Materials with novel functional properties

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Hydroxyapatite as a Mineral Matrix for Antibacterial Substances

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Abstract – A procedure has been developed for preparing a hydroxyapatite-based matrix with differently structured particles intended for manufacturing compositions for therapeutic and prophylactic use. Complexes of iron(III) and manganese(III) with tetraphenylporphyrin, known for their antibacterial activity, are used as modifying agents. The process of immobilization of metal complexes on the hydroxyapatite matrix proceeds under the action of ultrasonic field. The composition based on nanosized hydroxyapatite and tetraphenylporphyrin metal complexes is found to have a higher antibacterial activity than the composition based on the microorganized hydroxyapatite particles. The results of preliminary biomedical assays indicate potential benefits of using the modified hydroxyapatite-based composition for medical applications.

Keywords: hydroxyapatite nanoparticles, hydroxyapatite microparticles, antibacterial activity, porphyrin metal complexes.

Гидроксиапатит как минеральная матрица для антибактериальных веществ

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Аннотация – Предложена процедура получения гидроксиапатита с различной степенью организации частиц, предназначенного для изготовления композиций лечебно-профилактической направленности. В качестве модифицирующего агента выступили комплексы железа(III) и марганца(III) с тетрафенилпорфирином, известные своей антибактериальной активностью. Процесс иммобилизации металлокомплексов на гидроксиапатитовой матрице проходит в условиях действия ультразвукового поля. Композиция наноразмерного гидроксиапатита и металлокомплексов тетрафенилпорфирина обладает более высокой антибактериальной активностью, чем композиция, содержащая микроорганизованный гидроксиапатит. Результаты медико-биологических испытаний указывают на перспективу использования модифицированного гидроксиапатита в медицинских целях.

Ключевые слова: наночастицы гидроксиапатита, микрочастицы гидроксиапатита, антибактериальная активность, металлокомплексы порфиринов.

INTRODUCTION

Currently, an extensive search is being conducted for relevant composite materials (*i.e.* biocomposites) with a pronounced therapeutic and prophylactic activity with respect to biological systems. In general, these composites are combinations of organic and inorganic materials, in which an organic component is a therapeutic agent or bioactive substance, while an inorganic one is a mineral phase with a specifically organized surface structure [1–3]. The practical significance of such biocomposites is determined by their newly-created properties which are different from the properties of the starting substances. The created modifications are affecting a variety of physical and chemical processes, primarily, sorption processes. Thus, a targeted selective absorption of a specific substance can be achieved, as well as the changes can result in unique combinations of properties that cannot exist under other conditions. This is particularly true for complexes of plant origin, such as propolis [4]. The introduction of a biocomposite into medical practice is preceded by verification of its compliance with medical and biological purity, requirements for cell adhesion, an ability to exist in the body without damaging neighboring cells, regulated degradation, etc. [5, 6]. These properties can be effectively controlled by regulating such parameters as the diffusion rate and the ratio of hydrophobic and hydrophilic components.

A problem of providing biocomposite stability, *i.e.* achieving its inability to spontaneous phase separation for a long period of time is of high socio-economic significance. For example, an oxytetracycline-hydroxyapatite system has been used for reducing concentration of antibiotic in the environment [7]. Hydroxyapatite nanoparticles are known to be used as good adsorbents of fluoride ions concentrated in the environment and can be applied for monitoring environment status [8]. However, a goal of systematic separation of phases with controlled release of a bioactive substance under variable conditions still remains challenging. Thus, there is a challenging task to obtain a stable biocomposite containing an active component with bactericidal activity. In this work, complexes of iron(III) and manganese(III) with tetraphenylporphyrin which are known for their bactericidal properties [9] have been chosen as an active component for obtaining biocomposite material for biomedical applications.

EXPERIMENTAL PART

A composition for therapeutic and prophylactic use based on hydroxyapatite particles with differently organized structure and colloidal silver was the subject of our research. Hydroxyapatite (HAP) is the predominant mineral (apatite) component of human bones (45–70 wt.%), dentin (72 wt.%), cement (71 wt.%) and tooth enamel (96 wt.%). It has dimer composition of $Ca_{10}(PO_4)_6(OH)_2$ [10, 11]. A method for obtaining nano- and microorganized HAP (n-HAP and m-HAP) for therapeutic and prophylactic purposes, as well as its purification procedure, require the implementation of technological solutions depending on the specific purposes.

Complexes of iron(III) and manganese(III) with tetraphenylporphyrin (MTPP, where M = FeCl or MnCl) were provided by the staff of Ivanovo State University of Chemistry and Technology.

Two liquid-phase procedures based on mechanoacoustic treatment of a calcium phosphate mixture at pH > 9 were used for preparing HAP compositions [12].

Calcium-phosphate mixture (CPM No. 1) for obtaining n-HAP compositions contained calcium nitrate tetrahydrate, ammonium hydrogen phosphate, ammonia solution (up to pH > 9), and distilled water. The reagents of analytical reagent grade and high purity grade were obtained from KhIMMED company (Russia).

The basic reaction for the n-HAP-biocomposites preparation process is as follows:

$$6(NH_4)_2HPO_4 + 10Ca(NO_3)_2{\cdot}4H_2O + 8(NH_3{\cdot}H_2O) \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 46H_2O.$$

Generally, the obtained n-HAP product is of technical grade because of its contamination with an excess of nitrate ions. In [6], a combined approach to HAP purification was tested, which was based on decanting the centrifugated suspension followed by heat treatment of the precipitate at 350°C. An aqueous dispersion of pure n-HAP was obtained in rotary-pulsed apparatus from microorganized HAP obtained by mechanical grinding and sieve separation of heat-treated HAP.

The calcium phosphate mixture (CPM No. 2) for obtaining m-HAP contained anhydrous calcium hydrogen phosphate, calcium hydroxide (up to pH > 9), and distilled water. The reagents of analytical reagent grade and chemically pure grade were obtained from KhIMMED company (Russia) and Aldrich (USA).

The basic reaction for the m-HAP-biocomposites preparation process is as follows:

$$6CaHPO_4 + 4Ca(OH)_2 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6H_2O.$$

The volume ratio of reagents for CPM No. 2 was (2 : 1) in accordance with the reaction shown above. Sediment aging took place during 1 day.

The dispersed phase particle size was measured using the dynamic light scattering method on Zetasizer Nano ZS instrument (Malvern, England). Each sample was preliminarily mixed with distilled water in a ratio of 1 : 10, then averaging was carried out by vigorous stirring for 2 min.

The preparation of the n-HAP-MTPP and m-HAP-MTPP compositions was performed under ultrasonic treatment of the appropriate mixtures under an intensive dispergation mode of the instrument MOD MEF 391 (MELFIZ, Russia) working at an operating frequency of 22.4 kHz and a process temperature of not more than 60°C.

An industrial circulation-type generator known as a rotary-pulsed apparatus (RPA) with an electric motor drive (RPA installation) from Delta-Rotor (Aviation Technology, Russia) was used for producing mechanoacoustic treatment. The installation scheme is presented in Figure 1.

RPA is designed for preparing ultrafine dispersions (suspensions and emulsions), homogenization, low-temperature pasteurization and sterilization of liquid media. The specific design construction (composed of two titanium disks) involves complex fluid media treatment in the operating zone which combines mechanical-acoustic effects, large velocity gradients, vortex formation, and high-freqency pulsations [13]. Operating rotor speed is 2500 rpm. Unlike the traditional

HAP production method, the synthesis of HAP in the RPA reactor provides a fast and complete formation of dispersions with particles of the nanoscale range.

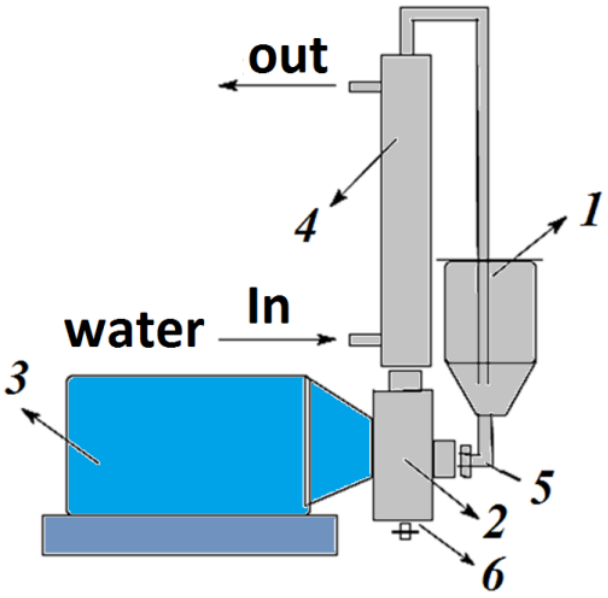

Fig. 1. RPA installation scheme: 1 – charging vessel; 2 – RPA; 3 – electric motor; 4 – pipe-in-pipe type refrigerator; 5 – thermocouple; 6 – drain cock.

Thus, it was of interest to prepare compositions of n-HAP (or m-HAP) and MTPP with the aim of obtaining HAP-based compositions with biocidal properties against bacteria and evaluating their antibacterial activity depending on the type of structure organization of HAP particles.

Synthesis and purification of n-HAP

Preliminary preparation of aqueous ammonia solutions of reagents was carried out as follows: 450 g of calcium nitrate tetrahydrate was dissolved in 5 l of distilled water, then a concentrated ammonia solution was added for adjustment of pH value to 10 (solution No. 1). Next, ammonium hydrogen phosphate in an amount of 151 g was dissolved in 2 l of distilled water, then the pH was adjusted to 10 by adding concentrated ammonia solution (solution No. 2).

The synthesis was carried out as follows: solution No. 1 was placed in the charging vessel of the RPA with cooling on (Fig. 1) and the apparatus engine was launched (2500 rpm). Then, at a rate of 0.5 l/min, solution No. 2 was injected into the charging vessel. The mixture was processed in the operating mode of the apparatus for 5 min, then the cooling was turned off and the dispersion was removed through a drain cock.

The dispersion was distributed in centrifuge tubes and separated under centrifugal forces on an Allegro 64R unit (Beckman, USA) at 6000 rpm for 30 min at 10°C. After the liquid was decanted, a new portion of distilled water was added under the action of ultrasonic field, and centrifugation, decantation and washing steps were repeated. Then, at a pressure of 2 kPa, the precipitate was filtered and transferred to a ceramic substrate and subjected to heat treatment in a muffle furnace for 30 min at

350°C. The dehydrated HAP was ground and the particles with a diameter of not more than 40 μm were separated by a sieve method.

The synthesis of pure n-HAP was carried out as follows: distilled water (10 l) was placed in the RPA charging vessel with cooling on, then, the apparatus engine was launched (2500 rpm). Next, microorganized HAP powder was introduced. The suspension was homogenized in the operating mode of the apparatus for 10 min, after which the cooling was turned off and the dispersion was removed through the drain cock.

Synthesis and purification of m-HAP

Saturated aqueous solutions of reagents were preliminarily prepared in the following way: 7 g of calcium hydroxide was dissolved in 5 l of distilled water, and filtered if necessary (solution No. 3). Next, calcium hydrogen phosphate in an amount of 0.5 g was dissolved in 2.5 l of distilled water (solution No. 4).

The synthesis was carried out as follows: solution No. 3 was placed in the RPA charging vessel with cooling on, and the apparatus engine was launched (2500 rpm). Then, at a rate of 0.5 l/min, solution No. 4 was introduced into the loading tank. The mixture was processed in the operating mode of the apparatus for 10 minutes, after which the cooling was turned off and the dispersion was removed through the drain cock. The precipitate was left for aging during 1 day.

The m-HAP dispersion was purified under the action of centrifugation forces with decantation of the supernatant without heat treatment.

Preparation of compositions of n-HAP (or m-HAP) with MTPP

In a 50% dispersion of n-HAP or m-HAP (weighing 100 g), 1 ml of MTPP solution was injected with a concentration of $1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$ or $1 \cdot 10^{-2}$ M/l under ultrasound conditions, with the temperature not raising above 60°C. Then, the dispersions were transferred onto inert membranes and filtered under reduced pressure. The MTPP content in the filtrate was determined by spectrophotometry using a DR/4000V instrument (HACH-Lange, USA) in the wavelength range of λ = 320–800 nm.

Bioassays

The compositions of n-HAP-MTPP and m-HAP-MTPP were subjected to biomedical testing on cell-culture material. Bacteria *S. aureus* p 209 (*Staphylococcus aureus*), *S. typhimurium* (*Salmonella*), *E. coli* 1257 (*E. coli*) were used as test cultures. Control samples were prepared from unmodified n-HAP and m-HAP. Test microorganisms were subcultured on meat-peptone agar and incubated for 17–18 h at 37°C. Then, a suspension of each microorganism was prepared in physiological saline and the concentration of microbial cells was established according to the turbidity standard of 10^{10} cells/ml. Samples of 100 mg suspension of n-HAP-MTPP or m-HAP-MTPP were placed in sterile Petri dishes, to which 1 ml of suspension of the test culture was added and kept at room temperature for 30 min. Then, 9 ml of sterile physiological saline was poured into dishes and kept for 15 min to elute the test culture from suspension particles. After the end of exposition period, 100 μl of the material from the dishes were subcultured on the surface of meat-peptone agar,

previously introduced into Petri dishes. Dishes were then incubated for 14–48 h at 37°C. Concomitantly, the control samples of suspensions of test cultures used in the experiment were subcultured in the same way for controlling the concentration of viable microorganisms. Then the count of the colonies of viable microorganisms grown on the agar surface was carried out.

RESULTS AND DISCUSSION

The size distribution of volume (mass) fraction of particles was determined which indicated that the mechanoacoustic treatment of aqueous dispersions of n-HAP resulted in formation of particles with a Z-average value of 40 nm, while for m-HAP dispersions – 1200 nm.

The results of bioassays of the n-HAP-MTPP and m-HAP-MTPP compositions are shown in Table 1. One can see from the data given in the table, that in the experiments with n-HAP-MTPP compositions with the maximum content of metal complexes $(0.5–0.6)\cdot10^{-4}$ mol/g, the number of viable microbial cells after 30 min exposure decreased for *Staphylococcus aureus* in 10 times, for *Escherichia coli* – more than 100-fold decrease was observed, while for *Salmonella* – 20-fold decrease was registered.

Table. Effect of n-HAP-MTPP and m-HAP-MTPP compositions on the viability of microorganism cultures depending on MTPP content per 1 g of suspension

Test culture	Viable microorganisms count (CFU/ml)		
	Starting test-culture	n-HAP-MTPP (m-HAP-MTPP)	Control sample
	M = FeCl, $0.9\cdot10^{-6}$ mol/g		
S. aureus p 209	$2.1\cdot10^{4}$	$4.1\cdot10^{3}$ ($1.1\cdot10^{4}$)	$4.5\cdot10^{3}$
E. coli 1257	$2.0\cdot10^{4}$	$6.5\cdot10^{2}$ ($4.5\cdot10^{3}$)	$9.5\cdot10^{3}$
S. typhimurium	$2.0\cdot10^{4}$	$4.0\cdot10^{3}$ ($2.0\cdot10^{4}$)	$6.0\cdot10^{3}$
	M = FeCl, $0.8\cdot10^{-5}$ mol/g		
S. aureus p 209	$2.1\cdot10^{4}$	$3.8\cdot10^{3}$ ($0.7\cdot10^{4}$)	$4.5\cdot10^{3}$
E. coli 1257	$2.0\cdot10^{4}$	$< 1\cdot10^{2}$ ($2.2\cdot10^{3}$)	$9.5\cdot10^{3}$
S. typhimurium	$2.0\cdot10^{4}$	$1.5\cdot10^{3}$ ($0.6\cdot10^{4}$)	$6.0\cdot10^{3}$
	M = FeCl, $0.5\cdot10^{-4}$ mol/g		
S. aureus p 209	$2.1\cdot10^{4}$	$2.0\cdot10^{3}$ ($0.3\cdot10^{4}$)	$4.5\cdot10^{3}$
E. coli 1257	$2.0\cdot10^{4}$	$< 1\cdot10^{2}$ ($1.7\cdot10^{3}$)	$9.5\cdot10^{3}$
S. typhimurium	$2.0\cdot10^{4}$	$1.0\cdot10^{3}$ ($1.0\cdot10^{4}$)	$6.0\cdot10^{3}$
	M = MnCl, $1\cdot10^{-6}$ mol/g		
S. aureus p 209	$2.1\cdot10^{4}$	$1.5\cdot10^{3}$ ($2.0\cdot10^{4}$)	$4.5\cdot10^{3}$
E. coli 1257	$2.0\cdot10^{4}$	$7.3\cdot10^{2}$ ($8.5\cdot10^{3}$)	$9.5\cdot10^{3}$
S. typhimurium	$2.0\cdot10^{4}$	$1.5\cdot10^{3}$ ($2.0\cdot10^{4}$)	$6.0\cdot10^{3}$
	M = MnCl, $0.9\cdot10^{-5}$ mol/g		
S. aureus p 209	$2.1\cdot10^{4}$	$1.5\cdot10^{3}$ ($0.5\cdot10^{4}$)	$4.5\cdot10^{3}$
E. coli 1257	$2.0\cdot10^{4}$	$4.2\cdot10^{2}$ ($1.2\cdot10^{3}$)	$9.5\cdot10^{3}$
S. typhimurium	$2.0\cdot10^{4}$	$1.5\cdot10^{3}$ ($0.9\cdot10^{4}$)	$6.0\cdot10^{3}$
	M = MnCl, $0.6\cdot10^{-4}$ mol/g		
S. aureus p 209	$2.1\cdot10^{4}$	$3.5\cdot10^{3}$ ($0.1\cdot10^{4}$)	$4.5\cdot10^{3}$
E. coli 1257	$2.0\cdot10^{4}$	$< 1\cdot10^{2}$ ($1.0\cdot10^{3}$)	$9.5\cdot10^{3}$
S. typhimurium	$2.0\cdot10^{4}$	$1.4\cdot10^{3}$ ($0.3\cdot10^{4}$)	$6.0\cdot10^{3}$

The bactericidal activity of the m-HAP-MTPP suspensions was substantially lower, in some cases, by an order of magnitude.

We believe that the antibacterial properties of metal complexes in HAP compositions are primarily associated with their effect on the cell walls of microorganisms by changing the charge of the bacterial cell. As a result, metal complexes become able to suppress the function of adhesion and colonization of pathogens. Apparently, MTPP complexes as part of HAP compositions are capable of disrupting the ionic balance of a living cell. This effect is particularly pronounced for nanosized suspensions. In addition, MTPP complexes containing mixed-valent metals, such as iron and manganese, stimulate the formation of reactive oxygen species in aqueous media, which, in turn, leads to suppression of the vital activity of pathogens.

The data obtained indicate that the proposed procedure for preparing compositions based on nanosized hydroxyapatite and tetraphenylporphyrin metal complexes can be a promising approach for producing advanced biocomposite materials used for sanitary and hygienic purposes and biomedical applications.

CONCLUSION

Thus, a procedure is developed for producing HAP-based compositions with differently organized structure of particles in a rotary-pulsating apparatus yielding a therapeutic composition containing complexes of tetraphenylporphyrin with iron(III) and manganese(III) ions. The composition based on nanosized n-HAP particles modified with metal complexes is found to have a higher antibacterial activity compared to the composition containing microorganized m-HAP particles. The performed bioassays confirm the benefits of using such compositions in medicine, veterinary medicine, as well as in food industry. In general, the results of the study show the relevance of creating hybrid systems based on porphyrin metal complexes which can be used for future development of disinfectants against pathogenic and potentially pathogenic microorganisms.

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