Study of the dependence of the structural defects and bulk inhomogeneities of nanocrystalline hydroxyapatite on the conditions of production using a biological source of calcium

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Abstract. The study of structural and bulk defects in nanocrystalline samples of hydroxyapatite with a developed surface was carried out. The XRD method established distortions in the crystal lattice of the synthesized samples of hydroxyapatite (HAP), which are associated with the inclusion of impurity atoms present in the eggshell of birds as a biological source of calcium. The presence of impurity atoms of magnesium, sodium, and fluorine in the obtained materials was determined by X-ray photoelectron spectroscopy. Applying ESR and Raman spectroscopy it was found the presence of CO₃ complex in the structure. Studies of the morphological organization of the samples found a large number of surface defects – nanopores – in crystalline HAP. At the same time, the size of these 3D defects depends on the conditions of preparation and is determined by the content of phosphate groups in the initial solution.

1. Introduction

Obtaining nanocrystalline samples of hydroxyapatite involving lattice defects, with a large specific surface area is one of the approaches to increase the resorption of this biogenic osteoplastic material when it replaces the damaged areas of human bone tissue [1]. Positive results in the use of microimpurities in the composition of synthetic hydroxyapatite applied for improving the processes of osseointegration of biocomposites are already known [1]. As secondary sources of calcium for the synthesis of biomaterials, it was shown that nutrient sources of calcium contain trace impurities with concentrations similar to those in the human body [2]. However, the type of impurities and their influence on the structure, composition and morphological organization are the subject of contemporary studies [3]. The purpose of this work was to establish the dependence of the structural and bulk defects of nanocrystalline hydroxyapatite on the conditions of its production using the eggshell of birds as a biogenic source of calcium.

2. Materials and methods

In the current study nanocrystalline HAP was obtained with the use of poultry eggshell according to the technique described in [4]. The number of all the reagents was calculated to obtain hydroxyapatite...
samples with a calcium phosphorous ratio similar to a stoichiometric one which is 1.67. A properly washed chicken eggshell was annealed at 950 °C and then the obtained calcium oxide was mixed with distilled water. The calcium hydroxide synthesized in such way was titrated by the 0.3M solution of phosphorus acid at a constant mixing speed. All the processes were exposed to the air, thus it was some amount of carbonate impurity was expected in the sample. A small change in the concentration of the carbonate-involved groups was controlled by the changes in the pH value of the solution from 9 to 7 with a step of 0.5 (pH-meter/ionomer IPL 111-1). Then the obtained precipitate was aged through 20 hours and washed, filtered and dried at 400 °C for the examination. The elemental analysis of the samples obtained was made by means of the X-ray spectral microanalysis (XRMA) (Inca – 250, JEOL JSM-6380LV). Determination of the phase composition as well as the sizes of the coherence scattering areas was performed by X-ray diffraction technique with the use of diffractometer DRON 4-07 (Co Kα λ= 1.7902 Å). The X-ray photoelectron spectroscopy (XPS) study was carried out with Thermo Fisher Scientific K-Alpha to clarify the type of impurities in the samples. Raman spectra were recorded with the use of Raman Microscope RamMics M532 EnSpectr, with the laser wavelength of 532 nm. In order to determine radicals in the structure of the obtained nanocrystalline HAP samples, spectra of the stationary and echo-detected electron spin resonance (ESR) were recorded using spectrometer Bruker E580. TEM technique was employed for a direct estimation of the sizes of nanocrystals for synthesized HAP samples as well as for obtaining microdiffraction from the local areas of nanocrystals (Libra 120 Carl Zeiss). During the preparation of the samples for the TEM study they were dispersed by ultrasonic exposure (~ 1 GHz).

3. Results
The results of the investigations showed that all of the samples obtained at pH values of the solution from 9 to 7 with a step of 0.5 and annealed at the temperature of 400 °C, are characterized by the formation of a single phase, calcium hydroxyapatite. The analysis of X-ray diffraction data and the data from the JCPDS – ICDD standard database (card No. 01-074-0565) shows that the crystal lattice distortions occur in the samples caused by the inclusion of impurity atoms in the unit cell of the hydroxyapatite precursor, i.e. biogenic calcium carbonate eggshell of the birds [2].

The X-ray spectral microanalysis data of the eggshell were averaged over 10 samples. As can be found from the data obtained, the composition of the raw reactant (eggshell) contains atoms of magnesium, sulphur, potassium and chlorine, which are able to incorporate into the structure of the synthesized material and form defects. Investigations of calcium hydroxyapatite samples obtained by the described technology using the XRMA method showed that only carbon and magnesium atoms, the last in the trace concentration, are found in the composition of synthesized materials, while sulphur, potassium and chlorine atoms are not detected by this method (table 1). It means that during the synthesis only carbon and magnesium atoms were incorporated into hydroxyapatite lattice.

| Samples | pH | Ca | P    | O    | C    | Mg |
|---------|----|----|------|------|------|----|
| S1      | 9  | 32.0 | 15.7 | 44.3 | 7.6  | 0.4 |
| S2      | 8.5| 29.3 | 15.5 | 46.3 | 8.7  | 0.2 |
| S3      | 8  | 31.3 | 17.7 | 46.0 | 5.2  | 0.4 |
| S4      | 7.5| 30.2 | 16.7 | 45.5 | 7.1  | 0.5 |
| S5      | 7  | 26.7 | 15.1 | 49.7 | 8.2  | 0.3 |

Thus, in the obtained samples, the ratio of calcium to phosphorus is higher than that taken as the stoichiometric one. The reason for this is the presence of low concentration carbon in the structure of the HAP as well as on the sample surface. Based on the data in Table 1 and the presence of carbon, the Ca / P ratio is above 1.7. XRMA data is in good agreement with XPS results. The analysis of the XPS spectral band profiles of the C-HAP showed that carbon is the main concomitant impurity in the HAP.
structure, and magnesium and fluorine are in trace concentrations. Raman spectroscopy was used to study the position of the carbonate anion in the HAP structure (figure 1a).

![Raman spectra of synthesized HAP samples](image)

**Figure 1.** (a) – Raman spectroscopy of synthesized HAP samples: curve 1 – pH=9, curve 2 – pH=7, (b) – ESR spectroscopy of the HAP samples (from the solution with pH=9).

It is known that if there is a distortion in the calcium hydroxyapatite crystal lattice and CO$_3^-$ anion is included in its structure, a shift of PO$_4$ bands is observed in the Raman spectra [5]. As can be seen from figure 1a, in the Raman spectra of the samples obtained at different pH values of the solution, in addition to the basic modes corresponding to phosphorus – oxygen group PO$_4$ and localized around 1049, 962, 591 and 433 cm$^{-1}$, an additional mode is observed in the region of ~ 1072 cm$^{-1}$. Its appearance in the spectra can be correlated with the carbonate anion CO$_3^{2-}$ (table 2).

| Raman band | Raman Shift, cm$^{-1}$ | Molecular group attribution | Reference data, cm$^{-1}$ | References |
|------------|------------------------|----------------------------|---------------------------|------------|
| $\nu_2$ PO$_4$ | 433 | O-P-O bending, $\nu_2$ | 430-433 | [5,8] |
| $\nu_2$ PO$_4$ | 448 | O-P-O bending, $\nu_2$ | 447-449 | [5,9] |
| $\nu_4$ PO$_4$ | 580 | O-P-O bending, $\nu_4$ | 579-581 | [5,8,9] |
| $\nu_4$ PO$_4$ | 591 | O-P-O bending, $\nu_4$ | 591-594 | [5] |
| $\nu_4$ PO$_4$ | 608 | O-P-O bending, $\nu_4$ | 607-608 | [5,9] |
| $\nu_1$ PO$_4$ | 962 | P-O stretching | 960-963 | [5,8,9] |
| $\nu_3$ PO$_4$ | 1030 | PO$_4$ | 1029-1033 | [5] |
| $\nu_3$ PO$_4$ | 1049 | PO$_4$P-O antysym stretching | 1047-1049 | [5,8,9] |
| $\nu_1$ CO$_3$ B-type | 1072 | B-type CO$_3$ | 1069-1077 | [5,8,9] |
| OH | 3570 | OH | 3570-3571 | [5] |

A detailed examination of the shape and intensity of the vibrations observed in the region of 1000–1150 cm$^{-1}$ and attributed to the PO$_4$ group (1030, 1049 cm$^{-1}$) and to the CO$_3$ group (1072 cm$^{-1}$) shows that with an increase in the intensity of PO$_4$ mode in the spectrum (sample obtained at pH 7, i.e. with a high content of phosphate groups), the intensity of the band of CO$_3$ group decreases.

To clarify the data on the inorganic radicals present in the samples, the ESR method was used. It should be noted that ESR signals in the obtained samples of carbonate-substituted calcium hydroxyapatite were not observed before their irradiation by electrons. To record the ESR signal, the samples dried at 400 °C (1 h) were subjected to electron irradiation with a dose of 10 kGy. The results were obtained by means of the techniques of stationary and echo-detected ESR seven days after irradiation (figure 1b).
To determine the nature of the radicals, the ESR spectra of all samples were simulated. This made it possible to establish that the total of five signals is observed in the ESR spectra: from the radicals $\mathrm{O}^-$, $\mathrm{CO}_2^-$, $\mathrm{CO}_3^{2-}$, $\mathrm{CO}_3^{3-}$ [6] and from the organic radical $\mathrm{CH}_3$ [7]. It was found that with a change in the content of phosphorus-oxygen groups in the samples, spin concentration of the paramagnetic carbonate radicals $\mathrm{CO}_2^-$, $\mathrm{CO}_3^-$, $\mathrm{CO}_3^{3-}$ (as well as the intensity of their paramagnetic centers according to Raman data) increases, while the spin concentration of $\mathrm{O}^-$ and $\mathrm{CH}_3$ radicals decreases.

In order to specify the crystal structure, as well as to establish the phase composition of the individual nanocrystalline in the synthesized samples, high-resolution TEM technique was used. The TEM experiment showed that the formed nanocrystals of all the studied samples have a rod-like shape, their diameter is of $\sim20$ nm and length is of $\sim50$ nm. A detailed study of carbonate-substituted calcium hydroxyapatite samples using the TEM method with a nanometer resolution showed the existence of a porous structure of these materials with the average pore size of $\sim3.5$ nm.

4. Discussion

The comparison of the results obtained by X-ray powder diffraction for all of the samples allows us to conclude that a change in the concentration of phosphate groups in the initial solution does not lead to that in the phase composition of the final material, but it affects the distortion of the crystal lattice of hydroxyapatite. Using the comparison of parameters of the unit cell, it can be seen that the materials synthesized in the work have smaller values than similar samples presented in the JCPDS – ICDD database. It is well known that the conditions for the synthesis of calcium hydroxyapatite affect the crystal growth processes, and this in turn can lead to a change in the parameters of the crystal structure [1]. Therefore, the smaller values of the unit cell parameters $a$, $b$, and $c$ of the samples obtained in the current work can be associated with the peculiarity of the method used to synthesize calcium hydroxyapatite from birds' egg shells, as well as with the presence of impurities in its composition. Based on the Raman, XRMA, ESR and XPS experimental data, it can be seen that calcium hydroxyapatite certainly contains magnesium in the calcium positions and carbonate complexes and fluoride in the position of $\mathrm{PO}_4$ group and $\mathrm{OH}$ group, respectively. These impurities and groups contribute to the creation of a biomimetic composite that is most similar in the elemental composition to hydroxyapatite of bone and dental tissue [2]. Small difference in the crystal lattice parameters between the samples can be referred to the changes in the content of carbonate groups in the process of obtaining samples detected by Raman and ESR methods.

Raman spectroscopic data (figure 1a, curves 1 and 2) showed that spectrum bands corresponding to the $\mathrm{PO}_4$ and $\mathrm{CO}_2^-$ change their intensity with the introduction of more phosphate groups in the raw solution. In this case the intensity of the mode (1073 cm$^{-1}$) of carbonate-anion vibrations decreases. It should be noted that Raman spectra of the dental tissue reveal the bands corresponding to the carbonate anion [8,9], therefore its presence in the samples confirms the possibility of using these materials as a basis for creating dental biomimetic composites.

The ESR method, which is sensitive to various structural defects and is often used to study calcium hydroxyapatite and its substituted forms [6,7], confirmed the formation of carbonate-substituted B-type hydroxyapatite for all of the obtained samples. Moreover, according to the ESR data, the decrease in Cu/P ratio in the samples with pH 9, 8.5, 8, 7.5, 7 increases the intensity of the signals from the $\mathrm{CO}_2^-$ and $\mathrm{CO}_3^{3-}$ radicals. This fact supports the Raman spectroscopy data on the dependence of the carbonate anion content in hydroxyapatite lattice.

The developed surface in the obtained samples of carbonate-substituted hydroxyapatite, detected by the TEM method, can be used to increase the sorption capacity of materials and the formation of bioactive composites. The porous structure is characteristic of calcium hydroxyapatite samples obtained by chemical precipitation methods [11]. Nanopores in carbonate-substituted calcium hydroxyapatite prove to be 3D defect. The TEM method showed their average size of $\sim3.5$ nm.

5. Conclusion

Structural and bulk defects of nanocrystalline samples of hydroxyapatite with a developed surface was performed. The XRD method revealed distortions in the crystal lattice of the synthesized samples of HAP, which are associated with the inclusion of impurity atoms present in the eggshell of birds...
employed as a source of biogenic calcium and the change in the content of phosphate groups in the process of synthesis. The presence of impurity atoms of magnesium, sodium, and fluorine in the obtained materials was unambiguously determined by X-ray photoelectron spectroscopy. In addition, the XPS method revealed the changes occurring in the structure of carbon bands, which are due to the presence of CO$_3$ complexes in the samples. It was found that the CO$_3$ complex was a structural defect that affects the parameters of the unit cell of hydroxyapatite to a greater extent than all other impurities. The structural defects in c-HAP nanocrystals detected by means of the ESR technique are attributed to the impurities that are present in the raw reactant, i.e. eggshell. The presence of these impurities in the structure of the final material results in the change of parameters for the elementary cell in the crystal lattice of c-HAP. The presence of carbonate-ions in the structure of c-HAP of B-type is confirmed using the Raman spectroscopy techniques. Their occurrence does also provide a considerable contribution to the observed surface morphology. Studies of the morphological organization of the samples found a large number of surface defects, namely nanopores in crystalline HAP. At the same time, the size of these 3D defects depends on the conditions of preparation and is determined by the content of phosphate groups in the initial solution. Thus, owing to their characteristics c-HAP samples synthesized according to our technology from the biogenic calcium source are potentially significant for developing biomimetic materials that are similar in their structure to the native hard dental tissues of a human.

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