

Spectral analysis of allogeneic hydroxyapatite powders

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Abstract. In this paper we discuss the application of Raman spectroscopy to the *in vitro* analysis of the hydroxyapatite powder samples produced from different types of animal bone tissue during demineralization process at various acid concentrations and exposure durations. The derivation of the Raman spectrum of hydroxyapatite is attempted by the analysis of the pure powders of its known constituents. Were experimentally found spectral features of hydroxyapatite, based on analysis of the line amplitude at wave numbers $950\text{--}965\text{ cm}^{-1}$ ($(\text{PO}_4)^{3-}$ (ν_1) vibration) and $1065\text{--}1075\text{ cm}^{-1}$ ($(\text{CO}_3)^{2-}$ (ν_1) B-type replacement). Control of physicochemical properties of hydroxyapatite was carried out by Raman spectroscopy. Research results are compared with an infrared Fourier spectroscopy.

1. Introduction

At the present stage of medicine development in the field of surgery and dentistry for the restoration of human bone tissue increasingly are widely used biomaterials based on hydroxyapatite (HA). According to its physical and chemical properties and physiological reactions in the body, a finely divided crystalline synthetic hydroxyapatite can be compared with the inorganic component of bone tissue [1]. However, osseointegration properties and biological activity of these materials can vary significantly depending on method of their preparation, and consequently, there are ways to increase their effectiveness.

Injection of the hydroxyapatite to the bone implant increases the physico-mechanical properties of the composite: increases the flexural strength, elastic modulus and hardness, approaching to the lower property values of natural bone [2].

Naturally occurring calcium hydroxyapatite is also interesting because of the large number of substituent elements (Na, Mg, K, Fe, Zn, Cu, Ba, F, Cl, C, S) as part of the HA crystals. The impurity concentration is not more than 3-5%, but affects the chemical-biological and physico-mechanical properties of biogenic HA due to distortions in the crystal lattice caused by the presence of vacancies, interstitial and substitutional impurities. Due to the large number of isomorphic substitution, it is possible to change the properties of hydroxyapatite directed and getting materials in composition close to the native bone [2, 3]. Since the bone matrix serves as a buffer system for the atoms of different metals, synthetic biomaterials based on HA must have different concentration of impurities for best correspondence to the replaceable area of bone [4-7].

At work with complex objects such as calcium phosphate and hydroxyapatite, as well as composites applying in dentistry and medicine, it is necessary to evaluate their composition. Control of physicochemical properties can be implemented by Raman spectroscopy method.



In work [8] was carried out determination of biocompatibility and efficacy of hydroxyapatite in clinical application to replace bone tissue using multislice computed tomography (MSCT). In a comparative analysis of tomograms, authors of Ref concluded that the application of hydroxyapatite to fill defects in bone tissue during surgery accelerates bone repair processes. However, the CT method is time consuming and expensive method.

Infrared spectroscopy method is actively used in the analysis of biomaterials based on hydroxyapatite. Through this technique, it is possible to make conclusion about composition of the molecular groups in the sample, to detect distortion in the crystal structure of HA and to determine the influence of defects on the molecular composition of hydroxyapatite. The advantage of this method is the relative simplicity of the experiment and a high selectivity. In addition, advantage of the infrared method is the possibility to use in the analysis of multi-component mixtures [9, 10].

For example, in ref [11-14] points to related changes in the frequency shifts corresponding PO_4^{3-} relations, during the formation of hydroxyapatite. The intensity redistribution of the main peak PO_4^{3-} group corresponds to a broadening of the X-ray diffraction lines, which confirms the influence of crystallite size on the IR spectra calculated theoretically. However, this method can not distinguish between types of minerals in the composition of the implant [10].

In Raman spectroscopy realized high spatial resolution (up to 1 micron), combined with ease of sample preparation, the lack of destruction after analysis and reduced demands to the sample (which may be opaque, hydrated, etc.) [15].

In the works of authors [16, 17] have shown that the line of symmetric stretching vibration PO_4^{3-} (ν_1) has a high sensitivity to the mineral encirclement: the frequency and form of this line depends on the local surroundings and change as a result of substitution of anionic groups and changes in the degree of crystallinity. The substituted carbonate-apatite B-type phosphate ν_1 line appears in the range of $955\text{--}959\text{ cm}^{-1}$. In a crystalline unsubstituted hydroxyapatite line ν_1 shifted to $962\text{--}965\text{ cm}^{-1}$. Finally, at a frequency line $945\text{--}950\text{ cm}^{-1}$ indicates the presence of a disordered lattice phosphate apatite. In general, the phosphate band ν_1 is a superposition of three components; it usually has an asymmetric shape due to the disordered vibrations contribution of phosphate and unsubstituted hydroxyapatite.

In addition to phosphate mineral component, in the Raman spectra also reflects the region $1065\text{--}1075\text{ cm}^{-1}$, corresponding to fluctuations in the carbonate ion B-type [18, 19].

In the region $1400\text{--}1800\text{ cm}^{-1}$, the most pronounced peaks of protein bonds are amide groups -CO-NH- . Following bands recorded in the spectrum: Amide I ($1655\text{--}1675\text{ cm}^{-1}$), which is mainly expressed by carbonyl groups C=O ; Amide II ($1555\text{--}1565\text{ cm}^{-1}$); Amide III ($1230\text{--}1289\text{ cm}^{-1}$), which includes C-N stretching and N-H deformation vibrations; deformational twisting oscillation CH_2 ($1455\text{--}1465\text{ cm}^{-1}$). Low-intensity peak at 1620 cm^{-1} , authors of the article [15] correlated with fluctuations in the side chain of tyrosine (Y8a).

In the papers [20-22] have also used Raman spectroscopy for the study of changes in the composition of hydroxyapatite obtained from dental hard tissues in various pathological processes. The results of experimental studies indicate the information content of the method of Raman spectroscopy to study the crystallinity, structural features of biomaterials based on HA.

The aim of this study was to analyze the spectral composition of the hydroxyapatite powders obtained from different bone tissue types of various animal species by Raman spectroscopy.

2. Materials and methods of research

Objects of study were 30 samples of hydroxyapatite powder obtained from a mixture of compact and cancellous bone tissue of different animal species. To obtain a demineralized biomaterial, bone was placed in a solution of hydrochloric acid, the concentration of which depends on the type of bone (for birds' bone tissue samples 1.8H HCl solution was chosen, as 2.4H and 3.6H hydrochloric acid solution respectively used for bone tissue of sheep and cows). The obtained demineralized solution is used to produce hydroxyapatite. Difference thus obtained against synthetic hydroxyapatite powder lies in the fact that the final addition of hydroxyapatite powder contains organic components, and small

concentrations of other apatite in the bone tissue. Measurements were taken of each sample at three different points.

The main method of research hydroxyapatite powder was used Raman spectroscopy method (Figure 1), implemented by the high-resolution digital spectrometer Andor Shamrock sr-303i with built-in cooling camera DV420A-OE, which provides spectral resolution of 0.15 nm, and fiber optic probe for Raman spectroscopy RPB785, combined with the laser module LuxxMaster LML-785.0RB-04 (up to 500 mW, wavelength 785 nm). This wavelength well proven in Raman spectroscopy in biological applications, due to the lower level of autofluorescence.

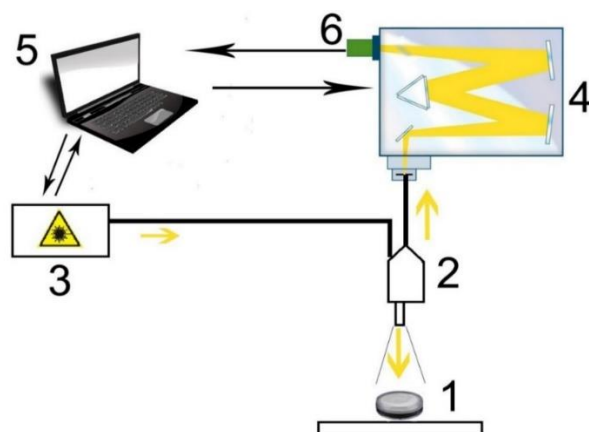


Figure 1. Experimental stand:

- 1 – studied object;
- 2 – Raman probe RPB785;
- 3 – laser module LuxxMaster LML-785.0RB-04;
- 4 – spectrometer Shamrock sr-303i;
- 5 – computer;
- 6 – cooling camera DV420A-OE.

Error of method does not exceed 4%. Processing spectra carried out in a software environment Wolfram Mathematica 9 and consisted in the removal of noise by the smoothing median filter. Then at the selected range of 400-2200 cm^{-1} using an iterative algorithm determined approximating line (a polynomial of the fifth degree) of autofluorescence component and subtracted it, yielding a dedicated Raman spectrum.

An additional method of research method was FTIR spectroscopy. IR - spectra of the samples were obtained on the console of frustrated total internal reflection (FTIR) from Pike Technologies Inc., which connected to the FTIR spectrometer SPECTRUM 100 (PerkinElmer, USA) in the range of wave numbers 550-4000 cm^{-1} with spectral resolution of 4 cm^{-1} and the signal accumulation of 20 scans. To record the spectra of hydroxyapatite powders used, which was applied directly onto the FTIR crystal consoles.

3. Results and discussion

Figure 2 shows the typical Raman spectra of hydroxyapatite powder samples obtained from biological materials with varying degrees of processing.

Registered Raman bands correspond to vibrations shown in Table 1. It is seen that these Raman peaks are presented in all samples.

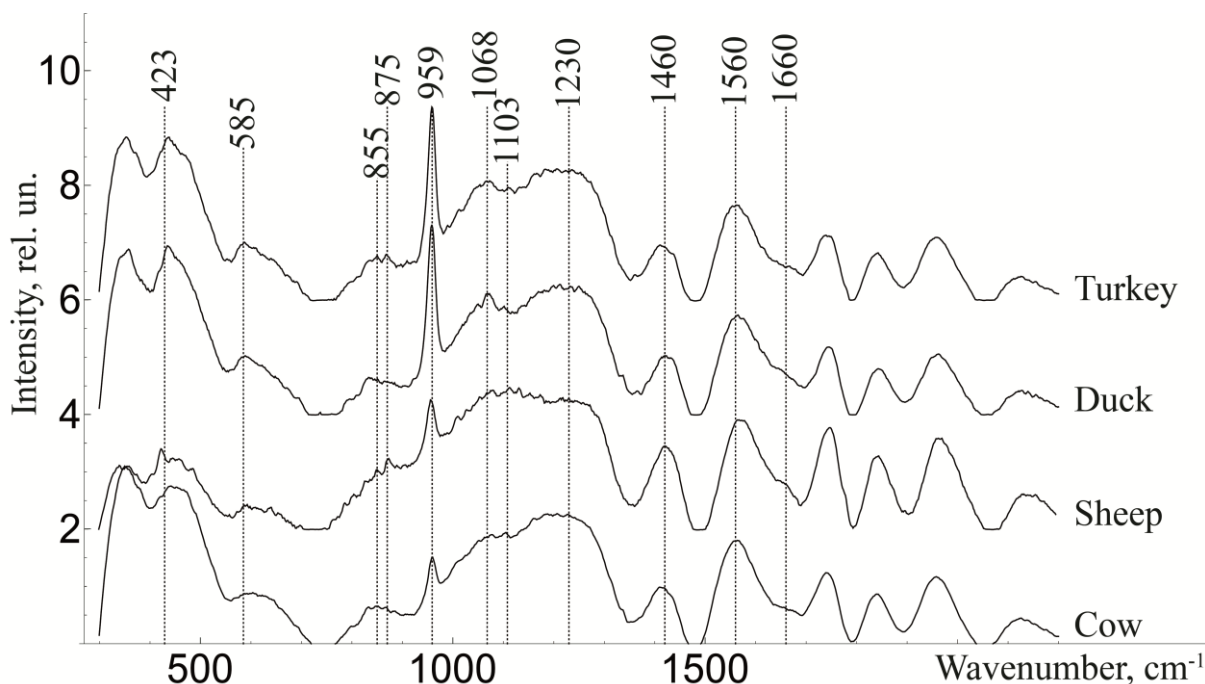


Figure 2. Averaged Raman spectra of hydroxyapatite powder samples.

The degree of substitution of the atoms and ions in the crystal of hydroxyapatite carbonate ions during the life changes, particularly there is an intensive substitution of the hydroxyl group with age corresponding hydrated CO_3^{2-} A-type ($1095\text{--}1103\text{ cm}^{-1}$). Therefore, in the production of composite materials based on HA seek to obtain mainly product with a predominant content of carbonate-B-type. [27, 28].

Figure 2 shows that the line corresponding to the intensity carbonate inclusion in the crystal lattice of hydroxyapatite ($1065\text{--}1075\text{ cm}^{-1}$, $(\text{CO}_3)^{2-}$ B-type substitution), predominates over Raman line, reflecting the concentration of the hydrated carbonate ion-type A. Hence the predominant content of carbonate-apatite B-type in studied HA samples is correspond to biomaterial close in chemical composition to the young bone tissue [18, 19].

Also, there is a relatively high content of organic components. Apart from Raman lines of proline and hydroxyproline, collagen component is represented by the Amide III groups (in region $1230\text{--}1289\text{ cm}^{-1}$), Amide II ($1555\text{--}1565\text{ cm}^{-1}$) and Amide I ($1655\text{--}1675\text{ cm}^{-1}$) [17].

The observed differences in the intensity of the lines at wavenumbers of $950\text{--}965\text{ cm}^{-1}$ (vibrations PO_4^{3-} (ν_1)) are the result of processing samples in hydrochloric acid solution with a normality varying depending on the animal species. Also, the differences can be explained by significant fluctuations in the content of amorphous calcium phosphate, depending on the individual and the age of the donor bone tissue. Amorphous calcium phosphate prevails at an early age; in the mature bone crystalline hydroxyapatite becomes predominant. Accordingly, on the composition of material affects both the type of bone tissue, and the age of the organism [24].

IR spectra of hydroxyapatite samples obtained from cancellous bone tissue of sheep, cows, turkeys and ducks are presented in Figure 3.

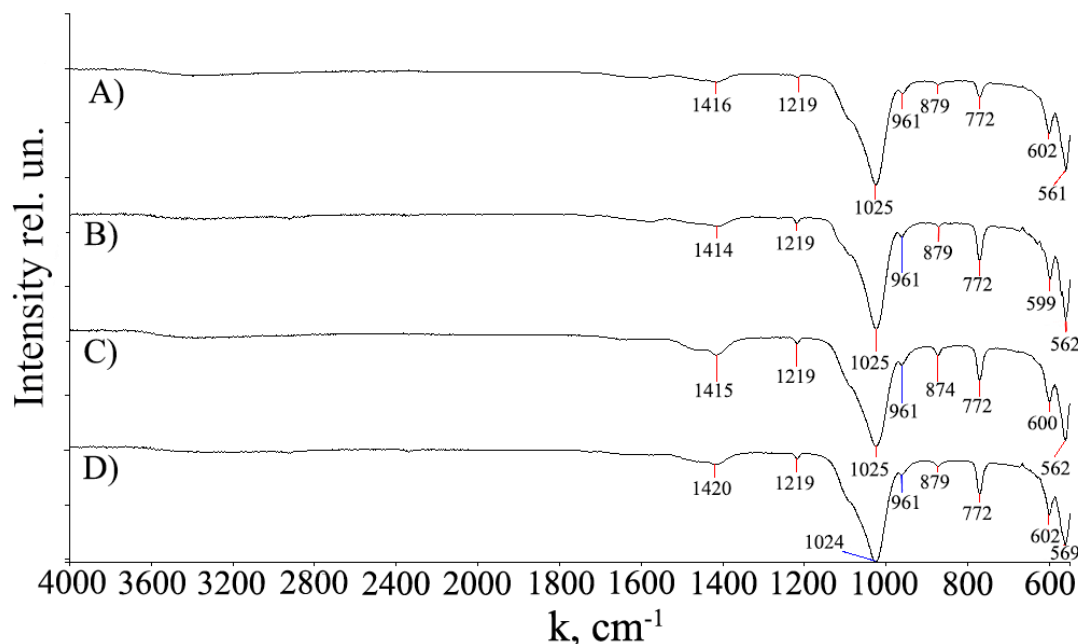


Figure 3. IR absorption spectra of hydroxyapatite for sample extracted from sheep cancellous bone tissue (A), cow (B), turkey (C) duck (D).

No significant difference in IR spectra of HA, obtained from different animal cancellous bone were found. Main intervals of wave numbers 950-965, 1020-1080, 520-660 cm^{-1} correspond to the vibrations of the phosphate ion [25]. The hydroxyl groups are shown in regions 3000-3600 and 1550-1700 cm^{-1} , the frequencies are overlapped with the frequencies of bound water. Frequencies 1415-1420, 1065-1075 and 870-880 cm^{-1} correspond to the presence of carbonate ions, which precipitates together with phosphates [26].

For all absorption spectra of hydroxyapatite, bands associated with organic components (Amide III, Amide II, Amide I) is significantly weaker than the line associated with the mineral component.

Table 1. Raman bands for hydroxyapatite powders.

Raman shift, cm^{-1}	Assignments
423	PO_4^{3-} (ν_2) (P-O stretching)
561, 585, 602	PO_4^{3-} (ν_4) (P-O bending)
855	Benzene ring of proline
875	Benzene ring hydroxyproline
880	CO_3^{2-} (ν_2) (C-O stretching)
950-965	PO_4^{3-} (ν_1) (P-O symmetric stretch)
1023,1030,1045,1052	PO_4^{3-} (ν_3) (P-O asymmetric stretch)
1065-1075	CO_3^{2-} (ν_1) B-type substitution (C-O in plane stretch)
1079-1080	Amorphous potassium carbonate (ν_1)
1095-1103	CO_3^{2-} (ν_1) A-type substitution (C-O in plane stretch)
1230-1289	Amide III (C-N-H stretch) (bending N-H)
1455-1465	CH_2 wag
1555-1565	Amide II (C-N-H stretch) (bending N-H)
1610-1620	Tyrosine Y8a (resonance-enhanced side-chain vibrations)
1655-1675	Amide I (C=O stretch)

The composition of the hydroxyapatite powders depends on the method of their preparation. HA, obtained from bone tissue, contains mineral components and the relative concentration of which depends on the degree of processing in the hydrochloric acid solution. Hence, for the most convenient control of changes in hydroxyapatite composition, analyze its main components, corresponding to mineral structure, by intensity of characteristic Raman lines: $950-965\text{ cm}^{-1}$ (symmetric stretching vibration $\text{PO}_4^{3-}(\nu_1)$) and $1065-1075\text{ cm}^{-1}$ (planar stretching vibration $\text{CO}_3^{2-}(\nu_1)$) [16-19].

For a comparative analysis of the results obtained using methods of Raman spectroscopy and FTIR - spectroscopy, we were built according shown in Figures 4-5.

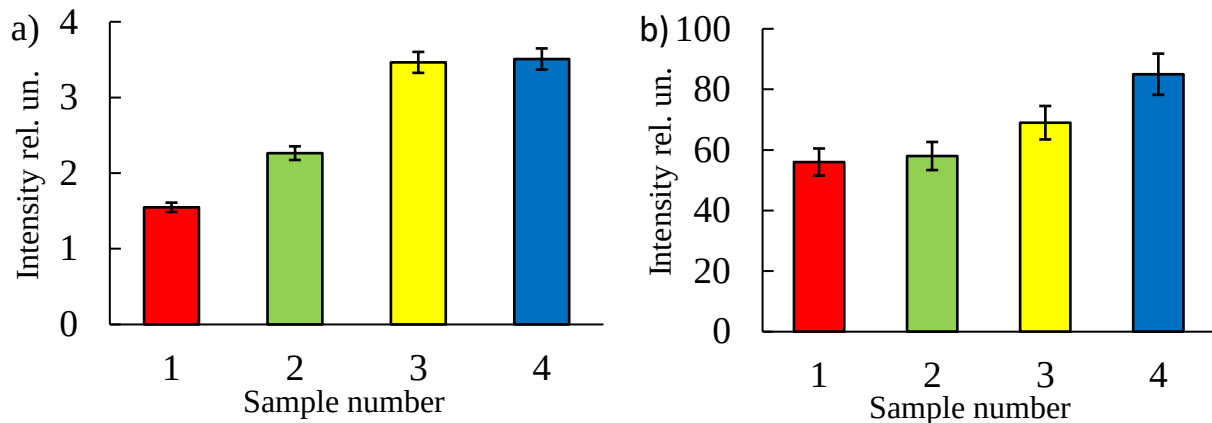


Figure 4. The intensity of the lines at the wavenumber of phosphate component for samples with different degrees of processing based on the data: a) Raman spectroscopy; b) FTIR spectroscopy.

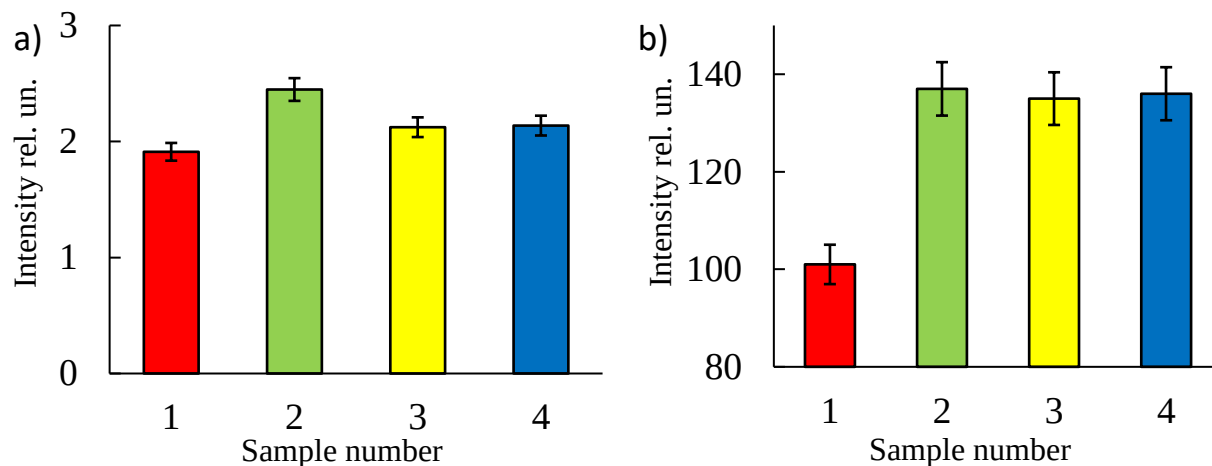


Figure 5. The intensity of the lines at the wavenumber of carbonate component for samples with different degrees of processing based on the data: a) Raman spectroscopy; b) FTIR spectroscopy; where HA samples obtained from the bone, are designated: 1. Cow, 2. Sheep, 3. Duck, 4. Turkey.

From the histograms presented in figures 4-5 it is seen that the intensity ratio of the amplitudes of the peaks Phosphate and carbonate ions at wave numbers $950-965\text{ cm}^{-1}$ and $1065-1075\text{ cm}^{-1}$ obtained by the method of Raman spectroscopy have a positive correlation with the results FTIR spectroscopy and reflects similar relative concentration dependence of substances.

The calculated data correlation coefficient of the two methods for of phosphate and of carbonate component is $R_{\text{PO}_4} = 0,86$ and $R_{\text{CO}_3} = 0,76$ respectively.

4. Conclusions

Were experimentally detected features of Raman spectra for hydroxyapatite powder samples obtained from a mixture of compact and cancellous bone tissue of different donors during demineralization, based on analysis of amplitude of $(\text{PO}_4)^{3-}$ line, integral line intensity $(\text{PO}_4)^{3-}$, integral line intensity $(\text{CO}_3)^{2-}$.

It was found that the degree of processing is characterized by change in the spectrum at wavenumbers of $950\text{--}965\text{ cm}^{-1}$ and $1065\text{--}1075\text{ cm}^{-1}$ corresponding phosphate and carbonate ions.

Research results are compared with Fourier transform infrared spectroscopy and represent a similar dependence of the relative concentrations of substances from the intensity amplitudes ratio for the major Raman peaks and the absorption bands in the infrared spectra. The calculated data correlation coefficient of the two methods for of phosphate and of carbonate component is $R_{\text{PO}_4} = 0,86$ and $R_{\text{CO}_3} = 0,76$ respectively.

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