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CHEMOMETRIC ANALYSIS OF BIOIMPLANTS OF BONE TISSUES DURING THEIR MANUFACTURE

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ABSTRACT

The results of expanded spectral analysis of donor bone implants using the Raman spectroscopy method are presented in the work. Mathematical methods of improving resolution of spectral contours and chemometric analysis are used for evaluation of component composition of the implants. It is shown that the Raman spectroscopy can be used for evaluation of relative concentration of mineral and organic components in extracellular matrix of bone tissue. Spectral features of the samples of bone tissues demineralized and processed with ultrasound with different degree are found as a result of the study. The criteria allowing evaluating the component composition during manufacture of bioimplants are suggested in the work.

Keywords: Raman spectroscopy, spectral analysis, bone bioimplants, chemometrics, demineralization, "Lioplast".

INTRODUCTION

Ensuring full regeneration of bone tissues in a damaged area of a bone is one of the acutest problems of modern medicine, despite the accumulated knowledge in this field. It can be solved by creating optimal conditions for regenerating processes in the areas of its resorption. One of the ways is using bone plasty materials [1, 2]. Among them are allogeneic implants made of human tissues that are optimal materials for reconstructing the damaged musculoskeletal system. The use of such implants does not disrupt homeostasis and metabolism of connective tissues and function of life support system of recipient, unlike autoplasty and xenoplasty and using synthetic materials [3]. After special processing allogeneic materials almost completely lose their antigenicity and when put into the body do not have a negative effect on it. They serve as a matrix, a conductor, gradually fully dissolves, and in their place new bone tissue is formed [4].

The success of such surgeries depends on their quality and the technology of bioimplant manufacture, aimed to maintain the necessary biological substances, involved in regenerative process, as hydroxyapatite, collagen, glycosaminoglycans [5], and to remove the cellular components (DNA, RNA) – the main factor of antigenicity.

The process of bioimplant manufacture requires constant monitoring of its quality and evaluating the organic compound. The quality of bioimplants can currently be assessed in vitro using a series of morphological, morphometrical, biochemical studies, but their disadvantage is the long process of information receiving and the destructive impact on a subject. Therefore the use of optical methods is rather promising as they can be used as screening tests, they are performed rapidly, low-cost and do not destroy the presented samples [6, 7].

Among the physical methods the widely used for control of the implants made of bone tissues are scanning electron microscopy [8], X-ray spectroscopy [9] and the Raman spectroscopy method [10, 11], that has certain advantages and allows making real time nondestructive qualitative and quantitative analysis of composition of biological objects and provides information about molecular structure with high spatial resolution.

The method of Raman spectroscopy is used in the work [12] for studying the crystal structure of human hard dental tissues in the dental caries process. The spectral features of samples of hard dental tissues in different pathological processes were found as a result of the experimental study. The authors also concluded that the method is informative for studying the composition and structural features of biomaterials.

The aim of this work: evaluation of mineralized bone implants during the process of their manufacture using the method of Raman spectroscopy and the methods, making the spectra more informative.

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MATERIALS AND METHODS OF RESEARCH

Raman spectroscopy method, implemented by the experimental stand, including the Raman probe RPB-785 (focal length of 7.5 mm), combined with the laser module LuxxMaster LML-785.0RB-04 (power up to 500 mW, wavelength of 784.7 ± 0,05 nm) and the high-resolution digital spectrometer Shamrock sr-303i, providing spectral resolution of 0.15 nm, with the build in cooling camera DV420A-OE (spectral range of 200-1200 nm), was used as the main method of analysis of bioimplants.

The subjects of the study were 48 samples of cancellous bone bioimplants in the form of a cube with sides measuring 5*5*5 mm, made using "Lioplast"® technology (technical specifications TU-9398-001-01963143-2004). All samples were divided in two groups according to the way of their manufacture: allogenic (cadaverous) and intra-operatively resected. The samples in every group were divided in four subgroups according to the amount of demineralization and ultrasound processing (eight groups of samples total). The spectra of each of six sides of sample were taken in different points and averaged.

The technological process of bone bioimplant manufacture included low frequency ultrasound processing of tissue (24-40 kHz for 2-3 minutes), that ensured maximum spongious degreasing and removal of stroma and bone marrow cells from trabecular area of the bone. Demineralization of the bone tissue samples was made using mild solution of hydrochloric acid. The final stage of processing included bone lyophilisation using ALPHA 2-4 LSC device, which is freeze-drying the samples. Then the sealed material was finally sterilized using radiation techniques.

The spectra processing was made in the software Wolfram Mathematica 10 and included clearing up noises by the smoothing median filter (7 points). Then in the chosen area of 300-2200 cm^{-1} approximating line (an eighth order polynomial) of autofluorescent component was determined using iteration algorithm [13] and then this component was subtracted receiving allocated Raman spectrum. The error of the used coefficients did not exceed 4% [11].

RESULTS

Figure 1 shows the distinctive Raman spectra of mineralized and demineralized samples, obtained from different sources in the area of 750 – 2000 cm^{-1}. The main differences are seen in the Raman lines of 814, 850, 956, 1001, 1026, 1068, 1239, 1272, 1560 and 1735 cm^{-1}.

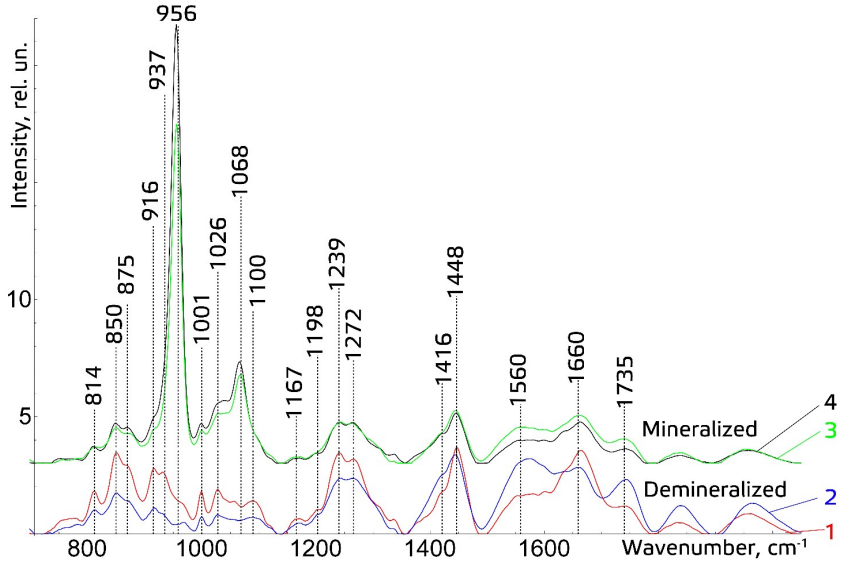

Figure 1 – Averaged normalized Raman spectra of mineralized and demineralized samples of bioimplants, taken from cadaverous (1,4) and antemortem (2,3) bone tissue

Figure 1 shows that there are significant differences of intensities of the Raman lines of bioimplants manufactured from the differently processed bone tissues. The significant decrease of intensity of the lines 956 and 1068 cm^{-1}, corresponding to PO_4^{3-} (ν_1) (P-O symmetrical valence fluctuation) and CO_3^{2-} (ν_1) B-type substitution (C–O planar valence fluctuation)

was characteristic for demineralized bioimplants of both groups. After demineralization the decrease of intensity of these lines in the samples obtained from both cadaverous, and antemortem resected bone tissue was observed.

The Raman line of 814 cm^{-1}, corresponding to phosphodiester bonds in DNA / RNA, is also in all groups of samples, which probably indicates the destruction on nuclei and incomplete removal of remnants of DNA / RNA from the samples.

The degree of processing and the quality of implants depends on whether the cellular components (DNA, RNA) are fully removed and the formed extracellular matrix is preserved; the main components of extracellular matrix are hydroxyapatite, collagen, glycosaminoglycans, proteoglycans [10]. The quality of implant depends directly on content of these components in it.

When analyzing the Raman spectra the most interesting are the spectral lines of 1416, 916, 974 and 814 cm^{-1} (RNA / DNA), 850 and 937 cm^{-1} (proline), 1167 cm^{-1} (GAGs, CSPGs), 1230-1280 cm^{-1} (amide III), 1560 cm^{-1} (amide II), 1660 cm^{-1} (amide I), 1448 and 1738 cm^{-1} (lipids and fatty acids), 956, 1048, 1068, 1084, 1100 cm^{-1} (hydroxyapatite).

It should be noticed that nonlinear regressive analysis of spectral curve has limitations, related to resolution of spectral lines and reliable division of the lines with overlapping less than 10 cm^{-1} is not possible.

For relative quantitative assessment of component composition of the surface of bioimplant on the basis of bone tissue we have introduced relative coefficients. Relatively permanent component in the studied samples of bone tissue was Amide I [10] (Figure 1), corresponding to the Raman shift of the frequency of 1660 cm^{-1}, therefore the amplitude of this divided line was used as denominator (I_{1660}) in the introduced coefficients (k):

$$k_i = \frac{I_i}{I_{1660}},$$

where I_i – intensity of spectral lines of the analyzing components.

The results of PCA are shown in the form of a set of data: the graph of counts (Figure 2), the graph of loads (Figure 3).

In our case the main components PC-1 and PC-2 provide information, describing the 99.75 % of the model, therefore describing the PCA of all eight samples we will consider only PC-1 – PC-2. Analyzing the further principal components PC-3 – PC-6 would be difficult as there is no information allowing interpreting their physical meaning.

The graph of counts describes the structure of the data and generally shows differences or similarities between the groups of samples. Figure 2 shows that the main differences between all the groups of samples are described by the main component PC-1, and these differences are the most significant. Positive values of PC-1 mainly characterize the demineralized samples, and vise versa negative values mainly characterize the mineralized samples that were not processed using hydrochloric acid.

Positive values of PC-2 characterize intra-operatively resected materials, and negative values – cadaverous. Therefore, we can conclude that PC-1 describes the difference of component composition, arising after bone tissue demineralization, and PC-2 shows spectral features of bone tissue samples, obtained from different sources. Influence of ultrasound processing on spectral characteristics of samples is also significant, but is less than the process of demineralization and the influence of the way of biomaterial obtaining, therefore, the further analysis will be focused on finding the features of only demineralized bioimplants.

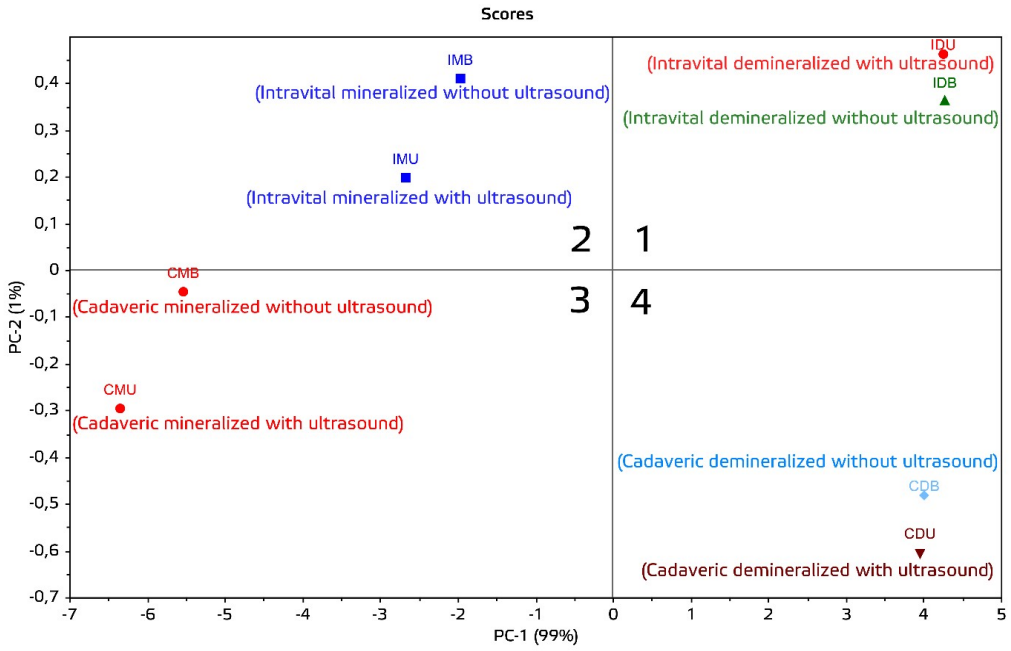

Figure 2 – The graph of PCA counts of averaged Raman spectra of eight samples of bone tissue

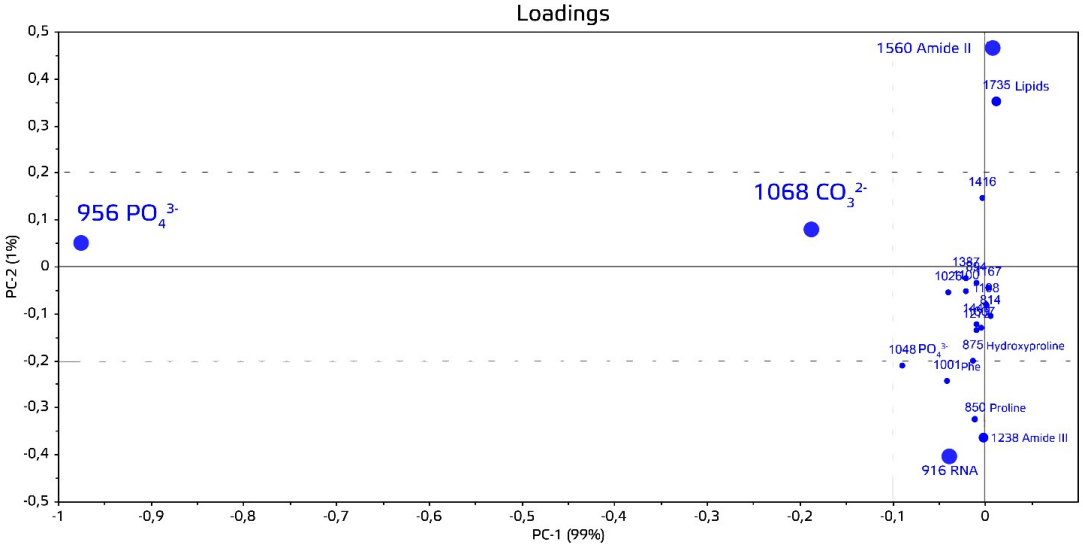

Figure 3 – The graph of PCA loads of spectral lines of the Raman spectra of the samples of bone tissues

The loads describe the structure of data in a form of contribution of variables to the load in every PC and show how well PC considers variation of the values of variables.

Analysis of figures 3 and 4 and the output PCA data allows to make the following conclusions:

1. The differences between the mineralized and demineralized samples are described by the principal component PC-1 and components, showing relative intensity of the lines 956 and 1068 cm^{-1}, corresponding to PO_4^{3-} (ν_1) (P-O symmetrical valence fluctuation) and CO_3^{2-} (ν_1) B-type substitution (C–O planar valence fluctuation). These variables are very important in mineralized samples, and we can see that the cadaverous materials have higher concentration of hydroxyapatite, than the intra-operatively resected ones.

2. In this, the higher the value of PC-1 for the variable, the more it influences the observed difference of component composition, e.g., which can be seen from the value of the coefficient k_{956}. Figure 2 shows that intensity of

the spectral line of 956 cm^{-1}, corresponding to the fluctuations of phosphate ion in hydroxyapatite, is higher for cadaverous samples.

3. PC-2 describes the difference between the cadaverous and intra-operative samples and is characterized by the values of two groups of coefficients, describing the relative intensity of the lines:

1 - 1560 cm^{-1} (amide II) and 1735 cm^{-1} (phospholipids);

2 - 850 cm^{-1} (proline), 1238 cm^{-1} (amide II), 1001 cm^{-1} (phenylalanine) and 916 cm^{-1} (RNA)

The variables of the first group are very important for intra-operative samples. The cadaverous samples are characterized by the higher values of variables of the second group.

4. Therefore, data field in Figure 2 was divided in four quadrants.

Influence of the process of demineralization on hydroxyapatite concentration in bone tissue is thoroughly described in [14]. The increase of demineralization time during bone bioimplant manufacture leads to decrease of relative concentration of minerals (phosphates 956 cm^{-1} and carbonates 1068 cm^{-1}) with regard to organic components.

Then we will consider the influence of ultrasound processing and the source of bone tissue on component composition of biomaterials without taking into account the process of demineralization. In this case principal components PC-1 and PC-2 provide information, describing the 79.9 % of the mathematical model (Figure 3), therefore, the further analysis will consider only these two principal components. The components PC-3 – PC-6 are not included in the further analysis, as their analysis is difficult, because of complex data structure and influence of the other factors not considered, influencing the quantitative component composition (noise).

Figure 4 shows that the main differences between cadaverous and intra-operative samples are described by the principal component PC-1, and these differences are the most significant. The positive values of PC-1 mainly characterize the cadaverous samples.

The positive values of PC-2 characterize the samples without ultrasound processing. Therefore, we can conclude that PC-1 describe the difference of component composition of bone tissue, obtained from two different sources, and PC-2 shows spectral features of bone tissue samples depending on the influence of ultrasound processing.

The detailed analysis is shown in Figure 4, where two-dimensional diagrams of the introduced coefficients are showing differences and similarities of the samples of four groups.

Figure 4a shows the characteristic areas of the groups of samples and it is seen that the coefficients k1735, k1448, indicating the relative concentration of fatty acids, are lower for the samples with ultrasound processing than for the group of samples without it, which indicates the destructive influence of ultrasound processing on fatty acids. The values of k1735 for cadaverous samples are also lower, than for intra-operative ones. The coefficient k1448 does not depend on the source of bone tissue.

Figure 4b shows values of optical coefficients k1001, k1026 indicating the relative concentration of phenylalanine in collagen and proteins, which are part of bone tissue biomatrix. Regarding the data in Figure 4, we can conclude that there are significant differences between the groups, i.e. ultrasound processing and the source of biomaterial influence the relative concentration of phenylalanine.

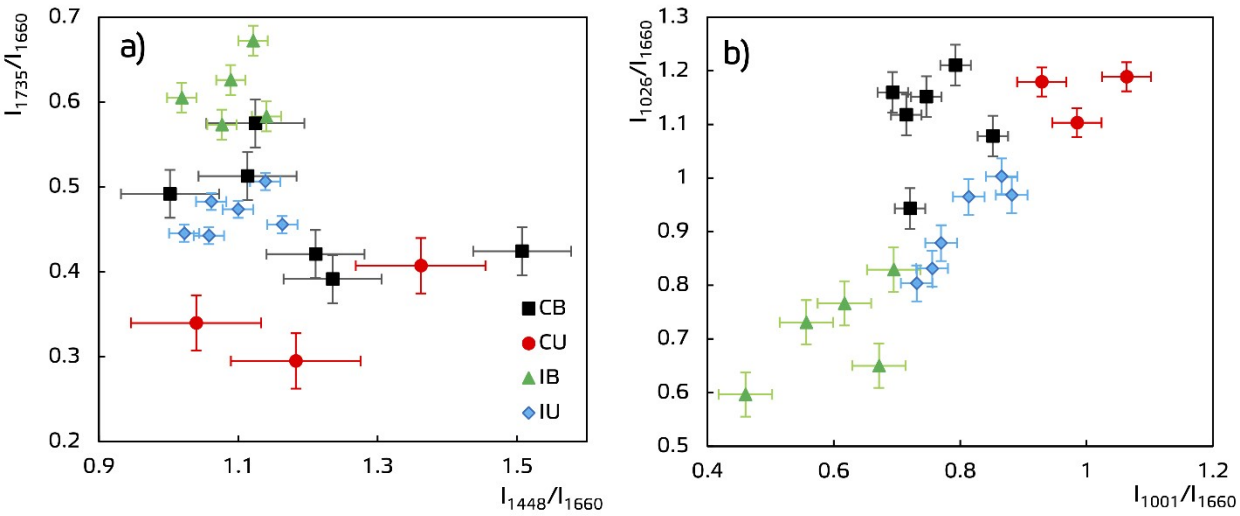

Figure 4 – Two-dimensional diagrams of the introduced coefficients: CB – Cadaveric without Ultrasound; CU – Cadaveric with Ultrasound; IB – Intravital without Ultrasound; IU – Intravital with Ultrasound

Therefore, with the use of spectral analysis of Raman spectra with mathematical methods of increasing the resolution of spectral contours and chemometric PCA analysis for assessment of implants obtained from bone tissue, it was shown that the processing allows to remove the components affecting their quality but preserves the necessary amount of mineral components and extracellular matrix: glycosaminoglycans, collagens, prolines, hydroxyprolines and phenylalanine [15].

CONCLUSION

The main criteria of evaluation of component composition of mineralized bone grafts made using "Lioplast" technology during their manufacture were established.

The introduced coefficients allow evaluating the degree of influence of ultrasound processing, demineralization and the source of bone tissue.

It was established that the main differences are seen in the Raman lines of 1448, 1735 (lipids & fatty acid), 850 and 875 cm^{-1} (proline & hydroxyproline), 1001 and 1026 cm^{-1} (phenylalanine) and 1272, 1560 cm^{-1} (amide II, amide III).

It was shown that optical method of bioimplant assessment by the introduced spectral ratio can be further used for optimization of the process of its manufacture due to improvement of quality of the manufactured material and selection of individual parameters of its processing.

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