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Hydroxyapatite for Filling Bone Tissue Defects

A. T. Teleshev[1], V. N. Gorshenev[2], M. A. Yakovleva[2]*, V. A. Fomichev[1], R. S. Fadeev[3], V. V. Minaychev[4], and V. S. Akatov[3]

Hydroxyapatite was synthesized in conditions of mechanoacoustic treatment of an aqueous reaction mixture consisting of ammonium hydrogen phosphate and calcium nitrate using a commercially available rotary pulsation apparatus. Mechanoacoustic treatment of the reaction mixture was found to lead to the formation of nanosized hydroxyapatite particles with a predominant mean diameter of about 20 nm. Purification of technical hydroxyapatite to remove traces of ammonium nitrate by thermal processing at 350°C is proposed. The crystalline phase of hydroxyapatite was found not to be dominant in the structure. The porosity of hydroxyapatite amounted to about 75% with a mean pore diameter of 2.8 × 103 nm. Thermal processing of hydroxyapatite in aqueous medium was found to yield a paste-like form. Hydroxyapatite paste had no cytotoxicity and did not prevent cell adhesion, though it almost completely inhibited cell spreading.

Introduction

Bone tissue is a living heterogeneous material consisting of cells and extracellular matrix, which is made up of a combination of collagen fibrils and a mineral phase (hydroxyapatite, HAP, carbonate apatite), along with water [1-3]. The organic fibrillar material consists mainly of type 1 collagen fibers [4]. Collagen fibers are elastic and resistant to breaking, in contrast to the mineral phase, which determines the hardness of bone tissue [5, 6]. The combination of high impact strength and the high rigidity of the mineral component generates the excellent mechanical properties of bone [7]. Artificial constructs made of composite materials including organic and mineral parts are of great interest for the development of the manufacture of biomaterials simulating the structure and properties of natural bone tissue and suitable for regenerating the musculoskeletal apparatus. Many data [8-10] have demonstrated the high biocompatibility of compositions combining HAP and collagen. The technical issues of controlling the formation of the structure of the material while creating artificial biocompositions remain unresolved. The extent of dispersity of the mineral part of the composition has significant influence on mechanical strength. Synthetic HAP in which the particles have high dispersity may better support the osteoblast differentiation of mesenchymal cells [11, 12].

The aim of the present work was to optimize the synthesis of HAP suitable for filling defects in bony tissue and developing osteoconductive and osteoinductive materials.

Experimental Section

HAP was prepared using a rotary pulsation apparatus (RPA) (OOO NPP Aviatekhnika) [13]. From the point of view of monitoring the process, the best method of preparing HAP of the required purity is the well-known (for example, [9, 14]) exchange reaction between diammonium phosphate and calcium nitrate. HAP synthesis using the RPA was performed as follows. Calcium nitrate tetrahydrate (450.6 g) was dissolved in 5 liters of distilled water, and concentrated ammonia solution was

[1] Moscow Pedagogical State University, Moscow, Russia.
[2] N. M. Emanuel Institute for Biochemical Physics, Russian Academy of Sciences, Moscow, Russia; E-mail: lina.invers@gmail.com
[3] Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, Russia.
[4] Pushchino State Institute of Natural Sciences, Pushchino, Moscow Region, Russia.
* To whom correspondence should be addressed.

added to reach pH 10.1 (solution No. 1). Solution No. 2 was prepared by dissolving 151 g of diammonium phosphate in 2 liters of distilled water and adding concentrated ammonia solution to reach pH 10.0. With refrigeration switched on, solution No. 1 was placed in the load vessel and the rotor was switched on at 2500 rpm. Solution 2 was added to the load vessel at a rate of 0.5-1 liter/min. The mixture was treated in the working mode for 2 min and decanted.

Purification of HAP by decantation. The resulting HAP-containing pulp was left to stand, the liquid was decanted, and the residue was washed with distilled water and the process of standing, decanting, and washing was repeated. The residue was transferred to a vacuum filter and filtered, and the retained material was washed with ethanol and hot water to the pH of the washing water, which was 7.5. The residue was dried at 120°C for 3 h.

Purification of HAP by centrifugation was performed on an Allegro 64R centrifuge (Beckman) at 6000 rpm for 15-20 min at 10°C.

Thermal purification of HAP was performed by heating the filtered pulp to 350°C for 30 min.

Particle size in the disperse phase was assessed by dynamic light scattering on a Zetasizer Nano instrument (Malvern). IR spectra were taken in reflection mode on a Nicolet 380 spectrometer with ZnSe glass. Elemental analysis was run on an Eager 300 CHN analyzer. Calorimetric curves were obtained on a Netzsch apparatus, model DSC-204 F1. X-Ray analysis was performed on an Emma diffractometer (GBC Scientific Equipment Pty Ltd.). A Neon 40 EsB scanning electron microscope was used.

In Vitro Studies of the Biocompatibility of HAP Paste

NIH/3T3 mouse embryo fibroblasts were obtained from the All-Russian Collection of Cell Cultures (Institute of Cytology, Russian Academy of Sciences, St. Petersburg). Cells were cultivated in DMEM/F12 liquid nutrient medium (Sigma-Aldrich, USA) supplemented with 10% embryonic calf serum (Gibco, USA) and 80 μg/mL gentamicin sulfate (Sigma-Aldrich, USA) at 37°C in a 5% CO_2 atmosphere. The in vitro biocompatibility of HAP paste was tested by seeding cells on a microscope slide of thickness 170 μm. Half of the slide was precoated with HAP paste and the other half remained uncoated. Cells were seeded on the surface of the slide at a density of 1×10^4 cells/cm^2. Cytotoxicity and mitotic activity were evaluated 72 h after seeding, when cells were also photographed. The reference sample was commercial ReproBone novo paste (Ceramisys, UK).

Cytotoxicity Test

The numbers of live and dead cells were determined by fluorescent microscopy after staining with fluorescent stains calcein AM (Sigma-Aldrich, USA) and propidium iodide (Sigma-Aldrich, USA). Cells were stained in culture medium containing 1 μg/mL calcein AM and 2 μg/mL propidium iodide for 25 min at 37°C. Analysis was performed by counting at least 500 cells. The numbers of live and dead cells were analyzed using a DM 6000 fluorescence microscope (Leica, Germany). Images were also obtained by staining cells with fluorescent stains Hoechst 33342 (Sigma-Aldrich, USA) and acridine orange (Sigma-Aldrich, USA) at concentrations of 1 and 2 μg/mL respectively for 10 min at 37°C. Images were obtained using a TCSSP5 confocal microscope (Leica, Germany).

Analysis of Mitotic Activity

Numbers of mitotic cells were determined by fluorescence microscopy using vital staining with the fluorescent nuclear stain Hoechst 33342 (Sigma-Aldrich, USA) at a concentration of 1 μg/mL. Mitotic cells were detected from the distributions of chromatin typical of prophase, metaphase, anaphase, and telophase using a DM 6000 fluorescent microscope (Leica, Germany). At least 500 cells were used for the analysis. The mitotic index (MI) was calculated as $MI = ((P + M + A + T)/N) \times 100\%$, where $(P + M + A + T)$ is the total number of cells at the prophase, metaphase, anaphase, and telophase, respectively, and N is the number of cells analyzed.

Statistical analysis

Experimental results are presented as means ± standard errors ($M \pm SEM$). Experiments were performed in at least three repeats ($n \geqslant 3$). Statistically significant differences were identified using the Mann–Whitney test.

Results and Discussion

Mechanoacoustic activation of the exchange reaction between diammonium phosphate and calcium nitrate allows nanosized HAP particles to be formed. It should be noted that a decrease in the rate of delivery of diammonium phosphate solution into the reactor and an increase in the rate of mixing of reagents led to an increase in dispersity. Thus, a rate of rotation of the RPA

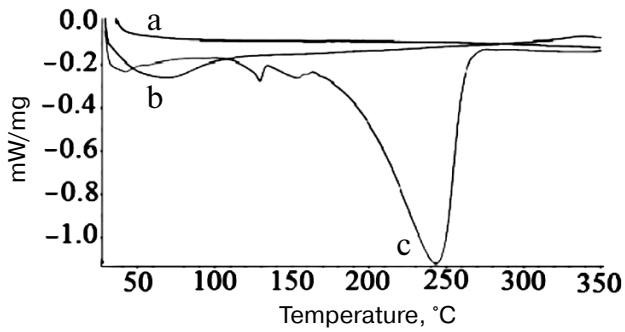

Fig. 1. Calorimetric curves: a) HAP heated to 350°C; b) HAP purified by washing; c) ammonium nitrate.

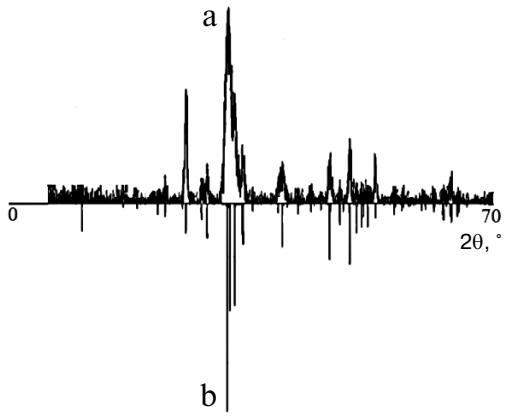

Fig. 2. Diffractograms: a) thermally treated HAP; b) published diffractogram of HAP [15].

of 3000 rpm and a rate of addition of diammonium phosphate solution to the reactor of 0.5 liter/min produced a dispersion characterized by particles with a mean diameter of about 20 nm. The proportion by weight of these particles in the reaction was ≈ 90%. The following variations on the preparation of pure HPA were used: purification of HAP by decantation and purification of HAP by centrifugation (this approach, in contrast to sedimentation in the gravitational field, increased the rate of the purification process). Purification of HAP by washing involved using large quantities of water and could not be regarded as effective. Thermal treatment of HAP to purify it from contaminants as used in [14] was of interest. HAP samples contaminated with ammonium nitrate could be purified successfully by thermal treatment at 350°C for 30 min. Thus, filtration of pulp and single washing of the retentate with water followed by drying at 120°C for 3 h produced a sample of technical HAP contaminated with ammonium nitrate (absorption bands at 1330 and 820 cm^{-1}). These bands were not seen in the IR reflection spectra of the thermally treated HAP sample. Elemental analysis data for purified HAP confirmed the complete absence of nitrogen-containing contaminants in thermally treated HAP (using ammonium nitrate as the standard). Additional confirmation of the absence of ammonium nitrate in purified HAP samples was provided by colorimetric studies (Fig. 1).

HAP prepared by the exchange reaction and subjected to heat processing formed a xerogel with a typical blue luminescence. The diffractogram of the thermally treated HAP sample is shown in Fig. 2. The crystalline phase of HAP, with an elementary hexagonal cell of dimensions:

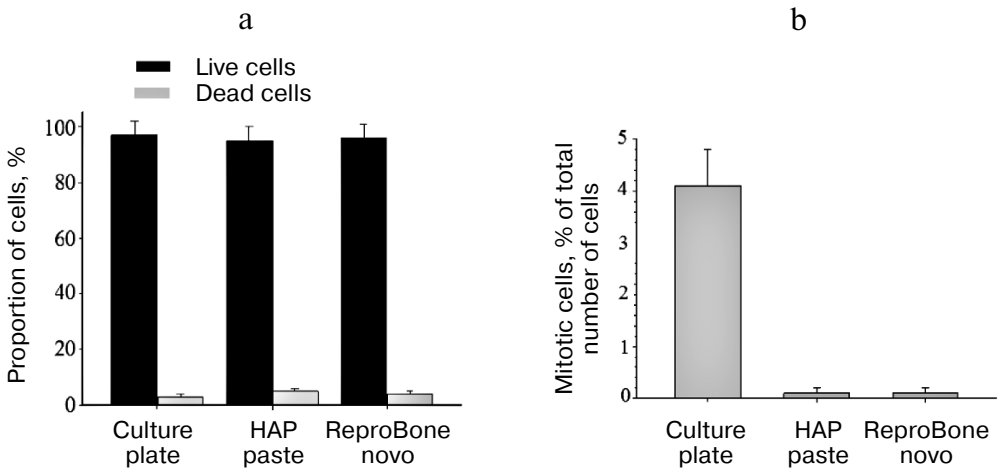

Fig. 3. Cell viability of cells on HAP paste and ReproBone novo paste (a); absence of mitotic activity of cells cultured on the surfaces of HAP and ReproBone novo (b) pastes.

a) = 9.4257 Å, b) = 6.8853 Å, is identified. The space group was P63/m.

Standard contact porometry in terms of changes in the weights of samples treated in vacuo with octane showed that the porosity of heat-treated HAP was about 75% with a mean pore radius 2.8×10^3 nm. HAP purified from contaminants and dispersed in water to form the paste showed changes in properties over time. These changes were in particular directed towards increases in the sizes of particles in the disperse phase. Thus, after holding the dispersion of purified HAP in aqueous medium for 30 days at 20°C, the mean particle diameter increased to 44 nm.

The ζ potential dropped from +30 mV for the dispersion of unpurified hydroxyapatite to about +5 mV, typical of the dispersal of purified hydroxyapatite.

Studies of the biocompatibility of HAP paste were performed 72 h after starting cell cultivation. Figure 3a shows the results of studies of biocompatibility. HAP paste, like ReproBone novo paste, had no cytotoxic action. The numbers of dead cells on cultivation on these samples were no different from the numbers of dead cells on cultivation on the surface of the culture plate. The mitotic activity of cells cultured on slides coated with HAP paste was assessed 72 h after the beginning of cultivation. Cells on the HAP paste surface, as on the ReproBone novo paste, were unable to divide. Cultivation of cells on culture plates showed that the mitotic index after 72 h of cultivation was (4.1 ± 0.8)% (Fig. 3b). These data led to the suggestion that the pastes could prevent adhesion and spreading of cells on the paste surfaces. This suggestion was tested by morphological analysis of adhesion and spreading of cells on the surfaces of HAP and ReproBone novo pastes. Cells were found to attach to the paste surfaces, though only a proportion of cells were able to spread on pastes, this proportion being about (35 ± 5)% of the total.

This characteristic was found for both the HAP paste developed here and for the ReproBone novo paste.

Conclusions

Mechanoacoustic processing of the reaction mix was found to lead to the formation of nanosized hydroxyapatite particles mostly with a mean diameter of about 20 nm. Technical HAP was purified from traces of ammonium nitrate by thermal treatment at 350°C. HAP paste was found to have no cytotoxic activity and not to prevent cell adhesion, though it significantly inhibited spreading of cells and reduced their mitotic activity.

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