Comparative spectral analysis of the extra-cell matrixes surface of heart valves before and during the process of their decellularization

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Abstract. There are presented the results of the application of Raman-scattering spectroscopy method (RS) for the qualitative analysis of rams' heart valves surfaces before and during their decellularization. While analyzing RS spectra, it was found that basic differences appear at wave numbers 812 cm⁻¹, 1062 cm⁻¹, 1340 cm⁻¹ and 1440 cm⁻¹, corresponding to phosphodiester linkage of RNA; OSO₃ corresponds to symmetrical stretching of glycosaminoglycans and chondroitin-6-sulfate; corresponds to deformation mode of proteins and nucleic acids (DNA); proteins, lipids. Optical analysis has shown that while analyzing decellularization on the valves surfaces, the content of glycosaminoglycans, proteins and lipids decreases; retains a high DNA content. It was found that with the aid of entered optical numbers it is possible to control the process efficiency of decellularization of the heart valves.

1. Introduction

The issue of treating people's heart valve diseases is one of the priorities of modern medicine. One of the most radical methods of treatment is the replacement of valves [1, 2]. However, the quality, design and properties of the prosthetic cardiac valve are constantly improving; they can not be compared with native valves in their properties. Thuswise, clinical cardiosurgery is in need of creating new types of implants and improving the technology of their production. [3, 4].

Because of generous amount of complications while using valve bioimplants there is a need of high-quality processing of biomaterials. Decellularization is one of the supplementary methods of tissue engineering of heart valves. This process is aimed at removing cells from the tissue with preservation of extra-cellular matrix and three-dimensional material structure [5, 6]. A number of authors consider that in order to reduce the tissue antigenicity during the process of decellularization there is a necessity for absolute elimination of cellular components, in particular: membranes and connected membrane proteins with it, bioplasts, nuclei and nucleic acids that contained in them [7, 8].

Currently there is no commonly used methodology for decellularization of heart valves. Furthermore, there is no universally received ways to control its effectiveness. With this object in mind, histological, histochemical, biochemical and immunological methods are currently used. Their main disadvantage is the destruction of the analyzed samples along with the labor-consuming nature.
and high cost [5-8]. Consequently, the search for appropriate way of analyzing the qualitative composition of heart valves in the process of decellularization is the priority.

The Raman-scattering spectroscopy method can be effective in assessing the decellularization effectiveness of the surface of heart valve samples, since it is able to determine the content of the main matrix components and does not require destruction of the biomaterial during the study [9-11].

The goal of this research is to analyze the qualitative composition of the heart valves surface by using the Raman-scattering spectroscopy method before and after their decellularization.

2. Materials and methods of research
Aortic valves of sexually mature rams are used as a research material. Valves decellularization was carried out in the modification according to the protocols [11, 12, 13] in Samara State Medical University (SamGMU). There is a phase 1 of decellularization before fermentative treatment and a phase 2 after fermentative treatment. Biomaterials samples were stored in phosphatic-salt solution with antibiotics at a temperature of 4 °C before the study.

Spectral-response characteristics of the samples were studied using an experimental stand including a high-resolution digital spectrometer Shamrock sr-303i with built-in cooling chamber DV420A-OE, a fiber-optics probe RPB-785 for Raman spectroscopy, combined with laser module LuxxMaster LML-785.0RB-04 (with controlled output up to 500 mW, and a wavelength of 785 nm). The radiation power of 500 mW of the laser within the exposure time up to 300 seconds does not cause destructive changes in the samples.

RP probe focused laser radiation on the object at a distance of 7.5 mm from the output window with a focal spot diameter of less than 0.2 mm and collected radiated emission.

3. Results and discussion
Figure 2 shows the average RS spectra for the surfaces of aortic valve samples before decellularization (control), and RS spectra of valve surfaces in the process of performing decellularization before (step 1) and after (step 2) fermentative treatment of the samples.
Figure 2 - Average spectra of surfaces of aortic heart valves before and during their decellularization

While carrying out RS spectroscopy of the valve surfaces before and during their decellularization, we obtained qualitatively identical Raman bands corresponding to certain oscillation modes (Table 1).

Table 1. The results interpretation of RS spectroscopy of aortic valves surfaces

| Wave number, cm⁻¹ | Substance, oscillation                                      |
|-------------------|------------------------------------------------------------|
| 812               | Phosphodiester linkage RNA[15]                             |
| 855               | ν(C–C) Proline, oxyproline, tyrosine [16, 17]               |
| 935               | ν(C–C) Proline, oxyproline [18]                            |
| 988               | C-H Lipide bend [19]                                       |
| 1003              | Phenyllalanine [18]                                        |
| 1033              | CH oscillation of phenylalanine [20], CN stretching in protein [20,21] |
| 1062              | OSO-3 symmetrical stretching of glycosaminoglycans, chondroitin-6-sulfate [18] |
| 1082              | C-H in a mode of a stretching of fibers (and a mode of lipids to a lesser degree) [21] |
| 1202              | Hydroxyproline, tyrosine [23]                              |
| 1246, 1271        | Amide III [21]                                             |
| 1340              | Deformation of proteins and nucleic acids (DNA) [20]       |
| 1376              | CH oscillations [12]                                       |
| 1440              | δ (CH2) proteins, lipids [18]                             |
| 1556              | Elastin (protein) [20]                                    |
| 1625, 1661        | Amide I (C = O) [22]                                       |

From Fig. 2 it can be seen that there was a reduction of intensity during the first phase of decellularization at wave numbers 812 cm⁻¹, 1062 cm⁻¹ and 1440 cm⁻¹, corresponding to the phosphodiester linkage RNA; OSO-3 symmetrical stretching of glycosaminoglycans of chondroitin-6-sulfate; proteins, lipids. As the second phase of decellularization completed there was noted a little
decrease in the intensity at a wave number of 1340 cm\(^{-1}\), corresponding to the deformation mode of proteins and nucleic acids (DNA).

The relatively constant surface samples component of the study before and during decellularization was amide III, corresponding to the line intensity at a wave number of 1246 cm\(^{-1}\) [14]. Therefore, the number magnitude was used as a denominator at calculating the entered optical number (coefficient) \(f\):

\[
f = \frac{I_i}{I_{1246}}
\]

\(I_i\) - is the intensity value at the wave number of the analyzed component, \(I_{1246}\) - is the intensity value at the wave number of amide III.

Figure 3 presents two-dimensional diagrams of optical numbers (coefficients) reflecting a change in the composition of the main surface components of aortic heart valves before and during the process of decellularization at different phases.

![Diagram](image)

**Figure 3** - Two-dimensional diagrams of the entered optical numbers (coefficients) (Control - samples before decellularization, step 1 - samples before fermentative treatment, step 2 - samples after fermentative treatment)
The two-dimensional dependence analysis showed that in the phases of performing decellularization there was observed a gradual decrease of the optical numbers (coefficients) I_{1246} / I_{1340} , I_{1062} / I_{1246} and I_{1440} / I_{1340} in comparison with the values obtained during the study of the surfaces of intact aortic valves. The optical number (coefficient) I_{1340} / I_{1246} slightly decreases even after the second phase of decellularization.

4. Conclusions
During the study of surface of aortic valves before and during their decellularization by using RS spectroscopy, it was found that even after the first phase of decellularization there occurred intensity at the wave numbers 812 cm\(^{-1}\), 1062 cm\(^{-1}\) and 1440 cm\(^{-1}\) corresponding to the phosphodiester linkage RNA ; OSO-3 symmetrical stretching of glycosaminoglycans of chondroitin-6-sulfate; proteins, lipids. After the second phase of decellularization was completed there was noted a little decrease in the intensity at a wave number 1340 cm\(^{-1}\) corresponding to the deformation mode of proteins and nucleic acids (DNA).

Entering optical numbers (coefficients) and two-dimensional analysis, we elucidated the effectiveness of the decellularization process of aortic valves. This effectiveness was indirectly manifested as a decrease in the content of lipids, proteins and glycosaminoglycans on the surface. Nevertheless, the DNA content in the valve samples insignificantly decreased even before the completion of the second phase of decellularization.

With the help of the entered optical numbers (coefficients), it is possible to control the effectiveness of decellularization process of the heart valves.

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References
[1] Astapov D A, Nazarov V M, Zheltovsky Y V, Isayan M V, Demidov D P, Kaganskaya N A 2013 A xenobiological prosthesis in the left heart (Siberian Medical Journal - No. 1) pp. 13-17
[2] Bokeria L A, Kagramanov I I, Kokshenev I V, Britikov D V 2009 Biomaterials in Cardiovascular Surgery (Moscow) p. 350
[3] Gendlin G E, Storozhakov G I, Vavilov P A et al. 2008 Indications for surgical treatment of patients with heart valve diseases (Heart) Vol. 7, № 2, pp. 113-117
[4] Dohmen P M, Konertz W 2009 Tissue-engineered heart valve scaffolds (Ann. Thorac. Cardiovasc. Surg.) 15(6), pp. 362–367
[5] He M, Callanan A 2013 Comparison of methods for whole-organ decellularization in tissue engineering of bioartificial organs (Tissue Eng Part B Rev) Vol. 3, № 19, pp. 194-208
[6] Zia S, Mozafari M, Natasha G, Tan A, Cui Z, Seifalian A M 2015 Hearts beating through decellularized scaffolds: whole-organ engineering for cardiac regeneration and transplantation (Crit Rev Biotechnol)
[7] Lavresin A V, Nasredinov A S, Kurapeev D I, Anisimov S V, Mitrofanova L B, Beshchuk O V 2014 Decellularization of aortic allografts and their morphological evaluation (Genes and cells) Vol. 9, № 1
[8] Assmann A, Delfs C, Munakata H, Schiffer F, Horstkötter K, Huynh K, Barth M, Stoldt V R, KAMIYA H, Boeken U, Lichtenberg A, AKHYARI P 2013 Acceleration of autologous in vivo recellularization of decellularized aortic conduits by fibronectin surface coating (Biomaterials) Vol. 25, № 34, pp. 6015-6026
[9] Krafft C, Dietzke B, Popp J 2009 Raman and CARS microspectroscopy of cells and tissues (Analyst Vol. 6, № 134, pp. 1046-1057
[10] Enrique Uceda Otero, Sokki Sathaiah, Landulfo Silveira Jr., Pablo Maria Alberto Pomerantz S, Carlos Augusto Gonçalves Pasqualucci 2004 Raman spectroscopy for diagnosis of calcification in human heart valves (Spectroscopy IOS Press) Vol. 18, pp. 75–84
[11] Sara Mangialardo, Valentina Cottignoli, Elena Cavarretta, Loris Salvador, Paolo Postorino, Adriana Maras 2012 Pathological Biominerals: Raman and Infrared Studies of Bioapatite Deposits in Human Heart Valves (Applied Spectroscopy) Vol. 66, pp. 1121-1127

[12] Lichtenberg A et al. 2007 Biological scaffolds for heart valve tissue engineering (Methods Mol. Med.) Vol. 140, № 2, pp. 309-317

[13] Chan, J.W., Taylor, D.S., Zwerdling, T., Lane, S.T., Ihara, K., and Huser, T. 2006 Micro-Raman spectroscopy detects individual neoplastic and normal hematopoietic cells (Biophysical Journal) Vol. 90, pp. 648–656

[14] Lichtenberg A, Tudorache I, Cebotari S et al. 2006 A Preclinical testing of tissue-engineered heart valves re-endothelialized under simulated physiological conditions (Circulation) Vol. 114, pp. 1559-1565

[15] Chan J W, Taylor D S, Zwerdling T, Lane S T, Ihara K, Huser T 2006 Micro-Raman spectroscopy detects individual neoplastic and normal hematopoietic cells (Biophysical Journal) Vol. 90, pp. 648–656

[16] E Brauchle, S Noor, E Holtorf, C Garbe, K Schenke-Layland, and C Busch 2014 Raman spectroscopy as an analytical tool for melanoma research (Clinical and Experimental Dermatology) Vol. 39, pp. 636–645

[17] Nguyen T T, Gobinet C, Feru J, Brassart–Pasco S, Manfait M 2012 Piot Characterization of Type I and IV Collagens by Raman Microspectroscopy: Identification of Spectral Markers of the Dermo-Epidermal Junction (Spectroscopy) Vol. 27, pp. 421–427

[18] Aliz Kunstar, Anne M Leferink, Paul I Okagbare, Michael D Morris, Blake J Roessler, Cees Otto, Marcel Karperien, Clemens A van Blitterswijk, Lorenzo Moroni and Aart A van Apeldoorn 2013 Label-free Raman monitoring of extracellular matrix formation in three-dimensional polymeric scaffolds (Journal royal society)

[19] Xu H, Xu B, Yang Q, Li X, Ma X, Xia Q, Zhang Y, Zhang C, Wu Y, Zhang Y 2014 Comparison of Decellularization Protocols for Preparing a Decellularized Porcine Annulus Fibrosus Scaffold (PLoS ONE) Vol. 1, № 9

[20] Sana Tfaili, Cyril Gobinet, Gwendal Josse, Jean-François Angiboust, Michel Manfaita and Olivier Piot 2012 Confocal Raman microspectroscopy for skin characterization: a comparative study between human skin and pig skin (The Royal Society of Chemistry Analyst) Vol. 137, pp. 3673–3682

[21] Kristy L Cloyd, Ismail El-Hamamsy 2012 Sauvimon Boorungsiman Characterization of Porcine Aortic Valvular Intersstitital Cell ‘Calcified’ Nodules (PLoS ONE) Vol. 7, pp. 1-9

[22] Enrique Uceda Otero, Sokki Sathaiah, Landulfo Silveira Jr, Pablo Maria Alberto Pomerantzeff, Carlos Augusto Gonçalves Pasqualucci 2004 Raman spectroscopy for diagnosis of calcification in human heart valves (Spectroscopy IOS Press) Vol. 18, pp. 75–84

[23] E Brauchle, S Noor, E Holtorf, C Garbe, K Schenke-Laylandl, and C Busch 2014 Raman spectroscopy as an analytical tool for melanoma research (Clinical and Experimental Dermatology) – Vol. 39, pp. 636–645