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## Hydrothermal synthesis of bioactive calcium silicate glass

### ABSTRACT

*This work presents the synthesis of bioactive glass 60SiO<sub>2</sub>-40CaO (wt.%) by the hydrothermal method without using acid catalysts in shortened synthesis time. The precursors Si(OC<sub>2</sub>H<sub>5</sub>)<sub>4</sub> (TEOS), Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O were introduced in a hydrothermal system, and heated at 150 °C for 24 hours. The resulting gel was dried at 150 °C for 24 hours, then calcined at 800 °C for 3 hours to achieve bioactive glass. Several physical-chemical methods such as TG-DSC, XRD, SEM, and ICP-OES were used to evaluate the synthetic material. The bioactivity and biocompatibility of synthetic glass were evaluated by in vitro experiments in SBF solution and in cell culture environment. The obtained results show that the synthetic glass is an amorphous material, presenting the bioactivity through the formation of a hydroxyapatite mineral layer after 10 days of soaking in SBF solution, and also showing good biocompatibility with cells L-929.*

**Keywords:** Bioactive glass, bioactivity, artificial bone, hydroxyapatite, cell viability

### 1. INTRODUCTION

Bioactive glasses (bioglasses) are a type of material that is researched and applied as an artificial bone material used as a component in dental filling cement, culture powder and bone graft in orthopedic surgery to restore and repair damaged and diseased bones [1]. The bioactivity of the material is the ability to form a new layer of hydroxyapatite Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub> (HA) mineral on the surface when the material is implanted in bone defects or damaged bones in the human body. The HA mineral layer has a composition similar to the inorganic composition of human bone, so it is the bridge between the glass graft and natural bone, through which damaged bone parts can be repaired and filled [2,3]. The bioactivity of bioglasses can be tested through “in vitro” experiments according to the method of T. Kokubo & H. Takadama [4]. The glass sample is soaked in SBF solution (Simulated Body Fluid) - a solution with the inorganic ionic components similar to human blood and synthesized in the laboratory. Bioactive glass is synthesized by melting method, or sol-gel method; in which the sol-gel method has been commonly used in recent years.

The sol-gel method is undergone through two main stages: hydrolysis of precursors to create sol, and conversion of sol particles into gel. The resulting gel is processed at a high temperature to obtain a glass material. The sol-gel method overcomes the disadvantages of the melting method such as synthesizing the material system at lower temperatures, the resulting glass systems have a larger specific surface area, leading to higher bioactivity [5 -7]. However, the sol-gel synthesis process requires a long synthesis time because the conversion process from sol to gel usually takes several days. In addition, toxic inorganic acids are often used in the hydrolysis of precursors in the sol-gel synthesis process. In this work, we used a unique method to synthesize two-component bioactive glass SiO<sub>2</sub>-CaO. Synthesis experiment was conducted in a hydrothermal system at high temperature, so that the reactions of sol and gel formation could take place quickly. The glass material synthesized in this study was evaluated for their physical-chemical properties, bioactivity and cell biocompatibility.

### 2. MATERIALS AND METHOD

#### 2.1. Materials

Chemicals for synthesis of bioactive glass 60SiO<sub>2</sub>-40CaO (wt.%) consist of calcium nitrate tetrahydrate Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O (99%, Sigma-Aldrich); tetraethyl orthosilicate Si(OC<sub>2</sub>H<sub>5</sub>)<sub>4</sub> (TEOS) (98%, Sigma-Aldrich). Chemicals (over 99%, Merk) for

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synthesizing SBF solution (Simulated Body Fluid) include  $(\text{NH}_4)_2\text{HPO}_4$ ;  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ ;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ;  $\text{HCl}$ ;  $\text{NaCl}$ ;  $\text{KCl}$ ;  $\text{NaHCO}_3$ ;  $\text{CaCl}_2$ ;  $\text{C}_{14}\text{H}_{11}\text{NO}_3$ .

### 2.2. Synthesis process

The 60SiO<sub>2</sub>-40CaO (wt.%) glass system was synthesized by a non-catalytic hydrothermal method in this study. Investigation of the temperature factor shows that the hydrothermal reaction did not occur when the reaction mixture was carried out below 140 °C. Gel was formed at 150 °C while above 160 °C the gel was burned due to the decomposition of organic precursors. The H<sub>2</sub>O/TEOS molar ratio was chosen to be 10 based on previous researches on bioactive glasses synthesized by the conventional sol-gel method [5-7]. The hydrothermal time was chosen to be 24 hours based on surveys of the reactions according to the above temperatures. According to chemical principles, high temperature leads to the evaporation of water in a closed hydrothermal system, causing high pressure for the reaction system. The pressure of the hydrothermal system, which was not measured in this study, is referred to by the term "self-generated pressure or spontaneous pressure". The value of self-generated pressure will change according to the time of the reaction occurring in the closed system. After investigating factors such as temperature and reaction time, the glass material synthesis process is briefly summarized as follows: mixture of TEOS,  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  and distilled water was placed in a hydrothermal system (autoclave) and heated at 150 °C for 24 hours. The resulting gel was dried at 150 °C for 24 hours. From thermal analysis data, the dried gel powder was heated at 800 °C for 3 hours to create a bioglass system.

### 2.3. In vitro in SBF solution

Glass powder was tested in vitro in SBF solution (Simulated Body Fluid) to check whether it meets the requirements of a biomedical material; that is, to check the bioactivity of the material. This is a quick and simple experiment, intended to carry out a process or reaction in a test tube, in a culture dish outside the living body. In vitro experiment was conducted by soaking glass powder in a solution of simulating human body fluid SBF to investigate the possibility of new bone mineral formation [4]. The SBF solution is a simulated body fluid with a composition of ions similar to blood in the human body, which is synthesized from salt precursors in the laboratory. The glass material powder was soaked in SBF solution for different time periods. The temperature of soaked samples was kept at 37 °C similar to body temperature. The shaking speed for soaked samples was 50 rpm. After soaking periods, the glass material powders

were separated and washed with distilled water to remove excess ions and then rinsed with pure alcohol to completely remove free ions. The powder samples were dried and reserved for analysis of physical-chemical characteristics. The solutions were checked for the content of elements such as Ca, Si, P exchanged between the material and the SBF solution environment.

### 2.4. In vitro with cells

The glass system was tested in a cell culture environment to evaluate the biocompatibility of the synthetic material. Standard culture medium DMEM (Dulbeco's Modified Eagle Medium – Sigma Aldrich) was used in the experiment [8]. The fibroblast line (L-929 fibroblast) was cultured in a standard environment of 37 °C, with 5% CO<sub>2</sub> and 95% humidity. The existence of cell lines was determined by the MTT colorimetric method. MTT compound with the chemical formula of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is a yellow tetrazole, which is converted into purple formazan when interacting with mitochondria in living cells. Quantification of formazan through absorbance is determined by measuring at wavelengths from 500 to 570 nm using a UV-Vis spectrophotometer. Formazan quantification allows direct determination of the number of viable cells.

### 2.5. Methods of analysis and evaluation

TG-DSC (Thermal Gravimetric Analysis-Differential Scanning Calorimetry) is used to determine the calcination temperature that converts dried gel into a glass system. X-ray diffraction (XRD - X Ray Diffraction) is served to determine the phase composition of materials. Scanning electron microscope (SEM - Scanning Electron Microscopy) is a popular tool in materials research, allowing microstructure analysis from the surface of a specimen with high resolution without the need to destroy the sample. The ICP-OES (Inductively Coupled Plasma – Optical Emission Spectroscopy) method is used to analyze the content of Ca, Si, and P elements exchanged between the material sample and the "in vitro" testing environment, thereby evaluating the chemical interactions occurring between the bioglass material sample and the human physiological environment.

## 3. RESULTS AND DISCUSSION

### 3.1. Thermal analysis

The result of TG-DSC thermal analysis of the dried gel sample 60SiO<sub>2</sub> – 40CaO (wt.%) is shown in Figure 1. Thermal analysis diagram shows three mass loss ranges on the TG curve. The initial mass loss range of 30 °C to 220 °C with an endothermic

peak at 145.2 °C characterizes water removal [9-10]. The second mass loss in the range from 220 °C to 470 °C with an exothermic peak at 280 °C, is considered to be the dehydration of ethanol because some ethanol may still exist inside the pores of the dried gel [11]. The third mass loss between 470 °C and 660 °C with an endothermic peak at 508.9 °C is attributed to be the decomposition of  $\text{NO}_3^-$  groups [12-13]. According to

previous studies on bioactive glasses synthesized by the sol-gel method, at a temperature about 900 °C, a phase transition often occurs to form  $\text{CaSiO}_3$  mineral [12-14]. Observation show that the  $60\text{SiO}_2 - 40\text{CaO}$  (wt.%) glass system is structurally stable in this temperature region without any phase transition occurring. From the TG-DSC thermal analysis, the appropriate temperature for glass heating was chosen to be 800 °C.

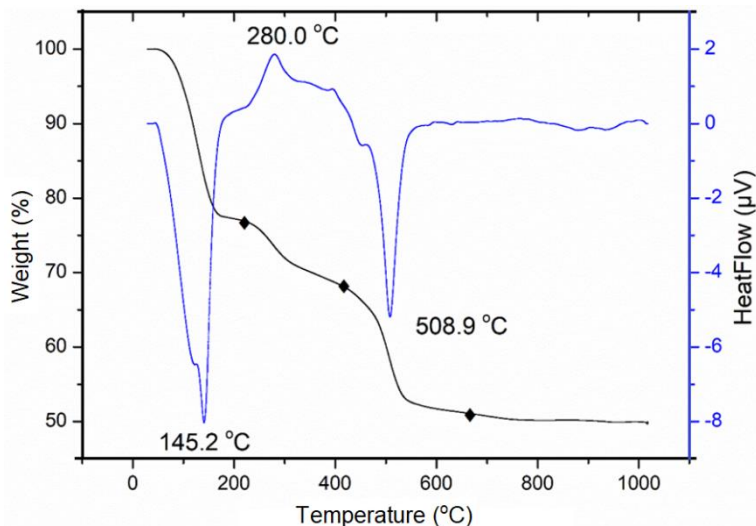


Figure 1. TG-DSC analysis of glass sample  $60\text{SiO}_2 - 40\text{CaO}$  (wt.%)

### 3.2. XRD analysis

Figure 2 shows the XRD pattern of  $60\text{SiO}_2 - 40\text{CaO}$  (wt.%) glass material before and after *in vitro* testing in SBF solution. The XRD pattern of the bioactive glass shows that a broad diffraction halo is observed, the resulting spectral pattern is characteristic of amorphous structural materials. XRD analysis results show that the

$60\text{SiO}_2 - 40\text{CaO}$  (wt.%) bioactive glass synthesized by the non-acid-catalyzed hydrothermal method still maintains amorphous property like the glass prepared by the conventional sol-gel method [15-17]. After 5 days of soaking in SBF solution, the interaction of the material with the SBF environment did not change the feature of the XRD diagram.

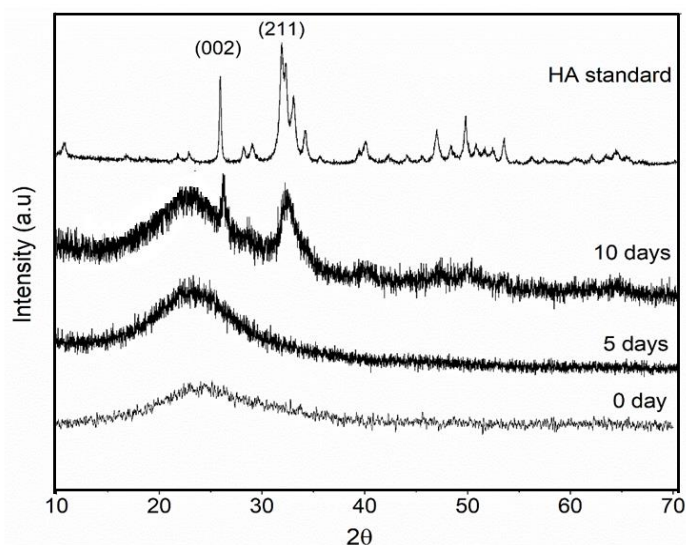


Figure 2. XRD diagrams of glass sample  $60\text{SiO}_2 - 40\text{CaO}$  (wt.%) before and after *in vitro* experiment in SBF solution

This result shows that the 60SiO<sub>2</sub> - 40CaO (wt.%) glass material has not shown bioactivity after 5 days of in vitro experiment. Compared with the previous studies, the glass system synthesized by the modified sol-gel method in this study has lower bioactivity [13, 15-17]. This may be due to the strong structure of the synthetic glass in accordance with the TG-DSC analysis, which gave a fairly high heating temperature to form the glass at 800 °C, and no glass melting at about 900 °C. The durable structure leads to a slow interaction of the material and the SBF environment, which means lower bioactivity. After 10 days of immersion in SBF solution, the hydroxyapatite (HA) mineral phase was identified by the clear appearance of two characteristic peaks at 2θ = 26° (002) and 32° (211) (JCPDS: 09432). The obtained result confirm the bioactivity of the synthetic glass system after 10 days of in vitro experiments in SBF solution.

### 3.3. SEM observation

Figure 3 presents SEM images of 60SiO<sub>2</sub> - 40CaO (wt.%) glass sample before and after immersion in SBF solution. The original glass material clearly shows irregular aggregation of small particles, creating a rough surface, and a porous structure (Fig. 3a). After 1 day of soaking, the surface changed due to the dissolution of the glass material in the SBF solution (Fig. 3b). The surface appeared small crystals quite uniformly after 5 days of soaking and became clearer after 10 days of soaking (Fig. 3c-3d). After 10 days of immersion in SBF solution, the mineralization process was determined by the formation of new HA crystals covering the surface of the glass samples compared to the original sample. The HA crystals are uniform, and cover the entire surface of the 60SiO<sub>2</sub> - 40CaO (wt.%) bioactive glass material as observed in Fig. 3d.

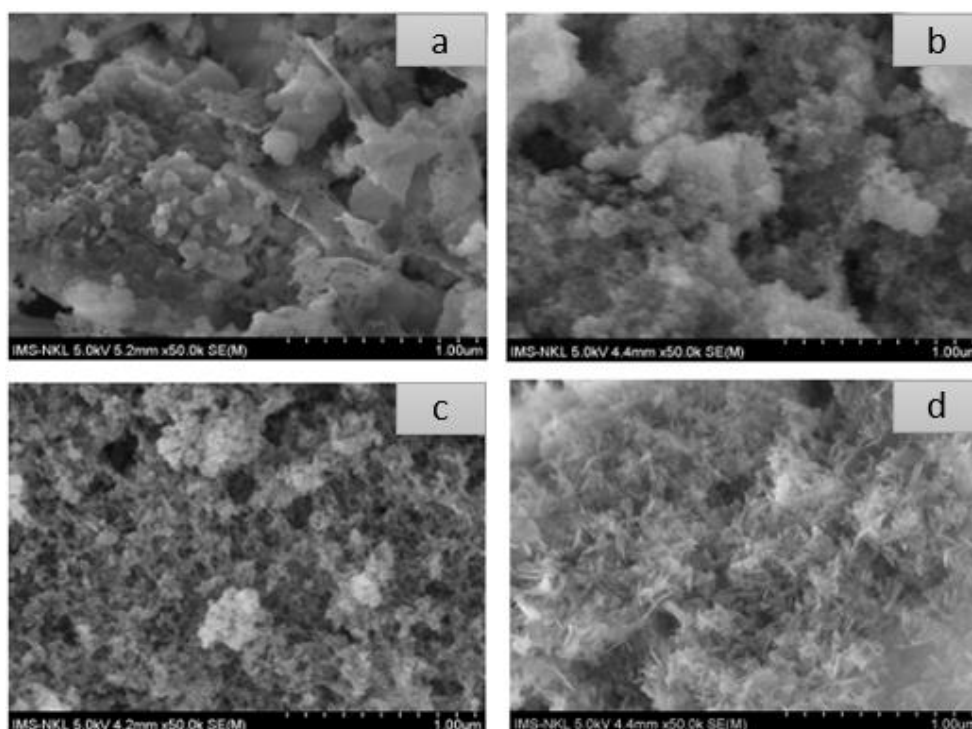


Figure 3. SEM images of glass samples before and after in vitro experiment in SBF solution: a-initial sample; b-sample after 1 day; c-sample after 5 days; and d-sample after 10 days

### 3.4. Dissolution of glass in SBF solution

The physical-chemical interactions of bioactive glass 60SiO<sub>2</sub> - 40CaO (wt.%) with the experimental environment lead to changes in the concentration of ions in the SBF solution analyzed by the ICP-OES method, as shown in Fig. 4. The elemental concentrations of Si, Ca, and P in the initial SBF solution were 0 ppm, 100.1 ppm, and 31.2 ppm, respectively. The Si concentration

increased rapidly at the beginning of soaking (from 0 to 3 days), then increased moderately from 3 to 5 days, then reached saturation value at the period of 5 to 10 days of soaking. According to previous studies, the increase of Si concentration is explained by the dissolution of the glass network through the release of Si(OH)<sub>4</sub> silicic acid, while the saturation process corresponds to repolymerization of the above acids to create a silica SiO<sub>2</sub> layer [18-21]. The Ca concentration increased at the

beginning of the experiment, due to the rapid exchange of  $\text{Ca}^{2+}$  released from the glass network and  $\text{H}^+$  in the SBF solution [22-24]. Then, the Ca concentration decreased sharply after 3 days and reached saturation after 5 days. The decrease in Ca concentration is explained by its consumption to create the HA mineral layer on the surface of the

bioactive glass [23-24]. No increase in P concentration was observed after the in vitro experiment, which can be explained by the absence of P in the initial glass, as well as the consumption of Ca and P in solution to form the hydroxyapatite mineral layer during the in vitro experiment.

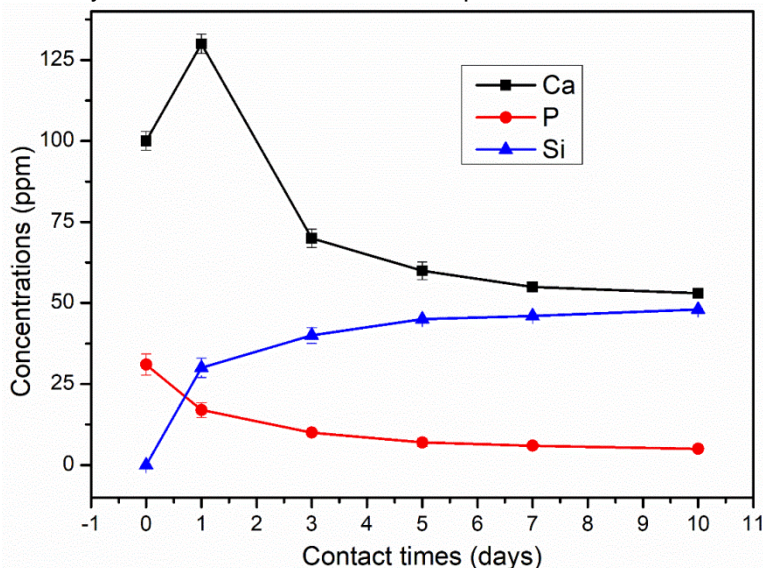


Figure 4. Ionic exchange between glass sample 60SiO<sub>2</sub> - 40CaO (wt.%) and SBF solution

### 3.5. Biocompatibility with cells

The 60SiO<sub>2</sub> – 40CaO (wt.%) glass system was tested for biocompatibility in cell culture medium.

The viability of fibroblast-like cells (L-929 fibroblast) directly exposed to bioactive glass powder for 24 h is presented in Fig. 5.

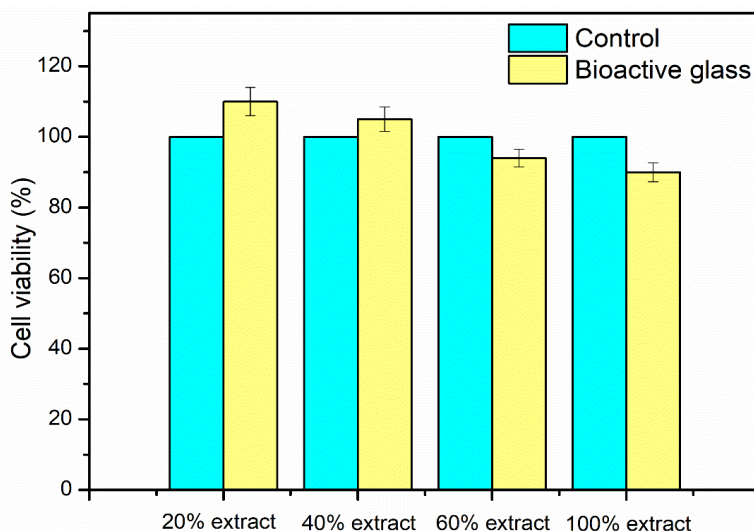


Figure 5. Biocompatibility of glass sample 60SiO<sub>2</sub> - 40CaO (wt.%) in cell culture environment

Cell viability without exposure to bioactive glass was chosen as the control (100%) [8]. According to ISO 10993-5 (Section 5: Cytotoxicity testing, in vitro methods 2009), cell viability is calculated as a percentage compared to the control. In cases where the average cell viability is less than 70%,

the material is cytotoxic. The results obtained showed that cell viability was 110%, 105%, 94%, and 90% for the glass extracts of 20%, 40%, 60% and 100%, respectively. The 20% extract showed the highest cell viability value, while the 60% and 100% extracts showed little difference. The cell

viability values for bioactive glass in this study are equivalent to those of previously studied glass systems synthesized by the sol-gel method [25]. The obtained results show that 60SiO<sub>2</sub>-40CaO (wt.%) bioactive glass synthesized by hydrothermal method without catalytic acid exhibits good biocompatibility in cellular environments even in high-concentrated extract.

#### 4. CONCLUSION

Bioactive glass material 60SiO<sub>2</sub>-40CaO (wt.%) has been successfully synthesized by the hydrothermal method without using acid catalyst with shortened synthesis time. Synthetic material exhibits an amorphous structure similar the natural structure of glass materials. The bioactivity of the 60SiO<sub>2</sub>-40CaO system was confirmed through the formation of a hydroxyapatite (HA) mineral layer after 10 days of in vitro experiment in SBF solution; therefore, it exhibits slower bioactivity than similar glass systems synthesized by the conventional sol-gel method. The diversity of bioactivities when using the new synthesis method in this study brings different application values of bioactive glass materials. Experimental evaluation of biocompatibility in cell culture medium shows that the bioactive glass material in this study has good biocompatibility with the L-929 fibroblast cell line.

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## IZVOD

### HIDROTERMALNA SINTEZA BIOAKTIVNOG KALCIJUM SILIKATNOG STAKLA

U radu je prikazana sinteza bioaktivnog stakla 60SiO<sub>2</sub>-40CaO (tež.%) hidrotermalnom metodom bez upotrebe kiselih katalizatora za skraćeno vreme sinteze. Prekursori Si(OC<sub>2</sub>H<sub>5</sub>)<sub>4</sub> (TEOS), Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O uvedeni su u hidrotermalni sistem i zagrevani na 150 °C tokom 24 sata. Dobijeni gel je sušen na 150 °C tokom 24 sata, zatim kalcinisan na 800 °C tokom 3 sata da bi se dobilo bioaktivno staklo. Nekoliko fizičko-hemijskih metoda kao što su TG-DSC, XRD, SEM i ICP-OES korišćeno je za procenu sintetičkog materijala. Bioaktivnost i biokompatibilnost sintetičkog stakla su procenjene in vitro eksperimentima u rastvoru SBF i u okruženju ćelijske kulture. Dobijeni rezultati pokazuju da je sintetičko staklo amorfni materijal, koji pokazuje bioaktivnost kroz formiranje mineralnog sloja hidroksiapatita nakon 10 dana namakanja u rastvoru SBF, a takođe pokazuje dobru biokompatibilnost sa ćelijama L-929.

**Ključne reči:** bioaktivno staklo, bioaktivnost, veštačka kost, hidroksiapatit, vitalnost ćelija

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