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Detailed analysis of the structural changes of bone matrix during the demineralization process using Raman spectroscopy

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Abstract

The results of experimental research of human cortical bone tissue depending on demineralization time were represented using Raman spectroscopy. Depending on demineralization time the ratio of the mineral (PO₄³⁻ and CO₃²⁻) and organic components (amide I) of bone tissue, as well as changes in the spectral regions responsible for the structural integrity of the collagen fibers in bone tissue (1200-1460 cm⁻¹ and 2880-3000 cm⁻¹) were investigated. The observed changes show a decrease in mineral components: thus, the value of Raman band intensity at 956 and 1069 cm⁻¹ for 5 minutes demineralization is 68.5 and 77.3 %, for 20 minutes - 55.1 and 61.1 %, for 120 minutes - 32.8 and 37 % from Raman intensity values of not demineralized tissue objects respectively.

Keywords: Raman spectroscopy; cortical bone; hydroxyapatite; collagen; scanning electron microscopy.

1. Introduction

In surgical and traumatology practice the alteration of the bone tissue integrity, associated with the diseases of musculoskeletal system, with the occurrence of microfractures amid weakening of the bone remodeling process with a consequent increase of bone fragility and porosity, is specific for a significant percentage of patients. Currently, for the replacement of bone defects is accepted to use the "gold standard" - autogenous bone grafts. At early stages of the study and use of autogenous tissues in transplantation process, the main problem with infectious complications following transplantation has forced specialists to refuse of this kind of grafts. Over time, the development of

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biomedical sciences in conjunction with innovative processing techniques of autogenous tissue resulted in the creation of new standards for the safe use of autogenous grafts [Flierl M.A. et al. (2013)].

The use of demineralized lyophilized bone tissue of donor that almost is not inferior to a bone graft based on patient's tissue, is one of the such standards. The important step in obtaining of demineralized bone biomaterial is the processing of tissue in acidic solution that led to loss of most of the mineral components [Kirillova I.A. (2004)] while maintaining of collagen and proteins proportion necessary for tissue regeneration [Timchenko P.E. et al. (2011)]. It is also known that the recovery speed of bone is higher in the transplantation of partially demineralized biomaterial as compared with a completely demineralized [West P.A. et al. (2005)]. The effect of demineralization incompleteness is explained by strong links of osteoinductor (responsible for the osteogenesis) with the mineral component of bone tissue [Slutsky L.I. et al. (1986)]. As a result, correctly selected the demineralization degree of bone matrix provides the perfect combination of osteoinductive and osteoconductive properties for effective bone regeneration.

Used biochemical and morphological methods to determine the demineralization degree often require significant time and resource costs that directly affect on the quality of patient care. At the moment, among the possible methods to solve this problem are the optical methods, and authors of paper [Tarnowski C.P. et al. (2002)] have proposed the method of Raman spectroscopy (RS) as a fast and efficient method for investigating the bone mineral components.

The aim of this work was the establishment of dependence for mineral and organic components of human bone biomaterial in the demineralization process using Raman spectroscopy.

2. Materials and methods

As objects of study the 36 samples of three-dimensional cortical human bone tissue of the "Lioplast" series (TU-9398-001-01963143-2004) were used. The first step in technological preparation process of grafts was degreasing and complete removal of bone marrow and blood from bone scaffolds by brief low-frequency ultrasonic treatment (24 - 40 kHz during 2-3 minutes), while the destruction of bone was not occurring. Then, to obtain the demineralized bone biomaterial we placed bone tissue in a 1.2 % solution of HCl while maintaining a constant solution temperature at +4 °C. The next step was lyophilization of samples on the ALPHA 2-4 LSC which consists in freeze-dried of tissue.

Spectral characteristics of the samples were studied using an experimental setup (Fig. 1), includes a high-resolution digital spectrometer Shamrock sr-303i with an integrated cooled camera DV420A-OE CCD (spectral resolution of 0.15 nm (~ 1 cm⁻¹)), an RPB785 fiber-optic probe combined with the LuxxMaster LML-785.0RB-04 laser module (785 nm wavelength).

![Experimental setup](image)

Fig. 1. Experimental setup: 1 – object; 2 – Raman probe RPB785; 3 – laser module LuxxMaster LML-785.0RB-04; 4 – laser power supply module; 5 – spectrometer Shamrock sr-303i; 6 – CCD camera ANDOR DV-420A-OE; 7 – computer; 8 – transmitting fiber; 9 – receiving fiber
Due to the low level of autofluorescence the 785 nm wavelength of the visible wavelength range is well-proven in Raman spectroscopy for biological applications. Procedure description for work with the experimental stand earlier was presented in the article [Timchenko E.V. et al. (2014)].

3. Results

Before data analysis, the obtained Raman spectra of research objects were preprocessed in MatLab’7 for deletion of setup noise and fluorescence background using an approximation of the spectral curve by a fifth order polynomial, based on the algorithm proposed in the paper of [Zhao J. et al. (2007)]. A feature of the algorithm is a sequential and many iterative comparisons of the initial spectrum with a polynomial approximation of autofluorescence component, and the preservation of spectral contours and intensity of the initial Raman spectrum positively proved the algorithm in spectroscopy for biomedical research [Huang Z. et al. (2011)]. However, during the spectra study was found that at the large spectral range (exceeding the 1500-3000 cm\(^{-1}\)) the approximation of spectral curve led to cuts of Raman band near more intensive neighboring band such as 1003 cm\(^{-1}\) (ν (C-C) of phenylalanine) near 956 cm\(^{-1}\) (ν1 (PO\(_4\))\(^{3-}\) of hydroxyapatite). Therefore, the processing was carried out at the spectral regions 400-1150, 1200-1500, 1600-1800 and 2800-3000 cm\(^{-1}\) with appropriate criteria of approximation for sensitivity increase and maximum display without loss of informative bands.

Informativeness of Raman spectra of bone tissue samples depends on the complexity and structure of studied biomaterial (on the number of bands in Raman spectrum), because the resulting spectrum is a superposition of several spectral bands that leading to distortion of the real values of intensity. Therefore, to extract the Raman bands against the background of spectral curve, the decomposition of obtained spectra into spectral contours using deconvolution of Gauss-Lorentz function was conducted by PeakFit software (Systat Software, Inc.). Specific Raman spectrum for non-deamineralized cortical human bone is presented in Fig. 2. The determination coefficients of the resulting spectrum (generated by the spectral contours) from the initial spectrum for 790-1140 cm\(^{-1}\), 1200-1480 cm\(^{-1}\), 1590-1790 cm\(^{-1}\) and 2840-3020 cm\(^{-1}\) regions were \(R^2 = 0.9999, 0.9978, 0.9998\) and 0.9993 respectively.

The spectral region (Fig. 2a) contains the Raman bands responsible for the mineral structure of bone matrix, such as ν1 (PO\(_4\))\(^{3-}\) vibrational mode in the molecule of hydroxyapatite at 956 cm\(^{-1}\), ν3 (PO\(_4\))\(^{3-}\) at 1030 and 1045 cm\(^{-1}\), ν1 (CO\(_3\))\(^{2-}\) (B type substitution where (PO\(_4\))\(^{3-}\) a substituted (CO\(_3\))\(^{2-}\)) at 1069 cm\(^{-1}\) and ν1 (CO\(_3\))\(^{2-}\) vibration mode (A type substitution where a substituted OH- (CO\(_3\))\(^{2-}\)) at 1098 cm\(^{-1}\) [Yamamoto T. et al. (2011), Crane N.J. et al. (2013)]. Band at 956 cm\(^{-1}\) is dominant by the intensity throughout all Raman spectrum of bone tissue, which determines the content of hydroxyapatite in bone matrix, and also possible to study the content of carbonate (CO\(_3\))\(^{2-}\) in the process of demineralization. Less intense bands at 816, 852, 920, 941 and 870 cm\(^{-1}\) corresponding to the stretching vibration of C-C in proline and hydroxyproline [Yamamoto T. et al. (2011), Crane N.J. et al. (2013), Rehman I. et al. (2012), Połomska M. et al. (2010)] and 1003 cm\(^{-1}\) responsible for C-C stretching vibration in phenylalanine [Crane N.J. et al. (2013)] represent the organic component of bone matrix.

Further, Fig. 2b shows the spectral region responsible for structural integrity of bone collagen, where 1239, 1264, 1284, 1316, 1338 and 1381 cm\(^{-1}\) bands correspond to amide III (modes of ν (CN) and δ (NH)) [Yamamoto T. et al. (2011), Crane N.J. et al. (2013), Rehman I. et al. (2012), Polomska M. et al. (2010)] and 1396, 1418 and 1450 cm\(^{-1}\) correspond to mode of δ (CH2) and δ (CH3) [Rehman I. et al. (2012), Polomska M. et al. (2010)]. Spectral region of 1600-1700 cm\(^{-1}\) (Fig. 2c) in [Crane N.J. et al. (2013)] is a defining in the assessment of structural integrity of the collagen fibers. This region corresponds to amide I (stretching of C=O) with Raman bands at 1634, 1660, 1681 cm\(^{-1}\) (α-helix and β-sheet [Polomska M. et al. (2010), Shen J. et al. (2010)]), and 1604 cm\(^{-1}\) (stretching vibration of C=C in tyrosine [Rehman I. et al. (2012)]). The most remote region of the spectrum (Fig. 2d) at 2879, 2910, 2938 and 2980 cm\(^{-1}\) corresponds to the stretching vibrations of CH\(_2\) and CH\(_3\) in collagen [Polomska M. et al. (2010), Ali S.M. et al. (2013)].
In this article, the relative concentration of organic compounds in bone matrix was evaluated by the intensity of the amide III band (1239 cm\(^{-1}\)), CH\(_2\) deformation band (1450 cm\(^{-1}\)) and amide I region (1660 and 1681 cm\(^{-1}\)). In turn, Raman band at 956, 1069 and 1098 cm\(^{-1}\) were used for investigation changes in the mineral components of bone matrix.

Further, Fig. 3 shows the intensity change of Raman bands for bone matrix samples at different demineralization time relative to intensity values of Raman bands for non-demineralized samples. The intensity value at 2938 cm\(^{-1}\) were used for intensity normalization of all spectral bands:

\[
\ln(k) = \frac{I(k)_t}{I(k)_0 \cdot I(2938)_t}
\]

where \(I(k)_t\) – Raman intensity at a \(k\) wavenumber with a \(t\) demineralization time of sample, \(I(k)_0\) – Raman intensity at a \(k\) wavenumber without demineralization of sample \((t = 0)\), and \(I(2938)_t\) – Raman intensity at 2938 cm\(^{-1}\) wavenumber with a \(t\) demineralization time of sample.
As can be seen from Fig. 3a, bands at 956, 1069 and 1098 cm\(^{-1}\) are characterized by a significant intensity decrease with demineralization time: at 5 minutes of demineralization the values of In(956), In(1069) and In(1098) are 68.5, 77.3 and 83.2 %, at 20 minutes - 55.1, 61.1 and 76.2%, at 120 minutes - 32.8, 37 and 66.4% respectively. Fig. 3b shows the intensity changes in Raman bands of amide I region, indicates the cross-links in collagen: the main band at 1660 cm\(^{-1}\) is characterized by an intensity decrease to 89.2% at 5 minute demineralization and to 83.2% at 20 minutes, but 1681 cm\(^{-1}\) is characterized by an intensity increase to 19.4% at 5 minutes and 37% at 20 minute demineralization.

Raman intensity at 1239 cm\(^{-1}\), responsible for the amide III group, during all demineralization time varies by less than 5.6% (Fig. 3b), unlike the band at 1450 cm\(^{-1}\) whose intensity after 90 minute demineralization is decreased to 89.1% relative to Raman intensity of non-demineralized bone matrix sample (Fig. 3d). Practically unchanged in intensity the Raman band of collagen informs about an optimal parameters of the demineralized medium (HCl acid temperature and concentration) that does not lead to deformation and destruction of collagen. Availability of Raman bands, that correspond to the collagen structure and whose intensity increases with increasing demineralization time,
can be explained by the restructuring into chemical composition collagen, associated with a recovery or oxidation of CH-bond. Thus, the band intensities at 1284 and 2879 cm\(^{-1}\) grow with demineralization time and reach their maximum of 117.2 and 117.1% at 20 minutes demineralization relatively non-demineralized samples with subsequent intensity relaxation at 45-120 minutes demineralization.

The mineral composition change of investigated samples parallel was monitored by scanning electron microscopy. Micrographs of sample surface structure for cortical bone matrix depending on the demineralization time are presented in Fig. 4.

![Micrographs of sample surface structure for cortical bone matrix](image)

Fig. 4. The structure of cortical bone surface at different demineralization time for biomaterial: (a) 0 min, (b) 2 min, (c) 5 min, (d) 8 min. The accelerating voltage - 20 kV, beam current - 1 nA, number of points - 16

In Fig. 4 can observe as the demineralized samples have a certain degree of bone surface roughness (Fig. 4c) with the appearance of pores at 5 and 8 minutes demineralization. These changes are the result of "washing out" apatite crystals under an acid action on the object. That causes the bones to lose most of their hardness and compressive strength, and at a large demineralization time leads to deformation and destruction of the trabeculae.

4. Conclusion

As a result, Raman spectra of human cortical bone with a different demineralization degree were obtained. Shown that the demineralization process can be quantitatively controlled using the ratio \(\text{PO}_4^3^-\) and \(\text{CO}_3^2^-\): for 956 and 1069 cm\(^{-1}\) at 5 minutes demineralization the intensity value is 68.5 and 77.3%, for 20 minutes - 55.1 and 61.1%, at 120 minutes - 32.8 and 37% respectively. The spectral features for bone collagen depending on demineralization time were investigated by analyzing spectral regions of 1200-1460 cm\(^{-1}\) and 2880-3000 cm\(^{-1}\).

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