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Synthesis, structure, bioactivity and biocompatibility of melt-derived P_2O_5-CaO-B_2O_3-K_2O-MoO_3 glasses

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ABSTRACT

New glasses following the P_2O_5-CaO-B_2O_3-K_2O-MoO_3 composition were prepared using the classical melt quenching method in order to investigate and correlate their structural peculiarities with the composition and, moreover, with the bioactivity and biocompatibility behavior. The structure and surface morphology of samples were characterized by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). Two complementary techniques were used to determine the elemental composition inside samples and on the outermost layer of their surface, namely energy dispersive X-ray (EDX) and X-ray photoelectron spectroscopy (XPS), respectively. For assessing *in vitro* the bioactivity of the synthesized glasses, the ability to form hydroxyapatite crystallites on their surface upon immersion in simulated body fluid (SBF) was checked using SEM and EDX analysis. The *in vitro* cytotoxic effect of samples was evaluated using a mitochondrial activity assay.

The results reveal that the increase of MoO_3 content in the P_2O_5-CaO-B_2O-K_2O composition generates a continuously disordering of the local structure and the increase of Mo^{6+}/Mo^{5+} ratio values on samples surface. No bioactivity was observed for this glass system after 15 days of immersion in SBF. The *in vitro* toxicity studies show that samples containing MoO_3 up to 7mol% have a good biocompatibility with healthy human immortalized keratinocyte (HaCaT) cell line.

1. Introduction

The massive presence of glass and glass-ceramic compositions on the market of biomaterials is no longer a novelty. The already classical Hench's Bioglass 45S5 opened a huge interest on bio-glass/glass-ceramics as promising materials for bone repair, coatings for medical implants, scaffold design, drug delivery, gene transfection and cancer treatment [1]. This large area of applications is given by their flexible composition and controllable properties (mechanical, chemical, biological, etc.) as a consequence of structural peculiarities. The lack of long range order in the case of glasses, and the controlled crystallization process in case of glass-ceramics make possible to reach great characteristics for these classes of materials.

Biological performance of glasses is strongly influenced by the chemical composition, synthesis techniques, particles size and distribution, surface morphology and reactivity. Despite the volatilization issue and high demanded temperature, the traditional melt quenching method is still used for the synthesis of bioactive glasses but only two melt-derived compositions have been accepted for clinical use: 45S5 (Hench and Paschall, 1973) and S53P4 (Andersson et al., 1990) [2]. Both compositions are limited to four oxides, SiO_2, Na_2O, CaO and P_2O_5, that restricted their use to certain clinical applications. Generally, a great number of elements can be dissolved in glasses. The effect of Al_2O_3, B_2O_3, Fe_2O_3, MgO, SrO, BaO, ZnO, Ag_2O, CuO, Li_2O, K_2O, CaF_2 and TiO_2 on the *in vitro* or *in vivo* properties of bioactive glasses has been reported [3–7]. However, the effect of the composition on the properties of bioactive and biodegradable glasses is not fully understood.

Calcium and phosphorus are essential minerals found in the bone, blood and soft tissue of the body and have a role in numerous body functions. The right ratio between the level of phosphorus and calcium is a key factor for a good human body activity. Phosphate-based glasses have gained high interest as bone filling material and for fabrication of scaffolds for bone tissue engineering because of their high solubility and chemical similarity with the inorganic phase of human body [8]. P_2O_5 and CaO are the constituents of almost all phosphate-based glasses but very few compositions containing B_2O_3 have been reported. Recent studies showed that the boron atoms exhibit the catalytic effect, which enhances bioactivity of glasses [9,10].

In general, borate glasses present low chemical durability, some of them being able to convert faster and more completely to hydroxycarbonate apatite when immersed in an aqueous phosphate solution, such as simulated body fluids [3,11]. Potassium plays a significant role not only in the melting process [12] but in bioactive glasses potassium also helps to control the proper balance of fluids in cells and body fluids [3,13].

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In vitreous systems, molybdenum oxide may be either a glass forming component, or a network modifier, depending on the composition [14]. The glasses containing molybdenum were often studied as they have technological potential in electro-chemical and optical applications but no biological studies were done on such glasses. Molybdenum is an essential trace mineral for human body supporting healthy enzyme system function, normal cellular replication and airways healthy function [15]. It was also proved that the addition of molybdenum to bioactive glasses improves their biocompatibility in terms of protein adherence enhancement [16]. All these function are related with the Mo ions valence state since Mo ions are multivalent, existing in at least two valence states as Mo^{5+} with one paired electron and Mo^{6+} with a fully filled 3d shell. The Mo^{5+}/Mo^{6+} ratio depends on composition, the oxide activity and the basicity strength of the glass matrix [17].

In the present study, new glasses following the composition $xMoO_3\cdot(1\text{-}x)[48P_2O_5\cdot45CaO\cdot2B_2O_3\cdot5K_2O]$ (x = 0, 1, 3, 5, 7 mol%) have been synthesized using melt quenching method. The glass structure and morphology were characterized using XRD, FTIR, SEM/EDX and XPS. Having in view that the formation of a bone-like apatite in simulated body fluid (SBF) is often taken as an indication of *in vivo* bioactivity of the glass [18], the synthesized glasses were soaked in SBF for 15 days. The surface morphology changes imposed by this protocol were followed using different techniques.

One common method of testing the biocompatibility of biomaterials is the *in vitro* cytotoxicity test (Watana and Lockwood). This method is based on the cell culture studies, which provide useful information about the biological properties of materials. In this study, WTS-1 (water soluble tetrazolium salt) assay was conducted on HaCaT cells for different molybdenum concentrations. Finally, the glass structural particularities and their biological performances have been followed in connection with the molybdenum content in the glasses composition.

2. Experimental procedure

2.1. Glass preparation

The studied glasses have the composition expressed by the formula $xMoO_3\cdot(1\text{-}x)[48P_2O_5\cdot45CaO\cdot2B_2O_3\cdot5K_2O]$ with x = 0, 1, 3, 5 and 7 mol%. They were prepared using the conventional melt quenching method. Appropriate quantities of reagent grade MoO_3, $NH_4\cdot H_2PO_4$, $CaCO_3$, H_3BO_3 and K_2CO_3 were mixed in an agate mortar. The batches were melted in air, in sintered corundum crucibles, in an electric furnace at 1100 °C for 25 min. The melts were quickly cooled at room temperature by pouring and stamping between two copper plates previously cooled with liquid nitrogen. The glass samples were ground to powder in a Retsch Planetary ball mills, type PM 100. The average size of the obtained grains was about 30 μm.

2.2. Methods of glass characterization

The samples structure was investigated by X-ray diffraction using a Shimadzu XRD-6000 diffractometer with a monochromator of graphite for CuK_α ($\lambda = 1.54$ Å). The diffractograms were recorded in 2θ range from 10° to 80° with a speed of 1°/min.

A scanning electron microscope (SEM) type Jeol JSM 5510 LV of 3.5 nm resolution with 100 kV accelerating voltage and 3×10^5 magnification was used to investigate the surface morphology. For performing the SEM measurements the samples were glued on the sample holder with conductive carbon cement and then coated with gold using Edwards S 150 sputter coater device. The elemental composition of the samples was investigated using energy dispersive X-ray analysis.

For Fourier transform infrared (FTIR) measurements identical amounts of glasses were mixed with KBr in order to obtain thin pellets containing approximately 1 wt% glass powders. The pellets thickness was about 3 mm. The spectra were recorded at room temperature in the 350–4000 cm^{-1} range with a 6100 Jasco spectrometer with a maximum resolution of 0.5 cm^{-1} and signal/noise ratio 42,000:1.

X-ray photoelectron spectroscopy (XPS) analysis was performed using a SPECS PHOIBOS 150 MCD instrument. The base pressure in the analysis chamber of the spectrometer was below 5×10^{-9} mbar. XPS spectra were obtained using a monochromated Al Kα radiation, operated at 280 W. All spectra have been corrected for the charging effect. Survey scans were acquired with 1 eV/step and high resolution spectra with 0.05 eV/step. Analysis of the data was carried out with Casa XPS software [19].

2.3. In vitro assays in SBF solution

In vitro bioactivity of the glasses, reflected in their capability for self-assembling of hydroxyapatite layer onto their surface [20], was investigated by samples immersion in SBF solution at 37 °C. The SBF solution was prepared using the Kokubo recipe [21], which is the closest to the human blood plasma with respect to ionic concentration, buffered at pH = 7.38 by tris–hydroxymethyl-aminomethane (Tris, 50 mM) and hydrochloric acid. The powdered samples (0.2 g) were dipped in the SBF solution (30 ml) kept in clean bottles. The sample-SBF mixtures were stored in a thermostat at 37 °C for 15 days. The SBF solutions were not exchanged during the experiment. After 15 days, the samples were filtered, rinsed with doubly distilled water, and dried in air. The surface changes were then studied through XPS and SEM/EDX. X-ray diffraction and FTIR measurements were also performed on immersed samples.

2.4. Cell line and cytotoxicity test

Human immortalized keratinocyte cells (HaCaT, Cell LineService, Germany) were cultured in Dulbecco's modified Eagle's medium (Lonza) supplemented with 2 mM L-glutamine, Penicillin/Streptomycin 100 U/ml and 10% Fetal Calf Serum as monolayer at 37 °C in a 5% CO_2 humidified atmosphere.

The cytotoxic effect of samples containing the highest investigated MoO_3 content, i.e. 5 and 7 mol%, was assayed using WST-1 dye (water soluble tetrazolium salt, Millipore), assay based on the enzymatic cleavage of the tetrazolium salt WST-I to formazan by mitochondrial dehydrogenases active in the living cells. Therefore, 10^4 cells/well were seeded in a 96-well plate. After 24 h, the culture medium was removed and fresh medium containing 15.6 and 500 μg/ml samples was added to the test wells and the cells were placed in the incubator for another 24 h. To ensure the reproducibility of the results, each experiment was conducted in triplicate and the mean value with standard deviation was reported. At the end of the incubation period, medium was removed from all wells and 100 μl of fresh medium containing 10% WST-1 solution was added to each well. Empty wells with medium containing WST-1 reagent were used as blank and cells without glass powders were used as positive control. After 30 min of incubation, the absorbance was measured at 440 nm, using a microplate reader (FluostarOmega, BMG, Germany). A reference wavelength was used at 650 nm.

3. Results

3.1. Structural and morphological samples characterization

All samples were obtained in a glassy state with the vitreous structure confirmed by X-ray diffraction patterns (Fig. 1). The diffraction pattern exhibit broad halo around 2θ = 26° for samples with x = 0–3 mol% MoO_3 and 2θ = 23° for x = 5 and 7 mol% MoO_3. According to our synthesis conditions, during the melting process large amount of Mo^{6+} ions were reduced to Mo^{5+} ions. In fact, it was proven that in rich P_2O_5 content glasses, the amount of Mo^{5+} is larger and its electronic configuration favors the formation of $Mo^{(v)}O^6$ octahedron, each of it having a free corner [17,22]. Therefore, it is expected that the Mo^{5+}