Copper-releasing, borate-based glasses with antibacterial properties: synthesis and *in vitro* characterization

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Abstract

In this study, glasses within the system (60-x) B_2O_3·x ZnO·34CaO·1CuO, with x=5, 10, 15, 20, 25 mol% and with B_2O_3/ZnO ratios 11; 5; 3; 2; 1.4 have been synthesized and characterized in vitro. After being immersed in simulated body fluid (SBF) and saline solution, weight loss reduction and pH measurments, followed by inductively coupled plasma optical emission spectometry (ICP-OES), scanning electron microscopy (SEM) and X-ray diffraction (XRD) analysis were performed, in order to evaluate the changes in glass morphology. In vitro biodegradation and surface reaction were observed in all of the glasses, especially in the x=10, 15, 20 samples. SEM and XRD results revealed the presence of a hydrotalcite-like structure (double layered hydroxid) at the aqueous solution-glass surface interface, while Cu, Zn, Ca and B ions, with proangiogenic properties, were detected in the immersion fluid.

Keywords: *copper, borate glasses, in vitro, biological ions;*

Introduction

Bioactive glasses are a category of vitreous biomaterials with remarkable bioactivity and biocompatibility, which makes them highly promising in the medical applications field. In recent years, there has been a significantly growing interest in the use of borate-based bioactive glasses due to their excellent properties in bone regeneration and beyond. [4,24]. Low chemical durability, faster and more complete degradation in comparison with silicate glasses, and a controllable conversion to hydroxyapatite (HA) are among the well known characteristics of boron-containing glasses [10]. Still, an extensive and ongoing process of research is required to provide prospects for their use in soft tissue engineering [20].

A controllable degradation rate of the bioactive glass is desirable, taking into account that it should be similar to the rate of new tissue formation, and a high chemical durability results in a longer and less complete degradation rate upon immersion in aqueous solutions. [10,18,20]. This process is accompanied by dissolution of ionic compounds in the fluid in which the bioactive glass was immersed, resulting in pH and ionic concentration changes of the aqueous solution over time [6]. Also, to form a strong bond with the surrounding tissue, the glass surface must undergo a specific conversion phenomenon when immersed in simulated body fluid (SBF), leading to the fomation of a hydroxiapatite (HA)-like surface layer [8]. Therefore, *in vitro* characterization of these processes is essential to predict the *in vivo* behavior of the materials, and their applicability in tissue engineering [20].

Weight loss measurements and pH monitoring of the aqueous immersion solution are simple and relevant methods for assessing glass degradation rate, as they accompany these processes by changes in ion concentration of the *surrounding* aqueous *medium* [10]. Scanning electron microscopy (SEM) coupled with energy-dispersive X-ray analysis (EDS) is used to measure microstructural surface changes after immersion [16], while newly formed crystalline

phases can be detected by X-ray diffraction (XRD) analysis [10]. Concentration of ions released into deionized water following immersion, can be accurately measured by using inductively coupled plasma optical emission spectometry (ICP-OES), being an essential tool for predicting effects of the biomaterial on the surrounding tissue, including: cell growth stimulation, growth-factor enhancement [22], angiogenesis promotion [15] and gene expression regulation [23].

The aim of this paper was to thoroughly characterize 5 compositions of bioactive glasses derived from the B_2O_3–ZnO–CaO–CuO system, by measuring the weight loss and pH changes of the glass, after being immersed in aqueous solutions at 37°C. Additional SEM-EDS and XRD analysis were performed to characterize microstructural surface changes, while ICP-OES was completed after immersion of the glass in deionized water at 37°C. The results are intended to anticipate the biological behavior of the glasses, and their applicability in tissue engineering.

Materials and methods

Glass synthesis

Glasses within the system (60-x) B_2O_3·x ZnO·34CaO·1CuO, with x=5, 10, 15, 20, 25 ZnO mol% and with B_2O_3/ZnO ratios 11; 5; 3; 2; 1.4 were prepared with high purity raw reagent-grade chemicals. In order to obtain five different compositions of borate-based bioactive glasses, zinc oxide (ZnO), copper oxide (CuO), boric acid (H_3BO_3) and calcium carbonate ($CaCO_3$) powders were weighed, mixed and homogenized before being melted at 1230°C for 25 minutes in sintered corundum crucibles. The compositions were rapidly cooled at room temperature by quenching the molten glasses; subsequently, the samples were crashed in an agate mortar and passed through a sieve in order to finally obtain particles with a diameter (d) < 0.075 mm.

Degradation behavior and pH assessment of the borate-based glass samples in 0.9% saline solution

In order to evaluate the degradation behavior of the bioactive glasses, similarly sized samples were weighed before being soaked in 5 ml of normal saline solution (0.9%) and incubated at 37°C for 7 days. The weight reduction was assessed by measuring the relative weight loss (Δm) in all samples, using the following equation: $\Delta m = 100 \cdot (m_i - mf)/mi$, where m_i is the initial mass, found in the samples before immersion and m_f is the measured weight after 7 days of soaking in saline solution. Each sample was removed from the solution and wiped gently with filter paper to eliminate the fluid from the surface before being weighed. Consequently, the glass surface was microscopically evaluated at the end of immersion time (Olympus BX51).

Ionic dissolution assessment

100 mg of glass powder from each sample were soaked in 5 ml of deionized water, being subsequently incubated at 37°C for 5 days. The concentration of ions (copper, zinc, borate, calcium and aluminium) released during the degradation process were measured by ICP-OES analysis (FMD-07, Spectro Analytical, Germany). Multielement calibration solutions prepared from a stock solution of 1000 mg L^{-1} (Merck, Darmstadt, Germany) were used to calibrate the instrument. The estimated error in the measurement rate was +/- 5%.

Evaluation of the surface microstructural and chemical composition changes

Changes in the chemical and mirostructural properties of the samples, resulting from the interaction with aqueous solutions, were assessed after immersing the glasses in normal saline solution 0,9% for 7 days, at 37°C. The surface texture and morphological evaluation was performed by SEM, while using XRD analysis (Bruker D8 Advance), the crystalline phases were quantificated following interaction with the aqueous medium.

Results and discussions

Five glass compositions belonging to the (60-x) B_2O_3·x ZnO·34CaO·1CuO system, with x=5, 10, 15, 20, 25 ZnO mol% and with B_2O_3/ZnO ratios 11; 5; 3; 2; 1.4 were obtained by melting technique (fig. 1). Samples were rapidly cooled at room temperature, being subsequently crushed and passed through a sieve, resulting in particles with a diameter of <0.075 mm (fig. 2).

Fig. 1 Macroscopic appearance of the (60-x) B_2O_3·x ZnO·34CaO·1CuO with x=10 sample, after being quenched and crushed

Fig. 2 Macroscopic appearance of the (60-x) B_2O_3·x ZnO·34CaO·1CuO with x=5 sample, after being crushed and sieved to <0.075 mm particles

In the present study, granular forms of glass particles were obtained in order to increase the glass surface area, thereby increasing the reactivity of the sample surface in the surrounding physiological fluid.

Macroscopic aspects of the samples following immersion

After 7 days of immersion in saline solution at 37°C, a newly formed layer of approximately 0.5 mm thickness, very brittle, whitish and easily ruptured was noted at the surface of the glasses (fig. 3), especially in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x= 10; 15; 20 samples. Microscopically, the newly formed layer exhibited a crystallized appearance, while the underlying surface layer presented a smooth aspect, lacking pre-existing discontinuities (fig. 4). Clusters formed via agglomeration of particles were significantly decreased in number and size, whilst in some regions, due to the thickness of the newly formed layer and its opacity, the microscopic evaluation could not be achieved.

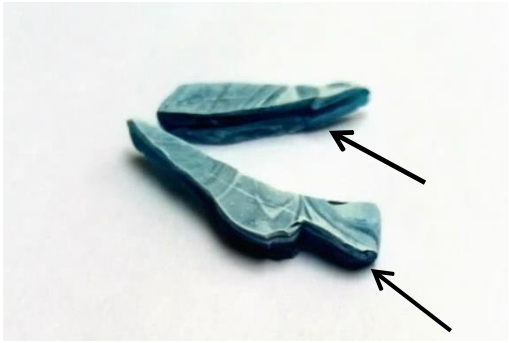

Fig. 3 Macroscopic appearance of the newly formed glass surface layer after 7 days of immersion in normal saline 0,9% in the (60-x) B_2O_3·x ZnO·34CaO·1CuO with x=10 sample

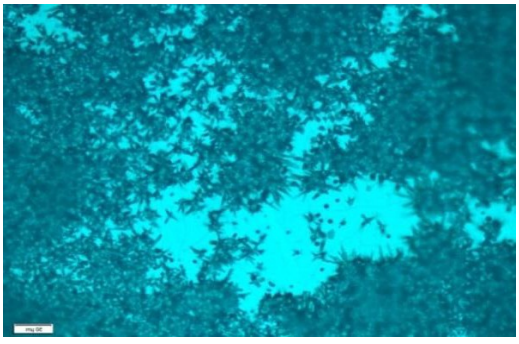

Fig. 4 Microscopic appearance of the surface layer after 7 days of immersion in normal saline 0,9% of the (60-x) B_2O_3·x ZnO·34CaO·1CuO with x=20 sample (100x)

A remarkable, dynamic process has been noted at the aqueous solution-glass surface interface on microscopic evaluation of the samples, consisting of an uninterrupted deposition of a thin layer, with a crystallized appearance (fig. 5-6).

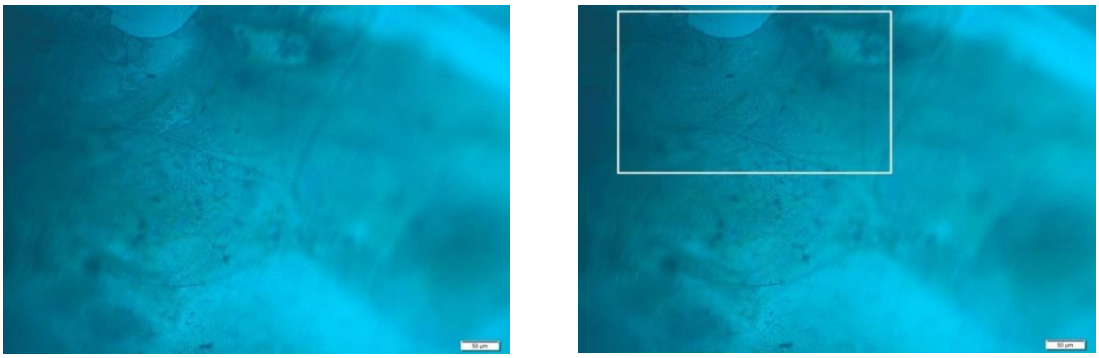

Fig. 5-6: Continuous deposition of a thin layer on the sample surfaces, after 7 days of immersion in SBF, at 37°C

Degradation behaviour and pH assessment of the bioactive glass samples

The average weight reduction (Δm) for all of the five samples after immersion in 0.9% saline solution, was found to be 0.86%. The highest weight reduction was recorded in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=10 sample (Δm= 1.76%), while the lowest weight reduction was identified in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=25 sample (Δm= 0.03%). Although weight losses are relatively low, they indicate the ocurrance of a degradation process; however, a reduced degradation and conversion rate is rather characteristic for silicate glasses, and represents a drawback by hindering the coordination between the bioactive glass degradation rate and tissue regeneration rate [7,20].

The starting pH value of the 0.9% saline solution at 37°C was 5.4. After 7 days of immersion, the most significant increase in the pH value was recorded in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=15 sample, with 3.5 units. The saline solution has undergone the smallest change in pH in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=25 sample, with 1.9 units. Changes in the pH take place due to changes in ionic concentrations, as a result of the degradation process [10,20]. A previous study has shown a correlation between increasing the B_2O_3 content in the sample and increasing pH of the immersion solution [7]. Huang [10] highlighted that, by increasing B_2O_3 content, a very rapid pH increase of the aqueous solution was caused, reaching a limit value in 50 hours; however, the final pH value was higher in the samples with a decreased B_2O_3 content, where the limit value was reached after 500 hours. In the present study, pH evaluation was performed after 168 hours of immersion and showed a lower pH increase in the sample with the highest B_2O_3 content. Increasing B_2O_3 content results in a lower chemical durability, which leads to a faster reaction with the aqueous medium, and therefore to the rapid change of the pH, but it's limiting value is detemined by the ionic concentrations released in the solution, as well as by their acidity and basicity [10].

Ionic dissolution products

In order to measure the ions concentrations released into the deionized water, the ICP-OES analysis was performed; results in all of the five samples are presented in Fig. 7.

Sample	Cu (mg/L)	Zn (mg/L)	B (mg/L)	Ca (mg/L)	Al (mg/L)
x=5	0.038	0.048	530	770	3.6
x=10	0.054	0.041	506	730	3.6
x=15	0.075	0.044	544	840	2.7
x=20	0.045	0.049	510	810	1.1
x=25	0.066	0.054	650	1270	1.2

Fig. 7: Ion concentrations of deionized water following immersion of the glass samples

The highest concentrations of B, Zn and Ca were found in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=25 sample (650 mg/L, 0.054 mg/L and 1270 mg/L respectively). Cu reached a maximum level in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=15 (0.075 mg/L) sample, while Al levels were found in all of the five samples, with the highest amounts being detected in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=5, 10 samples (3.6 mg/L). The presence of trace amounts of aluminium is explained by the high temperature and the long melting time during sample synthesis, which caused contamination from the walls of the crucible.

The biological significance of Cu is given by its anti-inflammatory, anti-infectious, antibacterial and proangiogenic properties [1]. A remarkable cellular distribution of Cu ions has been revealed in human endothelial cells, when induced to undergo angiogenesis [5]. Zn ions are required in various enzymatic activities and anti-inflammatory processes, possess a remarkable antimicrobial activity and are strongly involved in protein synthesis [2,12]. They appear to be actively involved in collagen synthesis and play a role in cell membrane stabilization, intracellular signaling and wound healing [14]. Increased levels of Zn were identified at the wound margins within the first 24 hours, while even higher levels were detected during epidermal granulation and proliferation [9,13]. Boron ions are apparently involved in the synthesis of collagen and proteoglycans, increase the turnover of extracellular matrix, and also promote protein phosphorylation [19]. Al has certain astringent, antacid and antibacterial properties, its toxic effects being dose dependent [17].

Microstructural characterization

SEM photomicrographs of the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=10, 15 samples are shown in fig. 10. Cauliflower-shaped agglomerates (fig. 8A), sphere clusters (fig. 8B), and also well defined, regular geometric shapes (fig. 8C-D) were observed at the surface of the outermost layer.

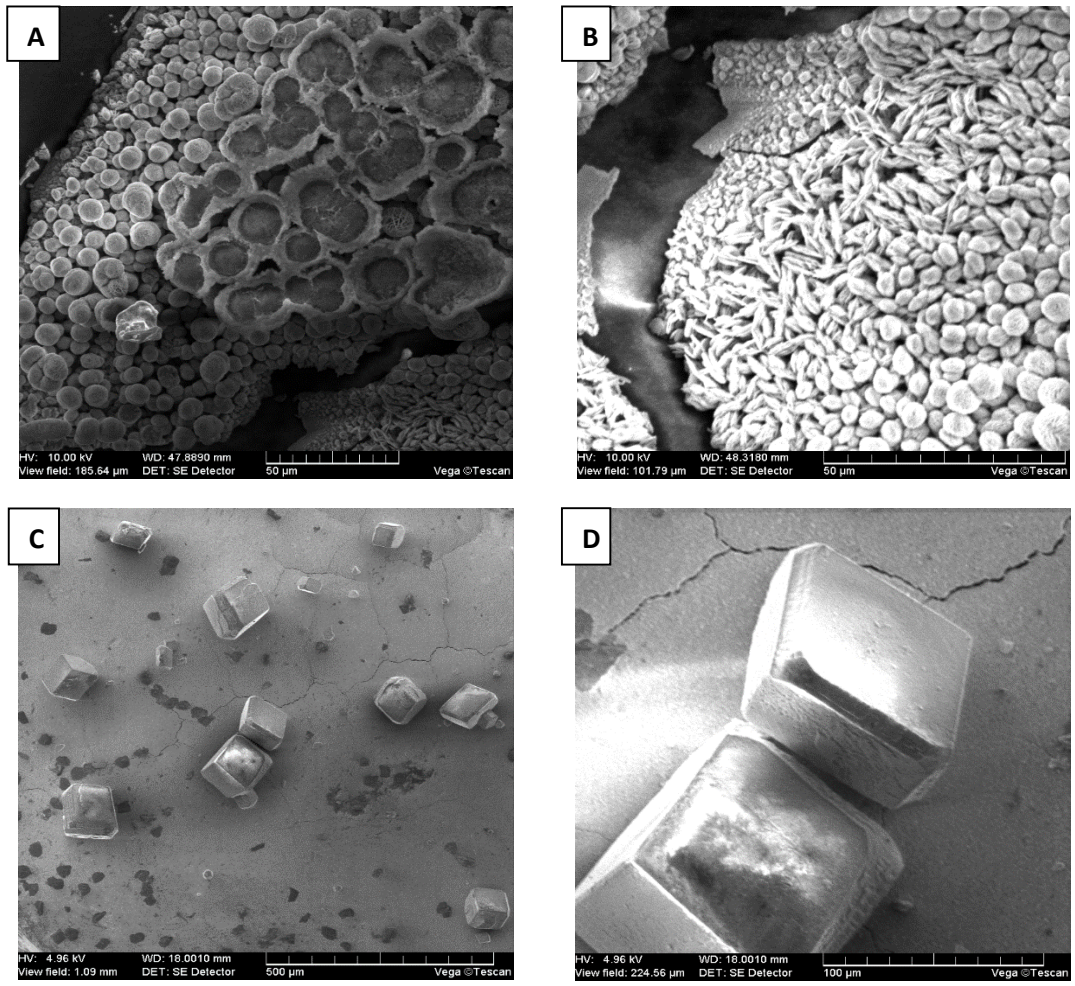

Fig. 8 SEM images of the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=10, 15 samples.
A : x=10, scale:50 µm; **B**: x=10; scale:50 µm; **C**: x=15, scale:500 µm;
D: x= 15, scale:100 µm

Following imersion in saline solution, XRD patterns of the samples were relatively similar (fig. 12), showing peaks corresponding to zinc aluminium carbonate hydroxide hydrate [$Zn6Al_2(OH)16CO_3.4H_2O$], a hydrotalcite-like structure, also known as double layered hydroxid (strongest line at $2\theta = 11.65°$). The importance of this compound lies primarily in its catalytic behavior, particularly being a substrate for the efficient immobilization of biological materials [3,21]. The development of this interface layer is a result of the interaction between Zn and Al ions, under alkaline hydrothermal conditions [11]. In his study, Koh [11] developed this chemical compound, and successfully used it as a synthetic substrate for hexagonal-patterned ZnO nanorods.

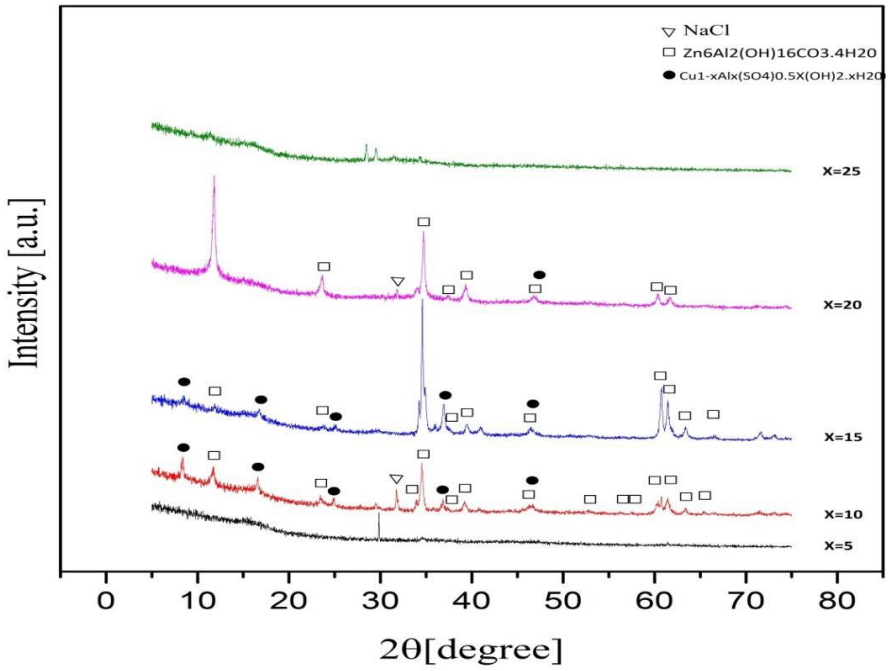

Fig. 12: X-ray diffraction patterns of the (60-x) B_2O_3·x ZnO·34CaO·1CuO system, with x=10, 15, 20 after immersion in SBF

Aluminum sulphate hydroxide hydrate is another compund detected by XRD analysis at the sample surfaces, the most intense peak being centered at 2θ = 8.31˚. The XRD scan also contained diffraction peaks corresponding to NaCl, originating form the immersion fluid.

Diffraction peaks could only be detected and assigened in the x=10, 15 and 20 samples.

Conclusions

Five different borate containg glasses with a composition in the (60-x)·B_2O_3·xZnO·34CaO·1CuO system, were synthesized and characterized by various methods, including ICP-OES, SEM-EDS, and XRD, in order to predict their applicability in tissue engineering. *In vitro* weight loss and pH measurements showed a lightly degradable behavior in all of the samples, while surface reactivity of the glasses was studied microscopically. None of these properties appeared to be linearly dependent on the boron concentration of the glass samples. SEM micrographs captured at the surface of the samples revealed the presence of various distinct, well-defined, regularly shaped structures, especially in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x = 10; 15 samples. The XRD patterns of the samples showed the existance of a newly formed, hydrotalcite-like structure at the aqueous solution-glass surface interface, with remarkable biological properties. These results indicate a highly promising stimulatory potential of these glasses, by releasing various ions with multiple activities, related to their proangiogenic and antibacterial properties.

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