

Formation of Nanocrystalline Hydroxyapatite in Presence of Some Aminoacids

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The influence of three amino acids on the hydroxyapatite formation in vitro under mild condition was investigated. The mineral obtained was studied by transmission electron microscopy and powder X-ray diffraction. The experiments suggest that the addition of these amino acids has a significant effect on the phase composition, crystal size and lattice microstrains of the resulting calcium phosphate mineral.

Keywords: Hydroxyapatite, Amino acids, Phase composition, Crystal size, Controlled growth.

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1. INTRODUCTION

The effect of the addition of organic molecules on the morphology of crystals has been studied long time to understand how crystals having classical geometry when formed in solution, can be so different when formed in organisms. Amino acids are biological molecules that are of particular interest due to their great importance for living organisms. Amino acids move freely in the internal environment of the organism with blood circulation after digestion of proteins. They are able to penetrate into the interior of the cells by simple diffusion, so their concentration is controlled by physiological mechanisms. So, the study of the effect of these biomolecules on calcium apatite crystal growth can be important for the understanding of processes of desirable or pathological calcification. Among the phases of calcium phosphates, hydroxyapatite (HAP) is the most stable phase and it serves as a model of the inorganic component of bones and teeth [1, 2]. Effect of amino acids on the crystallization of HAP has been the subject of a number of researches [3-4, 6-15]. Those studies, aimed to elucidate the mechanism of crystallization of HAP in biological conditions, have dealt mostly with the spontaneous deposition of sediment due to the high level of supersaturation of the mother solution. This led to poor reproducibility and lack of real understanding of the biological processes of calcification. On the other hand, the calcification is the most common cause of inefficiency in heart prosthetic valves made from porcine aortic valves. Mineral deposits of atherosclerotic human aorta consist mainly of calcium apatite (71 %), carbonate (9 %) and contain a high percentage and protein (15 %) [2]. The average ratio Ca / P is ~ 1.7 in the biomineral of a mature atherosclerotic plaque, as well as in mature skeletal biomineral [1, 2]. There is evidence that the processing with aminoacids prevents the calcification of heart valve bioprostheses [14].

Amino acids as potential inhibitors and modifiers of calcification are advantageous compared to other compounds that were used in the past, mainly because the amino acids are physiological substances that roam freely in circulation in all body tissues of vertebrates.

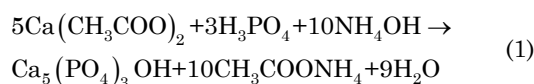
The main source of their inflow in the human body is the metabolic pathways converting protein molecules under digestion. Their concentration in the blood or tissues is locally controlled by physiological mechanisms such as penetration into a cell and subsequent metabolism, or via kidneys. This dramatically decreases the load on the liver, which is a major problem when using synthetic biologically active compounds or pharmaceuticals.

In our study, we investigated the effects of three amino acids (arginine, aspartic acid and alanine) on the formation of HAP nanocrystals in solutions that mimic physiological fluids.

2. MATERIALS AND METHODS

2.1 Sample Preparation

Hydroxyapatite was obtained by a standard reaction (1) as a starting material; calcium acetate and phosphoric acid were taken as source compounds. During the deposition of nanocrystals pH value was gradually accounted to 10.



Amino acids were added to the calcium acetate solution before the reaction had begun. The concentration of all amino acids was 150 mM to have the Ca²⁺/amino acid molar ratio in the solution equal to 1 / 2, since from mass spectrometry measurements [4] it is known that calcium-bound dimeric amino acid complexes are coordinated most strongly. Of course, further investigations with more amino acids and different molar ratios are planned. The resulting suspension was kept 24 h, the precipitate was filtered, washed and air dried. The reaction conditions were not specially adjusted for obtaining a monophasic sediment. Relatively mild conditions of synthesis and drying without very high pH and temperature values were used in order to eliminate the adverse effect of these parameters on the labile organic compounds and to make the reaction conditions closer to physiological.

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As model organic modifiers we used three amino acids with close molecular weights but differently charged (Fig. 1). Arginine, due to its guanidine groups is the most basic amino acid, at physiological conditions it has a positive charge and it is often a part of proteins that interact with DNA, and arginine is the only amino acid among 20 proteinogenic which is positively charged under our experimental conditions. Alanine is a nonpolar amino acid; aspartic acid is polar, both carrying negative charge at the pH level of the stock solution. According to [4], arginine has the highest relative Ca^{2+} -binding strength among proteinogenic amino acids, asparagine is in the middle third, and alanine is in the third having lowest calcium ion binding strength. Also, there are the experimental data on the influence of these three amino acids on the nucleation of biomimetic calcium-deficient hydroxyapatite [5], where the coupling of synthesis and functionalization has been used.

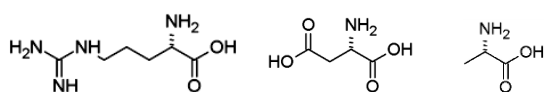


Fig. 1 – Arginine, aspartic acid and alanine

2.2 Data Acquisition and Processing

XRD investigations were performed using the diffractometer DRON4-07 (“Burevestnik”, Russia) connected to the computer-aided experiment control and data processing system. The Ni-filtered $\text{CuK}\alpha$ radiation (wavelength 0.154 nm) was used with a conventional Bragg-Brentano θ - 2θ geometry (2θ is the Bragg’s angle). The current and the voltage of the X-ray tube were 20 mA and 30 kV respectively. The samples were measured in the continuous registration mode (at the speed of 1.0 $^\circ$ /min) within the 2θ -angle range from 10° to 60° . All data processing procedures were carried out with the program package DIFWIN-1 (“Etalon PTC” Ltd, Russia). The separation of overlapping diffraction lines was done with the freeware program New_Profile 3.4 (<http://remaxsoft.ru/>). The phase analysis was carried out by comparing the diffraction patterns from the investigated samples and the reference JCPDS data.

Table 1 – Results of phase analysis of the samples

Sample	PDF-2 № 72-713 (brushite)	PDF-2 № 9-432 (apatite)
HAP, CaAc + H_3PO_4 , pH = 10	+ (54,6 %)	+ (45,4 %)
Ala HAP, CaAc + H_3PO_4 , pH = 10	–	+
Arg HAP, CaAc + H_3PO_4 , pH = 10	–	+
Asp HAP, CaAc + H_3PO_4 , pH = 10	–	+

Table 2 – Size of crystallites in the plane (002) according to Scherrer.

Name	Asp HAP	Ala HAP	Arg HAP	HAP
L , (0 0 2), nm	14,9575	18,7347	22,8231	25,8186
L , 3f conv, nm	14,083	17,6836	22,4573	24,546
$\langle e^2 \rangle^{1/2}$, 3f conv	0,00235	0,00169	0,00096	0,00085
L , (0 0 4), nm	18,0304	21,7889	22,4944	28,16

Table 3 – Evaluation of texture according to Harris. The calculation was performed on the texture reference sample JCPDS № 9-432

Plane	Asp HAP	Ala HAP	Arg HAP	HAP
(0 0 2)	0,92	1,11	1,18	1,24
(2 1 1)	1,08	0,89	0,82	0,76

The morphology of the deposits’ crystals was observed by transmission electron microscopes (TEM) JEM2010 (JEOL, Japan). Before TEM examinations the samples were annealed at 240°C to remove the organic part and then ultrasonically disaggregated (120 W, 22 kHz output frequency).

3. RESULTS AND DISCUSSION

X-ray diffraction data indicate that the addition of amino acids has a significant impact on the formation of hydroxyapatite crystallites in a model system. First of all, during the basic reaction under mild conditions (in the absence of amino acids), the second calcium phosphate phase has been formed in addition to nanocrystalline apatite, the brushite, with the crystallite size in the plane (020) greater than 50 nm. The presence of any of three amino acids prevents the formation of this brushite phase. In all three cases, in the presence of amino acids only nanocrystalline apatite was formed. The results of phase analysis are shown in the Table 1. An increase in preferred orientation in the c-plane can be observed in the samples HAP Asp, Ala HAP, Arg HAP, Hap (estimating from the intensity ratios of lines (002) and (211)).

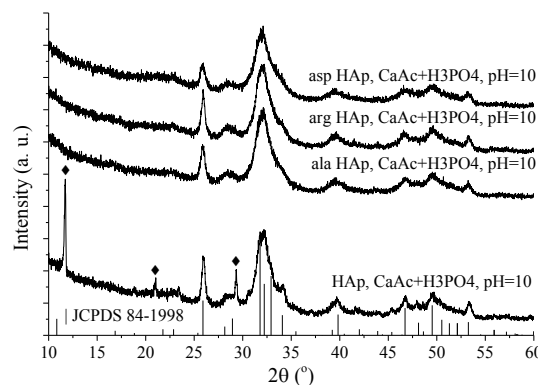


Fig. 2 – X-ray diffraction patterns of samples of HAP synthesized with three amino acids and without amino acids, but obtained using the same basic reaction. In the last sample (HAP without amino acid) also the phase of brushite is present (its main peaks are marked with ♦). Crystallite size of brushite according to Scherrer in the plane (020) is 54.7 nm

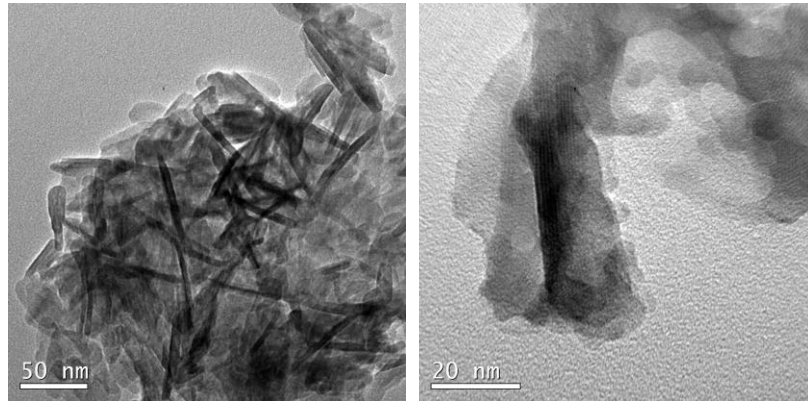


Fig. 3 – Reference sample of nanocrystalline HAP doped with brushite

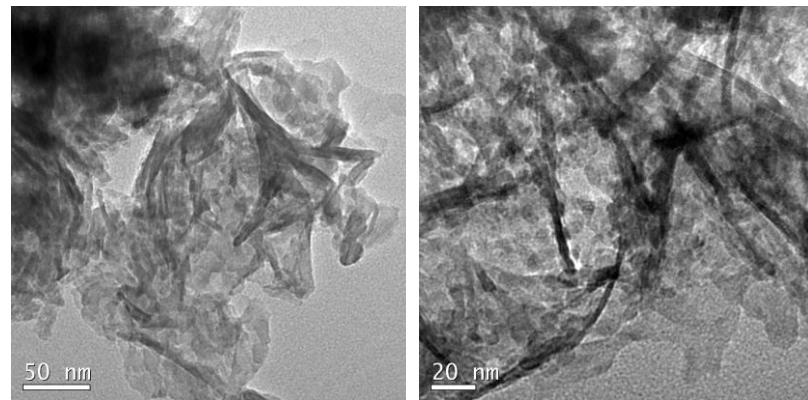


Fig. 4 – HAP synthesized in the presence of arginine

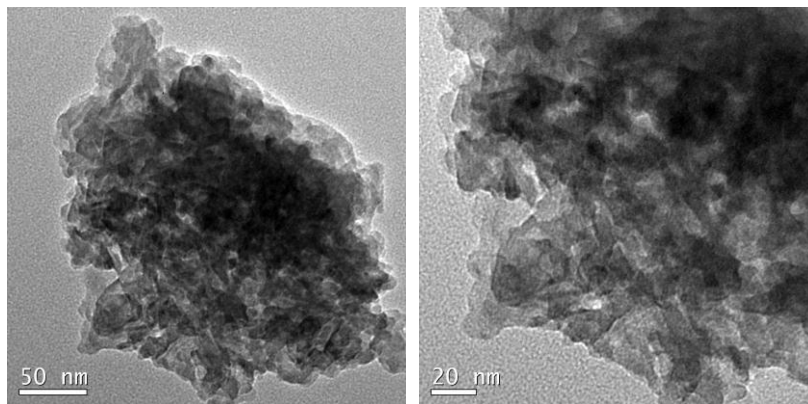


Fig. 5 – HAP synthesized in the presence of aspartic acid

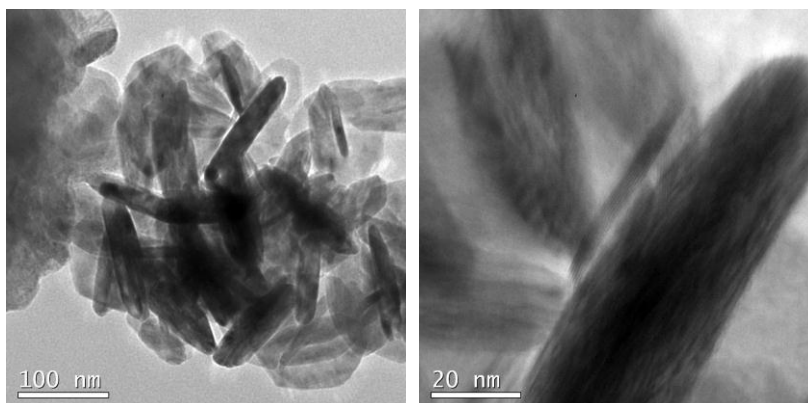


Fig. 6 – HAP synthesized in the presence of alanine

The difference in the intensities of the lines indicates a change in the crystal texturing of the samples depending on the influence of a certain amino acid, but the nanometer-scale size of crystallites makes it impossible to calculate textural characteristics accurately. Evaluation of texture according to Harris is shown in Table 3.

The morphology of the samples was investigated by transmission electron microscopy Fig. 3-5.

4. CONCLUSIONS

An example of the influence of three amino acids (arginine, aspartic acid and alanine) used as a co-reagent in the synthesis of nanocrystalline hydroxyapatite on its crystallographic and morphological properties has been shown. Noteworthy is the fact that the introduction of these amino acids in the synthesis of nanocrystals inhibits formation of non-apatite phases at a given base reaction and promotes the preferred orientation of crystallites in the c-plane, but only in the case of alanine a significant impact on the size and morphology of the crystalline blocks was observed.

Формування нанокристалічного гідроксиапатиту в присутності деяких амінокислот

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Було досліджено вплив трьох амінокислот на формування гідроксиапатиту *in vitro* у м'яких умовах синтезу. Отриманий мінерал було охарактеризовано за допомогою просвічуючої електронної мікроскопії та порошкової рентгенівської дифрактометрії. Експеримент свідчить, що додавання цих амінокислот має істотний вплив на фазовий склад, розмір кристалітів та мікродеформації решітки отриманого кальцій-фосфатного мінералу.

Ключові слова: Гідроксиапатит, Амінокислоти, Фазовий склад, Розмір кристалітів, Контрольований ріст.

Формирование нанокристаллического гидроксиапатита в присутствии некоторых аминокислот

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Исследовано влияние трех аминокислот на формирование гидроксиапатита *in vitro* при мягких условиях синтеза. Полученный минерал был охарактеризован с помощью просвечивающей электронной микроскопии и порошковой рентгеновской дифрактометрии. Эксперимент свидетельствует, что добавление этих аминокислот оказывает существенное влияние на фазовый состав, размер кристаллитов и микродеформации решетки полученного кальций-фосфатного минерала.

Ключевые слова: Гидроксиапатит, Аминокислоты, Фазовый состав, Размер кристаллитов, Контролируемый рост.

REFERENCES

1. J.C. Elliott, *Calcium Phosphate Biominerals* (M.J. Kohn, J. Rakovan, J.M. Hughes (eds) *Phosphates: geochemical, geobiological and materials importance. Series: Reviews in mineralogy and geochemistry, vol. 48.* (Mineralogical Society of America: Washington, DC: 2002).
2. Y. Pan, M.E. Fleet, *Compositions of the Apatite-Group Minerals: Substituting Mechanisms and Controlling Factors* (M.J. Kohn, J. Rakovan, J.M. Hughes (eds) *Phosphates: geochemical, geobiological and materials importance. Series: Reviews in mineralogy and geochemistry, vol. 48.* (Mineralogical Society of America: Washington, DC: 2002).
3. N. Roveri, E. Foresti, M. Lelli, I.G. Lesci, M. Marchetti, *Microscopic investigations of Synthetic Biomimetic Hydroxyapatite Microscopy: Science, Technology, Applications and Education*, 1868 (FORMATEX: 2010).
4. Y.P. Ho, M.W. Yang, L.T. Chen, Y.C. Yang, *Rapid Commun. Mass Spectrom.* **21** No 6, 1083 (2007).
5. B. Palazzo, D. Walsh, M. Iafisco, E. Foresti, L. Bertinetti, G. Martra, C.L. Bianchi, G. Cappelletti, N. Roveri, *Acta Biomaterialia* **5**, 1241 (2009).
6. S. Koutsopoulos, E. Dalas, *J. Crystal Growth* **216**, 443 (2000).

7. S. Koutsopoulos, E. Dalas, *J. Crystal Growth* **217**, 410 (2000).
8. S. Koutsopoulos, E. Dalas, *Langmuir* **17**, 1074 (2001).
9. S. Koutsopoulos, E. Dalas, *Langmuir* **16**, 6739 (2000).
10. S. Koutsopoulos, E. Dalas, *J. Colloid Interface Sci.* **231**, 207 (2000).
11. X. Chu, W. Jiang, Zh. Zhang, Y. Yan, H. Pan, X. Xu, R. Tang, *J. Phys. Chem. B* **115**, 1151 (2011).
12. M. Dutour-Sikiric, H. Füredi-Milhofer, *Adv. Colloid Interface Sci.* **128-130**, 135 (2006).
13. Z. Chen, Y. Fu, Y. Cai, J. Yao, *Mater. Lett.* **68**, 361 (2012).
14. G. Zhang, J. Chen, Sh. Yang, Q. Yu, Zh. Wang, Q. Zhang, *Mater. Lett.* **65**, 572 (2011).
15. M.T. Jahromi, G. Yao, M. Cerruti, *J.R. Soc. Interface* **10**, 20120906 (2013).