ISSN 1070-3632, Russian Journal of General Chemistry, 2008, Vol. 78, No. 4, pp. 521–526. © Pleiades Publishing, Ltd., 2008.
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Effect of Alizarin Red S on the Formation of Hydroxyapatite Crystals

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Received June 7, 2007

Abstract—Hydroxyapatite samples were prepared in systems calcium nitrate–diammonium hydrogen phosphate–ammonium hydroxide–organic component with addition of microamounts of Alizarin Red S. The participation of sulfo and hydroxy groups of the organic compounds in the formation of hydroxyapatite crystals was confirmed by X-ray phase analysis, IR spectroscopy, electron microscopy, and elemental analysis.

DOI: 10.1134/S1070363208040038

Numerous studies show that composites of hydroxy-apatite with organic biopolymers are better compatible with living tissues. Both collagen-containing hydroxyapatite forms and its composites with polysaccharides, e.g., Chonsuridum [1–5], are fairly actively used today. Furthermore, natural polysaccharides participating in the formation of bone tissues are also anticoagulants. For example, chitosan sulfate exhibits anticoagulant activity increasing with an increase in the degree of sulfation. Structurally related heparin is used in medicine for treatment and prevention of thromboses. It is known that noncollagenous proteins such as osteopontin, osteocalcin, osteonectin, etc., play a crucial role in mineralization and demineralization processes [7]. At the same time, osteopontin, for example, is a sulfated phosphoprotein binding calcium and forming an adsorption layer between crystals of hydroxyapatite and the biomaterial. It should also be noted that sulfo derivatives of aromatic compounds are often used as histological reagents owing to their capability to bind calcium ions into strong lacquers [8]. Presumably, anticoagulant activity of the above derivatives, as well as the participation of chondroitin sulfate and osteopontin in the formation of bone tissues, depend on the formation of calci-um complexes necessarily involving sulfo groups of these polymers. In this case, compounds with similar arrangement of the –OH and $-SO_3^-$ groups should control the growth of hydroxyapatite crystals. To evaluate this effect, we chose Alizarin Red S (sodium 1,2-dihydroxyanthraquinone-3-sulfonate) used as indicator for the determination of calcium in serum and urine and also known as histological reagent for revealing calcium in various cuts [9].

In this study we examined the effect of additions of Alizarin Red S and of the process conditions on the precipitation of hydroxyapatite from aqueous solutions containing calcium nitrate, diammonium hydrogen phosphate, ammonium hydroxide, and an organic component.

System $Ca(NO_3)_2$–$(NH_4)_2HPO_4$–NH_4OH–H_2O. As already noted in [10–15], the synthesis results depend on the order of mixing the reactants, rate and time of stirring, amount of ammonia added, temperature, and configuration of the temperature field in the initial step of the aggregation. Aiming to reduce the synthesis time, we performed a series of analogous experiments in which these conditions were varied. We found that prolonged stirring (>14 days) can be avoided by dropwise addition of the phosphorus precursor to the calcium precursor and careful introduction of an additional amount of ammonia only after noticeable decrease in pH of the solution (see Experimental). Under these conditions, hydroxyapatite was prepared within 24–36 h.

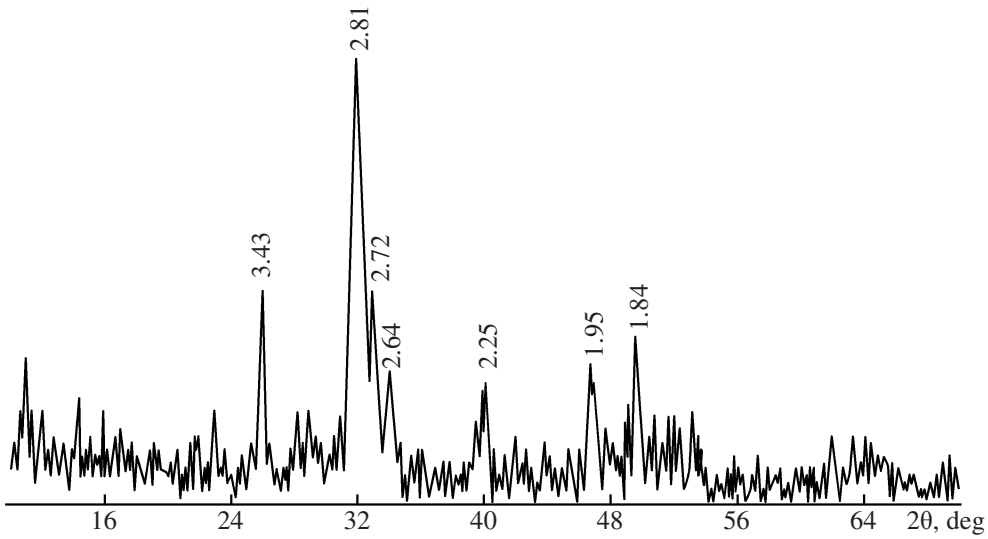

Fig. 1. Diffraction pattern of a hydroxyapatite sample with addition of Alizarin Red; synthesis time 24 h.

Single-step mixing of the reactants followed by stirring under similar conditions always yielded amorphous products.

The temperature conditions affected only the size and degree of hydration of the particles formed, i.e., the outward appearance of the hydroxyapatite precipitates. Gradual increase in the temperature from 15 to 55°C in the course of the synthesis resulted in coarsening of the crystals.

Examination of the effect exerted by an excess of the calcium or phosphate precursor on the composition and subsequent transformations of the phases was beyond the scope of this study. In all the experiments we used the optimal molar ratio Ca : P = 1.67 recommended by the majority of authors [10–15].

We performed a comparative synthesis of hydroxyapatite in the aqueous system $Ca(NO_3)_2$–$(NH_4)_2HPO_4$–NH_4OH without additions (I) and with addition of microamounts of Alizarin Red S (I*).

Addition of the above modifier resulted in crystallization of the precipitate. Precipitate I was a gel-like mass, whereas precipitate I* consisted of pale pink, readily filterable crystals.

The IR spectra of sample I* exhibit no noticeable bands of functional groups of Alizarin Red S and do not appreciably differ from the spectra of straight hydroxyapatite. The stretching vibration band of the hydroxy group $\nu(OH^-)$, characteristic of hydroxyapatite, is observed at 3617 cm^{-1}; the stretching and bending vibration bans of phosphate groups $\nu(PO_4^{3-})$ at 1047, 604, and 569 cm^{-1} and of carbonate group $\nu(CO_3^{2-})$ at 1420 and 1385 cm^{-1} are also well pronounced. The results of elemental analysis confirm that the product is carbonatohydroxyapatite.

The samples obtained were not calcined. Nevertheless, they show fairly clear diffraction patterns characteristic of hydroxyapatite. Depending on the synthesis conditions, both crystalline and gel-like precipitates could be obtained, but, according to X-ray phase analysis, the gel-like precipitates were not amorphous. This fact is attributable to variation of the degree of hydration of the particles and to the size effect [16]. Coarser crystals always formed in system I*. Figure 1 shows a typical diffraction pattern of hydroxyapatite I* after stirring for 24 h. It is known from the literature that, under the conditions of our experiments, the formation of intermediate phases of β-tricalcium phosphate and octacalcium phosphate (see, e.g., [1, 7, 11] and references therein) could be expected. However, X-ray phase analysis revealed no side phases in our samples.

Significant alterations of the hydroxyapatite crystal lattice were not revealed, either. It is interesting that single-step addition of the phosphate precursor to the calcium precursor at the standard temperature and pH 9.5 leads to a decrease in the concentration of calcium ions in the solution in the first minutes of the reaction by a factor of 10. In the presence of Alizarin Red S, under equal other conditions, this concentration decreased by a factor of 25, which confirms the parti-

cipation of the dye in the aggregation step. We succeeded in fixing this initial step of hydroxyapatite formation in the presence of the modifier. Figure 2 shows the diffraction pattern of the intermediate compound of system I*, obtained by mixing the reactants and filtering and washing the precipitate within 15 min. Despite a low degree of crystallinity, strong diffraction maxima (≥50%) corresponding to interplanar spacings *d* 2.867, 2.788, 2.755, and 2.667 Å could be revealed against the background of the main amorphous phase. As noted above, single-step mixing of the calcium and phosphate precursors in an alkaline solution without additions of the modifier always yields an amorphous mass which transformed into hydroxyapatite very slowly, and no intermediate phases were isolated.

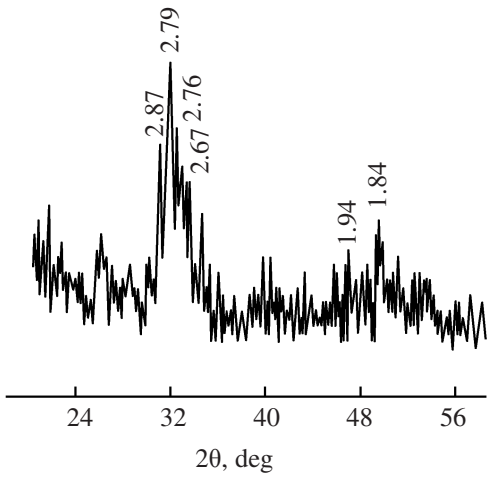

Fig. 2. Diffraction pattern of the intermediate compound of system I*. Synthesis time 15 min.

Thus, the organic functional groups (presumably –OH and $-SO_3^-$) affect the formation of hydroxyapatite crystals.

Systems $Ca(NO_3)_2$–$(NH_4)_2HPO_4$–NH_4OH–organic component. The systems both containing Alizarin Red S (samples marked with an asterisk) and without it were studied. As organic component we used gelatin (systems II*, II), glucosamine hydrochloride (systems III*, III), and low-molecular-weight polyvinylpyrrolidone (systems IV*, IV). Taking into account the fact hat the modern "aqueous" syntheses of hydroxyapatite are performed, as a rule, in simulated body fluid (SBF), we performed the synthesis of hydroxyapatite (IV*, IV) in the medium of Haemodesum blood plasma substitute falling into the category of SBFs. The choice of organic components was governed by the following facts: gelatin is the product of collagen hydrolysis and is frequently used in production of materials biocompatible with living body tissues; glucosamine hydrochloride affects the phosphorus and calcium exchange in bone tissues and favors formation of sound cartilaginous matrix; the pyrrolidone ring of polyvinylpyrrolidone is structurally similar to proline, which is a component of collagen and ensures the most important functions of this protein. The modifier, Alizarin Red S, was added to solutions (systems II*–IV*) to reveal its effect on the degree of crystallinity and rate of formation of the hydroxyapatite phase. In all the systems, we expected formation of composites of hydroxyapatite with the corresponding organic components and the presence of by-products.

We found that an additional phase identified by X-ray phase analysis as β-tricalcium phosphate [17, 18] is formed only at a noticeable decrease in pH (below 8) or at delayed introduction of ammonia. The diffraction patterns of such samples usually contain strong peaks at *d* 3.21, 2.80, 1.93, and 1.74 Å. The presence of the β-$Ca_2P_2O_7$ is also possible (the main reflections at *d* 3.23, 3.08, 3.04, 2.76 Å partially overlap with the lines of hydroxyapatite itself and of β-tricalcium phosphate). The content of the impurity phases is about 10% relative to hydroxyapatite after stirring the solution for 24 h. The synthesized crystals can be rapidly and readily filtered. The surface micrographs obtained with a scanning electron microscope demonstrate the presence of a mixture of spherical, platelike, and needle-like aggregates (Figs. 3a, 3b). When ammonia was present from the very beginning of the synthesis (pH 10) and this pH value was maintained throughout the synthesis, hydroxyapatite formed in all the cases as the only calcium-containing crystalline phase, with the amount of the amorphous constituent being different.

The studies showed that the organic components of systems II and IV, as well as prolongation of stirring to 14 days, do not noticeably affect the crystallinity of the hydroxyapatite formed. Product IV is characterized by very low degree of binding of polyvinylpyrrolidone with the hydroxyapatite surface. Gelatin in system III transfers the calcium hydroxyphosphate formed into a colloidal solution, i.e., promotes peptization of the precipitate. This fact is responsible for the low yield of the reaction product. As expected, the hydroxyapatite formed contains a large amount of the amorphous constituent.

Addition of Alizarin Red S affects the outward appearance and hardness of the composites, but does not noticeably affect the degree of adsorption of polyvinylpyrrolidone. Thoroughly washed precipitated do

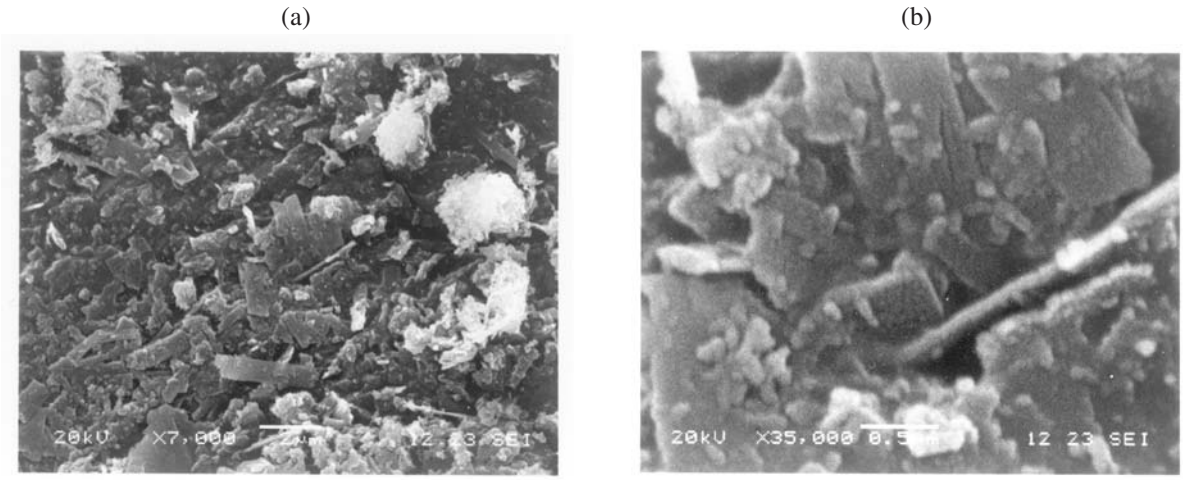

Fig. 3. Micrograph of a mixture of crystals of hydroxyapatite, β-tricalcium phosphate, and β-$Ca_2P_2O_7$. White bar: (a) 2 μm and (b) 0.5 μm.

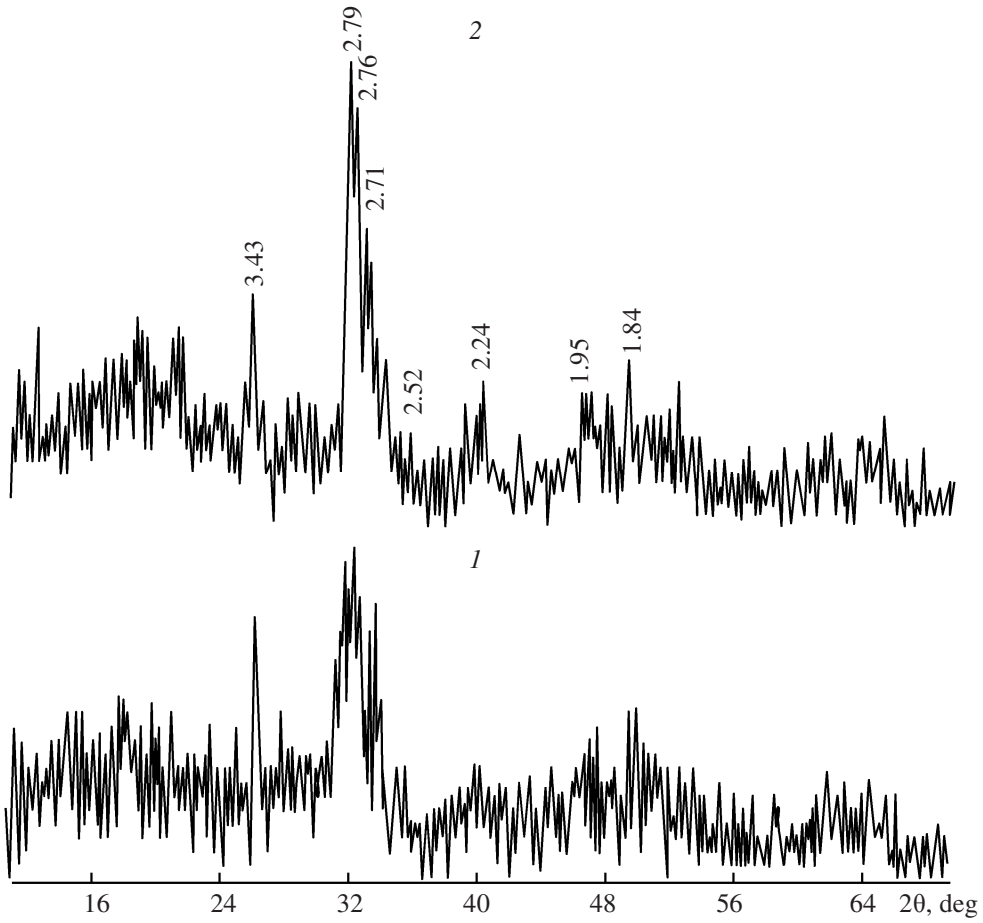

Fig. 4. Diffraction patterns of samples (*1*) II and (*2*) II*. Stirring time 24 h.

not show noticeable incorporation of SBF ions into the crystal lattice of hydroxyapatite, either. According to elemental analysis, the precipitates contain a small amount (0.78–0.88 wt %) of sodium.

Addition of Alizatin Red S to any of these systems (samples II*, III*, IV*) affects the perfection of the crystals and the time of their ripening. Figure 4 shows for comparison the diffraction patterns of samples II

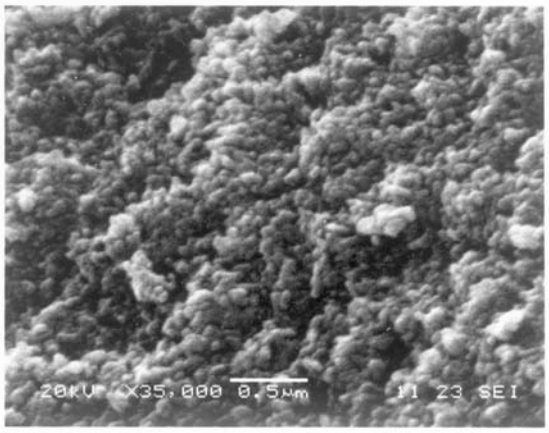

Fig. 5. Micrograph of the surface of hydroxyapatite sample IV; white bar: 0.5 μm.

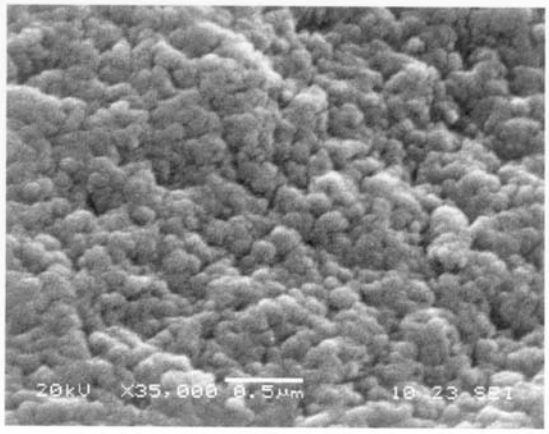

Fig. 6. Micrograph of the surface of hydroxyapatite sample IV*; white bar: 0.5 μm.

and II* after stirring the solutions for 24 h. In most cases, under various synthesis conditions, addition of Alizarin Red S led to an increase in the size of the crystals or to a decrease in the degree of hydration of the particles. Figures 5 and 6 show the micrographs of hydroxyapatite samples prepared in systems IV and IV*, respectively. The precipitates were not subjected to a high-temperature treatment. The particle size of hydroxyapatite IV is 50–100 nm, and that of IV*, 160–200 nm. According to the IR data, hydrogen bonds in samples IV* are formed more actively. The vibration band of the free hydroxy group, ν(OH), is observed at 3626 cm^{-1}, and that of hydrogen-bonded hydroxy group, at 3200–3600 cm^{-1}. Bonds belonging to structures with intramolecular hydrogen bonds (2800–3000 cm^{-1}) and apparently related to polyvinylpyrrolidone are manifested fairly clearly. The stretching and bending vibration bands of the phosphate (1044, 606, 568 cm^{-1}) and carbonate (1422 cm^{-1}) groups [19, 20] are well pronounced.

Thus, introduction of Alizarin Red S modifier into systems $Ca(NO_3)_2$–$(NH_4)_2HPO_4$–NH_4OH–organic component leads to a decrease in the crystallization time of hydroxyapatite and an increase in the particle size, but does not affect the degree of binding of hydroxyapatite with the organic component.

EXPERIMENTAL

As calcium precursor in the synthesis of hydroxyapatite we used a 0.1 M solution of calcium nitrate (Schering-Kahbaum, Germany), and as phosphorus precursor, 0.06 M diammonium hydrogen phosphate (Merck, Germany). The required pH was adjusted with an ammonia solution. Microamounts of Alizarin Red C modifier were introduced at the very beginning of the synthesis. To examine systems II and II*, we prepared a 0.5% solution of gelatin, dissolved $Ca(NO_3)_2$ in it, and performed the synthesis by the general procedure described below. In the case of systems III and III*, glucosamine hydrochloride was added in an amount of 20 g l^{-1}. The composite of hydroxyapatite with polyvinylpyrrolidone (systems IV and IV*) was obtained in SBF, a blood plasma substi-tute (Haemodesum) prepared according to the standard formulation (g l^{-1}): polyvinylpyrrolidone 60, NaCl 5.5, KCl 0.42, $CaCl_2$ 0.5, $MgCl_2$ 0.005, and $NaHCO_3$ 0.23. The solution was stirred with a magnetic stirrer. The phosphorus precursor was added to the calcium precursor dropwise over a period of 9 h. In the process, pH 10 was maintained; if pH decreased to 9.5, ammonium hydroxide was added. Various temperature modes were used: constant temperatures of 25 (T_1) or 55ºC (T_2); increase in the temperature from 15 to 55ºC (ΔT). The amount of calcium in the solution in intermediate steps of the synthesis was determined according to [21]. Then the precipitate was left without stirring and heating for 10 h, after which it was stirred for an additional 5 h. The precipitate was filtered off using a vacuum pump and a glass frit with the pore diameter of 40 and 15 μm, washed with boiled distilled water, and dried in a thermostat at a temperature not exceeding 100ºC. The samples were not calcined, to retain the organic constituent.

Electron-microscopic examination was performed with a JSM-6380 LV device (Japan), and elemental analysis, with an Energy-250 attachment. The IR spec-

tra in the range 4000–400 cm^{-1} were taken with Infralyum FT-02 and Specord IR-75 spectrometers from samples prepared as KBr pellets. The diffraction patterns were recorded with DRON-3M and DRON-7 devices (CuK_α radiation, scanning rate 2 deg min^{-1}).

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