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Features of Mineralization of Hydroxyapatite on the Surface of Calcium-Silicophosphate Glass-Ceramic Materials *in vivo*

O. V. Savvova[1], O. I. Fesenko[1], O. V. Babich[2], O. A. Nikolchenko[3], H. K. Voronov[1], and Yu. O. Smyrnova[1]

[1]*O. M. Beketov National University of Urban Economy in Kharkiv, 17, Marshal Bazhanov Str., UA-61002 Kharkiv, Ukraine*
[2]*SRI 'Ukrainian Scientific Research Institute of Ecological Problems', 6, Bakulin Str., UA-61002 Kharkiv, Ukraine*
[3]*Sytenko Institute of Spine and Joint Pathology, N.A.M.S. of Ukraine, 80, Pushkinska Str., UA-61024 Kharkiv, Ukraine*

The features of mineralization of hydroxyapatite (HAp) on the surface of calcium-silicophosphate glass-ceramic materials *in vivo* are analysed. It is found that the implantation of biomaterials based on BS-11 and ASZ-5 bioactive glass-ceramic materials characterized by a strengthened structure and an adjustable level of resorption implements the chemical and biochemical mechanisms of formation of the apatite-like layer. The peculiarities of compositions and technologies for obtaining bioactive glass–ceramic materials for replacement of bone defects are analysed. The conditions for the formation of an apatite-like layer *in vivo* on the surface of glass-ceramic materials are established. These ones include crystallization process of fine-dispersed HAp, ensuring the reactivity of glass-ceramic materials due to destruction of them, initiation of the nucleation of non-stoichiometric HAp on the surface of materials. As determined, the stimulation of the adsorption process of proteins on the surface of the ASZ-5 and BS-11 glass-ceramic materials is realized by ensuring the values of the surface microroughness index $R_a = 2.6$ and 6.0 μm and the surface free energy of 51.5 and 74.6 MJ/m^2, respectively. For the developed glass-ceramic materials based on calcium-silicophosphate glasses, the formation of a sitallized structure under conditions of low-temperature heat treatment makes it possible to provide their operational properties close to those for bone cortical tissue ($K_{1C} = 2.44$ and 2.8 MPa·m$^{1/2}$, $HV = 7800$ and 3800 MPa, $\sigma_{compr} = 160$ MPa). This fact allows us to consider them as promising when creating implants, which can be used to replace the statically and dynamically loaded areas of bone tissue in orthopaedics and

maxillofacial surgery. This one, together with the shortened periods of resorption and mineralization of bone tissue, will increase the efficiency of prosthetics by halving the rehabilitation period for patients and avoiding the necessity for repeated operations.

Проаналізовано особливості мінералізації гідроксиапатиту (ГАп) на поверхні кальцій-силікофосфатних склокристалічних матеріялів *in vivo*. Встановлено, що за імплантації біоматеріялів на основі біоактивних склокристалічних матеріялів БС-11 та АСЗ-5, які характеризуються зміцненою структурою та реґульованим рівнем резорбції, реалізується хемічний і біохемічний механізми формування апатитоподібного шару. Проаналізовано особливості складів і технології одержання біоактивних склокристалічних матеріялів для заміщення дефектів кістки. Встановлено умови формування апатитоподібного шару в умовах *in vivo* на поверхні склокристалічних матеріялів: реалізація процесу кристалізації тонкодисперсного ГАп, забезпечення реакційної здатности склокристалічних матеріялів за рахунок деструкції їх, ініціяція зародкоутворення нестехіометричного ГАп на поверхні матеріялів. Визначено, що стимулювання процесу адсорбції протеїнів на поверхні склокристалічних матеріялів реалізується за рахунок забезпечення значень показника мікрошерсткости поверхні склокристалічних матеріялів АСЗ-5 і БС-11 $R_a = 2{,}6$ і 6,0 мкм та вільної енергії поверхні 51,5 і 74,6 мДж/м^2 відповідно. Для розроблених склокристалічних матеріялів на основі кальцій-силікофосфатних стекол формування ситалізованої структури в умовах низькотемпературного термічного оброблення уможливлює забезпечити їхні експлуатаційні властивості, які наближені до таких властивостей для кісткової кортикальної тканини ($K_{1C} = 2{,}44$ та 2,8 МПа·м$^{1/2}$, $HV = 7800$ і 3800 МПа, $\sigma_{\text{стиск}} = 160$ МПа), дає змогу вважати їх перспективними для створення імплантатів, що можуть бути використані для заміни статично та динамічно навантажених ділянок кісткової тканини у ортопедії та щелепно-лицевій хірургії. Це разом зі скороченими строками резорбції та мінералізації кісткової тканини дасть змогу підвищити ефективність протезування за рахунок скорочення вдвічі періоду реабілітації пацієнтів і виключення повторних операцій.

Key words: glass-ceramic materials, biomaterials, *in vivo*, apatite-like layer, bone tissue.

Ключові слова: склокристалічні матеріяли, біоматеріяли, *in vivo,* апатитоподібний шар, кісткова тканина.

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1. INTRODUCTION

The development of medical science and technology requires the creation of materials for obtaining bone tissue substitutes of a new generation, intended for long-term functioning in the body [1]. Materials for the manufacture of implants must ensure biocompatibil-

ity for a long time, namely, do not change the physicochemical properties, do not cause chronic inflammation, and do not show a carcinogenic effect, not subject to calcification. However, it should be emphasized that, along with the ability to calcify, mineralization of calcium-containing compounds plays an important role in the restoration of the function of bone tissues and their formation [2]. Therefore, to ensure the biocompatibility of the implant, it is important to correct these processes, which are similar in their mechanism. The effectiveness of the functioning of bone endoprostheses is ensured by the formation of a strong adhesion layer in the implant–bone system, which is realized due to the mineralization of apatite-like structures. To achieve the latter, it is important to take into account the theoretical approach to substantiating the calcification of biomaterials.

The primary stages of calcification, in the cellular approach, are associated either with the presence of already dead cells (as in the case of treated biological tissues), or with the death of recipient cells that adhere to the biomaterial. At present, a theory based on the formation of calcium–phospholipid–phosphate complexes is being widely tested, the reason for the formation of which is the affinity of acidic phospholipids for calcium ions. It is envisaged that the presence of such complexes in metastable solutions of calcium and phosphates provokes the precipitation of insoluble crystals of hydroxyapatite (HAp) [3].

The concentration theory allows one to take into account and evaluate the role of the concentration of calcium and phosphate ions in the intercellular fluid. Known physicochemical theories associate the formation of heterogeneous nucleation centres of HAp crystals with the macromolecular stereoconfiguration of collagen or consider the transformation of crystalline HAp precursors such as amorphous calcium phosphate (ACP) [3]. The concentration theory is clearly realized in the implantation of biomaterials based on bioactive ceramic [4] and glass-ceramic materials characterized by a strengthened structure and an adjustable level of resorption [5].

The complexity of the simultaneous providing with high strength of the implant–bone bond for a short time depends primarily on the bioactivity of the glass material and its ability to form a thin apatite-like layer on its surface [6, 7]. This can be achieved by heterogeneous nucleation of apatite crystals by the presence of Si–OH, Ti–OH, Zr–OH structural elements on the surface of materials. At the stage of collagen fibre synthesis and at the initial stages of bone biomineralization, silicon is associated with calcium, initiating the process of deposition of bone minerals, and is an important ‘transitional’ element in the formation and development of cartilage and bone structures.

It should be noted that the identification of mineralization products is important from the point of view of the fact that the crystal-chemical structure of the resulting hydroxyapatite also depends on the composition of the physiological medium, because during the growth the crystals capture impurities of cations from the medium and form nonstoichiometric solid solutions. Therefore, it is worth talking about the appearance of apatite-like structures on the surface of calcium phosphate biomaterials, rather than pure hydroxyapatite. Obviously, these structures are close to bone ones, since the chemical composition of bone hydroxyapatite somewhat differs from the stoichiometric composition of natural and artificial analogues in the Ca:P ratio, as well as in the presence of ion impurities, the total content of which may exceed 5%.

The use of bioactive resorption glass-ceramic materials [8], which are close to bone tissue in chemical and phase composition, as bone tissue substitutes can significantly reduce the time of their fusion with bone tissue. However, the high level of resorption of such materials can lead to the formation of an inhomogeneous, fragile bonding layer between the implant and the bone, especially under stress. Therefore, it is a detailed study of the mineralization process of bioactive glass-ceramic materials during the entire *in vivo* period that will make it possible to assess the effectiveness of the use of implants with different resorption periods.

2. EXPERIMENTAL

2.1. AIM Setting and Research Methodology

The aim of this work is to determine the features of the mineralization of hydroxyapatite on the surface of calcium silicophosphate glass-ceramic materials *in vivo*.

The phase composition of the materials was studied using x-ray phase (DRON-3M diffractometer) and petrographic (NU-2E optical microscope) methods of analysis.

The structure of the surface layer of glass-ceramic materials (GCM), which was removed after implantation, was investigated using x-ray spectral analysis (scanning electron microscope RES Tesla 3 LMU with a resolution of 1 nm using an Oxford X-max 80 mm energy dispersive spectrometer).

Evaluation of the hydrolytic destruction of the developed materials was carried out in non-enzymatic media using the extreme (*ES*) and simulated solutions (*SS*) tests according to ISO 10993-14-2011 by weight loss in the corresponding B_{ES} and B_{SS} solutions.

The surface free energy (SFE) value of the experimental materials was determined by the Owens–Wendt–Rabel–Kaelble method,

according to which the surface energy of a solid includes two components: dispersive and polar ones. This method provides for the calculation of the SFE based on the contact angle between the material surface and various liquids, followed by the calculation of its two surface free energy components using the Mathcad computer software. To improve the accuracy of the obtained values, six liquids with known polar and non-polar components of surface tension were used. Surface microrelief was determined as the arithmetic mean of the deviation of the surface profile (R_a) using a Surtronic 3+ profilometer.

Vickers hardness (*HV*) and fracture toughness index (fracture viscosity, K_{1C}) were determined by indenting the Vickers pyramid under the load of 5000 g on it for 10 measurements using a TMV-1000 device. Compressive strength (σ_{compr} [MPa]) was determined according to GOST 8462-85.

Histological analysis of bone tissue regeneration was performed after the GCM-based implant was inserted into the distal metaphysis of the femur of rats on the first, 7^{th}, 14^{th} and 28^{th} days after implantation. After each term, the activity of alkaline phosphatase was determined by the kinetic method in the serum of rats. The studies were carried out on the base of the laboratory of connective tissue morphology of the Sytenko Institute of Spine and Joint Pathology N.A.M.S. of Ukraine.

2.2. Peculiarities of Compositions and Technology of Bioactive Glass-Ceramic Materials

To study the peculiarities of the formation of an apatite-like layer on the surface of bioactive glass-ceramic materials (GCM) *in vivo*, materials differing in structure and resorption capacity were chosen BS-11 [9] and ASZ-5 [10].

To obtain BS-11 and ASZ-5 GCM, the glasses based on calcium silicophosphate systems with SiO_2 content in the range of 47–55 wt.%, the degree of connectivity of the silicon-oxygen framework (f_{Si}) 0.28 and the CaO/P_2O_5 ratio 1.7–4.0 were synthesized (Table 1). This is to ensure their biological activity by finely dispersed crystallization of HAp and initiation of its nucleation on the surface of materials *in vivo*. The glasses contain Al_2O_3 and B_2O_3 to provide structural strength, which determines their ability to resorption and mechanical loads.

ZnO, ZrO_2, TiO_2, CaF_2 and CeO_2 as nucleation catalysts were added into the AS-5 glass to control the apatite formation processes. To approximate the glass composition to the composition of natural bone tissue in the process of mineralization, the trace elements (Cu_2O, V_2O_5, MoO_3, CoO, SrO, La_2O_3) of 0.1 wt.% were introduced.

TABLE 1. Generalized chemical composition of glasses (in [wt.%]) and the ratio of phase-forming components.

Marking of Glass	Glass-Forming Components		Phase-Forming Components		Modifiers			Doping Constituents
	SiO_2	$Al_2O_3 + B_2O_3$	$CaO + CaF_2 + P_2O_5$	CaO/P_2O_5	R_2O	RO	RO_2	
BS-11	55.0	10.0	25.0	4.0	10.0	—	—	—
AS-5	47.0	6.0	29.4	1.7	10.5	2.6	4.4	0.1

AS-5 and BS-11 experimental glasses were obtained by glass technology: melted under identical conditions at temperatures of 1523 and 1723 K in corundum crucibles, followed by the cooling on a metal plate. To obtain GCM by ceramic technology, there were used powders of model glasses milled to a residue on sieve No. 063 of no more than 5%. The samples were prepared by the method of semi-dry pressing, formed in the form of cylinders with a diameter of 4 mm and a height of 10 mm using a 2% solution of carboxymethyl cellulose as a temporary binder.

To increase the fracture toughness of the GCM, zirconia stabilized with yttrium oxide was additionally introduced into the AS-5 glass composition. Such a sample of GCM was marked with ASZ-5 and was used for further research. The transformation strengthening of the glass matrix was carried out due to the controlled transition of tetragonal ZrO_2 to the monoclinic phase, which is accompanied by volume increase of 3 vol.% [11, 12].

Heat treatment of materials was carried out at 1023 K during 30 min for ASZ-5 and at 1323 K during 30 min for BS-11. After heat treatment, the GCMs were characterized by insignificant porosity (up to 10%), which is a consequence of sintering of narrow-fraction glass powders with a particle size of ≤ 60 μm.

2.3. Characterization of Bioactive Glass-Ceramic Materials

Glass-ceramic materials are characterized by a fine-dispersed volume-crystallized structure with a HAp content of 55–60 vol.% (Table 2). Providing the sitallization process of the initial glasses under the conditions of low-temperature short-time heat treatment makes it possible to form a high-strength structure of GCM, which is capable of withstanding variable dynamic loads. Along with this, in the glass structure, in addition to the presence of calcium phosphates, sybotaxic groups of future apatite-like crystalline phases

TABLE 2. Characteristics of the GCM composition, structure and destruction.

Indicators		Marking of Samples	
		BS-11	ASZ-5
The crystalline phase content (HAp), vol.%		55.0	60.0
The degree of the silicon–oxygen framework bonding (f_{Si})		0.28	0.28
Roughness (R_a), μm		6.0	2.0
Surface free energy, mJ/m^2		51.50	74.59
Material destruction, wt.% (ISO 10993-14-2001)	B_{ES}	0.20	0.44
	B_{SS}	2.00	2.96

are formed, which are potential nucleators during the formation of a mineralized layer *in vivo* on the implant surface.

Stimulation of the protein adsorption process on the surface of the GCM is realized by ensuring the values of the structural strength index $f_{Si} = 0.28$ and the surface microroughness indicators (Table 2). This will provide an increase in the SFE index *in vivo* by increasing the proportion of the electrostatic component of the chemical bond in the glass.

The ASZ-5 GCM is characterized by the highest values of weight loss, despite its location in a low-silica area. With a simultaneously higher HAp content, this material, in comparison with BS-11, is characterized by a higher reactivity and ability to accelerate the formation of an apatite-like layer *in vivo*. These assumptions are the basis for studies on the features of nucleation and growth of non-stoichiometric hydroxyapatite (*n*HAp) on the surface of calcium silicophosphate glass-ceramic materials *in vivo*.

3. RESULTS AND DISCUSSION

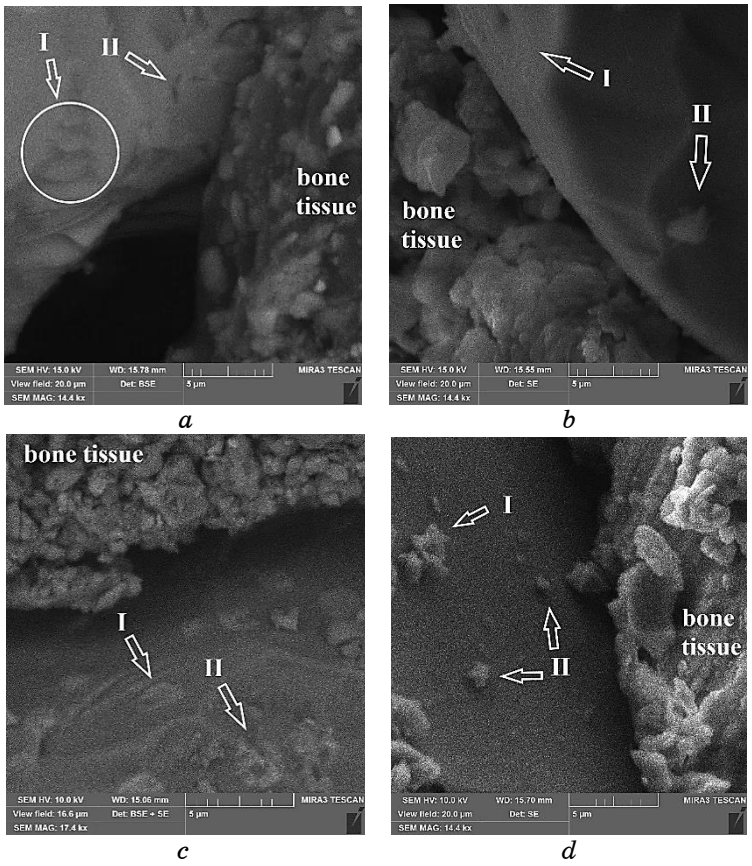
3.1. Hydration and Nucleation of Apatite on the Surface of Bioactive GCM *in vivo*

In the first hours of *in vivo* contact, hydrolysis and condensation processes occur on the GCM surface. The materials are hydrolysed with the formation of a gel-like silica-rich layer saturated with OH^- groups. The structural elements ≡Si–OH provide areas for heterogeneous nucleation of apatite crystals.

The study of the surface morphology of the implant material and bone tissue showed that, for the BS-11 GCM on the 1st day of exposure, the hydrolysis process with the formation of ≡Si–OH bonds and their subsequent polycondensation ≡Si–OH + HO–Si≡ → ≡Si–O–

Si≡ + H_2O are observed. The result is the formation of a thin gel-like layer of silicic acid in the form of nanoinhomogeneities over the entire surface of the material under study and individualized spheres of amorphous calcium phosphate (ACP) (Fig. 1, *a* I, II) as a precursor of *n*HAp crystals. For the ASZ-5 GCM, against the general background of spheres of the silica-gel layer (Fig. 1, *b* I), single crystals, which have a plate-like shape, are observed (Fig. 1, *b* II). A manifestation of the intensification of the nucleation process of *n*HAp crystals is a change in the parameters of the crystal lattice of apatite-like structures and the morphology of crystals, which change during the growth of crystals.

At the initial stages of growth (7–14 days), both materials under study are characterized by the formation of solid solutions with a structure that is very different from the structure of *n*HAp. How-



a *b* *c* *d*

Fig. 1. Surface structure of bioactive GCM: BS-11 (*a*); ASZ-5 (*b*) after 1 day of exposure; BS-11 (*c*); ASZ-5 (*d*) after 7 days of exposure.

ever, the growth of crystals for the experimental GCM, as well as the mechanism of nucleation, is significantly different.

Thus, on the 7^{th} day, BS-11 GCM is still characterized by the presence of a silica-gel layer and an accumulation of nanoinhomogeneities, which form flat (Fig. 1, *c* I) and convex spheres (Fig. 1, *c* II). A decrease in the size of inhomogeneities and an increase in their number indicate the formation of a significant number of crystallization nuclei. For ASZ-5 GCM, during the mentioned period, the simultaneous presence of a silica-gel layer and platy crystals (Fig. 1, *d* I) is observed; they form aggregates of different sizes (Fig. 1, *d* II).

3.2 Growth of Crystals on the Surface of Bioactive GCM *in vivo*

After 14 days of implantation, the BS-11 GCM is a multiphase system, which consists of individual spherical and platy crystals ranging in size from 0.5 to 5 μm (Fig. 2, *a* I) and their clusters of about 10 μm, which form a single crystalline block. The presence of a significant amount of spherulites is evidence of the formation of amorphous calcium phosphate (Fig. 2, *a* II), which is a precursor of the formation of native bone.

For the ASZ-5 GCM, inhomogeneities are represented by spherulites, which form ridges and spalls (Fig. 2, *b* I). This process is accompanied by a phase rearrangement of ACP, followed by levelling of the surface and the formation of a layer-by-layer structure of the material with the presence of platy crystals of *n*HAp (Fig. 2, *b* II).

After 28 days of implantation, the formation of *n*HAp aggregates is observed in the structure of the BS-11 and ASZ-5 GCM implants (Fig. 2, *c*, *d* I), which are similar to crystals for mature lamellar bone (Fig. 2, *c*, *d* II). At the implant–bone interface, it is observed the formation of a transition layer containing, like the implant material, crystals of the platy structure of carbonate hydroxyapatite (CHAp). This is due to the incorporation of carbonate ions into the apatite lattice, which affects the mineralization process.

The intensification of nucleation for the ASZ-5 GCM makes it possible to form CHAp on their surface even on the 28th day *in vivo*. They are crystals from prismatic to acicular of a hexagonal syngony, assembled into aggregates (Fig. 2, *d* II). Crystals of CHAp are present in the form of plates ranging in size from 50×20 nm to 25×(2–5) nm, which are oriented in a certain way with respect to the axis of collagen fibres.

3.3. Mineralization of Bioactive GCM *in vivo*

For experimental GCM, due to an increase in the calcium concentra-

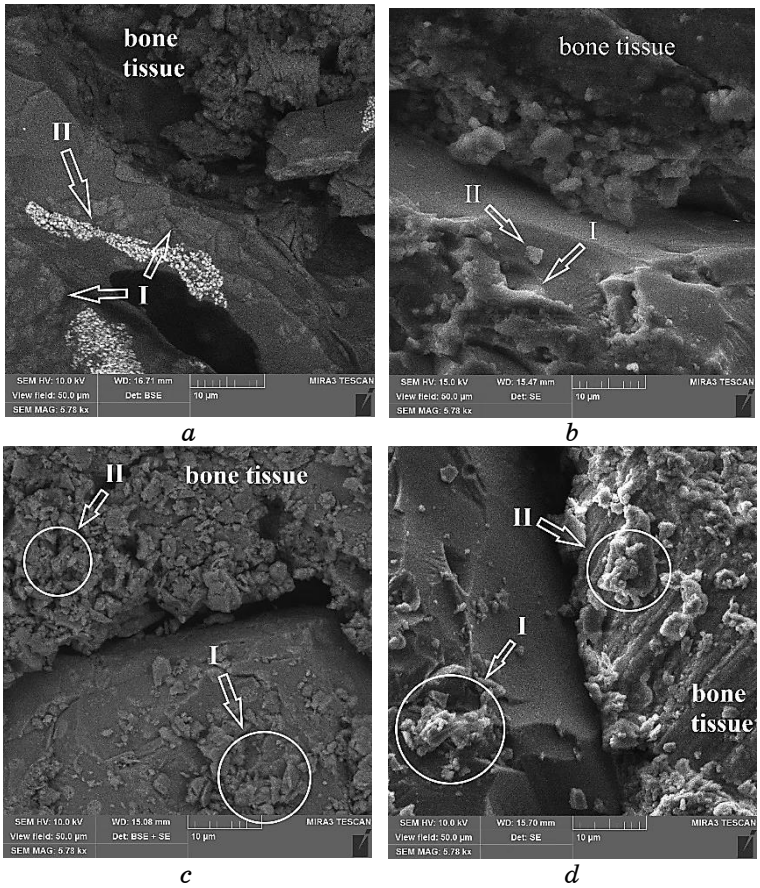


Fig. 2. Surface structure of bioactive GCM: BS-11 (*a*); ASZ-5 (*b*) after 14 days of exposure; BS-11 (*c*); ASZ-5 (*d*) after 28 days of exposure.

tion and accompanying factors in the environment, the process of appearance of CHAp crystals around the implant (osteoinduction) is 'triggered'.

The confirmation of the fact that, after the formation of the bioactive GCM–collagen bond, osteoblasts mineralize the osteoid area formed by the synthesis and secretion of matrix bubbles, is the appearance of an alkaline phosphate phase in high concentrations. The microenvironment inside the matrix bubbles promotes the formation of *n*HAp crystals.

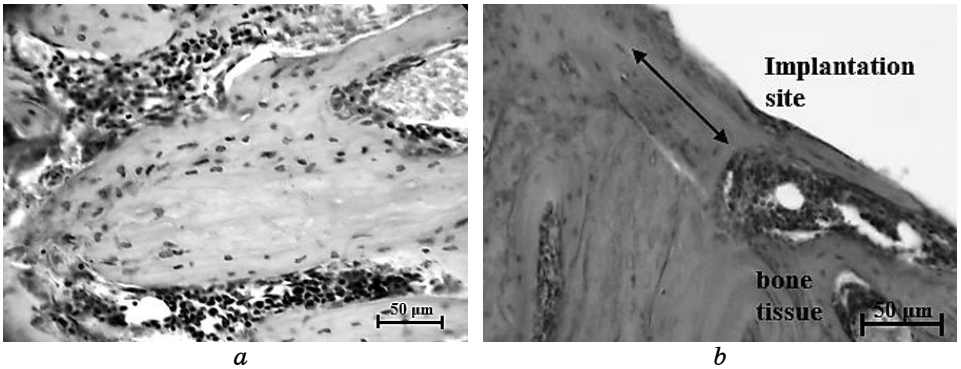
Thus, according to the results of biochemical analysis of the blood serum of rats after implantation of the BS-11 and ASZ-5 GCM samples, the dynamics of alkaline phosphatase activity was found in accordance with the stages of bone tissue regeneration of the implants:

on the 7th day, 411.80 ± 27.60 U/L; on the 14th day, 952.50 ± 63.30 U/L; on the 30th day, 828.00 ± 98.60 U/L. It was established that, on the 7th and 14th days, the activity of alkaline phosphatase increases that indicates its release by osteoblasts during bone formation. On the 30th day, a slowdown in this process shows the gradual completion of the process of bone tissue remodelling.

Mineralization of new bone tissue, as well as its growth (osteoconduction), on the surface of GCM-based implants is possible, when a significant surface area of contact between the biological fluids and the implant is provided, that is, with a sufficient porosity (≥ 30%) of the latter.

Even on the 30th day of implantation, microscopically, the area of removal of the pin on histopreparations of animals with an implant based on the BS-11 GCM was represented by a cavity, on the perimeter of which mainly newly formed trabeculae from coarse fibrous and lamellar bone tissue, areas of fibroreticulate tissue of an osteogenic nature and centres of dense connective tissue were determined (Fig. 3, *a*). In the bone tissue that forms the cavity paries for a given period, even in the cortical part, there is lamellar bone tissue, which has a significant density of osteocytes in narrow lacunae that surround the intercellular matrix. The boundary between the regenerate and the maternal bone is clearly defined.

30 days after the operation, the formation of lamellar bone tissue, the trabeculae of which were directed along the surface of the injected material, was noted around the site of implantation of the ASZ-5 GCM. It was on this basis the newly formed bone could be distinguished from the maternal one. The reorganization of the bone regenerate is evidenced by the presence of clastic structures of osteons, irregular cement lines (Fig. 3, *b*).



a *b*

Fig. 3. Fragment of the distal metaphysis of the rat femur after injection of BS-11 (*a*) and ASZ-5 (*b*) samples.

With the use of the developed materials as implants, regeneration has a more favourable course, as evidenced by the restoration of the bone structure on the 30th day of observation, when a part of the regenerate (50% for BS-11 GCM and 70% for ASZ-5 GCM) in the defect is represented by a mature lamellar bone.

Thus, taking into account the mechanical properties of BS-11 and ASZ-5 GCM, which are close to those properties for bone cortical tissue ($K_{1C} = 2.44$ and 2.8 MPa·m$^{1/2}$, $HV = 7800$ and 3800 MPa, $\sigma_{compr} = 160$ MPa), implants based on them can be used to replace the statically and dynamically loaded areas of bone tissue in orthopaedics and maxillofacial surgery.

The introduction of implants based on bioactive glass-ceramic materials BS-11 and AC3-5 with reduced terms of resorption and mineralization of bone tissue will increase the efficiency of prosthetics by halving the rehabilitation period for patients and eliminating repeated operations.

4. CONCLUSIONS

The regularities of mineralization of calcium-containing compounds during the formation of bone tissue on the surface of bioactive glass-ceramic materials with different resorption periods have been established. It has been determined that the implantation of biomaterials based on bioactive glass-ceramic materials characterized by a strengthened structure and an adjustable level of resorption implements the chemical and biochemical mechanisms of the formation of an apatite-like layer.

The features of the compositions and technologies for obtaining bioactive glass-ceramic materials for replacement of bone defects were analysed. It has been established that the provision of biological activity along with high strength of glass-ceramic materials can be realized by their sitallization and the formation of HAp crystals under conditions of low-temperature one-stage short-time heat treatment of glasses of calcium silicophosphate systems characterized by a SiO_2 content of 45–55 wt.%, $f_{Si} = 0.28$ and the ratio $CaO/P_2O_5 = 1.7–4.0$.

The conditions for the formation of an apatite-like layer *in vivo* on the surface of the GCM were analysed. These ones include crystallization process of finely dispersed HAp, ensuring the reactivity of the GCM due to their destruction, and initiation of the nucleation of nHAp on the surface of materials.

It was determined that the stimulation of the adsorption process of protein on the surface of the GCM is realized by providing the values of the surface microroughness index for ASZ-5 and BS-11 GCM $R_a = 2.6$ and 6 μm and SFE 51.5 and 74.6 MJ/m^2, respectively.

The features of hydration and nucleation of HAp on the surface of bioactive GCM *in vivo* were established, which consist in the hydrolysis process with the formation of ≡Si–OH bonds, their polycondensation and the formation of a thin gel-like layer of silicic acid in the form of nanoinhomogeneities and individualized spheres and ACP plates (1 day) and aggregates (7 days), forming ridges with the subsequent layer-by-layer structure of the material with the presence of lamellar crystals of *n*HAp (14 days), which are recrystallized into crystals of CHAp, oriented with respect to the axis of collagen fibres (28 days), followed by the formation of the bioactive GCM–collagen bond.

The activity of osteoblasts, which mineralize the osteoid area around the implant based on bioactive GCM, is determined by the activity of alkaline phosphatase. It is confirmed the formation of mature lamellar bone tissue, when using the developed bioactive glass-ceramic materials and the expediency of their use as biocompatible materials for bone tissue regeneration within one month.

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