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PROTEIN ADHESION TO BIOACTIVE MICROSPHERES INVESTIGATED BY FLUORESCENCE SPECTROSCOPY

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ABSTRACT. This study is focussed on protein functionalization of spray dried samples of $CaO\text{-}SiO_2\text{-}P_2O_5$ bioactive glass system calcinated at different temperatures. The protein functionalization of the samples surface was achieved in simulated body fluid enriched with bovine serum albumin. Fluorescence quenching of bovine serum albumin is used for a first evaluation of protein adhesion to bioactive glass samples. Differential thermal analysis was used to determine the heat treatment temperatures. Structural changes induced in the silicate network by calcination were evidenced by X-ray diffraction. The results show that the scaled intensity of tryptophan fluorescence signal recorded from protein solution after glass samples incubation depends on samples heat treatment temperature.

Keywords: *bioactive glass; surface functionalization; tryptophan fluorescence.*

INTRODUCTION

Bioactive glasses and glass-ceramics of $SiO_2\text{-}CaO\text{-}P_2O_5$ system are intensely studied because of their surface chemical reactivity in contact with body fluids, and also for their osteogenetic potential and applications in tissue engineering [1–3]. In physiological fluids, an interface layer is self assembled as a result of the interface ion-exchange mechanism known as bioactivity [4]. For *in vitro* study of bioactive glasses and glass–ceramics, Kokubo et al. [5] proposed a simulated body fluid (SBF) with ionic concentration almost equal to that of human blood plasma. *In vitro* tests in SBF are widely used as preliminary tests on new materials showing bioactive properties.

When biomaterials surfaces are in contact with biological media, such as blood or serum, they are immediately coated by protein and their adsorption on to the surface has a fundamental roll in the field of biomaterials [6]. With regard to biomaterials considered for scaffolds in tissue engineering,

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their most important role is to serve as support material for cells attachment and further grows and proliferation, but the cells do not attach directly to the culture substrate, they rather bind to proteins that are adsorbed to the surface of the scaffold [7, 8]. Bovine serum albumin is a protein commonly used for research purposes due to its stability, water solubility and versatile binding capacity [9].

The aim of this work was to synthesize a SiO_2-CaO-P_2O_5 system by spray drying method and to study the protein functionalization of the samples heat treated at different temperatures.

RESULTS AND DISCUSSION

The SEM images of the as prepared samples (Figure 1) show that the microspheres obtained by spray drying are still unseparated, they occur in aggregates, and there is a wide range of size distribution, from nanometers to micrometers. In this stage, the microspheres still contain traces of solvents. The thermal analysis (Figure 2) points out around 99℃ an endothermic peak that is attributed to the loss of residual water and ethanol. The two endothermic peaks observed in the range 240-390 ºC are associated with loss of alkoxy groups. The weight loss of 4.63 %, recorded around of 535℃, is associated with loss of hydroxyl groups [10]. According to these results, the as prepared samples were heat treated at 110^{o}C, 400^{o}C and 800^{o}C. It was considered the removal of water and ethanol after 110^{o}C treatment, the elimination of alkoxy groups after 400^{o}C treatment, and after 800^{o}C treatment the sample was expected to be completely free of other components than SiO_2, CaO and P_2O_5 oxides.

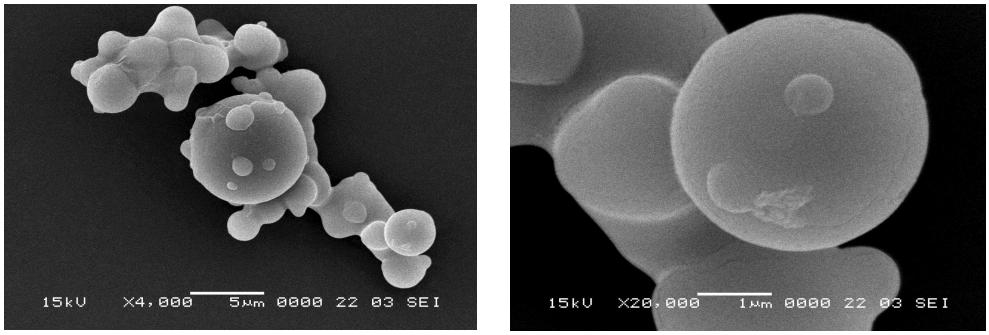

Figure 1. SEM images of the spray dried sample

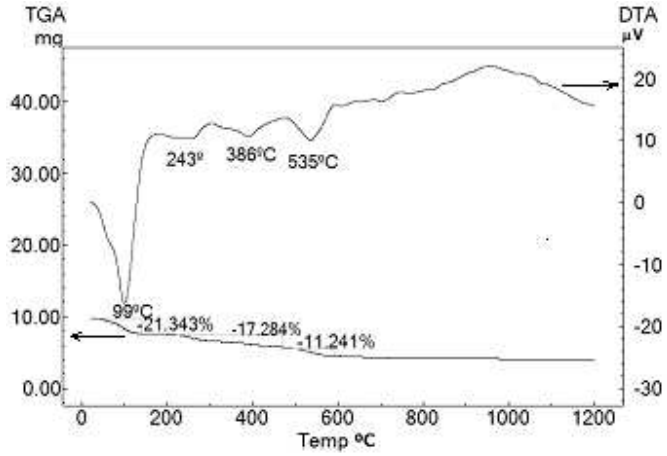

Figure 2. Differential thermal analysis (DTA) and thermal gravimetric analysis (TGA) runs of the spray dried sample.

X-ray diffraction patterns (Figure 3) of the thermal treated S_{110} sample show narrow lines of a crystalline phase identified as ammonium hydrogen phosphate, i.e. $(NH_4)_2HPO_4$ used as P_2O_5 precursor. With increasing the treatment temperature, the crystalline phase disappears, as observed in the diffraction pattern of samples S_{400} and S_{800}. No new crystalline phase is developed even after 800°C treatment. The X-ray diffraction pattern of S_{800} do not evidence any narrow line of crystalline phases, it consist of a broad line typical to amorphous systems.

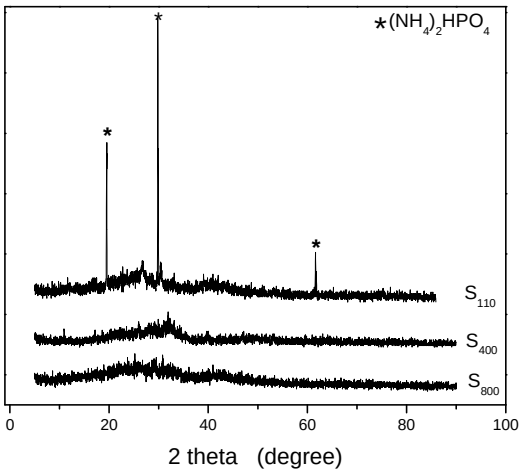

Figure 3. X-ray diffraction patterns of the thermal treated samples

Protein adsorption studies were made in the purpose to determine the interaction of the calcium-phospho-silicate system treated at different temperatures with the bovine serum albumin (BSA) protein. The protein concentration in BSA/SBF solution was 30 mg/ml. In the human blood the serum albumin concentrations are between 35 and 45 mg/ml [11-13], while in the interstitial fluid (tissue fluid) the concentration of albumin is much lower than in the blood [14, 15].

The fluorescence quenching of BSA in the incubated BSA/SBF solution was considered a measure of protein interaction with the glass microspheres [16]. The fluorescence is given by the tryptophan amino acid (which has much stronger fluorescence and higher quantum yield than the other two aromatic amino acids, tyrosine and phenylalanine), from the BSA [17]. This is an indirect method of determining the protein attachment to the sample surface, as the sample itself is not analyzed, but rather the liquid medium wherein the sample was incubated.

Quenching of fluorescence is a decrease in the fluorescence intensity and can occur due to non-molecular, intramolecular or intermolecular mechanisms. Non-molecular quenching can be due to attenuation of the incident light by the fluorophore itself or by other absorbing species; intramolecular quenching can occur if a quencher group interacts with the tryptophan aminoacid residue within a distance around 6-10Å; the intermolecular quenching processes can be either dynamic and/or static and require molecular contact between the fluorophore and quencher [18]. For this reason, quenching studies can be used to reveal accessibility of fluorophores to quenchers.

The samples were periodically measured in the first day, immediately after mixing (0 hour), after 2 hours respectively 4 hours immersion time. For the next six days the measurements were carried out once a day. The first 24 hours data (Figure 4) indicate a decrease in the fluorescence intensity given by the protein from the solution, which is supposed to happen because of protein adsorption on the samples surface. Protein adsorption onto the surface of the bioglass microspheres is further expected to mediate cell adhesion and tissue integration. Sample S_{110} takes up the highest quantity of protein compared to S_{400} and S_{800} samples. This fact is further remarked in the measurements carried out in steps of 24 hours for seven days.

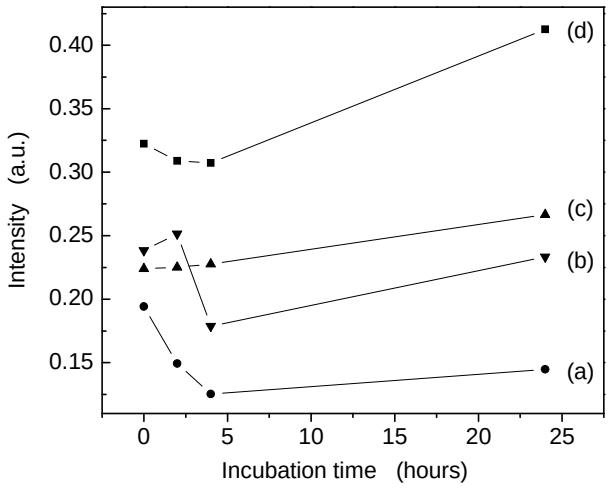

Figure 4. Time dependence of BSA fluorescence intensity during the first 24 hours in (a) SBF/BSA solution with S 110^{o}C; (b) SBF/BSA solution with S 400^{o}C; (c) SBF/BSA solution with S 800^{o}C, and (d) SBF/BSA reference solution.

In the first four days, for the sample treated at 400 ^{o}C the fluorescence signal directly related to the protein is almost at the same intensity level, but after five days, the intensity of the signal increases, suggesting the detachment of the protein from the sample surface, followed in the next two days by protein reattachment (Figure 5). A similar behavior is observed for the sample treated at 800 ^{o}C up to the fifth day, when the intensity of the tryptophan signal from BSA and sinks to a lower value denoting a depletion of protein molecules in solution due to their adhesion to the sample surface. The protein surface usually exhibits one or a few charged spots and therefore the protein adsorption on a substrate may be energetically favourable due to the protein-substrate electrostatic interaction. In some cases [19] it was shown that with increasing protein coverage, the protein desorption increases and there is critical fraction of the area covered by protein, and adsorption above this fraction is hindered both kinetically and thermodynamically. Beside the substrate charge dependence, the protein adsorption/desorption depends on protein size and shape, concentration, hydratation, substrate microstructure, hydrophobic behavior, solution pH, ionic strength, temperature, competitive adsorption with other proteins and ions, as well as the solvent motion relative to the substrate [20]. It is interesting to note that the fluorescence intensity of SBF/BSA reference solution and SBF/BSA solution with S 800^{o}C is increased after 24 hours. A similar effect was reported by Dai et al. [21], but the reason for this is unclear and requires further elucidation.

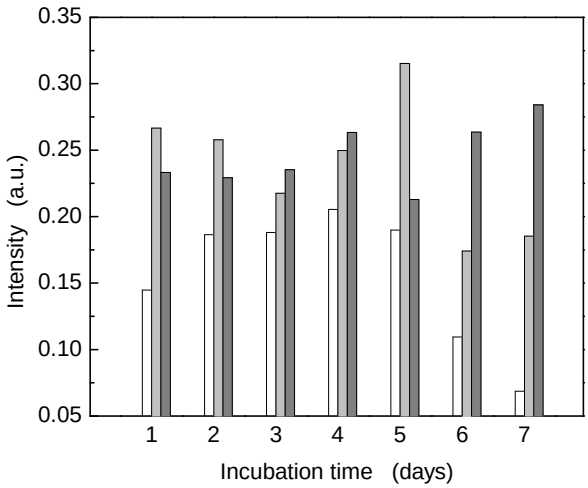

Figure 5. Time dependence of BSA fluorescence intensity during the first 24 hours in SBF/BSA solution with S 110^{o}C (white columns), SBF/BSA solution with S 400^{o}C (light gray columns), and SBF/BSA solution with S 800^{o}C (dark gray columns).

The results may be correlated both with the presence of the residual crystalline phosphate phase on S_{110} microspheres and residual hydroxyl groups on S_{110} and S_{400} microspheres.

CONCLUSIONS

The fluorescence quenching of BSA in BSA/SBF solutions after incubation of spray dried SiO_2-CaO-P_2O_5 glass microspheres heat-treated at 110 ºC, 400ºC and 800 ºC was used in order to investigate the protein attachment on these samples. The results obtained for several incubation times, up to seven days, show that the highest quantity of protein adheres on S_{110} sample. Different from S_{400} and S_{800}, the S_{110} sample contains a residual crystalline phosphate phase and a higher amount of residual hydroxyl groups. The heat treatment applied at 800 ºC completely removed the hydroxyl groups from S_{800} sample which after the long term–incubation attached less protein.

EXPERIMENTAL SECTION

The composition of the bioactive glass was $56Si0_2 \cdot 40CaO \cdot 4P_2O_5$ (mol %). The samples were prepared by spray drying method. The reason of using spray drying method instead of conventional sol-gel method was that the final product consists of microspheres with smooth surface, without edges or acicular shape. The reagents used were tetraethoxysilan $SiC_8H_{20}O_4$ (TEOS) - precursor for SiO_2, calcium nitrate tetrahydrate

$Ca(NO_3)_2 \cdot 4(H_2O)$ - precursor for CaO, and ammonium dibasic phosphate $(NH_4)_2HPO_4$ - precursor for P_2O_5 The mole ratio of the reactants $SiC_8H_{20}O_4$:$Ca(NO_3)_2 \cdot 4(H_2O)$:$(NH_4)_2HPO_4$ was 7:5:1. The calculated amount of tetraethoxysilan was mixed with the same amount of ethanol on a magnetic stirrer, and the other precursors were dissolved at room temperature in distillated water at saturation point. The compounds were mixed together at room temperature. The mixture was dried using Mini Spray Dryer B-290. Parts of the spray dried microspheres were separately treated at 110 ℃, 400 ℃ and 800 ℃ (noted S_{110}, S_{400} and S_{800}).

Protein adsorption on the surface of samples was analyzed in vitro. The SBF was prepared according to Kokubo protocol [5]. The ratio of S_{110}, S_{400} and S_{800} glass microspheres **:** BSA **:** SBF was 0.5 g **:** 0.9 g **:** 30 ml. The samples were incubated at constant temperature 36.5 ºC, up to 7 days, in polypropylene flasks. A reference solution consisting only of BSA and SBF was stored under the same conditions.

SEM imagines were recorded using a Jeol JSM 5510LV Scanning Electron Microscope. Differential thermal analysis (DTA) and gravimetric thermal analysis (GTA) were performed on Shimadzu analyzer DTG-60H, in air, using alumina crucibles, with heating rate of 10 ºC/min, from room temperature to 1000ºC. The structure of the samples was analysed by X-ray diffraction with a Shimadzu XRD-6000 diffractometer, using Cu Ka radiation (λ=1.5418 Å), with Ni-filter. The fluorescence spectra were recorded from 250 to 450 nm on Jasco spectrofluorimeter FP-6300. The excitation wavelength was 295 nm.

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