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Synthesis, characterization and antimicrobial properties of chemically modified apatite-related calcium phosphates

***O.V.Livitska*[1], *N.Yu.Strutynska*[1], *O.M.Vasyliuk*[2], *I.I.Grynyuk*[1], *S.V.Prylutska*[1], *N.S.Slobodyanik*[1]**

[1]T. Shevchenko National University of Kyiv,
64/13 Volodymyrska Str., 01601 Kyiv, Ukraine
[2]Zabolotny Institute of Microbiology and Virology National Academy of Science of Ukraine, 154 Zabolotnogo Str., 03143 Kyiv, Ukraine

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Na^+, CO_3^{2-}-HAPs and Na^+, M^{2+}, CO_3^{2-}-HAPs (M^{2+} — Zn^{2+}, Cu^{2+}) were synthesized by precipitation method and characterized. According to powder XRD and SEM data the prepared particles of all samples are of nano size and single phase. The quantitative elemental analysis showed that obtained calcium phosphates contain the following substituted elements: Na^+ (0.2–0.3 %wt), Zn^{2+} (1.1 %w/w) or Cu^{2+} (1.9 %wt) and CO_3^{2-} while the FTIR data confirm the partial substitution of phosphate by carbonate group (B-type) in HAP structure. The influence of particles size on phosphates properties was also determined for sample Na^+, CO_3^{2-}-HAP heated to 700°C. The antimicrobial properties of synthesized nanoparticles of chemically modified calcium phosphates against of opportunistic microorganisms — *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* were studied. The antimicrobial effect of modified HAPs (5–20 mM) on all tested reference strains was established. Prepared Na^+, Zn^{2+}, CO_3^{2-}-HAP had higher inhibitory activity against Gram-positive microorganisms — *S. aureus* and *S. pyogenes* than that for Gram-negative ones. Phosphate Na^+, Cu^{2+}, CO_3^{2-}-HAP had strong inhibitory effect on both Gram-positive and Gram-negative bacteria.

Keywords: nanoparticles, apatite, zinc, copper, antimicrobial activity.

Методом соосаждения синтезированы и исследованы химически модифицированные фосфаты кальция апатитового типа Na^+, CO_3^{2-}-ГАП и Na^+, M^{2+},CO_3^{2-}-ГАП (M^{2+} — Zn^{2+}, Cu^{2+}). По данным порошковой рентгенографии и сканирующей электронной микроскопии установлено, что синтезированные частицы всех образцов, имеющие наноразмер, не содержат примесей. Количественный элементный анализ показал, что полученные фосфаты кальция содержат Na^+ (0,2–0,3 масс.%), Zn^{2+} (1,1 масс.%) или Cu^{2+} (1,9 масс.%) и CO_3^{2-}. Данные ИК-спектроскопии подтверждают частичное замещение фосфатной группы карбонатной (Б-тип) в структуре апатита. Исследовано влияние размера частиц на свойства фосфатов для синтезированного Na^+, CO_3^{2-}-ГАП, нагретого до 700°С. Исследована антибактериальная активность синтезированных наночастиц химически модифицированных фосфатов кальция относительно условно-патогенных микроорганизмов *Staphylococcus aureus*, *Pseudomonas aeruginosa* и *Streptococcus pyogenes*. Установлено антимикробное действие модифицированных ГАП в пределах концентраций 5–20 мМ относительно всех исследуемых референтных штаммов. Na^+, Zn^{2+}, CO_3^{2-}-ГАП характеризовался высшей ингибирующей активностью относительно грам-положительных микроорганизмов - *S. aureus* и *S. pyogenes*, по сравнению с грам-отрицательными микроорганизмами. Самое высокое ингибирующее действие к грам-положительным и к грам-отрицательным бактериям показал Na^+, Cu^{2+}, CO_3^{2-}-ГАП.

Синтез, досідження та антимікробні властивості хімічно модифікованих кальцій фосфатів апатитового типу. *О.В.Лівіцька, Н.Ю.Струтинська, О.М.Василюк, І.І.Гринюк, С.В.Прилуцька, М.С.Слободяник.*

Методом співосадження синтезовано і досліджено хімічно модифіковані кальцій фосфати апатитового типу Na^+, CO_3^{2-}-ГАП та Na^+, M^{2+}, CO_3^{2-} -ГАП (гідроксиапатит) (M^{2+} — Zn^{2+}, Cu^{2+}). За даними порошкової рентгенографії та скануючої електронної мікроскопії встановлено, що синтезовані частинки всіх зразків знаходяться у нанорозмірному діапазоні і не містять домішок. Кількісний елементний аналіз показав, що отримані фосфати кальцію містять Na^+ (0,2–0,3 мас.%), Zn^{2+} (1,1 мас.%) чи Cu^{2+} (1,9 мас.%) або CO_3^{2-}, тоді як дані ІЧ-спектроскопії підтверджують реалізацію часткового заміщення фосфатної групи карбонатною (Б-тип) у структурі апатиту. Вплив розміру частинок на властивості фосфатів досліджено для синтезованого Na^+, CO_3^{2-}-ГАП, нагрітого до 700°С. Досліджено антимікробну активність синтезованих наночастинок хімічно модифікованих фосфатів кальцію щодо умовно патогенних мікроорганізмів *Staphylococcus aureus*, *Pseudomonas aeruginosa* та *Streptococcus pyogenes*. Виявлено інгібуючий вплив модифікованих ГАП у діапазоні концентрацій 5–20 мМ щодо усіх досліджуваних тест-штамів. Синтезований Na^+, Zn^{2+}, CO_3^{2-}-ГАП характеризувався вищою інгібуючою активністю щодо грам позитивних мікроорганізмів — *S. aureus* та *S. pyogenes*, порівняно з грам негативними мікроорганізмами. Найвищу інгібуючу дію як до грам позитивних, так і до грам негативних бактерій виявив Na^+, Cu^{2+}, CO_3^{2-}-ГАП.

1. Introduction

Members of hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$) structural row are widely used in many fields of industry, technology and medicine, in particular, dentistry and orthopedics [1–3], catalysis of important organic reactions [4, 5], creation of luminescent materials and pigments [6, 7], adsorption of heavy ions [8, 9]. Medicine is the main field of HAP use (bone substitution material, drug carrier, fluorescent probes) owing its biocompatibility, bioactivity and osteoconductive characteristics. It can be used in the form of ceramics, nanoparticles, composites based on organic polymers, coating of metal alloys [10–12] etc.

The apatite crystal structure is very flexible to incorporate foreign ions in its crystal framework. Such target substitution of cations and anions in apatite structure changes the chemical and thermal stability, mechanical properties of crystals and also can improve some biological or chemical characteristics [13]. For example, incorporation of carbonate ions in HAP structure framework affect the crystallinity of samples, their dissolution rates and the biological behavior creating lattice distortion and crystal defects. Synthetic carbonated hydroxyapatite can be used as bioactive and bioresorbable material for orthopedic because of its chemical similarity to a mineral component of human bones [14, 15].

However, the use of such materials has limitations that are mainly associated with the risk of infection. The development of inflammatory processes is the main cause of implant rejection. In this case using of doped hydroxyapatites by ions with antibacterial properties (copper (Cu^{2+}), zinc (Zn^{2+})) can inhibit bacterial infections. These ions are the key microelements in the organism and are included in many metabolic processes. Moreover, Zn^{2+} promotes metabolism and growth of bones, and has stimulatory effect on bone formation *in vitro* and *in vivo* as well as inhibitory effect on osteoclastic bone resorption *in vivo* [16]. The antibacterial nature of Zn^{2+} and Cu^{2+} ions is utilized in the synthesis of biomaterials used for bone tissue engineering. Several studies were devoted to its incorporation in apatite structures and such influence on biological behavior of materials [17–19].

The strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* are the most dangerous pathogens of infectious that cause disease of joint, bone, sepsis and tooth enamel destruction. However, the antimicrobial activity of modified hydroxyapatites against the strains *P. aeruginosa* and *S. pyogenes* has not been sufficiently studied, so evaluation of the antimicrobial activity of HAPs against these microorganisms is relevant.

The aim of this study was to synthesize and characterize nanoparticles of apatite-related chemically modified calcium phosphates and to investigate their antimicrobial action.

2. Experimental

Samples preparation. Chemically modified apatite-related calcium phosphates were obtained via the wet co-precipitation

method. The following substances (an analytical grade) were used as starting materials: $Ca(NO_3)_2{\cdot}4H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$, $Cu(NO_3)_2{\cdot}6H_2O$, Na_2CO_3 and $Na_3PO_4{\cdot}12H_2O$. Interaction was held at constant molar ratios Ca/P = 1.67, $CO_3^{2-}/PO_4^{3-} = 1$ and M^{II}:Ca = 1:50 ($M^{II} = Cu^{2+}, Zn^{2+}$) at the temperature 20°C. The mixture of nitrates ($Ca(NO_3)_2{\cdot}4H_2O$ and $M^{II}(NO_3)_2{\cdot}6H_2O$) was dissolved in water and added to container containing aqua solution of Na_2CO_3 and $Na_3PO_4{\cdot}12H_2O$. The obtained heterogeneous system was stirred for 15 min. and then amorphous samples were filtered and washed up few times with distillate water to eliminate any residual salts. Then powders were dried at 80°C for 72 h.

Methods of characterization. The phase analysis of synthesized samples was done by powder X-ray diffraction (XRD). Diffractometer Shimadzu XRD-6000 with Cu–Kα radiation (λ = 1.54178 Å) was used; 2θ = 5–90°, steps sizes = 0.01°. The Fullprof program was used for calculation of lattice parameters.

The vibration of different types of anions in samples was detected by Fourier transform infrared spectroscopy (FTIR) (KBr disks; PerkinElmer Spectrum BX spectrometer with frequency range 400–4000 cm^{-1} at 1 cm^{-1} resolution).

Surface characteristics (shape, size and elemental composition) of samples were observed using scanning electron microscopy (SEM; FEI Quanta 400 ESEM instrument) with energy-dispersive X-ray (EDX) analyzator (Genesis 4000 instrument).

Content of calcium, sodium, zinc, copper and phosphate was defined by atomic absorption spectroscopy (Thermo Electron M-Series instrument) while amount of carbon, hydrogen and nitrogen was measured using CHN elemental analysis (Elementar-Analysensysteme).

The weight loss and thermal behaviour of prepared Na^+, CO_3^{2-}-HAP were investigated using a Shimadzu DTG-60H simultaneous TGA/DTA analyzer. The sample (m = 13 mg) was heated in a platinum crucible at a rate of $10°{\cdot}min^{-1}$ from room temperature to 700°C (air atmosphere). α-Al_2O_3 was used as a standard sample.

The laboratory drying cabinet SingleDISPLAY and furnace SNOL-7.2/1100 with digital controller TermoPro-601 were used for drying and annealing of prepared samples.

Antibacterial activity test. The antibacterial activity of the obtained hydroxyapatites against three opportunistic microorganisms: *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 9027 and *S. pyogenes* ATCC 21059 was investigated as described by Z.Gang et al. [20] with some modifications. Opportunistic microorganisms are typically non-pathogenic microorganisms that act as a pathogen in certain circumstances.

Strains of conditionally pathogenic microorganisms were cultured during 24 h on tryptone soya broth at 37°C in duplicate. Obtained chemically modified calcium phosphates were sterilized by autoclaving at 0.75 atm (30 min at 112°C) before using.

Chemical samples in the different concentrations (5 mM, 10 mM and 20 mM) in sterile bottles with nutrient medium and 2 % the overnight culture (10^5 CFU/ml) — (*S. aureus or P. aeruginosa or S. pyogenes* strains) were added.

The growth of culture without hydroxyapatite was used as a control. The bottles were incubated at 37±1°C for 24 h and then suspensions of 24-hour bacterial culture were incubated on petri dishes containing tryptone soya agar. On the next day, the amount of bacterial colonies of tested strains on the surface of the agar medium was calculated.

Statistical analysis of data was performed using the program "Statistica 7.0". To assess the reliability of quantitative indicators of differences in different strains was used Post-hoc-test using the criterion of LSD. The difference was considered significant of $P \leq 0{,}05$.

3. Results and discussions

Samples preparation and their characterization. Chemically modified apatite-related calcium phosphates were synthesized via the wet precipitation method at fixed molar ratios Ca/P = 1.67, $CO_3^{2-}/PO_4^{3-} = 1$ and M^{II}:Ca = 1:50 (M^{II} = Cu, Zn). Interaction in the systems can be described as following schemes:

$$(10-x)Ca^{2+} + xNa^+ + (6-z)PO_4^{3-} + \\ + zCO_3^{2-} + H_2O \rightarrow \\ \rightarrow Ca_{10-x}Na_x(PO_4)_{6-z}(CO_3)_z(OH)_2;$$

$$(10-x-y)Ca^{2+} + xM^{2+} + yNa^+ + \\ + (6-z)PO_4^{3-} + zCO_3^{2-} + H_2O \rightarrow \\ \rightarrow Ca_{10-x-y}M^{II}_x Na_y(PO_4)_{6-z}(CO_3)_z(OH)_2.$$

The obtained amorphous precipitates after filtration and drying at 80°C were

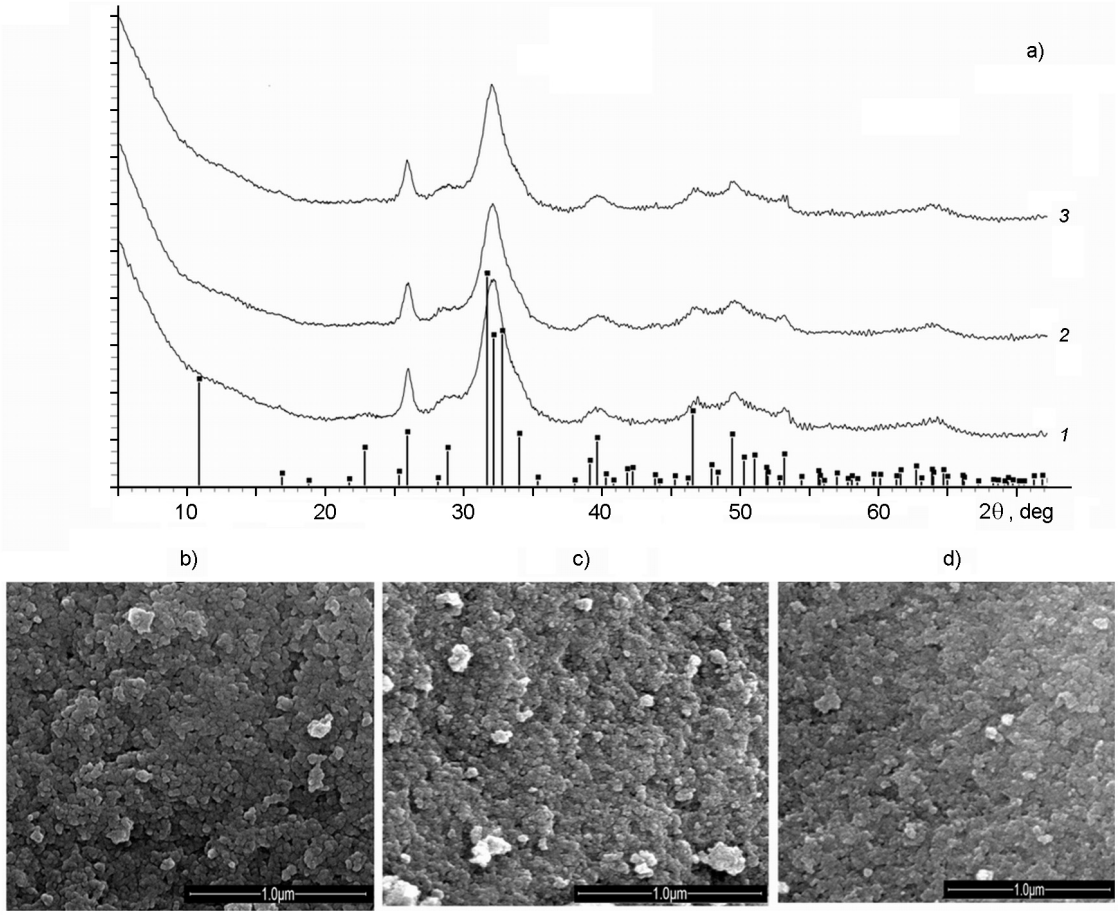

Fig. 1. XRD pattern and SEM image for prepared Na^+, CO_3^{2-}-HAP — (curve *1*, (a)), Na^+, Cu^{2+}, CO_3^{2-}-HAP — (curve *2*, (b)) and Na^+, Zn^{2+}, CO_3^{2-}-HAP — (curve *3*, (c)) dried at 80°C. ($Ca_{10}(PO_4)_6O$ — ICDD, #01-089-6495, vertical lines).

analyzed and their antibacterial activity was investigated.

Dried at 80°C powders belonged to poorly crystalline calcium phosphates on the base of XRD results. Its XRD patterns had wide reflections in all 2θ range (Fig. 1). At the same time, SEM images showed the formation of particles with diameter in the range of 30–50 nm (Fig. 1).

The FTIR-spectra for all samples are similar in respect of intensities and peaks positions (Fig. 2). Intensive modes in the ranges of 560–600 cm^{-1} and 1000–1100 cm^{-1} are related to symmetric and asymmetric stretching vibrations (ν_4, ν_1 and ν_3) of phosphate tetrahedron in apatite-type structure. The broad band in the range of 3200–3600 cm^{-1} is caused by sorbed water and stretching vibrations of structurally-bound OH-group in HAP framework, while corresponding mode of deformation vibration was found at 1600 cm^{-1}. Characteristic vibrations of carbonate groups in calcium phosphate framework were found at 870, 1430 and 1450 cm^{-1} (Fig. 2) and confirmed the realization of B-type substitution of PO_4^{3-} by CO_3^{2-} in HAP structure [21].

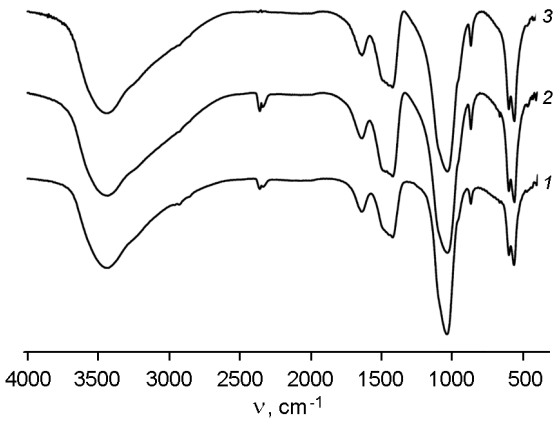

Fig. 2. FTIR spectra for prepared Na^+, $CO_3$2– -HAP — (curve *1*), Na^+, Cu^{2+}, CO_3^{2-}-HAP — (curve *2*) and Na^+, Zn^{2+}, CO_3^{2-}-HAP — (curve *3*) dried at 80°C.

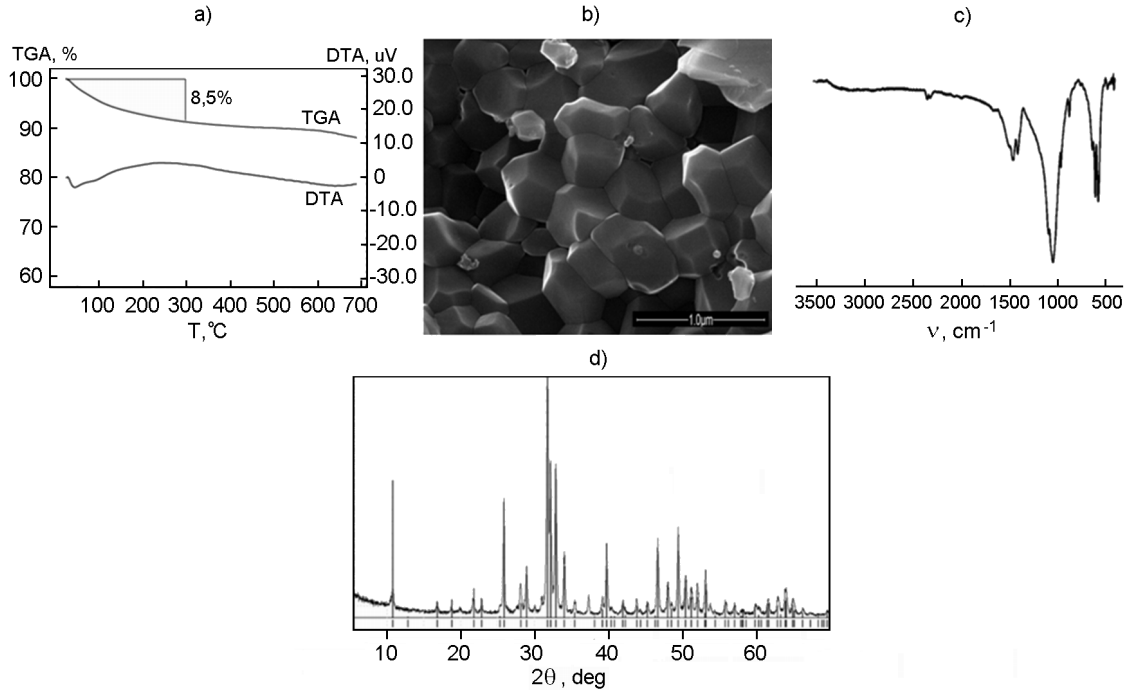

Fig. 3. TG/DTA for prepared Na^+, CO_3^{2-}-HAP dried at 80°C — (a), SEM image — (b), FTIR spectrum — (c) and XRD pattern — (d) (ICDD, #01-089-6495, vertical lines) for corresponding sample heated to 700°C.

According to atomic absorption spectroscopy and CHN analysis data and taking into account FTIR result (realization of B-type substitution PO_4^{3-} by CO_3^{2-}), the following formulas were assigned to obtained samples:

$$Ca_{9.32}Na_{0.26}(PO_4)_{4.9}(CO_3)_{1.1}(OH)_2 - Na^+,CO_3^{2-} - HAP;$$

$$Ca_{9.25}Cu_{0.18}Na_{0.19}(PO_4)_{5.05}(CO_3)_{0.95}(OH)_2 - Na^+,Cu^{2+},CO_3^{2-} - HAP;$$

$$Ca_{9.2}Zn_{0.27}Na_{0.16}(PO_4)_{5.10}(CO_3)_{0.9}(OH)_2 - Na^+,Zn^{2+},CO_3^{2-} - HAP.$$

It should be noted that EDX data also correlated with results of atomic adsorption spectroscopy that confirmed the homogeneity of samples in composition.

For the purpose of investigation of particles size influence on properties of phosphates, the obtained Na^+, CO_3^{2-}-HAP was heated to 700°C, analyzed and its properties were determined. According to TG/DTA data for this sample, the mass loss about 8.5 %w/w took place at its heating to 300°C that can be caused the elimination of sorbed water (Fig. 3a) [21]. SEM images confirmed the aggregation of particles with increasing of their size to 300–500 nm (Fig. 3b). At the same time, the intensity of bands related to the OH-group and H_2O for annealed at 700°C phosphate is significant less than that for corresponding sample dried at 80°C (Fig. 3c and Fig. 2, curve 1). The powder XRD data indicated that the apatite-related phosphate without impurities was obtained (Fig. 3d). The experimental pattern was indexed in the hexagonal system with the calculated lattice parameters: $a = 9.416(9)$, $c = 6.889(4)$Å.

It should be noted that, in the case of Na^+, Cu^{2+}, CO_3^{2-}-HAP and Na^+, Zn^{2+}, CO_3^{2-}-HAP the heating of samples to temperature 700°C led also to formation of single phase apatite-related phosphates. The calculated lattice parameters for these samples are following: $a = 9.434(6)$, $c = 6.897(3)$ Å for Na^+, Cu^{2+}, CO_3^{2-}-HAP and $a = 9.436(2)$, $c = 6.886(4)$ Å for Na^+, Zn^{2+}, CO_3^{2-}-HAP. Comparison of obtained values for Na^+, M^{II}, CO_3^{2-}-HAP with corresponding data for Na^+, CO_3^{2-}-HAP showed their increasing in the case of M^{II}-containing calcium phosphates that indicated on the intercalation of M^{II} ($M^{II} = Cu^{2+}$, Zn^{2+}) into an apatite structure.

Thus, four chemically modified apatite-related calcium phosphates: Na^+, CO_3^{2-}-HAP; Na^+, CO_3^{2-}-HAP (annealed at 700°C); Na^+, Cu^{2+}, CO_3^{2-}-HAP and Na^+, Zn^{2+}, CO_3^{2-}-HAP were prepared and used for investigation of their properties.

Antimicrobial activity. The investigation of size effect of Na^+, CO_3^{2-}-HAP and Na^+, CO_3^{2-}-HAP (annealed at 700°C) on biological properties showed antimicrobial activity of these samples against all tested reference

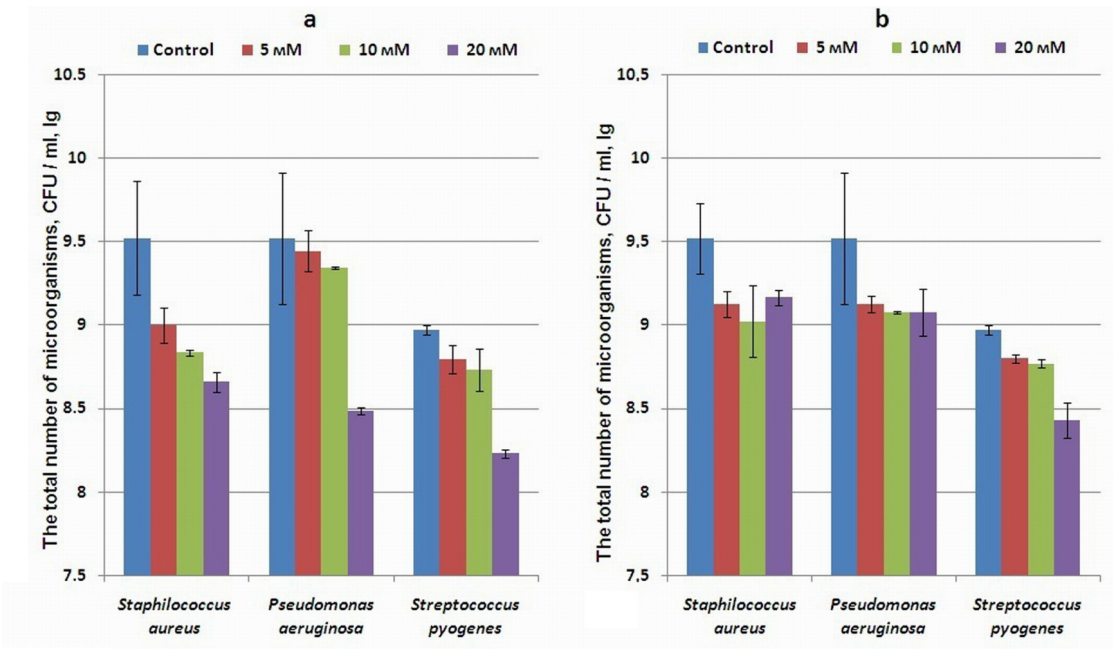

Fig. 4. The antimicrobial action of prepared Na^+, CO_3^{2-}-HAP (a) and corresponding heated to 700°C sample (b) at different concentrations.

strains. It was found that Na^+, CO_3^{2-}-HAP sample with the size of nanoparticles 30–50 nm has the highest antagonistic effect (Fig. 4).

After action of this sample on the growth of strain *S. aureus* the correlation between its concentration and the counts of microorganisms — colony-forming unit (CFU/ml) was found. Thus, increasing concentration of sample led to decreasing survival counts of the culture while for Na^+, CO_3^{2-}-HAP (annealed at 700°C) with the size of particles in the range 300–500 nm such effect was not observed.

Similar results were obtained at the investigation of influence of Na^+, CO_3^{2-}-HAP and Na^+, CO_3^{2-}-HAP (annealed at 700°C) on the growth of *P. aeruginosa* and *S. pyogenes* strains. It was found that high inhibitory concentration of samples on both strains *S. aureus* and *P. aeruginosa* is 10 mM. It should be noted that the literature data about influence of HAP on the growth *S. aureus* strain are controversial. Thus, authors [22] showed the antimicrobial effect of dry hydroxyapatite on strain *S. aureus*, while in [23] was not found antimicrobial action of apatite against this strain. Some authors have suggested that the degree of microorganism survival depends on the chosen investigation method as well as solubility degree of hydroxyapatite [24]. At the same time the significant difference at the action of investigated samples on Gram-positive and Gramnegative microorganisms was not observed.

The ions of zinc and copper are actively involved in metabolic processes in the body [25]. Moreover the zinc ions are present in the active centers of enzymes and play important role in the metabolism of bones. Due to high antibacterial activity of Zn^{2+} and Cu^{2+} the doped hydroxyapatites by such ions are used to decrease infections of prosthetic and implant caused by pathogenic and opportunistic microorganisms.

Taking this into account the antimicrobial action of Na^+, Cu^{2+}, CO_3^{2-}-HAP and Na^+, Zn^{2+}, CO_3^{2-}-HAP against strains of *S. aureus* and *S. pyogenes* (Gram-positive) and strain *P. aeruginosa* (Gram-negative) was investigated (Fig. 5). It was found that prepared Na^+, Zn^{2+}, CO_3^{2-}-HAP sample has a higher antibacterial activity against Gram-positive microorganisms — *S. aureus* and *S. pyogenes*, compared to Gram-negative bacteria. These data are in agreement with the results of other authors, that showed significant antimicrobial effect on strain *S. aureus* than that for *E. coli* culture [25]. It was showed, that increasing of zinc ions amount in apatite structure leads to decrease of the structure stability and formation of bonds with amino groups of microbial membrane proteins [26, 27]. This causes structural changes in microorganisms' membrane and finally led to cell death [18].

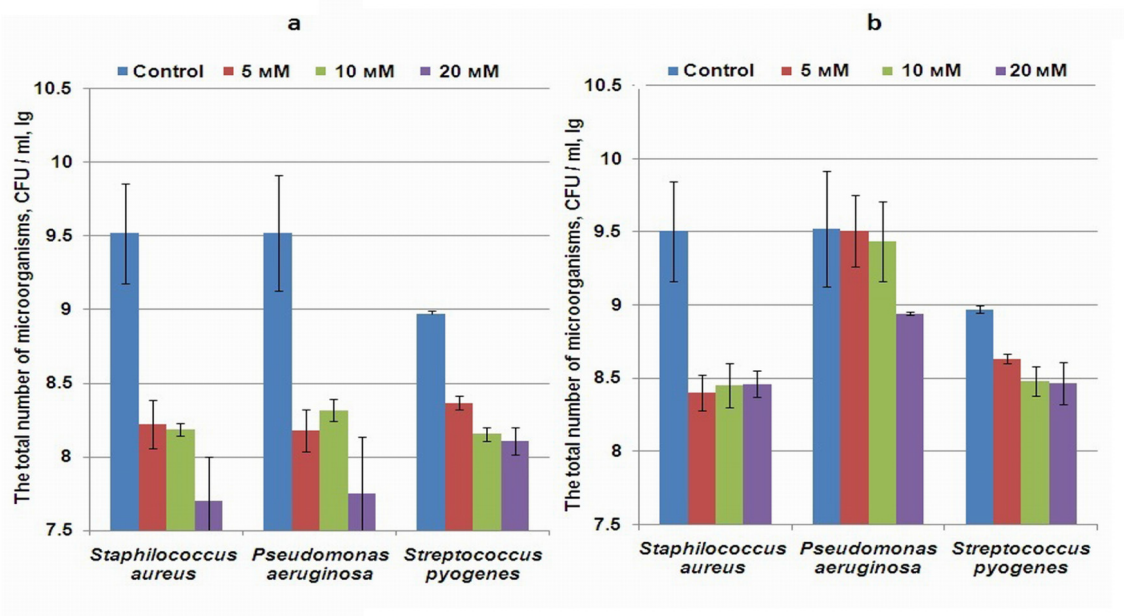

Fig. 5. The antimicrobial action of prepared Na^+, Cu^{2+}, CO_3^{2-}-HAP (a) and Na^+, Zn^{2+}, CO_3^{2-}-HAP (b) dried at 80°C at different concentrations.

The obtained results show that Na^+, Zn^{2+}, CO_3^{2-}-HAP at a concentration of 5 mM decreased in tenfold the growth of strain *S. aureus* in comparison with control ($4.3\pm0.19\cdot10^9$ CFU/ml and $2.9\pm0.49\cdot10^8$ CFU/ml, respectively). The strain *P. aeruginosa* had sensitivity only to 20 mM Na^+, Zn^{2+}, CO_3^{2-}- HAP, while there was no significant effect on the growth of this culture at sample concentrations of 5 and 10 mM (Fig. 5).

It was found that the sample Na^+, Cu^{2+}, CO_3^{2-}-HAP showed antimicrobial activity against all tested reference strains. The phosphate Na^+, Cu^{2+}, CO_3^{2-}-HAP at concentrations 5 mM and 10 mM inhibited in similar degree the growth of strains *S. aureus* and *P. aeruginosa* comparing with the control ($1.5–2.1\cdot10^8$ CFU/ml and $4.3–4.7\cdot10^9$ CFU/ml, respectively). At the same time, increasing the concentration of this phosphate to 20 mM led to 100-fold decrease of these strains growth in comparison with control. Among the investigated microorganisms, the survival of the strain *S. pyogenes* wasn't dependent on sample concentration. However, according to the literature data, copper ions have a low antimicrobial action against Gram-positive microorganisms. Copper ions, unlike other metal cations (Zn, Co, Ni, Mn, Fe and Sn), cause selective changes of the microorganisms membrane permeability [28]. Although the mechanisms of antibacterial activity of Cu-containing HAP have not investigated, but some authors suggest that copper ions cause DNA and RNA damage, promote structural changes of microorganism's membrane, increase its permeability, and, finally lead to cell death [18, 29]. According to the literature data, some microorganisms, such as *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Salmonella enterica*, become resistant to the toxic action of copper ions [30, 31], whereas Gram-negative microorganisms, in particular *Escherichia coli*, are sensitive to Cu-containing HAP.

4. Conclusions

According to XRD, SEM, FTIR and elemental analysis the Na^+, (Zn^{2+} or Cu^{2+}), CO_3^{2-} — containing apatite-related calcium phosphates were prepared. The particles of all samples are of nano size (30–50 nm) and have homogeneous compositions. It was found that heating of Na^+, CO_3^{2-}-HAP to temperature 700°C led to aggregation of particles and increasing of their size to 300–500 nm. Calculation of lattice parameters for Na^+, Cu^{2+}, CO_3^{2-}-HAP and Na^+, Zn^{2+}, CO_3^{2-}-HAP confirmed the intercalation of Cu^{2+} and Zn^{2+} into apatite-type structure.

An inhibitory effect of prepared samples on the growth of conditionally pathogenic microorganisms such as *S. aureus*, *P. aeruginosa* and *S. pyogenes* was found. It was detected inhibitory activity of Na^+, Zn^{2+}, CO_3^{2-}-HAP against Gram-positive microorganisms at 5 mM concentration while against Gram-negative microorganisms only at 20 mM concentration. The prepared Na^+, Cu^{2+}, CO_3^{2-}-HAP had the highest antimicrobial effect for both Gram-positive and Gram-negative bacteria.

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