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# Synthesis and Investigation of Physico-Chemical, Antibacterial, Biomimetic Properties of Silver and Zinc Containing Hydroxyapatite

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**Abstract.** In the work we carried out microwave synthesis of modified hydroxyapatites (HA) with different content of ions. A solid solution based on HA remains a single-phase sample when the calcium ions are substituted by silver and zinc ions up to 5 % by weight (0.5 mole fraction). The microstructure parameters, morphology and the particle powders size were studied by X-ray diffraction analysis, IR spectroscopy, and scanning electron microscopy (SEM). It is shown that the modification of HA by silver (AgHA) and zinc (ZnHA) ions increases the size of its particles, the degree of crystallinity, and the pore sizes of the samples while reducing their specific surface and uniformity of their forms. Elemental analysis and distribution of elements over the surface of HA, AgHA, and ZnHA powders were performed by X-ray spectral microanalysis (RSMA). The ratio of Ca/P is within the range of 1.66-1.77 and corresponds to the ratio of Ca/P in stoichiometric HA and the HA entering bone tissue. The ability of AgHA- and ZnHA-substrates to form on their surface a calcium-phosphate layer from the simulated body fluid (SBF) at 37 °C is determined. This ability decreases in the order: in ZnHA it is less than in AgHA, but greater than in HA. The antibacterial activity of the samples was analyzed. The AgHA sample has both bactericidal and persistent bacteriostatic properties in the case of direct contact with *Escherichia coli* cells.

## INTRODUCTION

At present of medicine development, the hydroxyapatite-based materials are extensively used for the restoration of human bone tissue in the field of surgery, traumatology, dentistry. Ideal implants, ceramics and dental cements from hydroxyapatites (HA) should have the structure, composition, and morphology identical to human bone tissue. Some scientists use chemical modification, in particular anionic and cationic substitution in its structure, to improve existing HA specific properties and to impart new ones [1].

As long as human bone matrix serves as a buffer system for the atoms of different metals, artificial HA-based biomaterials must have different impurity concentrations to fit the replaceable portion of bone [2, 3] in the best way. In most cases, the hydroxyapatite structure remains constant with such minor changes as the introduction of impurity atoms, the presence of vacancies or substitutions [4].

The HA samples containing silver and zinc ions are of particular interest. Owing to high biocompatibility and osteoconductivity, HA-containing materials absorb proteins, amino acids and other substances easily, which contribute to adsorption and bacteria colonization. Recently, to prevent bacterial growth on the surface of HA, various organic and inorganic components possessing antibacterial properties have been added. Some of such components are the Ag<sup>+</sup> and Zn<sup>2+</sup> ions.

The relative novelty of the study and insufficient scrutiny of the Ag<sup>+</sup> and Zn<sup>2+</sup> ions' impact on the HA structure, its physico-chemical and antibacterial properties make the investigations of the obtained samples advisable.

The HA structure can undergo isomorphous substitutions; hence, the factor of dimensional resemblance of cation substitutions with calcium ion is of great significance [1]. The capability of atoms or ions to enter the structure of other substance is determined by individual properties of atoms or ions (size, charge, electronic structure) as well as by peculiarities of the crystal structure of substances, forming solid solutions. The difference in atomic size of components must be insignificant and not higher than 8–15 % [4, 6]. Silver and zinc ions enter the HA structure only in small amounts, thus substituting calcium ions. From the viewpoint of vacancy formation, the biogenic and synthetic apatites, containing impurity atoms and groups of atoms in the positions of the HA structure, are considered as substitutional solid solutions [4, 7].

It was determined [8–12] that  $\text{Ag}^+$  and  $\text{Zn}^{2+}$  ions being in dissolved state in small amounts show an antibacterial oligodynamic influence on the cells of microorganisms. The  $\text{Ag}^+$  and  $\text{Zn}^{2+}$  ions influence many pathogenic microorganisms, such as *Staphylococcus aureus*, *Streptococcus*, *Escherichia coli* (*E. coli*); they have both a bacteriostatic antimicrobial effect (of inhibiting multiplication of microbes) and a bactericidal one (of killing microbes).

The authors [13] obtained and characterized porous composites HA–Ag. Hydroxyapatite was the main crystalline phase in the resulting composites. It has been established that the addition of silver ions to the composite material allows to reduce inflammation in the first stage of implantation and has a positive effect on the formation of bone tissue. The obtained composite materials containing silver are biocompatible, biodegradable and osteoconductive with a high level of porosity (75–85 %). These materials are recommended for orthopedic use.

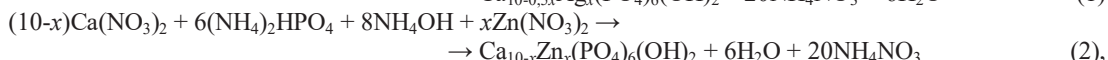
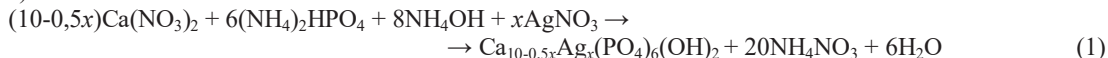
According to the most common theory of adsorption [14], explaining the mechanism of silver and zinc ions' influence on a bacterial cell, such cell loses vitality as a result of the interaction of electrostatic forces, arising between the cell membrane having a negative charge and the positively charged ions of silver or zinc during adsorption of the latter by a bacterial cell. At first, the ions are absorbed on the cell surface; then they penetrate into the cell, which leads to a decrease in enzyme activity, nucleotide damage and cell lysis.

The change in the elemental composition aimed at varying different HA properties, while maintaining its structure and phase composition, attracts the interest of a wide range of researchers [15, 16]. It is possible to vary crystallinity and porosity values of the material, to control its solubility, bioactivity and biocompatibility by introducing various elements.

The aim of the work is to study the physicochemical, antibacterial properties and biological activity of microwave synthesized silver- and zinc-containing hydroxyapatites with different content of  $\text{Ag}^+$  and  $\text{Zn}^{2+}$  ions.

## EXPERIMENTAL SETUP

Samples of hydroxyapatite with different mole content of ions  $\text{Ag}^+$  (AgHA) and  $\text{Zn}^{2+}$  (ZnHA) were obtained under microwave radiation by the method of precipitation from solutions in accordance with reaction equations (1, 2):



$x = 0.1; 0.3; 0.5$  (mole fraction)

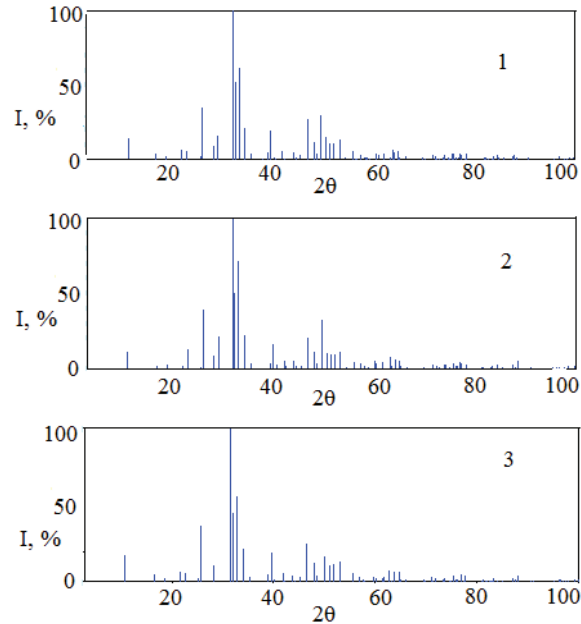
Synthesis and isolation of HA, AgHA and ZnHA samples were carried out in accordance with the protocol given in [2]. The concentrations of the initial aqueous solutions of calcium nitrate and ammonium hydrogen phosphate were 0.5 M and 0.3 M, respectively. Silver and zinc nitrates were added to a solution of calcium nitrate in the required quantities. During the synthesis solutions of reagents in a stoichiometric ratio were rapidly mixed, pH 10–11 was created by introducing ammonia solution ( $\rho = 0.907 \text{ g/sm}^3$ ), and immediately subjected to microwave radiation with a power of 110 watts for 40–50 minutes. A white precipitate separated from the solution was allowed to stand for 2 days in the mother solution and then filtered and dried in an oven at a temperature of 90 °C to constant weight. Portion of the resulting samples was subjected to calcination in a muffle furnace at 800 °C.

X-ray diffraction analysis (XRD) was performed on a Rigaku MiniFlex 600 diffractometer using the PCPDFWIN databases and the POWDER CELL 2.4 full-profile analysis program. Scanning electron microscopy (SEM) was used to study the morphology of the samples.

## RESULTS AND DISCUSSION

Comparison with an unsubstituted sample of HA showed (Fig. 1) that all AgHA and ZnHA samples are

single-phase solid substitution solutions based on hydroxyapatite phase  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  of the hexagonal system.



**FIGURE 1.** XRD patterns of calcined samples: 1 – HA; 2 – ZnHA ( $x = 0.3$ ); 3 – AgHA ( $x = 0.3$ )

The values of parameters  $a$ ,  $b$ ,  $c$  and volume  $V$  of elementary cells, obtained from the results of XRD samples using the Rigaku Data Analysis Software PDXL Version 2, are given in Table 1. Partial substitution of calcium ions by zinc ions of smaller sizes leads to a certain decrease in lattice parameters and the unit cell volume. At the same time, substitution of calcium ions with larger silver ions leads to an increase in the lattice parameters and the unit cell volume.

**TABLE 1.** Parameters and volume of unit cell of calcined HA, ZnHA and AgHA samples (by XRD results).

Sample	Lattice parameters			
	$a, \text{Å}$	$b, \text{Å}$	$c, \text{Å}$	$V, \text{Å}^3$
HA	9.434	9.434	6.880	530.3
ZnHA 0.1	9.427	9.427	6.881	529.2
ZnHA 0.3	9.420	9.420	6.872	528.7
ZnHA 0.5	9.414	9.414	6.845	527.1
AgHA 0.1	9.436	9.436	6.894	531.6
AgHA 0.3	9.451	9.451	6.892	533.2
AgHA 0.5	9.452	9.452	6.901	533.9

The IR spectra of the obtained samples were recorded on a Shimadzu FTIR-8300 IR Fourier spectrometer in the range of  $4000\text{-}400\text{ cm}^{-1}$ . The absorption bands were identified according to the tables of characteristic frequencies based on the literature and reference data [17, 18]. The spectra of the obtained materials with adjusted concentration of zinc and silver (Table 2) contain all absorption bands of HA functional groups vibrations. Narrow bands at  $3570\text{ cm}^{-1}$  correspond to stretching vibrations of hydroxyl groups. The doublet in the range of  $560\text{-}600\text{ cm}^{-1}$  and the triplet in the  $970\text{-}1060\text{ cm}^{-1}$  range correspond to the deformation vibrations and the stretching vibrations of the phosphate groups, respectively.

**TABLE 2.** Infrared modes of vibration of calcined HA, ZnHA and AgHA samples.

Modes of vibration	Vibrational frequencies, $\text{cm}^{-1}$						
	HA	ZnHA 0.1	ZnHA 0.3	ZnHA 0.5	AgHA 0.1	AgHA 0.3	AgHA 0.5
$\delta(\text{PO}_4^{3-})$	565 (s*)	562 (s)	561 (s)	564 (s)	561(s)	563 (s)	564 (s)
$\delta(\text{PO}_4^{3-})$	602 (m*)	599 (m)	599 (m)	598 (m)	599 (s)	598 (s)	599 (s)
$\nu(\text{PO}_4^{3-})$	965 (l*)	963 (l)	962 (l)	961 (l)	961 (l)	962 (l)	962 (l)
$\nu(\text{PO}_4^{3-})$	1021 (s)	1024 (s)	1023 (s)	1023 (s)	1022 (s)	1022 (s)	1022 (s)
$\nu(\text{PO}_4^{3-})$	1086 (l)	1088 (l)	1087 (l)	1089 (l)	1088(l)	1086 (l)	1088 (l)
$\delta(\text{OH}^-)$	630 (l)	630 (l)	629 (l)	629 (l)	629 (l)	629 (l)	630 (l)
$\nu(\text{OH}^-)$	3570 (l)	3570 (l)	3571 (l)	3568 (l)	3569 (l)	3572 (l)	3570 (l)

s\* – strong band; m\* – middle band; l\* – low band

Qualitative and quantitative elemental analyses as well as the distribution of elements over the surface of HA, AgHA and ZnHA powders were conducted by X-ray spectral microanalysis (RSMA) (scanning electron microscope Leo Supra 50VP with detector Oxford Instruments X-Max, X-ray spectral microanalyzer ShiftED 300), both at individual points and on the areas.

According to the RSMA results, the elemental composition of the modified HA was determined (Table 3): the ratio of Ca/P is within the range of 1.66-1.77, which is close to the ratio of Ca/P in the stoichiometric HA and the HA entering bone tissue. The elements of modifying ions are uniformly distributed over the surface of the samples; local inhomogeneous zones are absent.

**TABLE 3.** Content of Ag in AgHA and Zn in ZnHA (by RSMA results).

Sample	Content of Ag (Zn), mole fraction		Ca/P
	Experimental	Theoretical	
HA	-	-	1.66
ZnHA 0.1	0.080 ± 0.004	0.1	1.77
ZnHA 0.3	0.250 ± 0.013	0.3	1.68
ZnHA 0.5	0.420 ± 0.021	0.5	1.69
AgHA 0.1	0.040 ± 0.002	0.1	1.76
AgHA 0.3	0.060 ± 0.003	0.3	1.70
AgHA 0.5	0.110 ± 0.006	0.5	1.68

To assess the solubility of the HA, AgHA, ZnHA samples, the total concentration of calcium ions  $\text{Ca}^{2+}$  in the physiological medium ( $\omega(\text{NaCl}) = 0.9\%$ ), in which the samples were kept for 7 days to reach the saturation with respect to the solid phase, was determined. According to trilonometric determination of calcium ions in a saturated NaCl solution (0.9 % wt.), in the presence of eryochrom black T as the indicator, the values of solubility ( $C_{\text{Ca}^{2+}} \cdot 10^3 \text{ mol/l}$ ) were determined at 25 and 37 °C for both uncalcined and calcined powders at 900 °C HA, ZnHA and AgHA powders obtained by microwave synthesis. Solubility of all uncalcined AgHA samples in physiological saline at 25 °C and 37 °C is somewhat lower, and solubility of ZnHA samples is significantly higher compared to the unmodified HA. During calcination of samples, the solubility of ZnHA is greatly reduced, while in AgHA it remains virtually unchanged. The solubility of calcined ZnHA samples is greatly reduced, while the solubility of AgHA samples remains virtually unchanged (Tables 4, 5).

**TABLE 4.** Soluble powders values of silver- and zinc-containing hydroxyapatite and pure hydroxyapatite in 0.9 (wt. %) solution of NaCl (T 25 °C, pH 7).

Sample	$C_{Ca^{2+}} \cdot 10^3, \text{ mol/l}$	
	Uncalcined	Calcined at 900 °C
HA	1.59 ± 0.03	1.35 ± 0.08
AgHA 0.1	1.14 ± 0.13	1.19 ± 0.06
AgHA 0.3	1.26 ± 0.08	1.67 ± 0.03
AgHA 0.5	1.38 ± 0.04	1.44 ± 0.09
ZnHA 0.1	2.46 ± 0.07	0.52 ± 0.08
ZnHA 0.3	2.83 ± 0.08	0.63 ± 0.08
ZnHA 0.5	2.21 ± 0.03	0.42 ± 0.04

**TABLE 5.** Soluble powders values of silver- and zinc-containing hydroxyapatite and pure hydroxyapatite in 0.9 (wt. %) solution of NaCl (T 37 °C, pH 7).

Sample	$C_{Ca^{2+}} \cdot 10^3, \text{ mol/l}$	
	Uncalcined	Calcined at 900 °C
HA	1.72 ± 0.09	0.98 ± 0.08
AgHA 0.1	1.25 ± 0.04	1.39 ± 0.06
AgHA 0.3	1.35 ± 0.10	1.47 ± 0.09
AgHA 0.5	1.43 ± 0.11	1.52 ± 0.12
ZnHA 0.1	3.12 ± 0.04	0.86 ± 0.07
ZnHA 0.3	3.31 ± 0.02	0.72 ± 0.05
ZnHA 0.5	2.43 ± 0.09	0.59 ± 0.08

The morphology of samples surface and the dispersion were determined from the micrographs obtained on the energy dispersive microanalyzer at a magnification of 3000 times with the accumulation time of 600 s. Figure 2 shows SEM images of calcined samples, according to which the dispersion of HA powders was estimated by the “Secant method” [19] using Adobe Photoshop CS5. Figure 3 shows that the powders represent a mixture of agglomerates, varying in sizes and in the range of 1-12 microns. The conducted analysis of SEM images allows concluding that the samples exhibit a similar morphological organization, which means that for the selected materials, the introduction of silver and zinc ions into the crystal lattice of the HA does not change the morphology of materials ZnHA and AgHA.

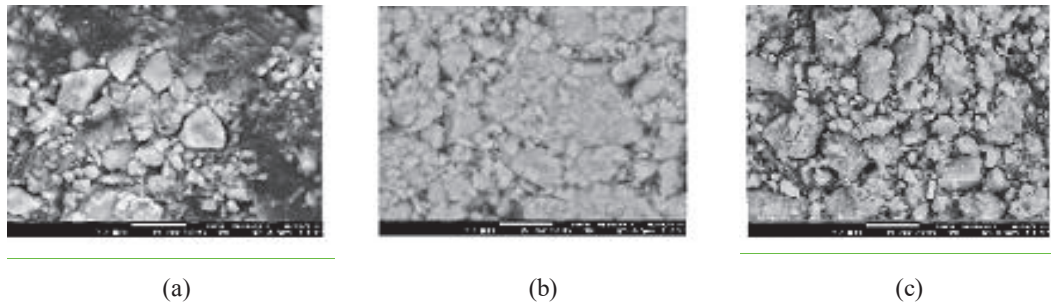


FIGURE 2. SEM images of the substrate surfaces of calcined powders AgHA ( $x=0.3$ ) (a), ZnHA ( $x=0.3$ ) (b) and HA (c); magnif. 3000

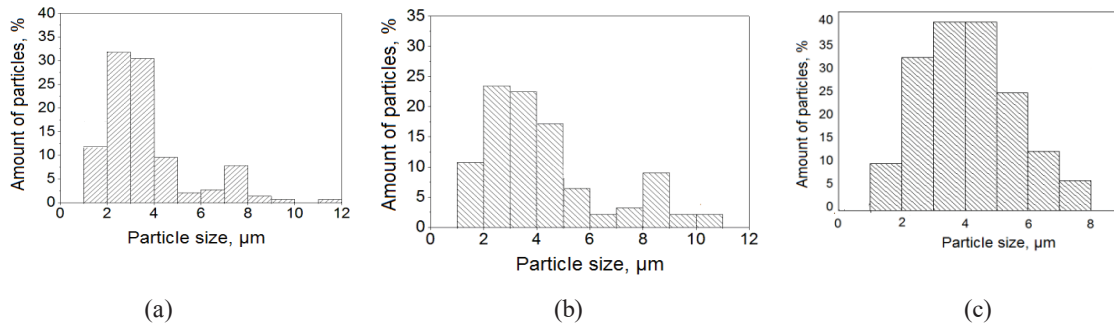


FIGURE 3. The histograms of particle distribution by sizes for the HA calcined samples of AgHA ( $x=0.3$ ) (a), ZnHA ( $x=0.3$ ) (b) and HA (c)

The specific surface area, the volume and the pore size of the HA powders were measured by the nitrogen adsorption method with subsequent degassing under the pressure of  $\sim 0.1$  Pa for one hour on a TriStar II Micromeritics device using the BET method. Analysis of the microstructure parameters shows that the modification by silver and zinc ions contributes to the increase in the particle size, the degree of crystallinity, the pore sizes of the samples, thereby reducing their specific surface and the uniformity of their shapes (Table 6).

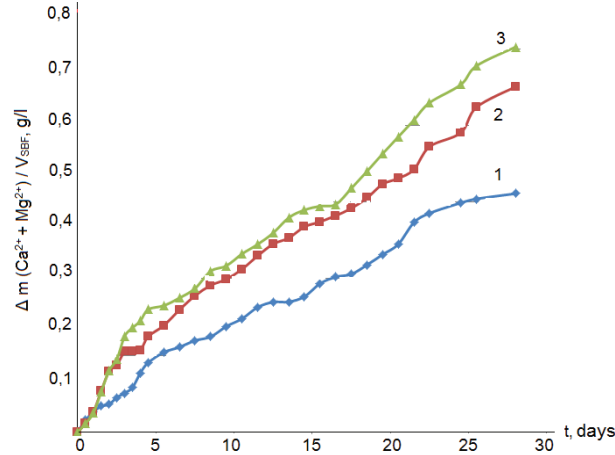
TABLE 6. Results of measuring specific surface area and porosity of HA, AgHA and ZnHA.

Sample	S, m <sup>2</sup> /g	Total pore volume, sm <sup>3</sup> /g	Average pore size, nm
AgHA ( $x=0.1$ )	79.3 $\pm$ 7.9	0.661 $\pm$ 0.066	34.3 $\pm$ 3.4
AgHA ( $x=0.3$ )	78.9 $\pm$ 7.8	0.610 $\pm$ 0.061	35.1 $\pm$ 3.5
AgHA ( $x=0.5$ )	76.0 $\pm$ 7.6	0.581 $\pm$ 0.058	36.7 $\pm$ 3.7
ZnHA ( $x=0.1$ )	45.7 $\pm$ 4.6	0.467 $\pm$ 0.047	43.4 $\pm$ 4.3
ZnHA ( $x=0.3$ )	44.8 $\pm$ 4.5	0.394 $\pm$ 0.039	44.8 $\pm$ 4.5
ZnHA ( $x=0.5$ )	34.6 $\pm$ 3.5	0.210 $\pm$ 0.021	58.1 $\pm$ 5.8
HA	106.2 $\pm$ 10.6	0.874 $\pm$ 0.087	22.8 $\pm$ 2.3

The formation of the calcium phosphate layer (CPL) on the HA surface is of great importance in the study of the bioactivity and biocompatibility of materials. Investigations of simulated body fluid (SBF) conducted by the method proposed by Kokubo [20, 21] are used as the test studies for prediction of biological activity of HA samples under the conditions of the body. Formation of CPL (the intake of calcium ions and phosphate ions, found in the SBF solution) is usually a long process, and the production of homogeneous layers on the HA substrates takes at least 7 days. To study the biological activity of the obtained calcined samples, a series of experiments on the formation of a calcium-phosphate layer from the simulated body fluid solution was

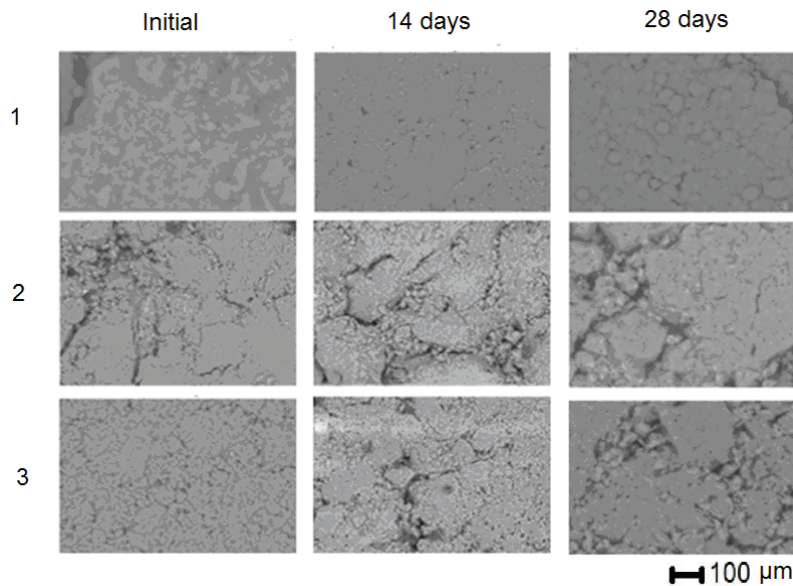
conducted for 28 days at  $T = 37\text{ }^{\circ}\text{C}$ . AgHA and ZnHA samples in the form of compressed tablets with a diameter of 5 mm, calcined at  $800\text{ }^{\circ}\text{C}$ , were placed in the container with SBF solution in such a way that the tablet was completely immersed in the solution. The constant temperature in the solution was maintained in an electric dry-air thermostat TS-1 SPU.

The quantitative assessment of the CPL formation process during 28 days is conducted by the change in the mass of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions in the solution and on the surface of the samples in accordance with the procedure presented in [22].



**FIGURE 4.** Curves of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions accumulation on the surfaces of calcined samples: 1 – HA; 2 – ZnHA ( $x=0.3$ ); 3 – AgHA ( $x=0.3$ )

The obtained kinetic curves (Fig. 4) show that  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions are adsorbed better on the surface of AgHA and ZnHA substrates than in the case of unsubstituted HA. This may be due to changing in the acid properties of the AgHA and ZnHA surface, as a result of which their samples adsorb more ions on their surface. CPL forms on the samples' substrates after 14 days of keeping them in the SBF solution. By the 28<sup>th</sup> day, the presence of CPL becomes apparent; the size of calcium phosphate grains on the surface layer of the newly formed CPL is 4-10  $\mu\text{m}$  (Fig. 5).



**FIGURE 5.** Electron micrographs of the substrate surface during the growth of the calcium phosphate layer: 1 – HA; 2 – AgHA ( $x=0.3$ ); 3 – ZnHA ( $x=0.3$ ); magnif. 3000



From the results of the SBF study, it can be concluded that AgHA and ZnHA samples are able to form CPL on their surface from the SBF solution and can bind to living bone. The investigation of the antibacterial properties of samples by the Koch method [23] consists of three stages: preparation of dilutions, seeding on a dense nutrient medium, counting of grown colonies.

The calcined samples of HA, AgHA ( $x=0.3$ ), ZnHA ( $x=0.3$ ) were tested. Bacteria *Escherichia coli* were used as a test strain. The standard culture medium for this test object is a lysogeny broth (LB) medium containing peptone 10 g/l, yeast extract 5 g/l, sodium chloride 10 g/l. The incubation was carried out in a thermostat in a 250 ml Erlenmeyer flask, with 100 ml of the lysogeny broth medium at 37-38 °C. The growth of the bacteria culture on the standard LB medium was taken as a control. Each sample was taken in an amount of 1 g, placed in a flask with the liquid nutrient LB medium and sterilized in an autoclave at 1 atm and 120 °C for 20 min. Sterilization of the solid nutrient LB medium for seeding and bacterial counting was carried out at 0.5 atm and 112 °C for 30 minutes. The results obtained during analysis of the bactericidal activity of the samples (Table 7) show that the HA sample, possessing antibacterial activity, reduces the number of *E. coli* 4.8 times.

**TABLE 7.** Bactericidal activity of the HA, AgHA and ZnHA samples concerning *E. coli* test strain

No	Option	Number of Colonies of <i>E. coli</i> Test Strain, CFU/ cm <sup>3</sup>
1	Control	$(6.0 \pm 1.1) \times 10^8$
2	HA	$(1.25 \pm 0.16) \times 10^8$
3	AgHA ( $x=0.3$ )	$(1.58 \pm 0.18) \times 10^8$ (In recalculation for zero dilution, where a large amount of powder was present – $(1.75 \pm 0.6) \times 10^2$ CFU/ml)
4	ZnHA ( $x=0.3$ )	$(2.1 \pm 0.65) \times 10^9$

The AgHA sample shows a more complex effect on the test strain. The number of *E. coli* at the end of the experiment decreased 3.8 times in comparison with the control sample, having antibacterial activity. However, the seeding of the bacteria suspension without dilution showed that the presence of the sample particles significantly suppresses the growth of bacteria, allowing one to grow only 0.0001% of the cells that are available in the sample. The bacteria demonstrate their viability, forming colonies on a dense nutrient medium in the dilution of the solution where the concentration of the initial sample decreases  $10^5$ - $10^7$  times. Thus, the AgHA sample demonstrates a persistent bacteriostatic property in the case of direct contact with cells, which suspends their development without killing them, that is, the AgHA sample has a bacteriostatic effect. No extraneous microflora was found on the nutrient medium: all the colonies were of the same type and they were related to the test strain under the study by morphological features.

## CONCLUSIONS

Based on the experimental and calculated data, obtained during the study using various physico-chemical, chemical-biological methods, we obtained results that allowed characterizing samples of silver- and zinc-containing hydroxyapatite and drawing the following conclusions.

AgHA and ZnHA samples with different contents of silver and zinc ions were obtained by the method of liquid-phase deposition under the action of microwave radiation. The main phase in all samples was hydroxyapatite  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  of the hexagonal system.

The distribution of elements of modifying ions over the surface of the samples occurs uniformly; local inhomogeneous zones are absent. The ratio of Ca/P in the samples is in the range of 1.66-1.77 that practically corresponds to the stoichiometric ratio in the biogenic HA.

The solubility of AgHA samples in physiological solution at 25 °C and 37 °C does not depend on the calcination stage, and its values are commensurable with the solubility of unmodified HA. Calcination of ZnHA samples lowers their solubility slightly, which does not change with the temperature increase of the physiological solution.

According to the scanning electron microscope data, AgHA and ZnHA powders represent a mixture of agglomerates with a particle size in the range of 1-12 microns and have an identical morphology. The modification of silver and zinc ions promotes an insignificant increase in the particle and pore sizes of the samples; at that, their specific surface area and uniformity of shapes decrease.

Samples AgHA and ZnHA show ability to form a calcium-phosphate layer on its surface from the simulated body fluid solution at 37 °C. Calcium and magnesium ions are better adsorbed on the surface of AgHA and

ZnHA than on the surface of the HA.

Samples HA and AgHA have bactericidal action (moreover, AgHA has persistent bacteriostatic action) when in direct contact with *E. coli* cells. The number of *E. coli* at the end of the experiment is reduced 3.8-4.8 times in comparison with the control sample.

## ACKNOWLEDGMENTS

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