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Effect of Copper and Zinc on the Bioactivity and Cells Viability of Bioactive Glasses

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Abstract

Bioactive glasses, doped with traces of copper (Cu) and zinc (Zn) were synthesized by fusion method. Cu and Zn present interesting functions for the biological metabolism through their antibacterial, anti-inflammatory and antifungal properties. Several physical methods were employed to characterize the bioactive glasses before and after immersion in a Simulated Body Fluid (SBF). The "*in vitro*" experiments showed that after soaking in SBF, the behavior of bioactive doped glasses are different compared to pure glass 46S6. Obtained results show that glass matrix undergoes some changes after 15 days of immersion. The non toxic character of doped glasses was confirmed after 24 hours of incubation. The kinetic of release of Cu and Zn was carried out. It highlights that Cu is more released than Zn. By SEM, the morphology of hydroxyapatite obtained with Zn-doped glass show a better crystallization compared to Cu-doped glass.

Keywords: Bioactive glass; Copper; zinc; Hydroxyapatite; Cells viability

Introduction

Synthetic biomaterials are considered like a great option to replace bone grafts. Bioactive glasses are able to form a hydroxyapatite layer $Ca_{10}(PO_4)_6(OH)_2$ on their surface when they are immersed in a simulated body fluid. This formation induces a bone bonding improving the bone growth [1,2]. Copper and zinc are interesting elements for biomedical applications because they present physiological properties. Copper has anti-inflammatory, anti-infectious and anti-bacterial properties. It is necessary for a lot of biological processes as angiogenic response [3]. Zinc introduces in several enzymatic processes, inhibits the bone resorption and promotes the proliferation of osteoblast [4]. The aim of this work is to study the effect of Cu and Zn on the formation of hydroxyapatite.

Materials and Methods

Melting synthesis of bioactive glasses doped with Cu and Zn

For elaboration of bioactive glasses, oxides (SiO_2, CaO, Na_2O, P_2O_5, CuO, ZnO) weighed and mixed in a polyethylene bottle for 2 hours using a planetary mixer. 0.1wt% of Cu and/or Zn were introduced to have the same quantities as in human bones. Three doped glasses were elaborated: 0.1% Cu in glass matrix named 46S6-0.1Cu, 0.1% Zn in glass matrix named 46S6-0.1Zn and 0.1% Cu + 0.1% Zn in glass matrix named 46S6-0.1CuZn.

The premixed mixtures were melted in platinum crucibles that were placed in an electric furnace. The first rise of temperature rate was 10°C/min and it was hold at 900°C for 1 hour to achieve the decarbonation of all products. The second rise of temperature rate was 20°C/min and it was hold to 1350°C for 3 hours. The samples were casted in preheated brass cylindrical molds (13 mm*8 mm high) and annealed at 565°C for 4h near the glass transition temperature of each glass. The obtained cylinders were reserved for the "*in vitro*" evaluations. Several cylinders were crushed using a mechanical crusher Retsch Fisher Block Scientific RM100. The obtained powder was sieved using Retsch Fisher Block Scientific AS200 sieve in order to have a powder presenting a granulometry of 40 µm. Cylinders were used for "*in vitro*" studies without cells and powder was used for "*in vitro*" studies with cells.

Physico-chemical characterizations

The bioactive glasses, before and after immersion in the SBF, were characterized by a powder X-ray diffraction analysis (BRUKER AXS D8 ADVANCE diffractometer by using Cu target, Bragg–Brentano geometry); FT-infrared spectrophotometer (Bruker Equinox 55); Scanning Electron Microscopy (Jeol JSM 6301F) and Inductively Coupled Plasma-Optical Emission Spectroscopy (Spectro Ciros Vision Ametek) to evaluate the structure, the morphology and the kinetic of the ionic exchanges respectively.

Bioactivity studies in the SBF solution

Cylinders were tested for long periods: 1, 2, 7, 15 and 30 days in 8 mL of SBF [5]. 30 mg of powders were tested for short times: 2, 4, 8 and 16 hours and long times: 1, 2, 7, 15 and 30 days in 60 mL of SBF at 37.0 ± 0.2°C with shake at 50 rpm.

Biological analysis

Cell proliferation evaluation: The viability of cells was determined by the standard colorimetric 3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Reactions were followed by measuring absorbance at 570 nm wavelength with a Dynatech MR 5000 microplate reader spectrophotometer. Each reading was carried out 3 times.

Statistical analysis: The quantitative results from the MTT tests were analysed using Statview V, one-way ANOVA followed by PLSD Fisher test to determine the significant differences layout.

Release of metals and pH of the conditioned medium: Concentrations of metal released in the bioactive glass conditioned medium were assayed using method described for SBF. The pH was measured with an electronic pH meter Mettler Toledo FE20/EL20.

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Results and Discussions

Physico-chemical results

The amorphous structure of all glasses was confirmed by XRD analyses before immersion in the SBF. The SEM micrographs showed that the surfaces of samples are smooth before immersion.

The XRD patterns for pure and doped glasses after 30 days of immersion in the SBF are presented in Figure 1. The formation of apatite is confirmed with three diffraction maxima at 25.8, 31.8 and 53.4 (2θ°) corresponding to the (002), (211) and (004) apatite reflections [6] for 46S6. Concerning doped glasses, the (002) apatite reflection decreases in intensity compared to 46S6. Moreover, the (211) reflection becomes more pronounced compared to XRD pattern before immersion. The disappearance of (004) apatite reflection is observed for doped glasses. Therefore, the crystallization of the apatite layer is slowed down when the doping elements were added.

The SEM micrographs of the bioactive glasses immersed for 30 days in the SBF are presented in the Figure 2. On pure and doped glasses, an apatite layer is formed. Morphologies of apatite crystals, their sizes and their reliefs are different according to the introduced doping element and their content. Indeed, for 46S6 (Figure 2a), the apatite layer is homogeneous. The apatite layer of 46S6-0.1Cu and 46S6-0.1CuZn (Figure 2b and 2c) have almost the same morphology and the same relief but the size of crystals is different and the apatite layer is more heterogeneous. For 46S6-0.1Zn (Figure 2c), balls of hydroxyapatite are formed on the surface. These micrographs confirm the XRD results showing the slowdown of the hydroxyapatite crystals growth. Moreover, a better crystallization of hydroxyapatite is observed with Zn-doped glass compare to Cu and CuZn doped glasses.

Results of ICP-OES are presented in the Figure 3. During the first hours of immersion, up to 2 hours of immersion, Cu is released very quickly to reach 0.25 ppm. Then, up to 16 hours, Cu of 46S6-0.1Cu is released more slowly compared to the concentration of Cu of 46S6-0.1CuZn which increases quickly. The concentration of Zn reaches 0.01 ppm and it is released continually and very slowly up to 16 hours. For longer times, from 16 hours up to 30 days, Zn element is released continually to reach 0.03 ppm. The same kinetic of release of zinc is observed for the glasses containing it. For copper element in 46S6-0.1Cu and 46S6-0.1CuZn, from 16 hours to 30 days, the concentration remains more or less stable. Moreover, 46S6-0.1Cu and 46S6-0.1CuZn present a similar kinetic of release for copper element from 1 day of immersion. Finally, the concentration of copper reaches 0.38 ppm and the one of zinc reaches 0.03 ppm after 30 days of immersion. The chemical environment of Cu and Zn in the glassy matrix and their chemical characteristics as electronegativity and atomic radius could explain their kinetics of release in the SBF and the different morphologies of hydroxyapatite crystals.

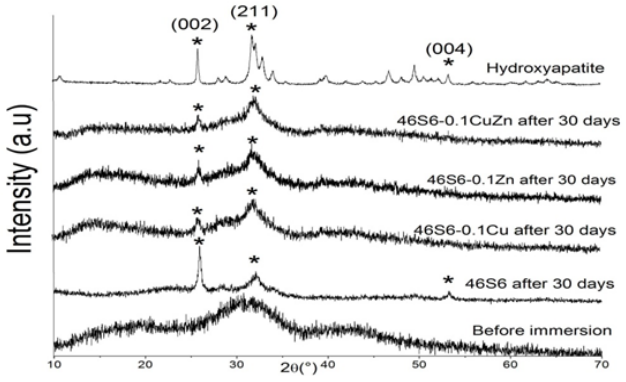

Figure 1: XRD patterns of pure and doped glass after 30 days of immersion in the SBF.

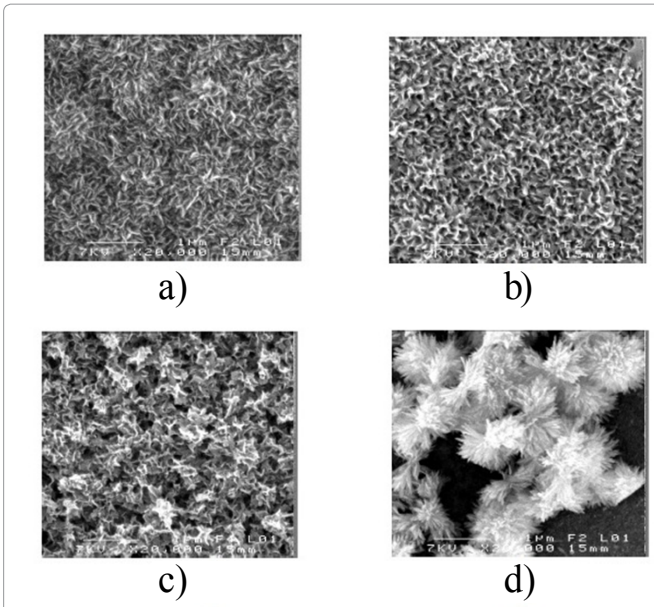

Figure 2: Morphologies of pure and doped glasses after 30 days of immersion. (a): 46S6; b): 46S6-0.1CuZn; c): 46S6-0.1Cu; d): 46S6-0.1Zn).

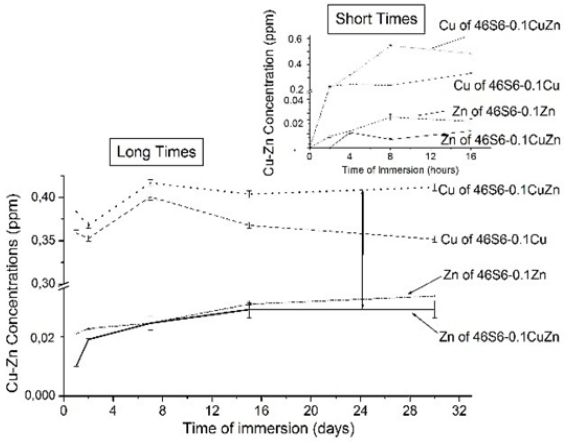

Figure 3: Cu and Zn concentration after immersion in the SBF at different periods.

Biological results

After 24 hours of incubation, tested biomaterials have a proliferative action on osteoblast cells ($p<0.0001$). This action is more acute with 0.1% Cu compared to 0.1% Zn. After 72 hours of incubation, this trend is reversed (Figure 4).

After 24 hours of incubation, bioactive glass 46S6 shows a cell proliferative effect on its own, whereas cell growth ($p<0.0001$) is not affected while using metal-enclosed materials. After 72 hours of incubation, cytotoxicity occurs but remains modest using copper as

metal (about 80% viability). Materials enclosing mixed copper/zinc assume a higher toxicity than single metal-enclosed glasses. 46S6 bioactive glass shows a significant proliferative effect (p=0.03) (Figure 5).

Using ICP-OES, the concentration of metals in the conditioned medium were measured. After 24 hours of incubation, 0.028 ppm of Zn and 1.203 ppm of Cu were released by 46S6-0.1CuZn; 1.123 ppm of Cu was released by 46S6-0.1Cu.

Bone cytotoxicity of the product is evaluated using human osteoblast SaOS2 cell lines. Assuming the biomaterial could be permeable to revascularization, its endothelial cytotoxicity is evaluated using human endothelial EAHY 926 cell line too.

While using bioactive glasses, a moderate cytotoxicity was observed after seventy two hours of incubation. The increased sensitivity of SaOS2 compared to EAHY 926 cells (cell growth induction as well as inhibition) suggests that the effects must be cell-and time dependent. Although metal ions cytotoxicity has not been described on the presently studied cell lines, examples in literature show a cell-and time dependent effect [7,8], which also appear to be dose dependent [8,9] for copper and zinc.

Dose dependent effect may not be imputable to the biomaterial itself since it is not influencing cell growth in its metal-free version (it is actually enhancing it).

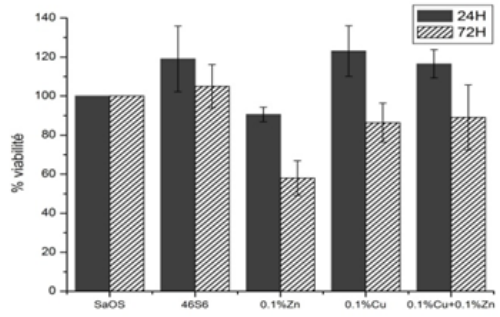

Figure 4: Cytotoxicity assays on osteoblast cells SaOS.

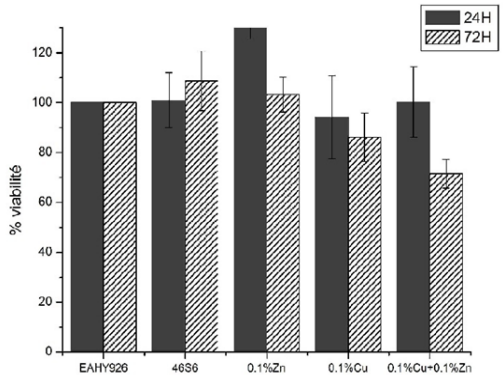

Figure 5: Cytotoxicity assays on endothelial cells EAHY926.

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Regarding copper, a lethal dose (LD50) has been reported as 46 $mg.L^{-1}$ after 24 hours of incubation on mice fibroblasts L929 [7] whereas zinc assume cytotoxic concentration variations from 10 to 250 $mg.L^{-1}$ [8,9]. Besides leached amounts well below the cytotoxic doses our bioactive glasses allows, after an incubation time of 24 hours, a release of 1.12 $mg.L^{-1}$ for copper and 0.028 $mg.L^{-1}$ for zinc. On the other hand the increasing toxicity observed after 72 hours must be related to an increase of pH values of the incubation medium while metal ions are added to the materials (pH is of 7.51, 7.6 and 7.52 for Cu, Zn and both respectively versus 7.48 for pure glass).

Conclusions

The physicochemical results show the formation of an hydroxyapatite for pure and doped bioactive glasses. These layers present different morphologies according to the introduced doping element. The cytotoxicity assays do not show toxicity of the biomaterials after 24 hours of incubation on osteoblast or endothelial cells. The mixture of Cu and Zn in the glassy matrix allows improving the cell proliferation. These results confirm the advantage of Cu and Zn for biomedical applications because they present different kinetics of release. These biomaterials offer an alternative for the orthopaedic or maxillo-facial surgery. Pure or doped glasses could be used according to several parameters as age, gender and site of implantation.

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