Chemometric analysis of the Raman spectra for determination of the composition of bones with different porosity

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Abstract. The results of application of the Raman spectroscopy method for evaluation of porosity influence on the process of bone fabric demineralization and for evaluation of component composition of bone grafts with different porosity in the process of their treatment are presented. The comparison has shown the difference in intensities of spectral lines of the mineralized samples on the wavenumbers 960 cm⁻¹ and 1070 cm⁻¹, corresponding to hydroxyapatite phosphate ion and carbonate ion, as well as of the demineralized samples on the wavenumbers 1000 cm⁻¹ (phenylalanine aromatic ring) and 1167 cm⁻¹ (GAG). The coefficients were implied and the two-dimensional analysis, showing the pore size influence on the demineralization process. This is due to the fact that the demineralization process doesn’t include acid mixing, so the areas of its non-uniform concentration appear in its interaction with the samples, particularly in the small-pored areas.

1. Introduction
Bone fracture healing is an important clinical and social issue in the modern world. Bone grafts are used in reconstructive surgery for bone structural integrity restoring and bone tissue osteogenic potential increase [1]. Complete regeneration of the tissue in defective areas of bones, despite of all the knowledge gained in this field, is one of pressing issues of modern medicine. In traumatology, orthopedics, stomatology, oncology and purulent surgery, the search of approach to treatment of bone and external protective tissues of the human body implies not only methods of treatment but also retaining the initial form, structure and functions of the traumatized and diseased bones [2]. It can be solved by creating optimal conditions for regeneration in the areas of bone resorption. One of the ways is to use the osteoplastic materials [3]. Among them, allogenic grafts received from human tissues are optimal materials for treatment of damages of the musculo-skeletal system. After special treatment, allogenic materials lose their antigenticity almost completely, and, placed into the body, they don’t have any negative influence on it [4]. They act as a matrix, conductor, gradually completely dissolves, and new bone tissue is formed in their place [5, 6].

To compare the quality of the grafts made from allogenic trabecular bones treated using the "Lioplast®" method, physical methods of analysis are reasonable to use.
The authors of the work [7] have also used the Raman spectroscopy method for research the changes in composition of hydroxyapatite received from dental hard tissues during different pathological processes. The results of the experimental study indicate that the method of Raman spectroscopy is informative for study of crystallinity, structural features of biomaterials on the basis of hydroxyapatite.

The Raman spectroscopy has certain advantages and allows real-time conduction of non-destructive, noninvasive analysis of biological subject composition and provides information about molecular structure with high spatial resolution. Thus the Raman spectroscopy method is used in the articles [8, 9] to estimate the main components of the human bones – hydroxyapatite and amid I-III. Comparing the amplitudes of the Raman peaks on wavenumbers in the range from 700 to 1800 cm\(^{-1}\), the authors have discovered significant changes of signals for the samples with different demineralization level.

The aim of this work is comparative analysis of component composition of the mineralized and demineralized bone grafts with different porosity, made by the "Lioplast®" method with large and small pores.

2. Materials and methods of research

The Raman spectroscopy method implemented with the use of experimental stand including the Raman probe RPB-785 (focal length 7.5 mm) combined with the laser module LuxxMaster LML-785.0RB-04 (power up to 500 mW, wavelength 784.7 ± 0.05 nm) and high-resolution digital spectrometer Shamrock sr-303i providing spectral resolution of 0.15 nm with the built-in cooling chamber DV420A-OE [10] (spectral range 200-1200 nm) was used as the main method of graft analysis. The spectra were taken from each sample on each size of the cube in seven different points.

The samples of trabecular bone grafts in a form of 5*5*5 mm cubes, made by "Lioplast®" method (TU- 9398-001-01963143-2004), were the subjects of the research. The samples were divided into four groups. The first group was made by the demineralized samples with large pores (KD). The second group was made by the demineralized samples with small pores (MD). The third and four groups were formed by the mineralized samples with large (KM) and small (MM) pores. The demineralized samples were received by bathing a bone tissue in hydrochloric acid solution with 2.4 normality after the pretreatment. At the final stage of the treatment the bones were lyophilized by the unit ALPHA 2-4 LSC which included the sample freeze-drying. The packed material was radiation sterilized at the final stage. The chemical factor applications were minimal in order to reduce allergic reactions and complications.

The spectrum processing was conducted using the software package Wolfram Mathematica 10 and included removing the noises using the smoothing median filter in 7 points. Then the approximation line (a seventh order polynomial) of autofluorescent component was determined on the chosen interval of 300-2200 cm\(^{-1}\) using an iteration algorithm [11], and then this component was subtracted, receiving the selected Raman spectrum. The error of the used coefficients did not exceed 4% [12].

The method of spectral contour modelling that increased the spectrum informativeness was implemented in MagicPlotPro 2.7.2 software, where spectra were decomposed to the lines described by the Gaussian function. The subsequent chemometric analysis using the principal component analysis (PCA) was implemented in the Unscrambler X software.

3. Results

For the research of the Raman spectra of bones the most interesting are intensities on the wavenumbers 960 cm\(^{-1}\) (PO\(_4\)^{2-} (v\(_s\))), 1070 cm\(^{-1}\) (CO\(_2\)^{2-} (v\(_s\)) B-type substitution), 855 cm\(^{-1}\) and 870 cm\(^{-1}\) (benzene ring of proline and hydroxyproline oscillation), 1000 cm\(^{-1}\) and 1028 cm\(^{-1}\) (phenylalanine oscillation), 814 and 914 cm\(^{-1}\) (DNA / RNA) [7, 9, 13].

The averaged Raman spectra of demineralized (Fig. 1) and mineralized (Fig. 2) samples of allogenic bone tissues are presented below.
Figure 1 – Averaged Raman spectra of demineralized allogenic samples of grafts: 1 – the sample with large pores; 2 – the sample with small pores

Apart from the Raman lines of proline and hydroxyproline the collagen component includes the groups of amide III (in the range of 1240-1277 cm\(^{-1}\)), amide II (in the range of 1555-1565 cm\(^{-1}\)) and amide I (in the range of 1655-1675 cm\(^{-1}\)), as long as the Raman lines 1000 and 1028 cm\(^{-1}\), that correspond to phenylalanine fluctuation.

All groups of samples also include the Raman lines at the wavenumber 814 cm\(^{-1}\), corresponding to phosphodiester bond of DNA/RNA, possibly indicating nucleus destruction and incomplete DNA/RNA removal from the samples.
Figure 2 - Averaged Raman spectra of mineralized allogenic samples of grafts: 1 – the sample with large pores; 2 – the sample with small pores.

The interpretation of Raman spectra of bone graft samples is presented in Table 1.

Table 1. Interpretation of Raman spectra of bone graft samples

| Raman shift, (cm⁻¹) | Assignments                                                                 |
|---------------------|-----------------------------------------------------------------------------|
| 814                 | Phosphodiesterase (DNA/RNA) (C’₅-O-P-O-C’‴) [13]                            |
| 855                 | Benzene ring of proline [14, 15]                                            |
| 870                 | Benzene ring of hydroxyproline [15]                                         |
| 914                 | Ribose (RNA) [13]                                                          |
| 931                 | Proline ν(C-C) skeletal of collagen backbone [15]                           |
| 960                 | PO₄³⁻ (v₁) (P-O symmetric stretch) [14]                                     |
| 1000                | Aromatic ring breathing of phenylalanine (protein assignment) [13]          |
| 1028                | Phenylalanine (CH₂CH₃ bending modes (collagen assignment)) [13]             |
| 1044                | PO₄³⁻ (v₂) (P-O asymmetric stretch) [15]                                    |
| 1062                | GAG’s (O-SO₃- symmetric stretch) [14, 15]                                  |
| 1070                | CO₃²⁻ (v₁) B-type substitution (C-O in-plane stretch) [14]                  |
| 1095                | CO₃²⁻ (v₂) A-type substitution (C-O in-plane stretch) [14]                  |
| 1167                | Tyrosine (DNA/RNA) [13]                                                    |
| 1230-1289           | Amide III (C-N-H stretch) [14]                                              |
| 1298                | Fatty acids (CH₂, CH₃ twisting and wagging (lipids)) [13]                   |
| 1420                | Deoxyribose (DNA/RNA) [13]                                                 |
| 1448                | Proteine & lipids, CH₂ twisting [14]                                        |
| 1555-1565           | Amide II (C-N-H stretch) [13]                                               |
| 1655-1675           | Amide I (C=O stretch) [14]                                                  |
| 1738                | Phospholipids (C=O stretch) [13]                                            |
Fig. 1 and Fig. 2 show, that there is a considerable difference between the intensities of wavenumber peaks of grafts with different pore sizes. The increase of the intensity on the wavenumbers 960 cm\(^{-1}\), 1070 cm\(^{-1}\), corresponding to PO\(_4^{3-}\) \((\nu_1)\) (P-O symmetric valent), CO\(_2\)\(^{2-}\) \((\nu_1)\) B-type substitution (C-O two-dimensional valent) was characteristic of the mineralized grafts of both groups. After demineralization there was an intensity decrease on these wavenumbers of the samples with large pores, as well as with small ones.

The nonlinear regressive analysis of Raman spectra was made using the MagicPlotPro 2.7.2 software, consisting of spectrum decomposition.

For estimation of component composition of bone grafts with different porosity we have implied the coefficients characterizing the relative concentration of key components. The relatively permanent component amide I \([16, 17]\), corresponding to the wavenumber 1660 cm\(^{-1}\) was used as a denominator of the implied coefficients \((k)\):

\[
k = \frac{I_i}{I_{1660}},
\]

were \(I_i\) – the intensities on wavenumbers of the analyzed components.

The principal component analysis (PCA) used for revealing hidden structures in big data sets was chosen to analyze these data.

![Figure 3 – PCA score plot of the Raman spectra of the mineralized bone samples](image-url)
Figure 4 – PCA loading plot of the Raman spectra of the mineralized bone samples

Fig. 3, Fig. 4 and PCA output data analysis indicates that:

1. The main differences between the groups of samples are shown by the coefficients representing relative intensity on the wavenumbers 960 cm\(^{-1}\) (PO\(_4^{3-}\) (\(\nu_1\))), 1070 cm\(^{-1}\) (CO\(_3^{2-}\) (\(\nu_1\)) B-type substitution), 1448 cm\(^{-1}\) (protein & lipids). These variables are very important in the samples with small pores.

2. The higher is the PC-1 value modulus of the variable, the more it influences the observed difference of component composition, e.g. which can be seen from the coefficient \(k_{960}\). Fig. 2 shows that the intensity of the spectral line 960 cm\(^{-1}\), corresponding to the hydroxyapatite phosphate ion fluctuations, is higher for the small pore samples.

A two-dimensional analysis of the introduced optical coefficients was carried out. Figure 5 shows the two-dimensional diagrams, representing quantitative amount of the main components of the mineralized and demineralized bone grafts with small and large pores.

Figure 5 – Two-dimensional diagrams of the implemented coefficients: a) mineralized bone tissue
b) demineralized bone tissue

Fig. 2 analysis shows that lower coefficients \(I_{960}/I_{1660}\) - mineral/organic matrix were characteristic of the mineralized samples with large pores in comparison with the small-pored samples. The coefficient \(I_{1070}/I_{1660}\) (Carbonate/Organic matrix ratio) was used for biomaterial mineralization level estimation. Fig. 5a shows that the coefficient \(I_{1070}/I_{1660}\) of the large-pored samples is also less than the one of the small-pored samples. This implies that the pore size influences the demineralization process. Apparently this
is due to the fact that the demineralization process doesn’t include acid mixing, so the areas of its non-uniform concentration appear in its interaction with the samples, particularly in the small-pored areas. The acid mixing should be ensured for the more uniform demineralization.

Fig. 5b also shows that higher coefficients $I_{1000}/I_{1660}$ and $I_{1167}/I_{1660}$, therefore higher relative concentration of phenylalanine and glycosaminoglycan, that are the main components of the extracellular matrix, involved in the process of graft acceptance, were characteristic of the demineralized samples with large pores in comparison with the small-pored samples.

The range of characteristics of the sample inside the groups resulting from the range of the initial parameters of the donor samples is worth noting.

4. Conclusion

The comparative spectral estimation of component composition of mineralized and demineralized bone grafts with different porosity made by "Lioplast®" method with large and small pores was made.

The comparison has shown the difference in intensities of spectral lines of the mineralized samples on the wavenumbers 960 cm$^{-1}$ and 1070 cm$^{-1}$, corresponding to hydroxyapatite phosphate ion and carbonate ion, as well as of the demineralized samples on the wavenumbers 1000 cm$^{-1}$ (phenylalanine aromatic ring) and 1167 cm$^{-1}$ (GAG).

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