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STRUCTURAL CHARACTERIZATION AND BIOACTIVITY (*IN VITRO*) OF SYNTHESIZED Cu-DOPED SOL-GEL GLASS 60S

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Great interest in different types of bioactive glass (BG) is due to high bioactivity, angiogenic, osteogenic properties. In this regard, the development of metal-alloyed BG as a material for bone tissue regeneration is attracting the attention of the scientific community. With this motivation, this study is focused on the investigation of the structure and bioactivity in vitro of copper-doped bioactive glass 60S.

Nanostructured samples of 60S glass with a composition (mol %) of 60 % SiO_2, 36 – x % CaO, 4 % P_2O_5 doped Cu (x = 0.25, 0.5 mol %) were synthesized by the sol-gel method in this research. The bioactivity of the synthesized material was evaluated by in vitro assays. The dynamics of the formation of hydroxyapatite (HA), contributing to the formation of effective connections with bones and soft tissues during immersion in a simulated body fluid (SBF Kokubo), was evaluated using FTIR, XRD, SEM-EDX and ICP-AES.

It has been found that BG samples with a higher content of an alloying element (a sample with 0.5 mol. % Cu) are more prone to hydrolysis, which leads to a higher activity of ion exchange processes involving the ionic components of SBF. In addition, the formation of weakly crystalline HA and the calcite phase upon contact with SBF is characteristic of both samples which confirms the bioactivity of the synthesized samples in vitro. The presented results are important for further development and research of BG doped with Cu as a promising material with osteoproductive, osteoconductive and antibacterial properties for tissue regeneration and tissue engineering.

***Keywords**: bioglass, sol-gel, copper-doping, bioactivity, bone regeneration*

INTRODUCTION

Despite the advantages of autografts and allografts, the limitations of each of them call for the need to find alternative biomaterials that have the ability to initiate osteogenesis, imitate natural bone along with the regeneration of fibroblasts, and firmly connect with surrounding tissues. Artificial materials such as demineralized bone matrix, coral hydroxyapatite, and phosphate-based ceramics have been used for decades to fill bony defects with virtually no concomitant soft tissue development. Hydroxyapatite (HA), β-tricalcium phosphate (βTCP) have only osseointegration and osteoconduction properties. Only bioactive glass (BG) has an osteogenic property that stimulates the proliferation and differentiation of osteogenesis cells and, in some cases, affects fibroblastic properties.

The clinical demand for bioactive glass is rapidly increasing day by day due to its versatility, namely bioactivity, resorbability, osteoproductivity, osteoconductivity, depending on the compositional range variations. As the number of combat injuries increases, so does the variety of implants required. A wide range of applications of BG includes implants for bone defects, for the restoration or replacement of damaged tissues, frameworks for bone plastic, preparation of bone cement, as a new drug carrier [1, 2]. In particular, the use of ion-doped BGs in the context of antibacterial applications without antibiotics is considered [3–6].

Over the past two decades, it has been discovered that different parts of our body require implants with different chemical and

physical properties. Properties such as resorption speed and mechanical strength can be corrected by ion alloying (Mg, Sr, Mn, Fe, Zn, Ag), which also has a positive effect on the formation of new bone [7]. The addition of active ions affects the crystallization, morphology, crystallinity, solubility, thermal stability and, mainly, the behavior under the influence of the physiological environment, promotes the proliferation of osteoblast-like cells and, as a result, encourages osseointegration *in vivo* [8–10].

Such doped biomaterials can serve as a framework for bone regeneration with appropriate mechanical properties, repair bone defects, and facilitate the healing process through soft tissue regeneration. Since it is known that many trace elements, such as Sr, Cu, Zn, Mg or Co, are present in the human body and their anabolic effect on bone metabolism is known, their inclusion in the composition of bioglass is relevant [11–15]. The release of these ions after exposure to a physiological environment tends to improve the bioactivity of the implant, which is associated with both osteogenesis and angiogenesis.

Initially, alloying additives were selected according to their valency similarity with elements already present in the composition of glass, but with the accumulation of information about the necessary trace elements in our body, interest in dopants was focused on some specific elements, their influence and possible synergistic effects [16–32].

Thus, the latest trend is to incorporate various ions into the composition of BG to improve physical characteristics and therapeutic effects. Some of the ions, such as Ga, Ge, La, Y, have already been introduced into the composition of BG, and the synthesized materials have been tested *in vitro* and *in vivo* [33, 34].

There are several reasons for the need for Cu doping of bioactive glass-ceramic materials. It is known that copper has an increased antimicrobial effect due to the ability to bind to imidazole, thiol, carboxyl and amine functional groups of microbial proteins, which leads to changes in the structure of the surface membrane of bacteria [35–40]. It has been proven that copper ions tend to support and generate endothelial cells [41], stimulate osteogenic activity and inhibit osteoclast behavior [42, 43]. In addition, copper stimulates elastin fibers and plays a key role in the deposition of collagen and elastin matrix [44, 45]. This is the best material for enhancing angiogenic properties, which also promotes the healing of bone tissue [46–49]. All of these attractive properties make copper a valuable additive for incorporation into bioactive ceramics and glasses for the fabrication of multifunctional biomaterials that combine osteoconduction/osteoinduction with novel therapeutic additional functions.

Sol-gel synthesis, which provides control over the homogeneity and purity of the synthesized material, is the optimal way to incorporate copper ions into the composition of bioactive glass [43, 46, 50–52]. Regarding the influence of copper on the physicochemical properties of bioglass and its bioactivity, conflicting data have been reported, as some authors have testified that the inclusion of Cu contributes to the stabilization of the structure [53] which prevents the dissolution of the glass and, thus, the release of ions. Others believe that doping promotes attenuation leading to lower glass transition and crystallization temperatures [54], and provides high bioactivity, as evidenced by the rapid formation of a HCA layer on surfaces in SBF. However, both groups of researchers agree that the inclusion of Cu does not prevent the formation of the hydroxyapatite (HA) layer, but affects the morphology of the layer and the size of the HA crystals [55–60].

When researching synthesized materials *in vitro*, the SBF Kokubo solution is most widely used, which makes it possible to study a number of properties: changes in the weight and morphology of the glass surface, physical and mechanical properties, as well as changes in pH and ionic concentrations of the solution. It has been proved that there is a correlation between the degree of bone ingrowth among glass particles and their capability to form apatite in SBF. Accordingly, the bioactivity of glass *in vivo* can be accurately predicted from its changes in SBF [60, 61].

This paper presents the results of comprehensive studies of BG 60S doped Cu, its structure and bioactivity *in vitro* as a promising material with osteoproductive, osteoconductive and antibacterial properties for tissue regeneration and tissue engineering.

EXPERIMENTAL PART

Materials. The composition (mol %) of BG

60S doped Cu was 60 % SiO_2, 36 – x % CaO, 4 % P_2O_5, Cu (x = 0.25, 0.5 mol %). The following reagents were used for sol-gel synthesis: tetraethyl orthosilicate (TEOS) $(C_2H_5O)_4Si$, triethyl phosphate (TEP) $(C_2H_5O)_3PO$, ethanol C_2H_5OH, calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), copper (II) nitrate ($Cu(NO_3)_2{\cdot}3H_2O$), 59 % solution of nitric acid (HNO_3). All reagents had "chemically pure" qualification (Merck Schuchardtohg, Germany).

Preparation of **BG 60S.** The synthesis of sol-gel glass 60S is described in detail in previous works [1, 33, 34]. BG 60S doped Cu was carried out similarly. The introduction of copper into glass panes was carried out at the stage of sol formation. An aqueous solution of calcium nitrate and copper nitrate was prepared separately by mixing the given amounts of components on a magnetic stirrer for at least 10 minutes. Then a solution of calcium - copper nitrates was added to the sol, stirred on a magnetic stirrer for at least 40 min, sonicated for 5 min and withstood the sol for 24 h at room temperature to complete the polycondensation processes. The produced glass was ground with a mortar and pestle to disagglomerate the particles.

For research the apatite forming ability *in vitro,* disks (d = 13 mm, h = 500 μm, pressure 10 tons) of the studied samples of sol-gel glasses (0.1 ± 0.005 g) were formed and the corresponding volume of SBF was calculated. Assessment of bioactivity was carried out using the standard *in vitro* procedure described by [60, 61] and ISO 23317:2014 using analytical reagent-grade chemicals NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, trishydroxymethyl aminomethane [Tris-buffer, $(CH_2OH)_3CNH_2$], and 1 M HCl.

Characterisations of BG 60S. Structural studies of the obtained samples were performed by powder X-ray diffraction method (XRD) using a DRON-4-07 diffractometer with Ni filtered CuK_α radiation, the Bragg-Brentano focusing, in 10–80° 2θ range with a step of 0.05°, exposure of 1 sec. Phase identification was performed with PDF-2 database.

Infrared spectra were recorded on a FTIR Spectrometer Tensor 27 (Bruker Optik GmbH, Germany) in the range 4000–400 cm^{-1} using KBr pellets with a resolution of 2 cm^{-1}.

The obtained samples were characterized by a scanning electron microscope MIRA 3 FE-SEM microscope (TESCAN, Czech Republic) equipped with an Energy-dispersive X-ray detector (EDX) Oxford Instruments, UK). EDX spectra of the prepared samples before and after immersion in SBF in 4, 8, 12 days were used to study changes in the elemental composition of the sample surface.

Determination of the elemental composition of samples before and after immersion in SBF in 4, 8, 12 days was carried out by the method of atomic emission spectrometry with inductively coupled plasma (ICP-AES) (Shimadzu ICPE-9000, Axial detection mode).

ICP multi-element standard solution IV (Merck, 1.11355) containing 1000 mg/l of Ag, Cu, Ca, Na, K, Mg was used to prepare calibration solutions; 96 % H_2SO_4 (Merck, 1.00732.2500) as S source and 85 % H_3PO_4 (Sigma-Aldrich, 04107) as P source. Calibration comparison solutions with concentrations of each of the elements of 0.01; 0.1, 0.5 and 2 mg/l were prepared.

For elemental analysis, all selected samples of bioglass were prepared as follows: 10.0 mg of the sample was placed in a 20 ml volumetric flask, 0.25 ml of concentrated nitric acid was added, mixed and kept in an ultrasonic bath for 30 min, the volume was brought up to the mark with water and mixed. 150 μl of the filtered solution obtained was transferred to a volumetric flask with a volume of 10 ml, brought to the mark with water and mixed. To determine Ca, Na and Mg, the obtained solutions were diluted 2 times.

In vitro apatite forming capability. Bioactive glass samples before and after immersion in SBF were examined by Fourier transform infrared spectroscopy (FTIR), X-ray thin film diffraction, and scanning electron microscopy after soaking for 4, 8, and 12 days, respectively. Samples were immersed in SBF in clean plastic bottles that had been prewashed with concentrated HCl and deionized water. The bottles were placed inside a thermostat at a controlled temperature of 36.5 °C. The SBF solutions were not refreshed during the immersion period for all XRD, EDXS, FTIR measurements. After immersion in SBF at 36.5 °C for various periods, the sample was removed from SBF and washed with deionized water. The samples were dried at 90 °C and stored in a desiccator.

The volume of SBF required for this study was calculated according to the equation:

$$V_s = S_a / 10,$$

where V_s is the volume of SBF (ml) and S_a is the apparent surface area (mm^2).

RESULTS AND DISCUSSION

Characterization of BG 60S

In vitro study of bioactivity.

X-ray structural analysis. Figs. 1, 2 show the XRD diffraction patterns of the synthesized BG samples before and after soaking in the SBF solution for different periods of time. On the diffractogram of the original BG 60S doped Cu (0.5 mol %) (Fig. 1 *a*) and doped Cu (0.25 mol %) (Fig. 2 *a*) in the area of diffraction angles ~ 22–30°, an asymmetric diffuse halo is observed, which indicates on the amorphousness of the material. Crystalline peaks related to the weakly crystalline phase of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (ICDD 86-740) and calcite $CaCO_3$ (ICDD 72-1214), are recorded already after 4 days of contact with SBF, and indicate the formation of at least of two crystalline phases in BG 60S (Figs. 1, 2 *b*). Soaking BG 60S in SBF leads to a change in its phase composition and the ratio of amorphous and crystalline components. After 8 days of soaking in SBF, the intensity of the diffraction peaks of both calcite and HA increases on the diffractograms of the samples which indicates an increase in their crystallinity–(Figs. 1, 2 *c*). The formation of calcite, which provides a potential ionic buffer for bone regeneration and has the ability to transform into hydroxyapatite (HA), is one of the signs of the high bioactivity of the synthesized samples. After 12 days of soaking the glass in SBF (Figs. 1, 2 *d*), a decrease in the width of the diffuse halo is recorded, which indicates the structuring of the amorphous phase. An increase in the intensity of the peaks of hydroxyapatite with practically unchanged intensity of the peaks of calcite is observed for the sample with a lower content of the alloying element (Fig. 2).

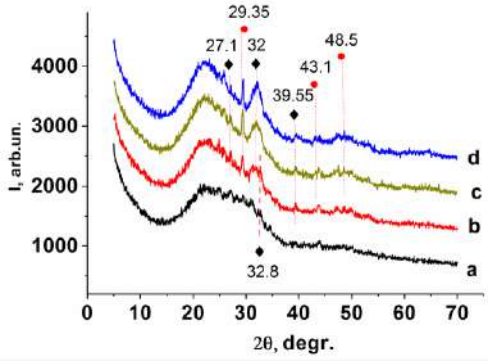

Fig. 1. XRD patterns of BG 60S doped 0.5 % Cu: *a* – before SBF test; after SBF test: *b* – after 4 days, *c* – after 8 days, *d* – after 12 days; ♦ – HA ($Ca_{10}(PO4)_6(OH)_2$), • – calcite ($CaCO_3$)

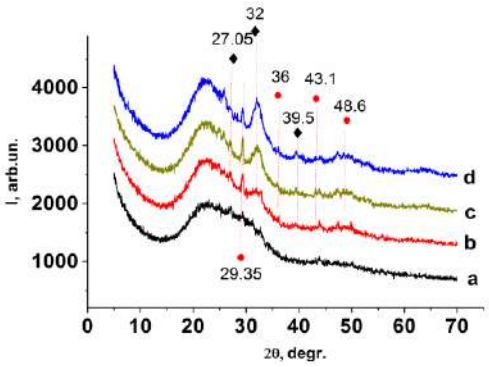

Fig. 2. XRD patterns of BG 60S doped 0.25 % Cu: *a* – before SBF test; after SBF test: *b* – after 4 days, *c* – after 8 days,*d* – after 12 days; ♦ – HA ($Ca_{10}(PO4)_6(OH)_2$), • – calcite ($CaCO_3$)

FTIR analysis. The changes that occurred with the bioglass during its immersion in the SBF solution were also observed using IR spectroscopy (Figs. 3, 4). Thus, the spectrum of the original glass illustrates a broad maximum at 1026 cm^{-1}, a low-intensity one at 875 and 800, 610, 560–587, and 440 cm^{-1}. Absorption maxima at 1026 cm^{-1} are associated with Si-O-Si and P-O stretching vibrations, and the bands at 800 and 440 cm^{-1} are associated with Si-O-Si bending vibrations (Figs. 3, 4).

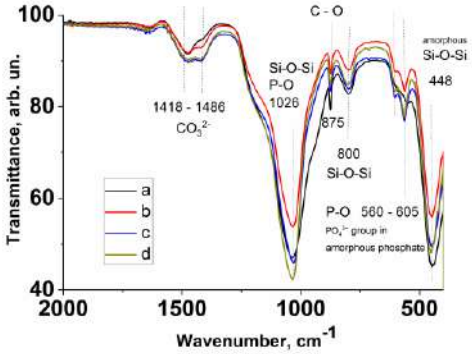

Fig. 3. FTIR-spectra of the BG 60S doped Cu 0.5 mol %: *a* – before SBF test; after SBF test: *b* – after 4; *c* – after 8; *d* – after 12 days

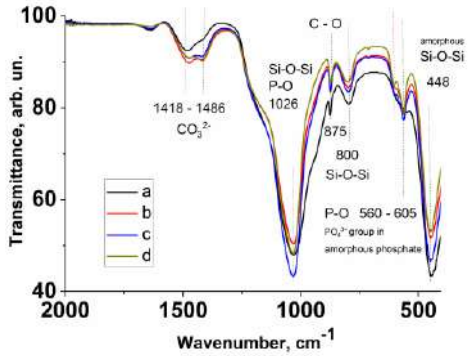

Fig. 4. FTIR-spectra of the BG 60S doped Cu 0.25 mol %: *a* – before SBF test; after SBF test: *b* – after 4; *c* – after 8; *d* – after 12 days

After 4 days of contact with SBF (Figs. 3, 4 *b*), an increase in the intensity of the bands at 1418−1486 and 875 cm^{-1}, which is also recorded for the sample during further exposure to SBF (Figs. 3, 4 *c*), is associated with active precipitation of $CaCO_3$ and is consistent with XRD data. The appearance of a new maximum and, at the same time, an increase in its intensity at 875 cm^{-1} after contact with SBF indicate the inclusion of CO_3^{-2} ions into the BG structure. An increase in the intensity of the bands at 800 cm^{-1}, associated with bending vibrations of Si-O-Si, indicates active processes in the structure of the siloxane network (-Si-O-Si-) of the glass. An increase in the intensity of the maximum at 600 and at 560−587 cm^{-1}, associated with P-O bonds, indicates active changes in the HA structure (Figs. 3, 4 *c, d*).

SEM-EDX analysis. SEM micrographs of sol-gel glass samples before contact with SBF are shown in Fig. 5, 6 *a*. EDX analysis confirms the presence of Si, Ca, P, Cu. Significant changes in surface morphology are recorded, respectively, during the period of 4−12 days of soaking in SBF, which is shown in Fig. 5, 6 *b*−*d*. The surface becomes homogeneous with a predominant Si content. After 4 days of immersion in SBF, the mass fraction (wt, %) of Ca and Cu decreases, which is a consequence of active desorption into the environment according to the theory of dissolution of bioactive glass in physiological fluids, at the same time, a slight increase in the mass fraction of P is recorded. For samples BG 60S doped 0.25 % Cu and P are

not fixed on the 8th and 12th day of contact with SBF, which can be explained by the absence (or minimal wt, %) in the near-surface gel-silicate layer (Tables 1, 2). At the same time, the increase in Ca wt, % correlates with the data of XRD studies (formation and preservation of the calcite phase) (Figs. 1, 2).

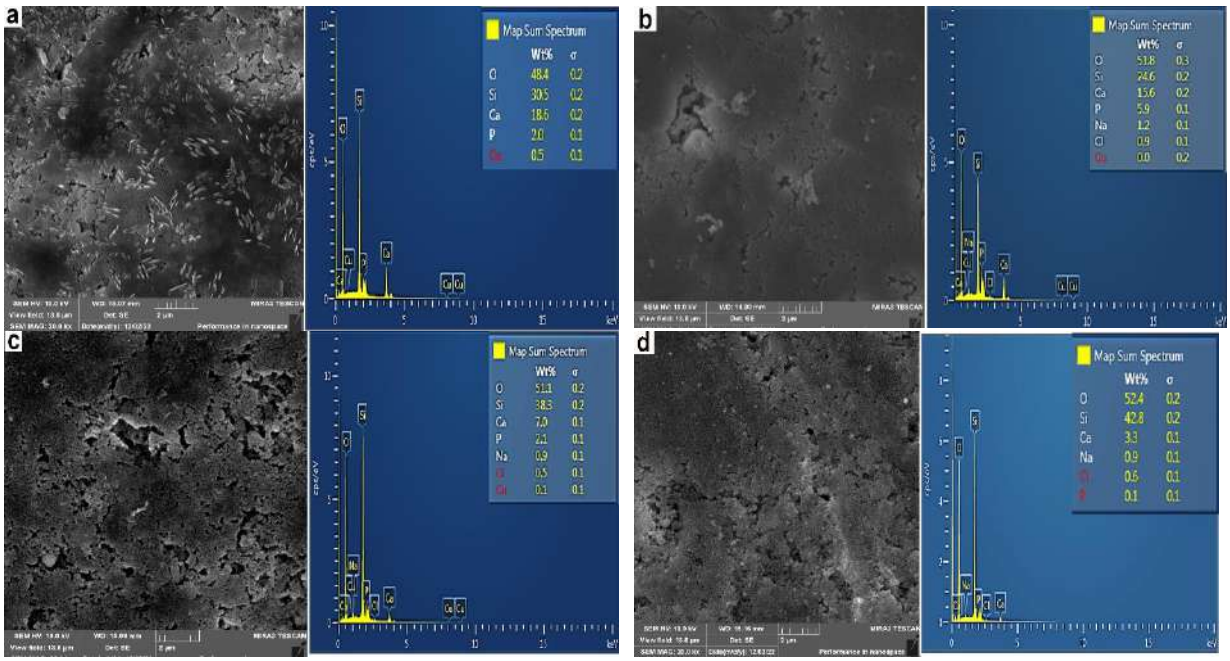

Fig. 5. SEM images and EDX spectra of the disc BG 60S doped with 0.5 % Cu: *a* – before SBF test; after SBF test: *b* – after 4; *c* – after 8; *d* – after 12 days

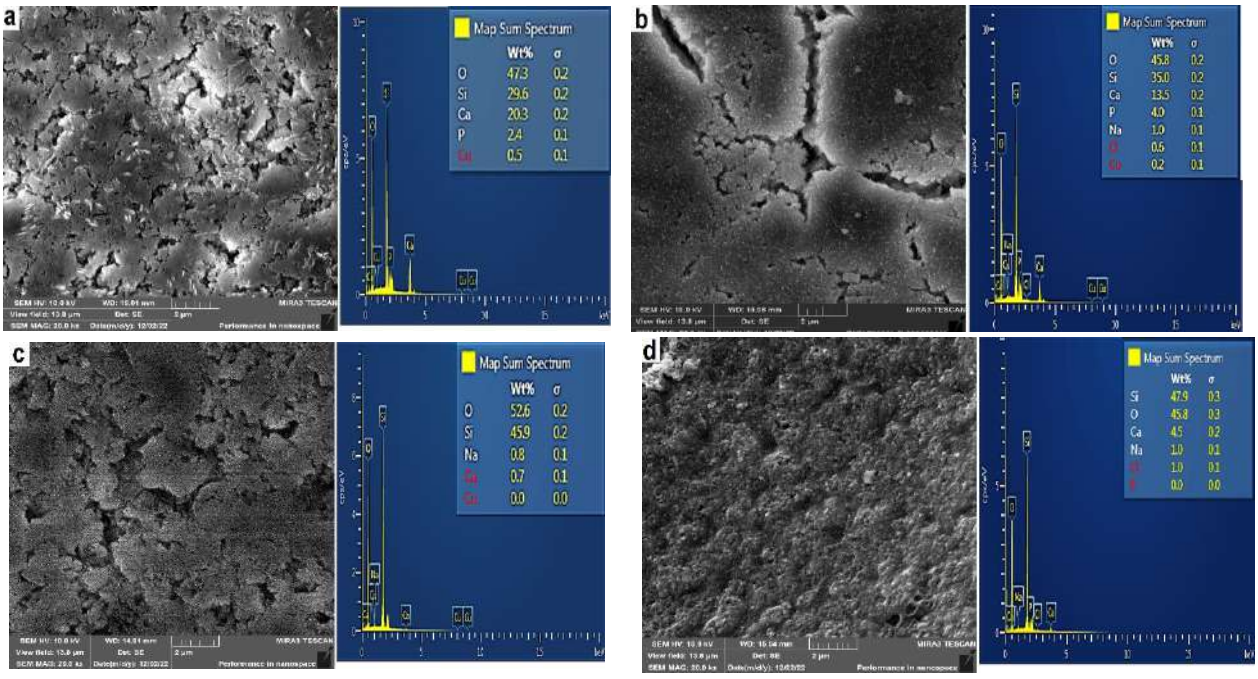

Fig. 6. SEM images and EDX spectra of the disc BG 60S doped with 0.25 % Cu: *a* – before SBF test; after SBF test: *b* – after 4; *c* – after 8; *d* – after 12 days

Table 1. EDX analyses of the samples BG 60S doped with 0.5 % Cu before and after in SBF solution

Contact time	Wt, % (EDX data)						
	O	Si	Ca	P	Cu	Na	Cl
0	48.4	30.5	18.6	2.0	0.5	0	0
4 days	51.8	24.6	15.6	5.9	0	1.2	0.9
8 days	51.1	38.3	7.0	2.1	0.1	0.9	0.5
12 days	52.4	42.8	3.3	0.1	0	0.9	0.6

Table 2. EDX analyses of the samples BG 60S doped with 0.25 % Cu before and after in SBF solution

Contact time	Wt, % (EDX data)						
	O	Si	Ca	P	Cu	Na	Cl
0	47.3	29.6	20.3	2.4	0.5	0	0
4 days	45.8	35.0	13.5	4.0	0.2	1.0	0.6
8 days	52.6	45.9	0.7	2.1	0	0.8	0
12 days	47.9	45.8	4.5	0	0	1.0	1.0

Determination of the elemental composition of the samples BG 60S doped Cu before and after SBF test. According to the obtained results, a decrease in Ca and Cu content is characteristic of both synthesized BG samples (Tables 3, 4). The high activity of ion exchange processes with the participation of these ions is recorded to a much greater extent for BG 60S doped with 0.5 % Cu. The decrease in the Cu content is consistent with the data of previous studies [56] and indicates the formation of a gel-silicate layer as a result of the depolymerization of silanol groups (≡Si-OH) in the glass structure and correlates with the results of EDX analyses. The inclusion of K^+, Na^+, Mg^{2+}, S (probably in the composition of SO_4^{2-} ions) is also associated with active processes of ion exchange of the hydrolyzed BG surface with the SBF medium. It should be noted that the absence of K, Mg, S for EDX results may be due to the low sensitivity of this method for these elements.

Table 3. Elemental analyzes of the samples BG 60S doped 0.5 % Cu before and after SBF test

time in SBF (days)	Wt, % (ICP-AES data)						
	Cu	Ca	P	K	Na	Mg	S
0	0.50	16.04	3.34	0.90	0.83	0.28	0.85
4	0.46	14.42	3.82	1.10	1.39	0.35	0.80
8	0.41	13.61	3.68	0.38	0.59	0.55	0.15
12	0.32	13.76	4.07	1.34	1.05	0.39	0.13

Table 4. Elemental analyzes of the samples BG 60S doped 0.25 % Cu before and after SBF test

time in SBF (days)	Wt, % (ICP-AES data)						
	Cu	Ca	P	K	Na	Mg	S
0	0.25	16.95	3.15	0.83	0.73	0.27	0.41
4	0.22	15.76	3.21	1.21	0.75	0.28	0.26
8	0.22	13.86	3.62	0.86	1.68	0.35	0.56
12	0.21	13.03	3.66	1.63	1.17	0.37	0.46

CONCLUSIONS

In summary, BG samples based on BG 60S doped with copper (0.5 and 0.25 mol %) were synthesized by the sol-gel method and fully characterized by SEM-EDX, FTIR, XRD, ICP-AES. The study of the bioactivity of these glasses *in vitro* was carried out with SBF Kokubo. It has been found that the sample with 0.5 mol % Cu is significantly more active in ion exchange processes with the participation of SBF. In addition, the formation of weakly crystalline HA and the calcite phase during immersion in SBF is characteristic of both samples. Overall, the presented results are fundamental and necessary for further development and research of Cu-doped BGs. In the future, it is necessary to carry out tests on gram-positive and gram-negative bacterial cultures, focusing on the antibacterial activity of copper-doped BGs.

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Структурна характеристика та біоактивність (*in vitro)* синтезованого золь-гель скла 60S, легованого Cu

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Великий інтерес до різних типів біоактивного скла (BG) пояснюється високою біоактивністю, ангіогенними, остеогенними властивостями. У зв'язку з цим розробка металолегованого BG як матеріал для регенерації кісткової тканини привертає увагу наукової спільноти. З цієї мотивації дане дослідження зосереджено на дослідженні структури та біоактивності в умовах in vitro легованого міддю біоактивного скла 60S.

У даному дослідженні золь-гель методом синтезовано наноструктуровані зразки скла 60S зі складом (мол. %) 60 % SiO_2, 36 – x % CaO, 4 % P_2O_5, легованого Cu (x = 0.25, 0.5 мол. %). Біологічну активність синтезованого матеріалу оцінювали методом in vitro. Динаміка утворення гідроксиапатиту (ГА), що сприяє формуванню ефективних зв'язків з кістками та м'якими тканинами під час занурення в симульовану рідину організму (SBF Kokubo), була оцінена за допомогою FTIR, XRD, SEM-EDX та ICP-AES.

Було виявлено, що збільшення вмісту легуючого елемента (зразок з 0.5 мол. % Cu) підвищує схильність BG до гідролізу, що проявляється в вищій активності іонообмінних процесів за участі іонних компонентів SBF. Крім того, утворення слабкокристалічного HA та фази кальциту при контакті з SBF характерно для обох зразків, що підтверджує біоактивність синтезованих зразків в умовах in vitro. Представлені результати є необхідними для подальших розробок та досліджень BG легованих Cu як перспективного матеріалу з остеопродуктивними, остеокондуктивними та антибактеріальними властивостями для тканинної регенерації та тканинної інженерії.

***Ключові слова**: біоскло, золь-гель, легування міддю, біоактивність, кісткова регенерація*

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