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Study of Physicochemical and Biological Properties of Calcium Phosphate Coatings Prepared by RF Magnetron Sputtering of Silicon-Substituted Hydroxyapatite

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Abstract—Coatings based on pure silicon and silicon-substituted hydroxyapatite were grown by RF magnetron sputtering. The coating surface morphology, phase and elemental composition were studied by scanning electron microscopy, energy-dispersive X-ray analysis, and infrared spectroscopy. It was found that coatings are X-ray-amorphous, their elemental composition being controlled by the sputtered target composition. The distribution of elements over the coating surface is homogeneous. Medical and biological properties of coatings were studied in vivo and in vitro. Osteogenic properties of coatings were studied. Coatings grown by sputtering of a stoichiometric hydroxyapatite target are biocompatible without osteoinductive activity. The introduction of silicate ions into the hydroxyapatite structure that forms an electrode target significantly enhances the in vivo effect of CaP magnetron coatings on the osteogenic activity and stromal bone-marrow stem cells.

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INTRODUCTION

The "regeneration" approach, i.e., the development and application of materials interacting with organism and stimulating neogenesis, is a modern trend in medical materials science. The necessary compatibility of the medical implant surface with biological tissues is achieved by using combined-structure implants with a rigid metal base and a biocompatible material coating, which can be a specially formed layer of calcium phosphates (CaP).

Various calcium phosphate material samples, depending on their physicochemical properties (crystallinity, porosity, solubility, surface roughness, elemental and phase composition, etc.), have different osteogenic activities [1]. Physicochemical and biomedical properties of implants are interrelated and strongly depend on the method for forming the surface artificial layer [2]. Nevertheless, it has not yet been possible to find a key combination of the structure, thickness, and dissolution rate of various coatings to materialize the osteogenic potential of stromal stem cells and to successfully integrate implants with bone. Currently, an optimum material for clinical practice is calcium phosphate ceramics based on hydroxyapatite (HA) $Ca_{10}(PO_4)_6(OH)_2$ (decacalcium hexaphosphate dihydroxide) and tricalcium phosphate $Ca_3(PO_4)_2$, the structures of which are close to the structure of the mineral component of bone tissue. Dense biocompatible hydroxyapatite ceramics is a low-active material; in the case of its use, the processes of implant resorption and growth kinetics of contact bone tissue are very slow [3–5]. The HA biological activity can be increased in two ways: by decreasing the crystallite sizes (to increase the specific surface area) and by changing physicochemical characteristics of the surface, i.e., to chemically modify it. In the latter approach, it becomes possible to develop materials that actively resorb when in contact with organism liquids.

Silicon-containing hydroxyapatite $Ca_{10}(PO_4)_{6-x}(SiO_4)_x(HO)_{2-x}$ (Si–HA) is considered to be a promising material, since silicon oxide anions (SiO_4^{4-}) are a natural component of intertissue liquid [5]. As a promising biomaterial, Si–HA has been studied relatively recently, mostly in the ex vivo system. It is indicated that the presence of silicon in glass ceramics and HA enhances their integration with bone tissue [6] and dissolution in vitro and in vivo [7–8]. For example, E.S. Thian et al. [9] described an in vitro positive response of osteoblast-like cells to Si–CaP coatings grown by magnetron deposition.

The basic technological methods for forming biocompatible coatings are plasma spraying [10–11], laser ablation [12], methods based on coating crystallization from various solutions [13–14], and RF magnetron sputtering [15–17]. The initial material is changed during deposition in some methods, and a coating represents a new, often multiphase, system. It is important that the used method be able to conserve

the chemical composition of an initial material when depositing the coating on the implant. The RF magnetron sputtering method fulfills this requirement; moreover, it allows variation of the coating elemental and phase composition by varying the initial composition of the target for sputtering and by varying the sputtering parameters (discharge power, working gas, and others) [18]. Another undeniable advantage of this method is a high adhesion strength of coatings. In [9, 19] Si–CaP coatings were grown using two separate targets for sputtering, prepared of silicon and unsubstituted HA.

The objects of this study were coatings grown by RF magnetron sputtering of targets prepared of pure HA and Si–HA. Biological compatibility of materials is caused by a certain level of their biological and physicochemical properties, among which are the toxicity, tumorogenesis stimulation, effect on blood, chemical composition, and geometrical property (relief). Therefore, the objectives of this research are to study the physicochemical properties of coatings grown by RF magnetron sputtering and the effect of the artificial surface relief on the response of cell culture and to study the osteogenic potential of coatings based on stoichiometric and silicon-substituted HA in a comparative respect.

EXPERIMENTAL

Coatings were deposited using a commercial 08PKhO-100T-005 system with a magnetron source (5.28 MHz). Coatings were grown at an operating pressure of 0.1 Pa (the limit pressure in the vacuum chamber is 10^{-4} Pa), a target–substrate distance of 40 mm, argon as a working gas, and an RF generator power of 250 W. The target was prepared of silicon-substituted HA powder (synthesized by the mechanochemical method) with a silicon content of 4.9 wt %, which corresponds to the formula $Ca_{10}(PO_4)_{4.28}(SiO_4)_{1.72}(OH)_{0.28.}$ This approach makes it possible to obtain composition-uniform coatings [20]. Previously, we showed [20] that, in contrast to the initial powder, the target material is two-phase bioceramics based on silicon-stabilized crystalline HA (the Si–HA phase composition do not differ from the phase composition of HA [7, 21]) and tricalcium phosphate (TCP). For comparative studies, a target of pure HA synthesized by the mechanochemical method was prepared. The target for RF magnetron sputtering (220 mm diameter, 10 mm thick) was prepared by ceramic technology; i.e., the powder was pressed at a pressure of 70 MPa, and then the pellet obtained was annealed at 1100°C for 1 h in air. As substrates for sputtering, KBr (for studying molecular bonds in coatings by IR spectroscopy) and technically pure titanium VT1-0 were used.

The morphology and elemental composition of coatings were studied using a Quanta 200 ESEM FEG (FEI Co.) scanning electron microscope (SEM) with a built in attachment for energy-dispersive X-ray analysis (EDX). The phase composition of formed Si–CaP and CaP coatings without silicon was determined by X-ray diffraction using a Shimadzu XRD-7000 diffractometer. Diffraction patterns were interpreted using the database of the International Center for Diffraction Data (ICDD): the card numbers for synthetic HA and titanium are 9-432 and 44-1294, respectively. The chemical bonds of phosphate and substitutional groups were determined by infrared (IR) spectroscopy. Optical absorption spectra were measured on a Bruker Vertex 70 device in the range of 400–4000 cm^{-1}.

Technique of Medical and Biologic Studies

To study the cell response to direct contact with the surface, a culture of fibroblast-like stem cells of a light man (Open Company Bank of Stem Cells, Tomsk) was used. Preparations represent a cell population with a limited lifetime, which retains an ex vivo stable karyotype during passage and is oncogenically safe. Cells are free of extraneous viruses (HIV, hepatitis, herpes, etc.) and bacterial agents (syphilis, mycoplasmas, clamydia, etc.). After defrosting, the cell viability determined according to ISO 10993-5 in the test with 0.4% trypan blue was 93%.

The objects under study were placed into wells of 24-well Costar plates, which were then filled with a cell suspension with a viable karyocyte (nucleus containing cell) concentration of 5×10^4 in 1 ml of an osteogenic medium with the composition: 20% of embryonic veal whey inactivated at 56°C, 50 μg/ml of ascorbic acid, 10 mM of beta-glycerophosphate, 10^{-6} M of dexamethasone, 50 mg/l of gentamycin, 280 mg/l of L-glutamine, 10 mm of HEPES buffer, and DMEM to 100 ml. The growth control was a fibroblast-like cell culture on plastic.

In four days implants were removed and dried in air. The samples with cells adhered to the surface were prepared for SEM according to [22]. Cells that had adhered to the coating were fixed in a 2.5% solution of glutaric aldehyde on a phosphate buffer for 30 min and then in a 1% solution of osmium tetroxide for 30–40 min, followed by double washing with a phosphate buffer (pH 7.2–7.4). Then cells were dehydrated in a series of aqueous solutions of ethanol with an ascending concentration of 30, 50, 70, 90, 100% for 15 min in each and twice in 100% acetone. The prepared samples were studied on a Phillips SEM 515 scanning electron microscope (SEM) at an inclination angle of 35°.

For a comparative analysis in vivo and in vitro, titanium samples 0.8 cm^2 in area with double-sided magnetron coating based on HA and Si–HA were prepared. According to ISO 10993-5, sterile extracts of objects with coatings were prepared under conditions of 7-day dissolution at 37°C in 4 ml of an isotonic solution of sodium chloride. To reproduce osteosynthesis conditions under clinical conditions (acidosis, the prescription of antibiotics), 30 mg/l of gentamycin antibiotic were added to the solution. In extracts pH

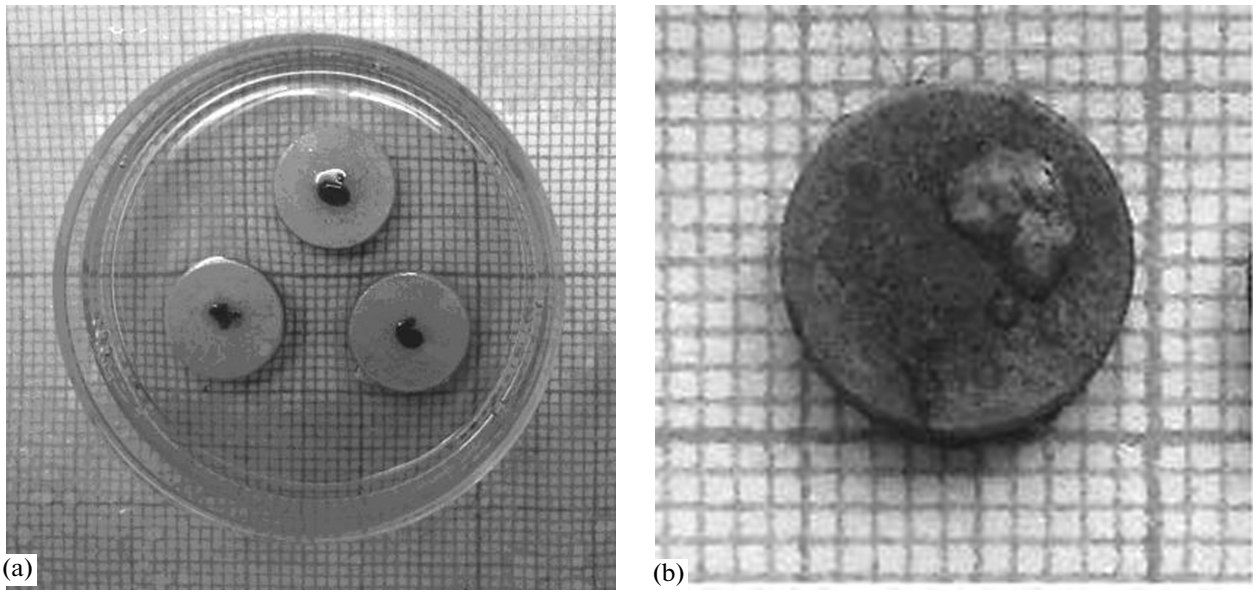

Fig. 1. (a) Organ culture of bone marrow of mice before hypodermic implantation and (b) the tissue plate grown on the magnetron CaP coating on mouse bone marrow after hypodermic implantation.

and the calcium ion concentration were also determined using kits for biochemical analyzers.

The cytotoxic test was performed using a cell material of BALB/c-line mice. Bone marrow cells were defrosted according to the standard procedure and brought to a concentration of 10^7 karyocyte/ml using DMEM. Samples to be tested were placed into the cell suspension and were cultivated in 2 ml of a cultural media for 3.5 h at 37°C. The control was a cellular suspension without samples. Cells were uniformly distributed over the medium volume by soft pipetting; the sample cytotoxicity was determined in the staining assay by 0.4% of trypan blue according to ISO 10993-5.

The osteogenic properties of HA and TCP that form the basis of the bone mineral matrix were convincingly proved by the phenomenon of ectopic osteogenesis, when the bone tissue is formed on the CaP surface of materials when HA and TCP are injected under the skin or into muscles. An adequate experimental approach to determining the possible osteogenic properties of CaP materials is a version of the phenomenon of eñtopic osteogenesis, in which an artificial sample is implanted under the skin or intramuscularly without using growth factors [23–24]. For example, in the case of porous CaP implantation, Yang et al. [25] observed ectopic osteogenesis of dogs and pigs, but not goats, rats, mice, or rabbits. Similar data on dogs were obtained by other authors [24, 26]. On the contrary, some researchers determined the parameters of osteoinduction (bone growth initiation) during intramuscular or hypodermic CaP implantation to rabbits and mice [23, 27]. We obtained reproducible results on hypodermic bone growth for mice from a syngenic bone marrow column preliminarily applied on CaP rough surfaces [2].

In the present experiments, 16 male mice of the BALB/c line from the collection fund of the Laboratory of Experimental Biomodeling of the Research Institute of Pharmacology, Siberian Branch, Russian Academy of Medical Science (Tomsk), were used. One implant with a syngenic (of the same animal breed) femur bone marrow column applied under aseptic conditions was hypodermically implanted to each animal under ether narcosis (the average bone marrow area was 7.5 mm^2). To provide cell adhesion, the bone marrow organ culture on the substrate (Fig. 1a) was cultivated at 37°C for 45 min in a cultural medium containing 95% of DMEM and 5% of embryonic veal whey. The bone marrow was a source of stromal stem cells and growth factors. In the case of separate hypodermic implantation of substrates or bone marrow fragments, the formation of tissue plates was not observed.

In 45 days implants were removed and tissue plates were cut (Fig. 1b) from disk surfaces; fixed in formalin, decalcified, and poured with paraffin; and thin (10 μm) slices perpendicular to the disk surfaces were made and stained with hematoxiline–eosine for histologic studies. Bone marrow and/or bone growth on the implant surface was considered a positive result; development of connective, muscular, or fatty tissue was a negative result.

RESULTS AND DISCUSSION

The typical morphology of coatings deposited by RF magnetron sputtering on the titanium substrate is

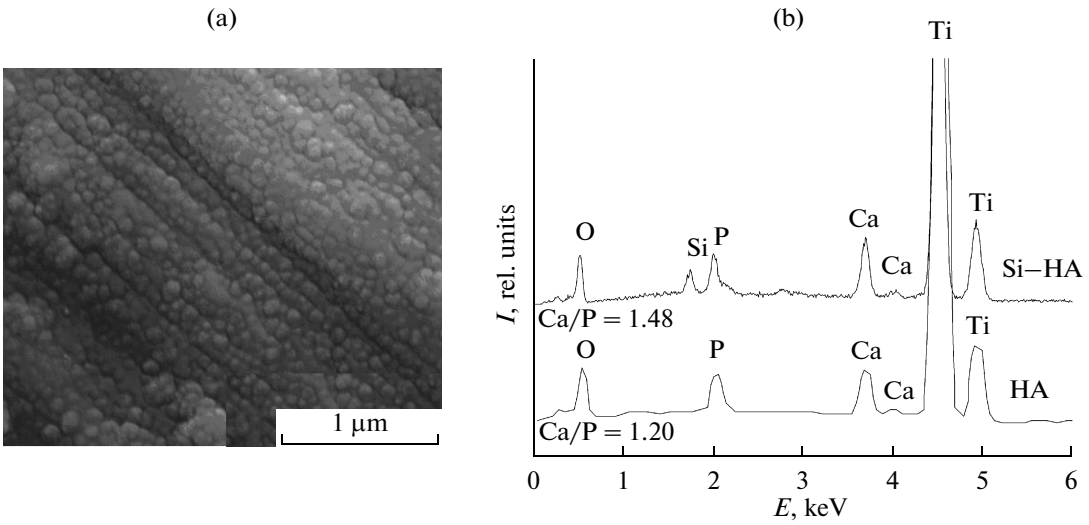

Fig. 2. (a) SEM image of the surface and (b) EDX spectra of coatings grown by RF magnetron sputtering.

shown in Fig. 2. It was found that the surface of coatings formed by this method is homogeneous, dense, and does not contain visible defects, cracks, and chips. Islands shaped as "dome" grains are major surface relief elements (Fig. 2a). The structure of coatings and dynamics of their growth are discussed in [28]. According to the EDX spectra shown in Fig. 2a, the coatings consist of calcium, phosphorus, and oxygen. The coatings grown by sputtering of a silicon-containing hydroxyapatite target contain silicon. The ratios Ca/P (Fig. 2b) for the formed coatings (Ca/P = 1.20–1.48) are lower than those of stoichiometric HA (Ca/P = 1.67) and Si–HA (2.34). As is known, the lower the ratio n(Ca)/n(P), the more soluble calcium phosphate [6]. In the case of Si–HA, it would be more correct to consider the ratio Ca/(Si+P), since the silicate ion SiO_4^{4-} partially substitutes the phosphate ion in the HA structure. For the coating grown by sputtering of a Si–HA target, the ratio Ca/(Si+P) is 0.96. Hence, silicon-containing coatings tend to a higher dissolution rate than CaP coatings not containing silicon.

Figure 3 shows the maps of element distributions in Si–CaP coatings deposited by RF magnetron sputtering on the titanium substrate. We can see uniform distributions of elements in the coating. The Si–CaP coatings grown by RF sputtering of the target prepared by simple mixing of silicon and HA [10] are characterized by nonuniform distributions of elements.

In this study uniform distribution of elements throughout the coating is achieved by using a target prepared from a material in which the silicate ion is incorporated into the HA lattice, substituting the phosphate ion. The distribution of elements in the coating grown by sputtering of stoichiometric HA is also uniform. The results of X-ray diffraction analysis of grown coatings are shown in Fig. 4a. An analysis of X-ray patterns showed the absence of crystalline phases corresponding to calcium phosphates. Only diffraction peaks of Ti substrate material were detected. Thus, the coatings grown by RF magnetron sputtering in the mode used in this study are characterized by an amorphous structure, which is in agreement with the data of [29].

The IR spectra (Fig. 4b) of the coatings grown by RF magnetron sputtering contain absorption bands caused by vibrations of P–O bonds of the phosphate tetrahedron in the apatite structure in the regions of bending and stretching vibrations at frequencies of 473 and 560 and 950 and 1024 cm^{-1}, respectively. Furthermore, there are also absorption bands characteristic of C–O bond vibrations. The band at 510 cm^{-1} in the absorption spectrum of coatings with substitution x = 1.72 (indicated by the arrow in Fig. 4b) corresponds to bending vibrations of Si–O bonds; stretching vibrations of these bonds can overlap the strong absorption band of the phosphate group in the frequency range of 900–1200 cm^{-1}.

Study of the direct interaction of fibroblast-like cells with magnetron CaP coatings showed that their shape depends on the artificial surface relief. Fibroblast-like cells arranged in coating pits had a round or oval shape typical of a liquid cell suspension. In regions without pits, cells are prolate (Fig. 5b). The formation of pseudopodia indicated their active growth in the region of contacts with artificial CaP coatings (Fig. 5).

Structural changes of calcium phosphate crystals in biological liquids, including simulated body fluid (SBF) [27], occur by the dissolution–precipitation mechanism [17]. At the same time, the features of the coating dissolution phase controlling in many respects (according to our data) the coating behavior in bones can be studied using an isotonic solution of sodium chloride [31–33].

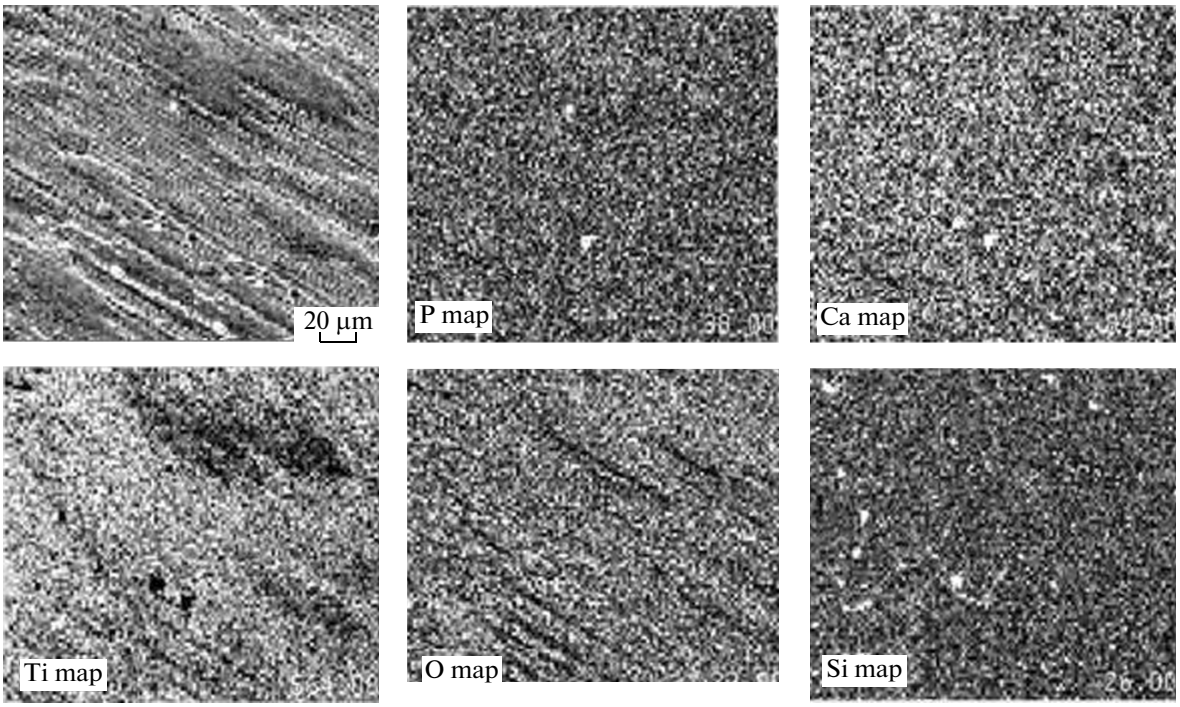

Fig. 3. Maps of element distributions in Si–CaP coatings grown by RF magnetron sputtering.

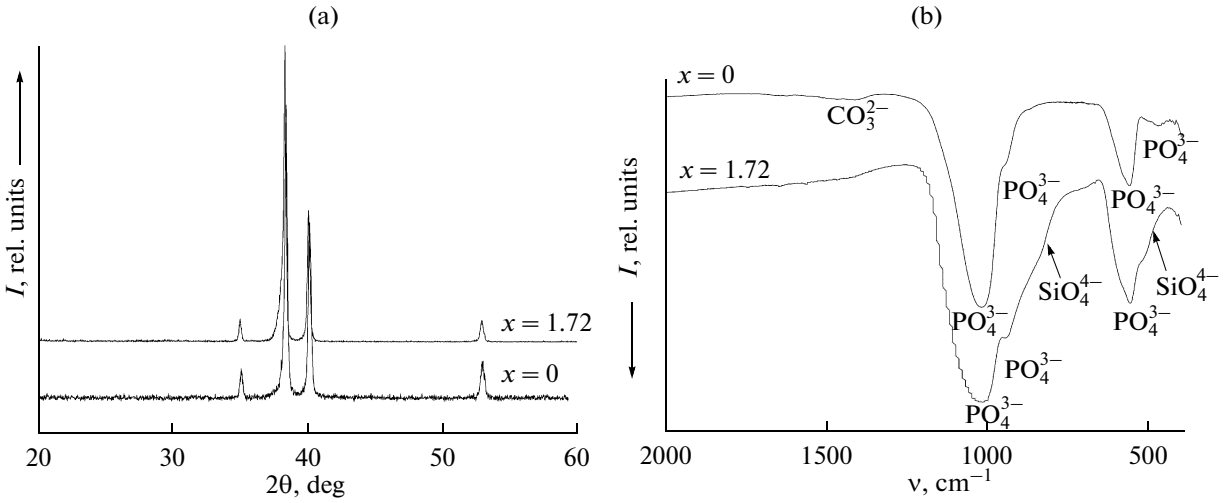

Fig. 4. (a) X-ray patterns and (b) IR absorption spectra of coatings grown by RF magnetron sputtering.

Chemical dissolution of magnetron CaP coatings grown by sputtering of an electrode target with various chemical compositions on titanium substrates showed an increase in pH of solutions by 9–25% in comparison with control substrates without deposition (Table 1). According to biochemical tests, this effect is caused by calcium ion diffusion from coatings into solution.

The presence of silicon in the magnetron CaP coating increased the calcium emergence from solid to liquid phase by a factor of 2.5 (Table 1). In this case, the molar ratio Ca/P in the solution was within 2.20–5.83, which is higher than that of coatings. Thus, according to the data obtained, the presence of silicon as a magnetron coating component significantly increases its biological activity, at least, due to an increase in the emergence of calcium ions to the solution, which play an important role in the regulation of the pool of stromal and hematogenic stem cells [34].

The results of the cultural study showed that magnetron CaP coatings deposited on metal substrates by sputtering of different targets had no statistically significant toxic effect on bone-marrow cells of mice in com-

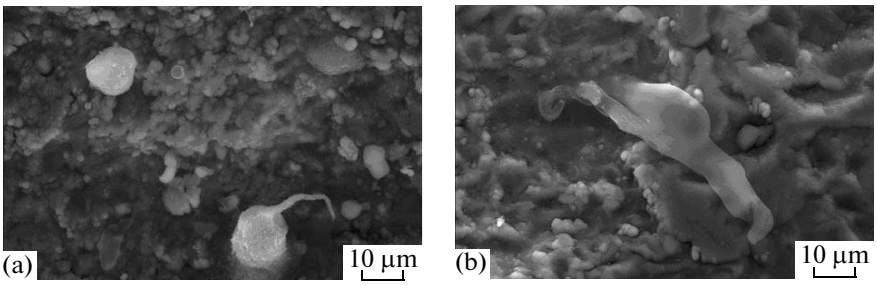

Fig. 5. SEM image of the fibroblast culture on CaP of the magnetron surface: (a) round fibroblasts and fibroblasts with pseudopodium and (b) prolate cell. Magnification is ×1250.

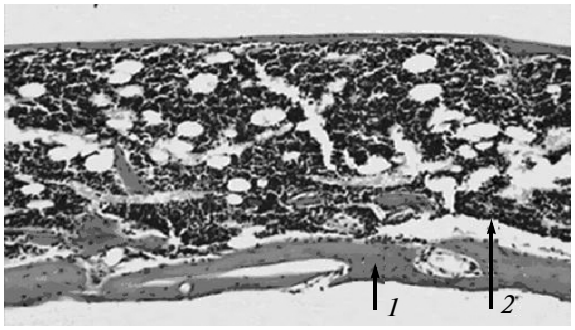

Fig. 6. Fragment of a histologic section of tissue plates grown on Si–CaP magnetron surfaces in the test of eñtopic osteogenesis of mice. The closed bone plate (*1*) with lacunas filled with red bone marrow (*2*) is determined. Stained by hematoxiline–eosine. Magnification is ×150.

parison with the medium toxicity control (Table 2). The absence of cytotoxicity of samples in vitro allowed the study of biocompatibility and specific activity of magnetron coatings in vivo in the hypodermic osteogenesis test.

The study of the tissue response to hypodermic implantation of the implants under study confirmed their high biocompatibility in vivo, since indications of inflammatory responses were not indicated in any observation groups in 45 days. Previously [2], we detected the direct correlation dependence ($r = 0.77$; $p < 0.05$) of the bone formation in the ectopic (hypodermic) osteogenesis test on the ratio Ca/P in CaP magnetron coating on the implant. In this case it was shown that, despite the fact that the ratio Ca/P is higher than the stoichiometric one, "smooth" magnetron coatings (with roughness class $R_a < 1$ μm) in principle have no osteoinducing ability. The probability of the formation of the tissue plate and bone growth from a bone marrow column on such coatings is zero despite their high biocompatibility.

Ectopic osteogenesis tests of magnetron CaP "smooth" coatings with a roughness class of $R_a < 1$ μm or better showed that the presence of silicon in magnetron coatings (Figs. 2a and 2b) increases the yield of tissue plates to 50%, the histologic composition of which corresponds to coarse-fibered bone tissue with cavities filled with red bone marrow (Fig. 6).

Thus, the presence of silicon in the composition of the CaP magnetron coating with surface roughness $R_a < 1$ μm transfers it from the class of biocompatible inert coatings to the class of bioactive osteoinductive materials.

Table 1. Results of 7-day dissolution of samples with magnetron CaP coatings grown by sputtering of different targets at 37°C in 0.9% sodium chloride with added gentamycin, $X \pm m$

Group no.	Samples, $n = 6$	Ca^{2+} emergence to the solution, % of control	pH of extracts, % of control
1	Magnetron deposition on Ti substrate, HA electrode	354 ± 68	109
2	Magnetron deposition on Ti substrate, HA (Si+HA) electrode	947 ± 74* <0.001	125

Note: Extracts of substrates without magnetron deposition were used as control.

Table 2. Average toxicity indicators of magnetron coatings in the case of cultivation in DMEM for 3.5 h, $X \pm SD(m)$, $n = 15$

Indicator	1	2	3
	Medium toxicity control	Magnetron coating, HA+Si target	Magnetron coating, HA target
Dead cells in the culture, %	9.45 ± 11.14 (4.98)	14.65 ± 9.28 (2.40)	8.88 ± 9.61 (2.48)

CONCLUSIONS

The coatings grown by RF magnetron sputtering are X-ray amorphous. The elemental composition of coatings (calcium, phosphorus, oxygen, and silicon in the case of Si–CaP) is controlled by the sputtered target composition. The coating is homogeneous, its surface morphology is controlled by the substrate surface relief, and the distribution of elements over the coating surface is uniform. The surface roughness class corresponded to $R_a < 1$ μm.

Magnetron coatings grown by sputtering of the stoichiometric hydroxyapatite target feature biocompatibility without osteoinducing activity. Silicate ion introduction into the hydroxyapatite structure forming the electrode target significantly increases the in vivo effect of CaP magnetron coatings on osteogenic activity of stromal bone marrow stem cells.

One of the mechanisms of the detected effect can be associated with an increased solubility of the Si–CaP layer formed on the titanium substrate. Magnetron deposition is a promising technology for biomedical applications.

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