat treatment with lower crystallisation
 6 temperatures (T_{c1}) there was predominantly amorphous with only a small amount crystals
 7 formation. However after latter heat treatments (T_{c2} and T_{c3}) occurred the formation of crystalline
 8 phases resulting in new silicate and borate glass-ceramics.

9 **Table 2** - Crystalline phases of BBG-Mg, -Ca and -Sr glasses obtained after heat treatments

Heat treatment	Heat temperature conditions	Crystalline phases
BBG-Mg_ T_{c1}	623 °C / 2 h + 726 °C / 2 h	Glass and SiO_2
BBG-Mg_ T_{c2}	623 °C / 2 h + 856 °C / 2 h	$\text{Mg}_2(\text{SiO}_3)_2$; $\text{Mg}_2\text{B}_2\text{O}_5$
BBG-Ca_ T_{c1}	622 °C / 2 h + 716 °C / 2 h	Glass and $\text{Ca}(\text{BO}_2)_2$
BBG-Ca_ T_{c2}	622 °C / 2 h + 827 °C / 2 h	CaSiO_3 ; $\text{Ca}(\text{BO}_2)_2$
BBG-Ca_ T_{c3}	622 °C / 2 h + 897 °C / 2 h	CaSiO_3 ; $\text{Ca}(\text{BO}_2)_2$
BBG-Sr_ T_{c1}	606 °C / 2 h + 697 °C / 2 h	Glass and SrSiO_3 ; $\text{Sr}_2\text{B}_2\text{O}_5$
BBG-Sr_ T_{c2}	606 °C / 2 h + 862 °C / 2 h	SrSiO_3 ; $\text{Sr}_2\text{B}_2\text{O}_5$

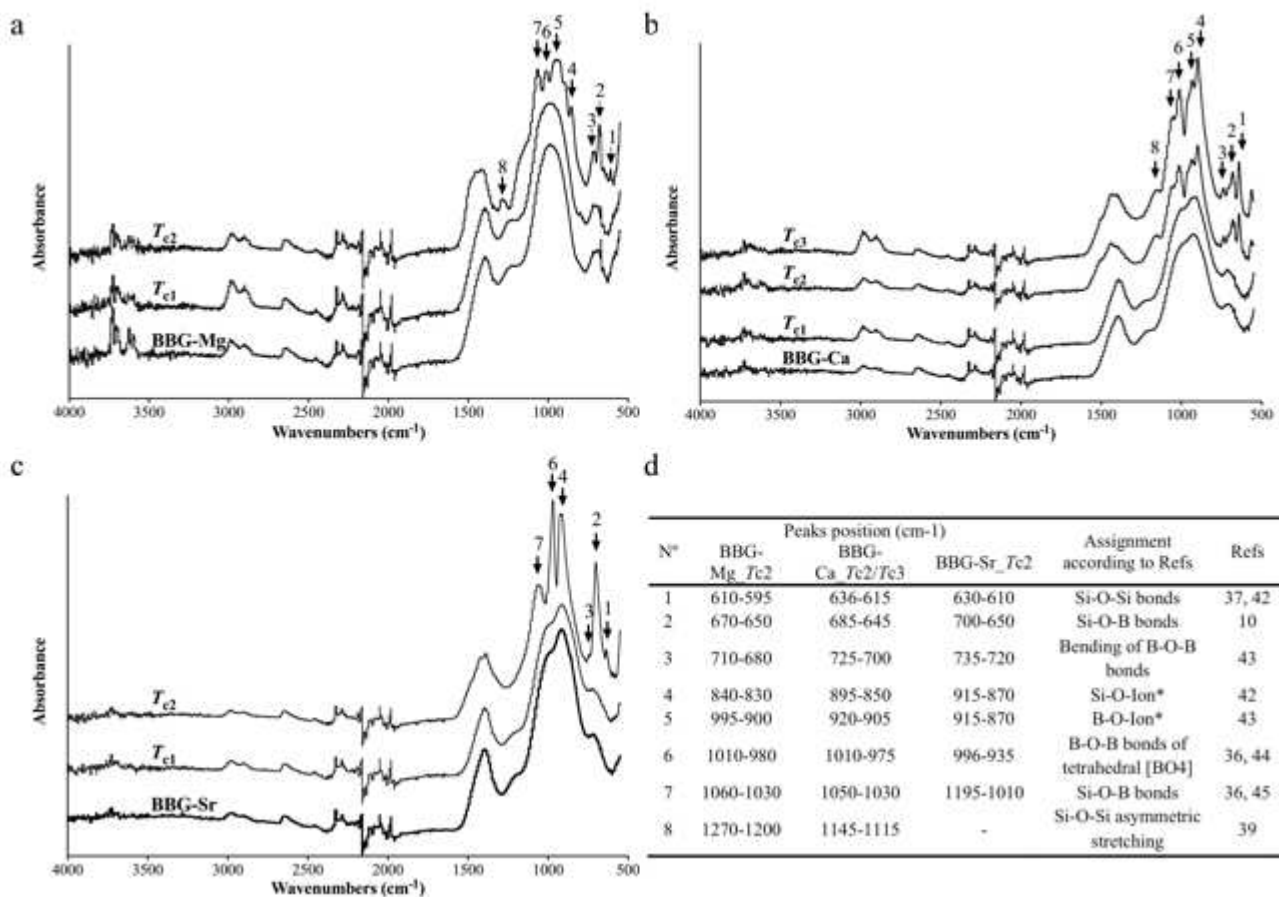


1 **Figure 2** - X-ray diffraction patterns of phase evolution over increasing crystallisation temperatures
2 for BBG-Mg (a), -Ca (b), -Sr (c) glasses (BBG-ion - before heat treatment, T_{c1} - after first heat
3 treatment, T_{c2} - after second heat treatment, T_{c3} - after third heat treatment).

4 The ATR-FTIR analysis was performed to evaluate the possible changes of vibrational spectra after
5 the heat treatments, because they can induce a process of structural grouping re-arrangements,
6 implying important modifications in the properties of the glasses^{30,36}. ATR-FTIR spectra of BBG-
7 Mg, -Ca, -Sr glasses and glass-ceramics are presented in Figure 3a, 3b and 3c, respectively. ATR-
8 FTIR spectra of all BBGs consist of dominant broad bands from 740-585 and 1210-740 cm^{-1}
9 generally attributed to B-O stretching of tetrahedral $[\text{BO}_4]$ units, and 1550-1260 cm^{-1} , which are
10 attributed to B-O stretching of trigonal $[\text{BO}_3]$ unit^{10,36}. On the other hand the same broad bands
11 from 740-585 and 1240-740 cm^{-1} can be also assigned to Si-O-Si symmetric and asymmetric
12 stretching, respectively³⁷⁻³⁹. The presence of these intense bands indicates the coexistence of
13 silicate and borate bonds, which supports the presence of borosilicate network structure in the
14 amorphous phase. There are two additional less intense bands ranging from 3000 to 2800 and 3750
15 to 3500 cm^{-1} in all spectra before and after heat treatment, which can be associated with water
16 content. The bands in the range 3000 to 2800 cm^{-1} have origin in hydrogen bonds and peaks from
17 3750 to 3500 cm^{-1} are due to OH- groups^{36,40,41}. Furthermore, there are no significant differences
18 comparing ATR-FTIR spectra of BBGs before and after T_{c1} heat treatment (comparing BBG-ion
19 with T_{c1} of Figure 3a, 3b and 3c). This fact suggests that there was no formation of detectable
20 crystalline phases. However, for the latter heat treatments (T_{c2} and T_{c3}) all BBGs showed a splitting
21 of the intense broad bands in several sharp peaks. The splitting of the broad bands might indicate
22 structural grouping re-arrangements (e.g. 1210-740 cm^{-1} broad band is splitting in 4 different peaks
23 for BBG-Mg glass-ceramic; Figure 3a). Thus, temperatures higher than 800 °C indeed affected the
24 glass network by rearranging the borosilicate structure. Moreover ATR-FTIR spectra of BBG-Ca
25 glass-ceramics (after T_{c2} and T_{c3} heat treatment – Figure 3b) did not show significant differences.
26 This analysis agrees with XRD data, indicating that the crystallisation temperatures of the double

1 exothermic peak found by DTA (Figure 1) did not introduce an evident different molecular re-
2 arrangement in glass structure and thereby their ATR-FTIR spectra will be analysed as one. The
3 correspondent assignments for sharp peaks formed after the latter heat treatments (T_{c2} and T_{c3}) are
4 presented in Figure 3d, from where vibrational peaks ranging from 636-595 and 1270-1010 cm^{-1}
5 can be attributed to the formation of Si-O-Si bonds and Si-O-Si asymmetric stretching, respectively
6 ^{37, 39, 42}. On the other hand peaks ranging from 735-680 and 1010-935 cm^{-1} can be assigned to
7 bending of B₂-O bonds in the borate glassy network and B₂-O bond of tetrahedral [BO₄] ^{36, 43, 44}.
8 Finally, peaks ranging from 680-645 and 1195-1010 cm^{-1} can be attributed to the formation of Si-
9 O-B bonds ^{10, 36, 45}. These assigned peaks confirmed the existence of new silicon and boron units at
10 the structure network as shown by XRD data. Of highlighting are the peaks found from 915-830
11 and 955-870 cm^{-1} that can be assigned to the formation of Si-O-Ion and B-O-Ion bonds. That
12 indicates the presence of silicate and borate structures bonding metallic ions, which is in agreement
13 with the formation of metal silicates and borates found by XRD analysis ^{10, 42, 43}.

14 The ATR-FTIR analysis showed that for the latter heat treatments there are peaks that can be
15 attributed to B-O-B and Si-O-Si bonds formation. The presence of those bonds supports the
16 formation of silicate and borate crystalline phases previously indicated by XRD data. Also, peaks in
17 the region of 950 to 850 cm^{-1} that are related with the formation either of Ion-O-Si and Ion-O-B,
18 strongly supports the presence of crystalline phases metal silicate and borate.



1

2 **Figure 3** - ATR-FTIR spectra of BBG-Mg (a), -Ca (b) and -Sr (c) glasses (BBG-ion - before heat
 3 treatment, T_{c1} - after first heat treatment, T_{c2} - after second heat treatment, T_{c3} - after third heat
 4 treatment). The numbers are referred to the type of bond found: 1 and 8 – Si-O-Si; 2 and 7 – Si-O-
 5 B; 3 and 6 – B-O-B; 4 – Si-O-Ion; 5 – B-O-Ion. (d) ATR-FTIR band assignments.

6 **3.2. *In vitro* cytotoxicity**

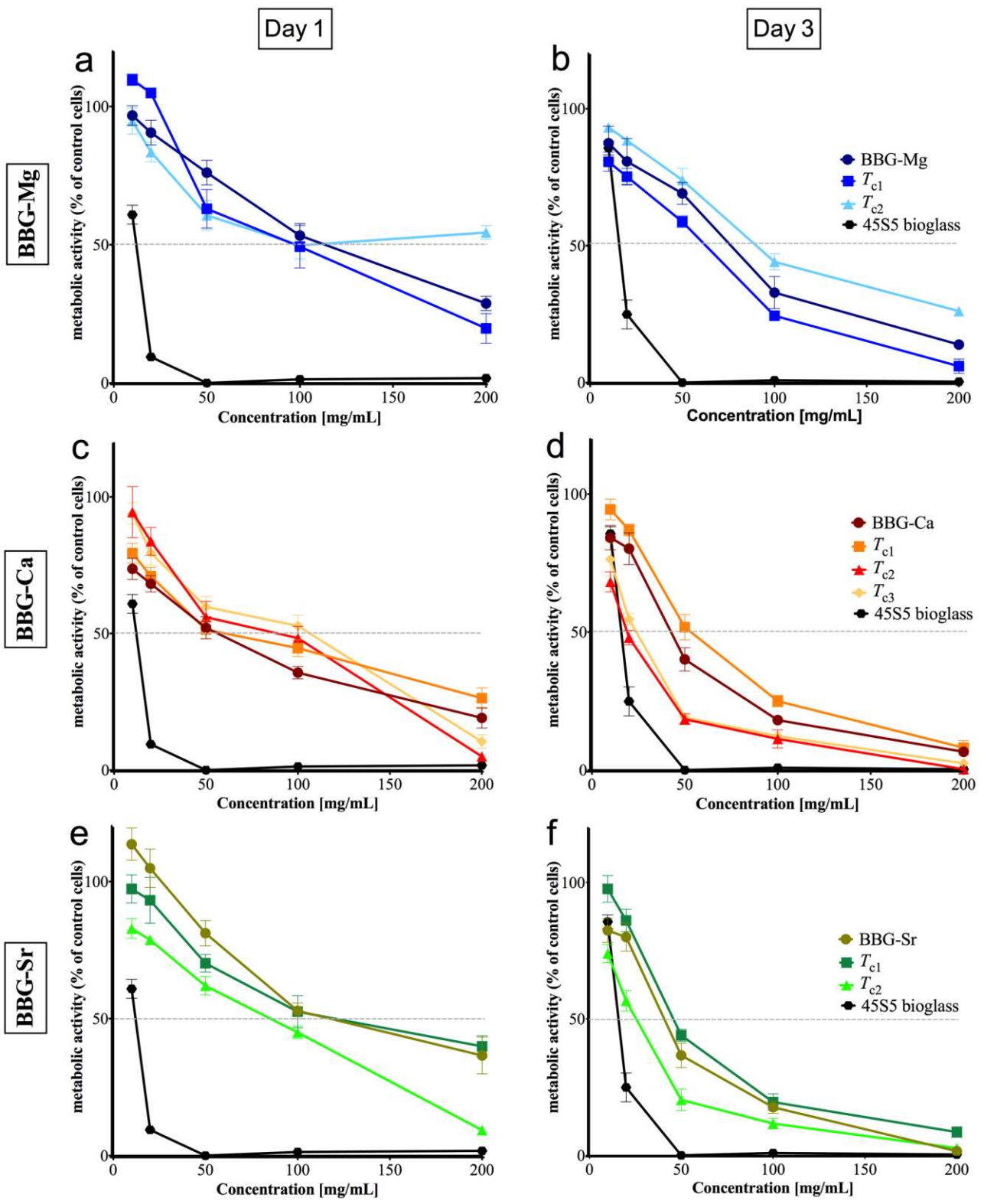
7 The cytotoxicity studies were designed to evaluate whether the degradation or dissolution of glasses
 8 and glass-ceramics affected the viability and proliferation of mouse lung fibroblast-like cells
 9 (L929). The experimental design was based on the international guidelines of ISO 10993-5:2009
 10 with some adjustments as described in the methods section³⁴. Each glass and glass-ceramic sample
 11 was pre-incubated in DMEM to give a glass-conditioned media that were added to the cell cultures
 12 for 1 and 3 days.

1 The PrestoBlue[®] viability data (Figure 4) showed that L929 cells in the presence either of glass- or
2 glass-ceramic-conditioned medium exhibited a metabolic activity higher than 50% for
3 concentrations lower than 50 mg/mL. In the case of BBG-Mg glass and glass-ceramics even after 3
4 days of culture, the cells displayed good metabolic activity for media conditioned by concentrations
5 of 50 mg/mL (e.g.: for BBG-Mg_ T_{c2} at 50 mg/mL concentration, cells present 74 % of metabolic
6 activity). However, all BBGs before and after heat treatment showed far higher metabolic activity
7 than the commercial 45S5 bioglass (e.g.: after 3 days of culture with 45S5 bioglass-conditioned
8 media, L929 cells showed only 1% of the metabolic activity observed in the control cultures).

9 Figure 5 exhibits the DNA measurements of L929 culture normalized to the control cultures
10 incubated in culture medium only. For all BBGs, before and after heat treatment, concentrations
11 below 50 mg/mL showed DNA levels indicating cell proliferation rates close to 100%, meaning that
12 the conditioned medium was not affecting cell proliferation. Similarly to the results observed in the
13 cell viability assays, BBG-Mg glass or glass-ceramics exhibited less cytotoxic effects than BBG-Ca
14 and -Sr glasses and glass-ceramics. Higher proliferation rates were observed with the media
15 conditioned with BBG-Mg than for the BBG-Ca or BBG-Sr-conditioned media (Figure 5).
16 Furthermore, culture medium conditioned by 45S5 bioglass was found to be significantly more
17 cytotoxic than culture medium conditioned with BBG glasses and glass-ceramics. Crystallisation of
18 the glasses was found to have no significant effect on cellular behaviour at day 1 however at day 3,
19 different cellular behaviour profiles were observed at all the concentrations tested. Specifically, for
20 the BBG-Mg glasses, an increase in cytotoxicity was detected for the first heat-treated glass-
21 ceramics (T_{c1}), which could be related to the formation of SiO_2 during the heat treatment. The
22 formation of SiO_2 molecules reduces the amount of silicate in the dominant amorphous phase,
23 which may increase the ratio of borate in the amorphous glass matrix. The borate-rich amorphous
24 phase has a faster degradation rate¹⁵, increasing the quantity of ions in solution as well as the pH (~
25 8.0-9.0)⁴⁶. On the other hand, the BBG-Mg glass-ceramics formed after the second heat treatment
26 (T_{c2}) showed a reduction in cytotoxic, which may have been due to the formation of two different

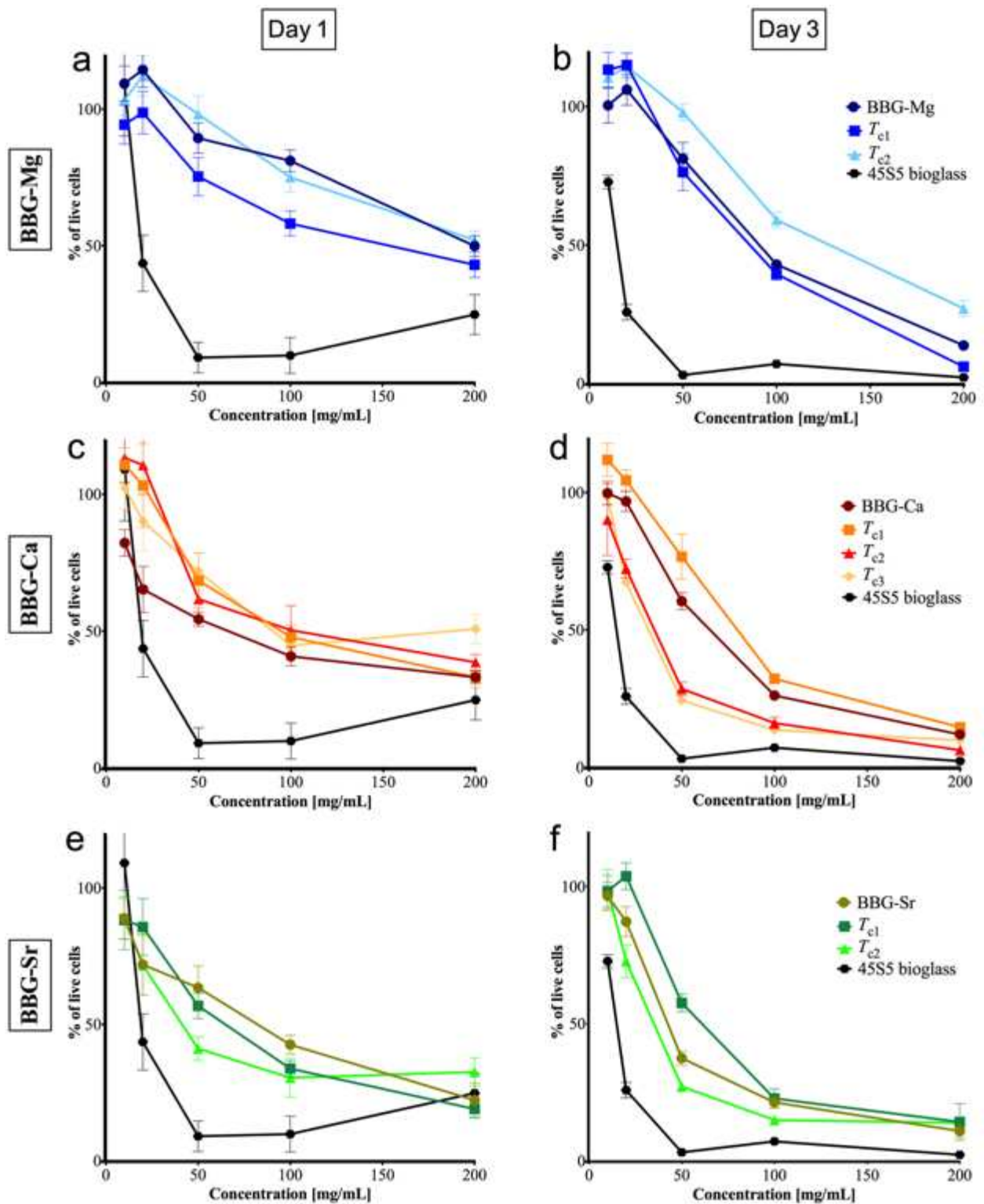
1 crystalline phases (magnesium silicate and magnesium borate) so reducing the availability of ions
2 contained largely within the stable crystalline phases^{27, 30}.

3 The glass-ceramics produced from heat-treated BBG-Ca and -Sr glasses also showed different
4 cellular behaviour from the parent glasses. A reduction in cytotoxicity was found for glass-ceramics
5 formed after the first heat treatment (T_{c1}) and an increase in cytotoxicity with further heat
6 treatments (T_{c2} and T_{c3}). These results may be explained by the formation of different crystalline
7 phases with higher availability for ion release, which may have increased the pH and can be directly
8 observed by colour changes in medium culture³⁰. The pH of the non-conditioned and conditioned
9 media was measured and found to range from 8.0 to 8.5 for all BBG glasses and glass ceramics.
10 The pH of conditioned media from 45S5 bioglass ranged between 8.5 and 9. While the addition of
11 boron to glass matrix is associated with bone healing and formation as well higher conversion rates
12 to HA^{6, 12}; the cytotoxic evaluation demonstrated that it is possible to modify the cytotoxic effects
13 of BBGs by a controlled crystallisation of the constituent glasses. Together, these features make
14 BBGs suitable candidates for bone tissue engineering application.



1

2 **Figure 4** - The cell viability of L929 cells after 1 and 3 days of culture with conditioned medium
 3 from BBG-Mg, -Ca and -Sr glass and glass-ceramics. (a, c and e – 1 day of culture, b, d and f - 3
 4 days of culture).



1

2 **Figure 5** - The proliferation of L929 cells in contact with BBG-Mg, -Ca and -Sr glass and glass-
 3 ceramic-conditioned media after 1 and 3 days of culture (a, c and 3 - after 1 day of culture, b, d and
 4 f - after 3 days of culture).

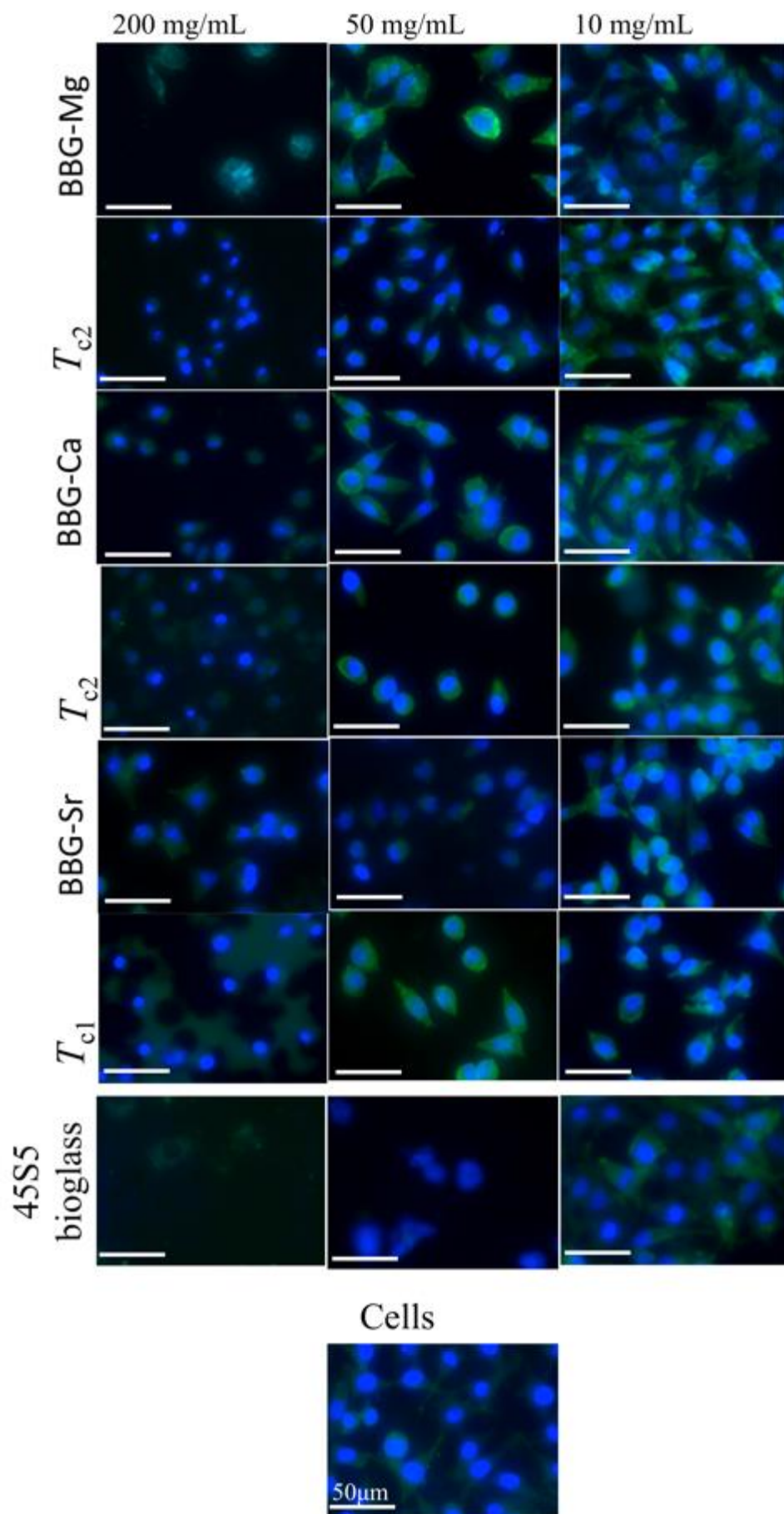
1 After 3 days of culture, morphologic assessment of L929 cells (Figure 6) revealed a concentration
2 dependent behaviour of the BBG-Mg-, BBG-Ca- and BBGCs- conditioned media. Where of 10
3 mg/mL of BBG-Mg-, BBG-Ca-, BBG-Sr- or BBGCs were used to prepare the conditioned media,
4 the cells, showed a characteristic fibroblast morphology with the formation of actin filaments that
5 was similar to that of the control cultures in the absence of glass-conditioned media. Use of 50
6 mg/mL of BBG-Mg-, BBG-Ca- and BBG-Sr- and BBGCs to prepare the conditioned media,
7 resulted in fewer cells with a classical elongated fibroblastic and a greater proportion exhibiting a
8 more rounded morphology. Use of BBG-Mg-, BBG-Ca-, BBG-Sr- or BBGCs concentrations of 200
9 mg/mL gave conditioned media in which only a few rounded L929 cells could be observed after 3
10 days. These results are in agreement with cell viability and cell proliferation data (Figure 4 and 5)
11 supporting a concentration dependence cytotoxic effect. In the case of the commercial 45S5
12 bioglass cultures, for concentrations higher than 10 mg/mL the in the conditioned media no live
13 cells were observed after 3 days of culture. At lower 45S5 bioglass concentrations (10 mg/ml),
14 culture of L929 cells in the 45S5 bioglass-conditioned media resulted in the cells having a
15 fibroblastic morphology similar to the control cell cultures. As showed by the viability and the
16 DNA assays, crystallisation of the glasses was found to have a positive or negative effect on cellular
17 morphology at day 3 of culture. Especially for a concentration of 50 mg/ml, it was observed that the
18 less toxic samples (i.e. BBG-Mg_Tc2, BBG-Ca_Tc1 and BBG-Sr_Tc1) presented higher number of
19 cells with a classical elongated fibroblastic morphology. This is consistent with the typical lower
20 solubility of the ceramic phases when compared to the glassy ones, limiting the release of ionic
21 components from the BBGCs and maintaining them at non-cytotoxic concentrations in the culture
22 medium.

23 Specifically, for the BBG-Mg glasses, an increase in cytotoxicity was detected for the first heat-
24 treated glass-ceramics (T_{c1}), which could be related to the formation of SiO_2 during the heat
25 treatment. The formation of SiO_2 molecules reduces the amount of silicate in the dominant
26 amorphous phase, which may increase the ratio of borate in the amorphous glass matrix. The

1 borate-rich amorphous phase has a faster degradation rate ¹⁵, increasing the quantity of ions in
2 solution as well as the pH (~ 8.0-9.0) ⁴⁶. On the other hand, the BBG-Mg glass-ceramics formed
3 after the second heat treatment (T_{c2}) showed a reduction in cytotoxic, which may have been due to
4 the formation of two different crystalline phases (magnesium silicate and magnesium borate) so
5 reducing the availability of ions contained largely within the stable crystalline phases ^{27, 30}.

6 The glass-ceramics produced from heat-treated BBG-Ca and -Sr glasses also showed different
7 cellular behaviour from the parent glasses. A reduction in cytotoxicity was found for glass-ceramics
8 formed after the first heat treatment (T_{c1}) and an increase in cytotoxicity with further heat
9 treatments (T_{c2} and T_{c3}). These results may be explained by the formation of different crystalline
10 phases with higher availability for ion release, which may have increased the pH and can be directly
11 observed by colour changes in the culture medium ³⁰. The pH of the non-conditioned and
12 conditioned media was measured and found to range from 8.0 to 8.5 for all BBG glasses and glass
13 ceramics. The pH of conditioned media from 45S5 bioglass ranged between 8.5 and 9.0.

14



1 **Figure 6** – Fluorescence microscopy of L929 cells morphology after 3 days incubation with BBGs
2 or BBGCs -conditioned media. Conditioned media were prepared with 10 mg/ml, 50 mg/ml or 200
3 mg/ml BBG glass or BBGCs. Cells cultured with non-conditioned medium was used as negative
4 control and 45S5 bioglass conditioned media was used as positive control (The blue colour (DAPI
5 staining) shows the nucleus of cells; Green colour (phalloidin staining) shows the actin filaments).

6 **4. Conclusions**

7 Three novel amorphous BBGs with different glass modifiers have been successfully synthesised by
8 the melt quench technique. The DTA analysis allowed the identification of two or more exothermic
9 peaks indicative of crystallisation. However, after a controlled heat treatment at these temperatures,
10 XRD and ATR-FTIR data indicated that only the samples that were treated at the higher
11 crystallisation temperatures produced significant crystalline phase(s). Also, different crystalline
12 phases were formed for the different substituted BBGs. Specifically for, BBG-Mg, BBG-Ca and
13 BBG-Sr glass-ceramics was detected the presence of magnesium silicate- $Mg_2(SiO_3)_2$ and
14 magnesium borate- $Mg_2B_2O_5$; wollastonite- $2M-CaSiO_3$ and calcium borate- $Ca(BO_2)_2$; and strontium
15 silicate- $SrSiO_3$ and strontium borate- $Sr_2B_2O_5$, respectively. To our knowledge, this is the first time
16 it is demonstrated that controlled crystallisation of BBGs might produce glass-ceramics with less
17 cytotoxic effects on cells. Moreover, neither BBGs nor BBGCs induced a cytotoxic effect on cells
18 at concentrations lower than 50 mg/mL and they exhibited less cytotoxicity compared to 45S5
19 bioglass at all concentrations used. Finally, interaction of BBGs with cells in a biological
20 environment is a complex process that is continuously under study from the scientific and medical
21 device communities. However, the great biodegradable properties of BBGs combined with the fact
22 that partial or total crystallisation permits cytotoxicity reduction. The BBGs can be used to achieve
23 medical-grade material for development of new generation bone tissue engineered implants.

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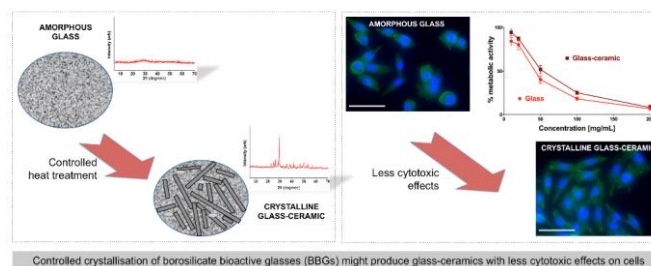
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The Design and Properties of Novel Substituted Borosilicate Bioactive Glasses and Their Glass-Ceramic Derivatives

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With this research the effect of crystallisation on the properties of borosilicate bioactive glasses (BBGs) and their suitability as biomaterials was evaluated. By *in vitro* cytotoxicity assay we demonstrated that the crystallisation of the BBGs reduced their cytotoxicity to L929 cell line, providing a method for the improvement of the biocompatibility of bioactive borosilicate glass-ceramics.