

## BIOMATERIALS

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### REACTION-ASSOCIATED RESORBABLE PHOSPHATE MATERIALS: PRODUCTION AND TESTING IN VITRO

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The properties of reaction-associated calcium-phosphate materials obtained by pressing paste based on  $\alpha$ -tricalcium phosphate with subsequent holding in different solutions are presented. The phase composition, solubility and mechanical characteristics of the materials obtained are studied and biological tests in vitro are performed.

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**Key words:** hydrosilicate, tricalcium phosphate, reaction-associated materials, strength, cytotoxicity, resorbability.

Dense ceramic based on hydroxyapatite  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  (HA) and/or tricalcium phosphate  $\text{Ca}_3(\text{PO}_4)_2$  (TCP) is finding wide application as implants in bone tissue replacement operations. However, these and similar implants fulfill only a support function, not permitting bone growth into the material, which is associated with its low solubility in the body's medium and the absence of a linked system of pores [1].

The so-called regenerative approach has been given more and more attention in recent years. In this approach emphasis is placed on complete replacement of the biomaterial of the growing bone while the material is relegated to the role of a support and guiding element, an active source of substances required for constructing bone tissue [2]. Thus, the use of bio-resorbable materials, whose resorption rate matches the growth rates of bone tissue, is trending in the field of orthopedic and maxillofacial surgery. In the case of calcium phosphate materials this can be attained first and foremost by obtaining implants at temperatures close to room temperature, on the basis of phases with better solubility than HA.

Calcium phosphate cements, which have studied recently as the main alternative to bioceramics, are characterized by higher solubility [3], while the technology for obtaining them is predicated on a significant number of methods for setting the porosity [4]. In addition, their relatively low consolidation temperatures make it possible to combine cements with physiologically active substances (for example, proteins initiating osteosynthesis) as well as with proliferating cell cultures (for example, stem cells) [5, 6]. Such properties open up the potential of calcium phosphate cements as a base for developing tissue-engineered structure for osteoplastics [7–9].

A series of works whose authors proposed and implemented simple and at the same time effective approaches to obtaining cements with improved mechanical properties have appeared in recent years. The specifics of the technological aspects of preparing these materials enabled the investigators to talk about a new class of materials (reaction- or chemically associated materials), which combine the advantages of ceramics and cements. The use of special modifying additives or pre-pressing of pastes makes it possible to significantly lower the porosity, making it possible to attain high strength [10]. The impossibility of using them in situ appears to be the only drawback of this group of materials.

Previously, we attempted to develop calcium-phosphate reaction-associated materials based on non-stoichiometric HA, but the samples obtained exhibited significant strength variance, low crack resistance and a high percentage of re-

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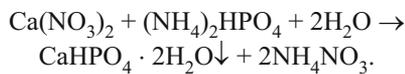
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jects [11]. In the present work an improved technology for obtaining reaction-associated materials was tested; specifically, the composition of the solutions used for solidifying the formed paste was expanded.

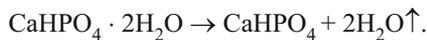
## MATERIALS AND METHODS

The precursor for obtaining reaction-associated material based on nonstoichiometric hydroxyapatite was  $\alpha$ -tricalcium phosphate ( $\alpha$ -Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>,  $\alpha$ -TCP), which possesses relatively high porosity and permits the cement paste to set rapidly and effectively.

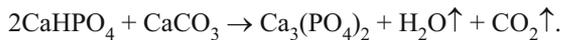
First, to synthesize this compound brushite (CaHPO<sub>4</sub> · 2H<sub>2</sub>O) was obtained by the solution method



The precipitate obtained was carefully washed, filtered and dried at temperature 260°C in 12 h to obtain monetite



The monetite was mixed with an appropriate amount of calcium carbonate in a ball mill under a layer of acetone in 15 min. The mixture formed was dried at room temperature in 24 h to remove acetone residues and annealed at 1400°C for 1 h to obtain  $\alpha$ -TCP



The  $\alpha$ -TCP powder was mixed with different liquids (water, 0.5 M solution of sodium nitrate, 1 M solution of NaHCO<sub>3</sub>), after which the paste obtained was placed into an 8-mm in diameter dismountable press mold and pressed under pressure 400 MPa for 1 min. The column obtained was held at room temperature for 24 h for initial setting and then placed in a hydrostat and kept in water or a solution at 60°C for 24 h, after which it was finally dried and prepared for tests (the technological scheme for obtaining reaction-associated material is presented in Fig. 1).

X-ray diffraction studies were performed with a Rigaku D/Max-2500 diffractometer using Chang's method [12]. The microstructure of the samples was investigated with a scanning electron microscope equipped with a LEO SUPRA 50VP autoemission source (CarlZeiss, Germany). The IR absorption spectra of the samples were recorded with a SpectrumOne IR spectrophotometer (Perkin-Elmer, USA).

Cylindrical samples with diameter-to-height ratio 1 : 1.5 (diameter 8 mm, height 12 mm) were prepared for mechanical tests to determine strength. The samples were subjected to uniaxial (along the axis of the cylinder) compression with strain rate 1 mm/min on a R-05 universal testing machine equipped with a Spider (Germany) multichannel measuring system.

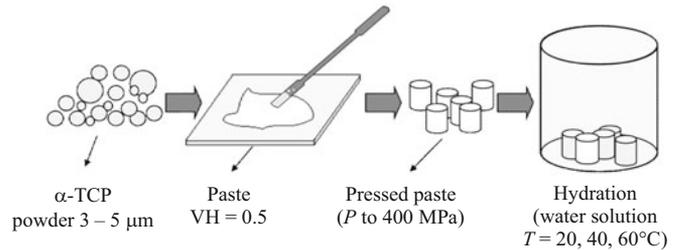


Fig. 1. Scheme for obtaining reaction-associated material.

The cytotoxicity was investigated using extracts and samples of compact materials following [13, 14]. The viability of the cells was assayed by the MTT test based on the reduction of the colorless salt of tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT) by mitochondrial and cytoplasmic dehydrogenases of live metabolically active cells with formation of light-blue formazan crystals dissolved in dimethylsulfoxide. Color development was recorded by measuring the optical density at wavelength 540 nm in the wells of a 96-well culture plate using a photometer (model 680 BIO-RAD, USA).

## RESULTS AND DISCUSSION

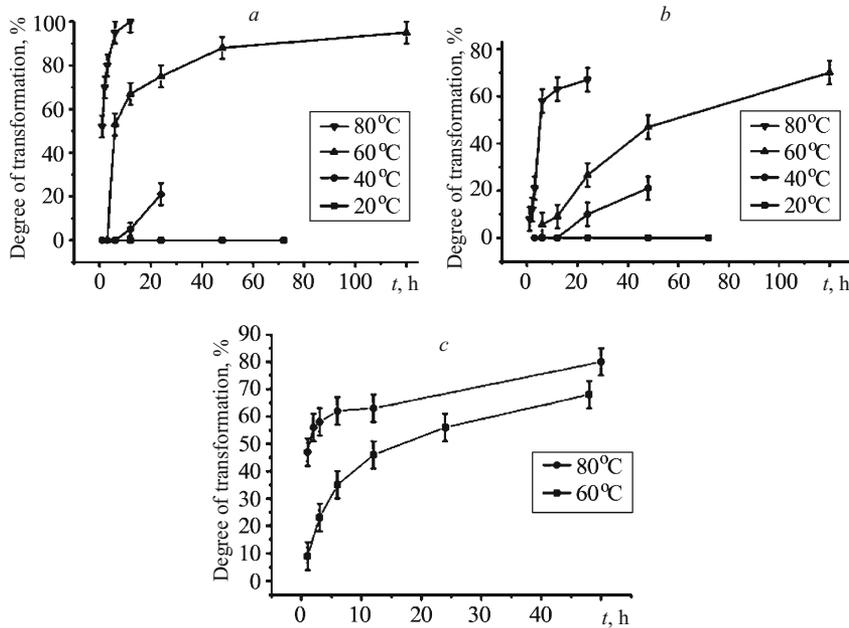
Our previous works on the preparation of reaction-associated calcium phosphate implants showed a bottleneck in the technological scheme — the stage of press-out of cement paste from a fixed cylindrical compression mold [11]. It should be noted that when paste is compressed it undergoes initial setting and loses plasticity. For this reason, during press-out, because of significant friction of the surface of the pressed paste against the wall of the matrix of the press mold cracking of the pressed part occurs (accompanied by a characteristic acoustic effect). Experience shows that the cracks formed do not close at the stage of solidification of the pressed part in a water medium. This results in the following:

- significant number of rejected (cracked) pressed parts (to 50%);
- low ultimate strength (in compression) to 60 MPa for the remaining whole solidified parts;
- Weibull distribution of the ultimate strength of parts in the same batch, indicating the presence of a mainline crack in seemingly high-quality parts.

The use of a press mold with a conical four-section die makes it possible to avoid the press-out stage and, correspondingly, eliminate the problems indicated above.

In the present work, aside from water, solutions of sodium citrate and hydrocarbonate were used as mixing liquids and solidification medium for the cement pastes formed.

Sodium citrate was used for several reasons. First, it is a complex-forming agent with respect to the cation Ca<sup>2+</sup>, as a result of which it increases the solubility of the initial  $\alpha$ -TCP (and, therefore, supersaturation of the solution with respect to HA), resulting in longer setting times for the cement paste

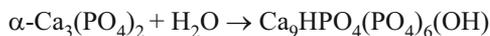


**Fig. 2.** Degree of transformation of  $\alpha$ -tricalcium phosphate into non-stoichiometric hydroxyapatite versus the hydrolysis time  $t$ : in water (a), sodium citrate solution (b) and sodium hydrocarbonate solution (c) at different temperatures.

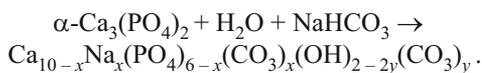
and finer microstructure of the hardened cement mix. Second, the citrate ions present in the solution can become adsorbed on the surface of the embryos of nonstoichiometric apatite crystals, significantly slowing their growth, which also contributes to obtaining material consisting of very small particles of HA, which has a positive effect on its solubility and, of course, on the strength. Third, the use of a solution of sodium citrate as mixing liquid makes it possible to significantly decrease the water/cement ratio in the paste obtained and thus it significantly it, playing the role of a plasticizing agent.

The use of a sodium hydrocarbonate solution as a mixing liquid and solidification medium results in the formation of carbonate-substitute hydroxyapatite (CHA), characterized by even higher solubility with respect to the nonstoichiometric apatite and is comprised of fine equiaxial particles. Thus, the chemical reactions occurring during the solidification of reaction-associated materials can be schematically written as follows:

in the cases of mixing with water and a sodium citrate solution



and mixing with sodium hydrocarbonate



From the chemical standpoint such reactions must be interpreted as hydrolytic.

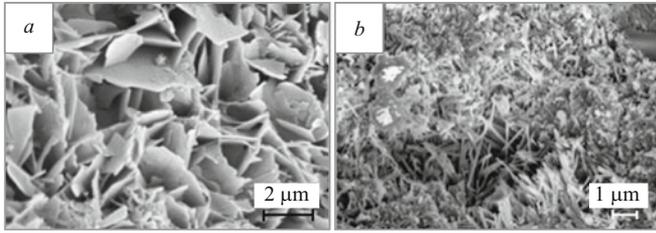
The results of a quantitative x-ray phase analysis of the samples obtained in different solidification media and at different temperatures are presented in Fig. 2. Evidently, of the time dependences of the degree of conversion of  $\alpha$ -TCP into

HA presented in the figure, the rate of the hydrolysis reaction at 20 and 40°C is too low for the reaction to be used in practice to create compact materials. In water at 60 and 80°C the reaction is practically completed in 24 and 6 h, respectively. The reaction slows down considerably when sodium citrate and hydrocarbonate solutions are used, which is probably associated with blocking of crystal growth in the first and formation of more soluble CHA in the second case.

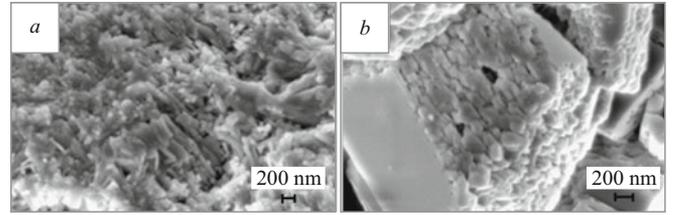
IR-Spectroscopy shows that nonstoichiometric apatite forms in the course of hydrolysis, since the spectrum contains bands corresponding to vibrations of  $\text{HPO}_4^{2-}$ , which are absent in pure HA. These bands are much weaker in the case of sodium citrate, since the alkaline medium formed by it results in a lower concentration of hydrophosphate ions in the solution and therefore displacement of the ratio Ca/P toward pure HA. In the case of hydrolysis in a sodium hydrocarbonate solution bands characteristic for vibrations of carbonate groups in the apatite structure are found to be present, which indicates the formation of CHA; in addition, as the synthesis temperature decreases, the degree of substitution in the formula for CHA increases.

Investigations of the microstructure of samples of reaction-associated materials have shown that the hydrolysis of TCP in water starts with the formation of fine needle-shaped apatite crystals on its surface, which subsequently intergrow, because of morphological selection of crystal embryos, into platelets at 60°C or continue to grow in the form of needles at 80°C (Fig. 3).

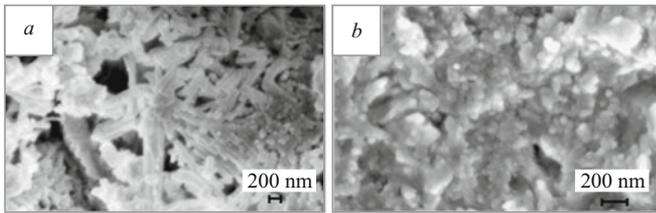
In the case of sodium citrate a gel-like structure forms initially and subsequently closely packed crystals of uniaxial shape of the order of 50 nm in size form at 60°C (Fig. 4a). At higher temperatures a combined morphology with the presence of uniaxial and prolate particles is observed (Fig. 4b).



**Fig. 3.** Photomicrographs of samples synthesized in water at temperatures 60 (a) and 80°C (b).



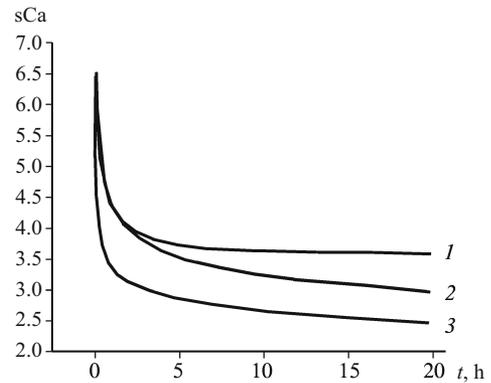
**Fig. 5.** Photomicrograph of samples obtained in a sodium hydrocarbonate solution at temperatures 80 (a) and 60°C (b).



**Fig. 4.** Photomicrograph of samples obtained in a sodium nitrate solution at temperatures 80 (a) and 60°C (b).

In the case of sodium hydrocarbonate spherical particles of the order of 100 – 200 nm in size are formed, and as the reaction proceeds they form an ordered structure comprised of hexagonal meso-crystals, which is not observed at higher temperature (Fig. 5).

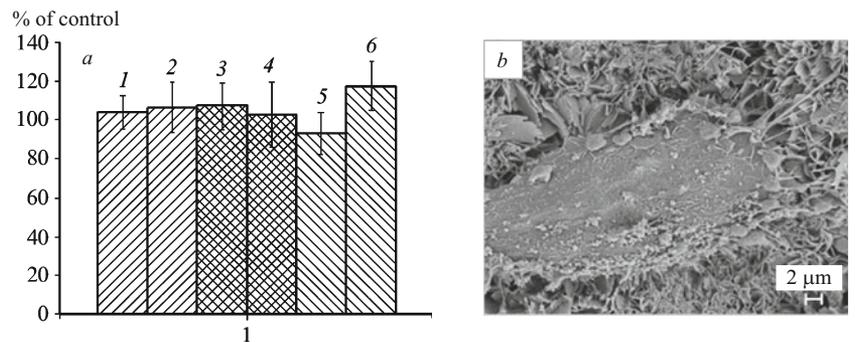
In vitro tests have shown that sodium citrate increases solubility (probably because of a change in particle size); the sample obtained using sodium hydrocarbonate exhibits the highest solubility because of the formation of the more soluble carbonate hydroxyapatite (Fig. 6). An investigation of the effect of extracts of materials on cells showed an absence of water-soluble fractions that have an accelerating effect on their metabolic activity, as indicated by the very small difference with the control based on the MTT-test data (the metabolic activity, %, of cells for samples were determined in comparison with the number of surviving cells on glass, taken as 100%). All materials maintained adhesion of the cells on the surface and supported their active spreading (Fig. 7).

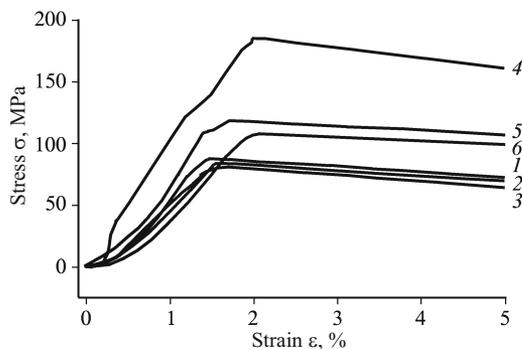


**Fig. 6.** Variation of the content of calcium ions sCa in solution with samples obtained using water (1), a solution of sodium citrate (2) and a solution of sodium hydrocarbonate (3).

The strength of the dense samples obtained under different conditions was studied (Fig. 8, Table 1). The slow decrease of the stress after the ultimate strength of the sample (fracture) is reached indicates quite high resistance to crack growth in the volume of the material probably because of branching of the cracks and dissipation of the elastic energy at the contact boundary of the HA crystals and in pores. The most interesting samples obtained using sodium hydrocarbonate at 60°C are the ones with strength of the order of 120 MPa and the best solubility as well as the ones obtained in sodium citrate at 80°C, which possess the highest strength — of the order of 180 MPa, higher than the strength of tubular bone.

**Fig. 7.** Viability (metabolic activity, %, according to MTT-test data) of NCTC L929 cells during incubation with extracts from materials: 1, 2) materials mixed with water with porosity 0 and 50%, respectively; 3, 4) materials mixed with sodium citrate with porosity 0 and 50%, respectively; 5, 6) materials mixed with NaHCO<sub>3</sub> with porosity 0 and 50%, respectively (a); characteristic form of the cell spreading on the surface of the material (b).





**Fig. 8.** Examples of loading curves for samples (to fracture) under different conditions: in water  $T = 60^{\circ}\text{C}$  (1),  $T = 80^{\circ}\text{C}$  (2); in sodium citrate solution  $T = 60^{\circ}\text{C}$  (3),  $T = 80^{\circ}\text{C}$  (4); in sodium hydrocarbonate solution  $T = 60^{\circ}\text{C}$  (5),  $T = 80^{\circ}\text{C}$  (6).

## CONCLUSIONS

Reaction-associated TCP/HA composite materials with strength in compression to 180 MPa and acceptable solubility, maintaining adhesion and spreading of cells on them, were obtained. An accelerating effect on the viability and metabolic activity of cells was observed, making it possible to talk about the prospects for using these composite materials as osteoplastic materials.

The kinetics of the main reaction  $\text{TCP} + \text{H}_2\text{O} \rightarrow \text{HA}$ , proceeding in reaction-associated composites TCP/HA at temperatures 20 – 80°C in water medium containing modifying additives (sodium citrate, sodium hydrocarbonate), was studied. Modifying agents slow down the main reaction considerably, and in the case of sodium hydrocarbonate also modify the chemical composition of the product.

An increase in the temperature of the main reaction changes the character of the agglomeration of the HA embryos with a change of the habitus of the crystals from plate- to needle-shaped in the case of the reaction in water. Modifying additives change considerably the morphology of HA embryos and give a denser microstructure with a different character of the agglomeration of the crystallites. The smaller size of the crystallites and the high density of the contacts of HA crystals with the use of modifiers make it possible to attain higher macroscopic strength.

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**TABLE 1.** Ultimate Strength of Samples Obtained under Different Conditions

| $T, ^{\circ}\text{C}$ /<br>mixing liquid | Water      | Sodium citrate | Sodium<br>hydrocarbonate |
|--|------------|----------------|--------------------------|
| 60                                       | $87 \pm 4$ | $77 \pm 3$     | $120 \pm 5$              |
| 80                                       | $84 \pm 4$ | $180 \pm 7$    | $110 \pm 5$              |

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