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Elena V. Timchenko  
Pavel E. Timchenko  
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Elena V. Timchenko,<sup>a,\*</sup> Pavel E. Timchenko,<sup>a</sup> Artur Lichtenberg,<sup>b</sup> Alexander Assmann,<sup>b</sup> Hug Aubin,<sup>b</sup> Payam Akhyari,<sup>b</sup> Larisa T. Volova,<sup>c</sup> and Svetlana V. Pershukina<sup>a</sup>

<sup>a</sup>Samara National Research University, Samara, Russia

<sup>b</sup>Heinrich Heine University, Clinic of Cardiovascular Surgery, Dusseldorf, Germany

<sup>c</sup>Experimental Medicine and Biotechnologies Institute of the Samara State Medical University, Samara, Russia

**Abstract.** We report the results of experimental studies on cardiac implants using a Raman spectroscopy method (RS). Raman spectra characteristics of leaves and walls of cardiac implants were obtained; the implants were manufactured by protocols of detergent-enzymatic technique (DET) and biological, detergent-free (BIO) decellularization, using detergents (group DET) or a detergent-free, nonproteolytic, actin-disassembling regimen (BIO). There were input optical coefficients that allowed us to carry out evaluation of the protocols of DET and BIO decellularization on the basis of the concentrations of glycosaminoglycans, proteins, amides, and DNA. It was shown that during DET and BIO decellularization, composition aberrations of proteins and lipids do not occur and the integrity of the collagenous structures is preserved. It was found that during the DET decellularization, preservation of glycosaminoglycans is better than during BIO decellularization. © 2017 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: [10.1117/1.JBO.22.9.091511](https://doi.org/10.1117/1.JBO.22.9.091511)]

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## 1 Introduction

At present, surgical treatment of cardiac valve disease occupies a considerable share of all cardiac treatment. In the last decade, the demand for heart valve transplants increased greatly. Even though quality, design, and properties of heart valve prostheses constantly improve, they are not comparable to the properties of native valves.<sup>1</sup> After a transplant of a native implant, the recipient will be at risk of chronic immune rejection and lifelong immunosuppression therapy.<sup>2</sup> That is why the overarching aim in the field of tissue engineering is the development and quality control of cell-free scaffolds suitable for the replacement of lesional tissues and organs. The large number of complications associated with implantation of an artificial cardiac valve necessitates high-quality processing of biomaterials. Decellularization is one of the helper methods of tissue engineering of heart valves and is aimed at the removal of cells from the tissue while maintaining the territorial matrix and the three-dimensional structure of the organ.<sup>2</sup> The most popular method of decellularization of heart valves is chemical treatment of biomaterials.<sup>3</sup> In particular, the detergent-enzymatic method is widely used with various detergent solutions and different duration of its exposure.<sup>4</sup> To reduce the antigenic potential of tissue, the detergent-enzymatic technique (DET) decellularization process involves removal of cell constituents such as membranes and the related soluble proteins, bioplasts, the nucleus and nucleic acids (DNA and RNA) in it. In the meantime, the capacity for remodeling is retained. Nevertheless, the use of DET decellularization as a method of heart tissue cleaning can cause damage to such components as proteoglycans and

glycosaminoglycans (GAGs), which are actively involved in cell growth, cell differentiation, and morphogenesis.<sup>4</sup> The problem with preservation of the territorial matrix structure after exposure to detergent-enzymatic agents is still relevant and necessitates the use of sparing decellularization [biological, detergent-free (BIO)] methods.<sup>4</sup> BIO decellularization effectively preserves GAGs, but it is difficult to ensure complete removal of cellular debris or unacceptable components of the territorial matrix using enzymatic treatment. Theoretically, in the extracellular matrix, researchers must preserve collagen, elastin, and GAGs, after which the main immunogenic components of cells, namely, lipid membranes, membrane-associated antigens, and soluble proteins, must be removed.<sup>5</sup> Most of the existing methods of quality control of cardiac implants may be used only with limitations. Magnetic resonance imaging (MRI) does not allow researchers to evaluate the composition of a biological tissue or to achieve the necessary space resolution of cardiac tissue samples within a short scan time. Furthermore, MRI scanners are expensive and time consuming.<sup>6</sup> Histology and scanning electron microscopy provide high-quality images of the microtexture of decellularization samples, and, as a result, they are the most popular methods for evaluation of a decellularization process, but they require destructive preparation of a sample.<sup>6</sup> Limitations of these methods of quality control of implants can be overcome by Raman scattering spectroscopy. This method is a simple, noninvasive, and rapid approach to quality control of materials for tissue engineering.<sup>7-9</sup> Thus, it was shown<sup>10</sup> that Raman spectroscopy (RS) allows researchers to identify the composition of cardiac tissue and the proportions of matrix components. Some authors<sup>7</sup> have conducted a comparative analysis of treated and control aortic valve cusps using

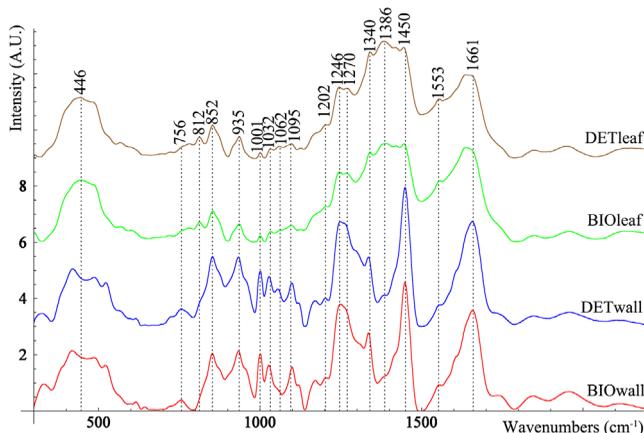
\*Address all correspondence to: Elena V. Timchenko, E-mail: [laser-optics.timchenko@mail.ru](mailto:laser-optics.timchenko@mail.ru)

Raman scattering spectroscopy. Comparing the Raman intensity of wave numbers in the region 400 to 1800  $\text{cm}^{-1}$ , those authors identified significant alterations in signal intensity for the extracellular matrix of the aorta. In one article,<sup>8</sup> researchers showed an estimate of cyto-reduction in decellularized bovine pericardia (during their lyophilization) using a Raman scattering method. Major changes in the spectrum were observed at wavenumbers 1004, 1240 to 1250, and 1665  $\text{cm}^{-1}$  corresponding to phenylalanine and type I and III amides. In one study,<sup>9</sup> the RS method was used for assessing protein dissolution of cardiac tissue during decellularization. The intensity of the Raman bands corresponding to the vibration of amide I at wavenumber 1665  $\text{cm}^{-1}$  allows researchers to reveal differences in the total secondary structure of a protein among tissues. For this reason, RS is a promising, noninvasive, and noncontact way to assess the extracellular matrix.

The aim of this study is to carry out an assessment of DET and BIO decellularization of cardiac implants by the Raman scattering spectroscopy method and to provide the typical spectra after the respective decellularization protocols.

## 2 Materials and Methods

We used 50 decellularized aortic valves of adult pigs. The native valvular samples have been obtained from healthy pigs from a local slaughterhouse near the University of Duesseldorf (Germany). DET and BIO decellularization of valves with retrieval of extracellular matrices was carried out using technologies of decellularization of Heinrich-Heine University Duesseldorf (Germany): DET decellularization based on detergent with sodium dodecyl sulfate and sodium deoxycholate as the main agents; BIO decellularization means physiological, detergent-free decellularization based on latrunculin B, high ionic strength of a saline solution, and DNase. A detailed description of the protocol based on DET and BIO decellularization is presented elsewhere.<sup>5</sup> The laboratory facility that was used in this work is described in Ref. 11 and includes a diode laser (LML-785.0RB-04), an optical Raman module (PBL 785), a spectrograph (Sharmrock SR-303i) with an integrated digital camera (ANDOR DV-420A-OE), and a computer. The use of



**Fig. 1** Raman spectra, normalized to the average intensity value of the extracellular matrix of the heart valve: the leaves and walls, obtained using DET and BIO decellularization (spectra are shifted vertically for illustrative purposes).

this spectrograph provides a resolution of 1.7  $\text{cm}^{-1}$  at a low level of intrinsic noise. To exclude the contribution of background to the Raman spectrum, we used the subtraction method of a background component of polynomial approximation with postfiltration of random noise effects. In this work, analysis of the Raman spectra was carried out in the range of 300 to 2200  $\text{cm}^{-1}$ . The laser radiation power of 400 mW within our exposure duration up to 200 s does not cause destructive changes in the samples. Registration of Raman spectra was carried out using an optical probe that was situated under the object at a distance of 7 mm.

Parts of this work, i.e., the peak values for GAG, amide I, and amide III structures, have been published in a previous article.<sup>12</sup>

## 3 Results

Let us consider typical normalized averaged Raman spectra for samples of the extracellular matrix of leaves and walls of cardiac implants obtained using DET and BIO decellularization (Fig. 1). Decoding of the Raman spectrum is shown in Table 1.

As seen in Fig. 1, the samples of the extracellular matrix of the heart valves (leaf and wall) have spectral differences. It is noteworthy that during DET decellularization of the walls of cardiac implants, the intense peak is 1340  $\text{cm}^{-1}$  [deformation of proteins and nucleic acids (DNA)], less than that of the samples manufactured by a BIO decellularization protocol.

At the same time, Fig. 1 shows that at wavenumber 1246  $\text{cm}^{-1}$  corresponding to amide III, 1661  $\text{cm}^{-1}$  corresponding to amide I (C=O), and 1450  $\text{cm}^{-1}$  corresponding to  $\delta(\text{CH}_2)$ ,

**Table 1** Decoding of Raman spectra.

Wavenumber, $\text{cm}^{-1}$	Substance, oscillation
756	Tryptophan <sup>13</sup>
812	Phosphodiester bond RNA <sup>14</sup>
852	$\nu(\text{C}-\text{C})$ proline, oxyproline, tyrosine <sup>13,15</sup>
935	$\nu(\text{C}-\text{C})$ proline, oxyproline <sup>16</sup>
1001	Phenylalanine <sup>16</sup>
1032	CH oscillation of phenylalanine, CN stretching in protein <sup>17</sup>
1062	$\text{OSO}_3^-$ symmetrical stretching of GAG of chondroitin-6-sulfate <sup>16</sup>
1095	Lipids, nucleic acids <sup>17</sup>
1202	Hydroxyproline, tyrosine <sup>13</sup>
1246, 1270	Amide III <sup>18,19</sup>
1340	Deformation of proteins and nucleic acids (DNA) <sup>13,17</sup>
1386	$\text{CH}_3$ oscillations <sup>17</sup>
1450	$\delta(\text{CH}_2)$ proteins and lipids <sup>16</sup>
1553	Elastin, tryptophan <sup>17</sup>
1661	Amide I (C=O) <sup>16,19</sup>

proteins and lipids did not show spectral differences. This finding allows us to suggest that there were no significant differences between DET and BIO decellularization influence on proteins and lipids as well as the integrity of collagenous structures. Nevertheless, to confirm this finding, we must conduct a detailed analysis of the bands at 852 and 935  $\text{cm}^{-1}$ , which correspond to  $\nu$  (C—C) proline and oxyproline. The intensity of the band at 1062  $\text{cm}^{-1}$  corresponding to the  $\text{OSO}_3^-$  symmetrical stretching of GAGs of chondroitin-6-sulfate is an important criterion for assessing DET and BIO decellularization. A reduction in peak intensity indicates leaching of sulfated GAGs during manufacture of the matrix.

With decellularization procedures amide I is quite stable (1661  $\text{cm}^{-1}$ ), so this wavenumber served as a denominator in the coefficients below. Therefore, the ratio of the Raman bands may be used as lead criteria for evaluation of potential immunogenicity of extracellular matrices of heart valves

$$f = \frac{I_i}{I_{1661}}, \quad (1)$$

where  $I_i$  is the intensity values at wavenumbers for the components being analyzed.

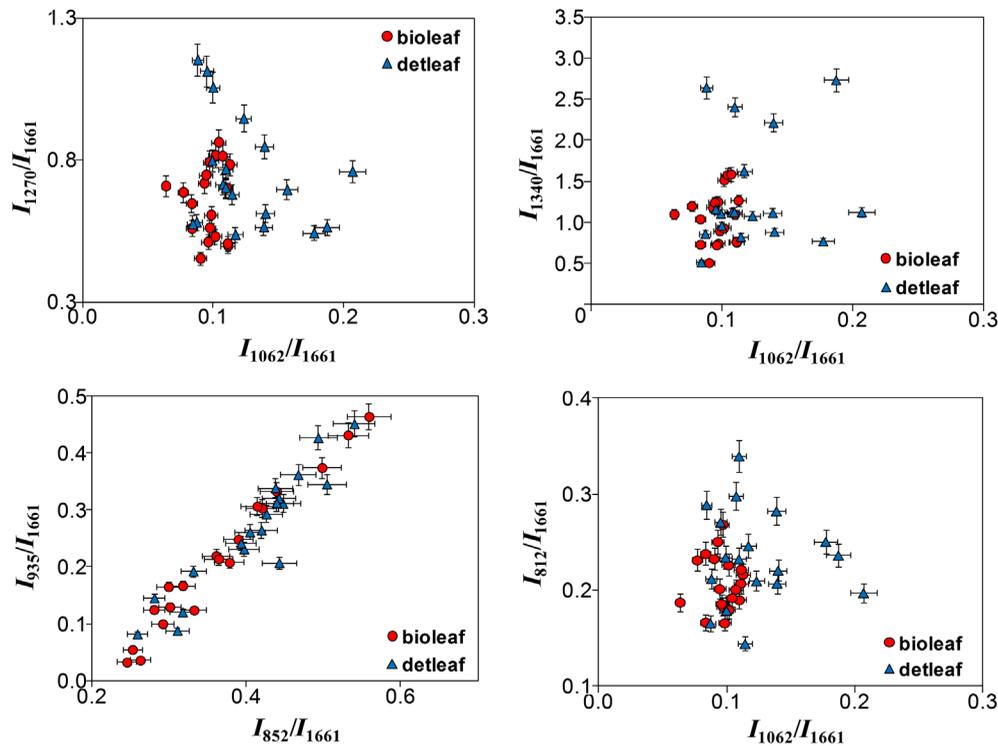
Recovery properties of implants are assessed by their structure and composition. Thus, preserved GAGs, proteins, proline, and hydroxyproline affect the ability to regenerate and elasticity. The residual nucleic acids (DNA) are capable of eliciting an immunological response in the recipient and rejection of the implants. The proposed coefficients allow us to assess these parameters and to evaluate these criteria.

Figures 2 and 3 show two-dimensional (2-D) diagrams of the input optical coefficients  $f$ , showing main changes in the

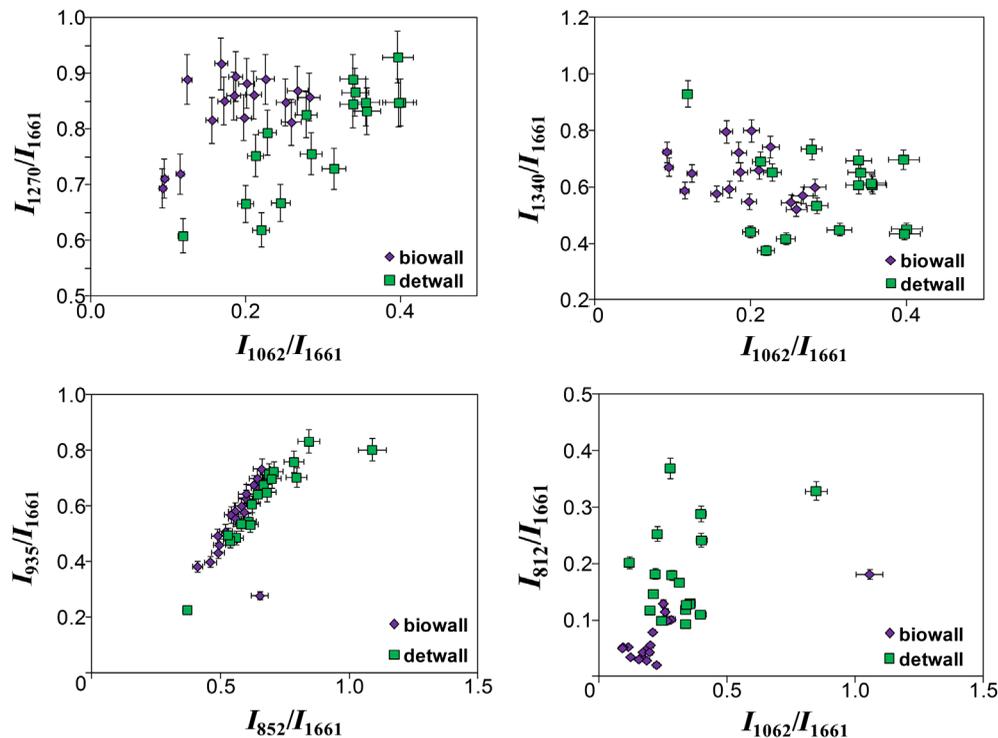
composition of the walls and the cusp samples of the heart valves using DET and BIO treatment. For separation of overlapped peaks within the range of 700 to 1790  $\text{cm}^{-1}$ , we used MagicPlotPro 2.5.1 software with the representation of individual peaks of the Gaussian function. The position of the peaks on the wave numbers' axis and peak width were preset. The spikes amplitude was determined by the iteration mechanism. The minimization of the residual amount or the achievement of an admissible number of iteration procedures (100) speak in capacity of stop-criteria. The method of PCA-analysis has proved itself to be good enough to identify the essential components during the data analysis, which includes Raman-scattering spectroscopy. Conversely, the component with the highest dispersion is not always the most informative, and the representation of components by linear combination of the original data is not always admissible.

As shown in Figs. 2 and 3, in the extracellular matrices of leaves and walls during DET and BIO decellularization, there are no significant differences in impact on proteins and lipids, as well as the impact on the concentrations of proline, oxyproline, and basic amino acid residues of collagen, which are used as a status indicator of collagen in the tissue.<sup>18,19</sup> There is a decrease in the intensity of band 812 during DET treatment of walls, which is responsible for the phosphodiester bond RNA (Fig. 3), but the difference is insignificant for leaves, probably because of the smaller thickness of the objects. In addition, analysis of 2-D diagrams revealed that DET decellularization yields better preservation of GAGs in comparison with BIO decellularization, as recently reported.<sup>12</sup>

On the other hand, during treatment of leaves by the protocol of DET decellularization, the amount of nucleic acids (DNA) is greater than in the samples subjected to BIO decellularization.



**Fig. 2** 2-D diagrams of the input optical coefficients for extracellular matrices of cardiac valves (leaves), obtained by DET and BIO decellularization.



**Fig. 3** 2-D diagrams of the input optical coefficients for extracellular matrices of cardiac valves (walls), obtained by DET and BIO decellularization.

## 4 Conclusion

Input optical coefficients enable a qualitative assessment of the protocols of DET and BIO decellularization in terms of the concentrations of GAG, proteins, amides, and DNA. Thus, the typical spectra of aortic and valvular tissues after DET and BIO decellularization are provided. It was found that the main spectral differences in extracellular matrices of cardiac walls between DET and BIO decellularization appear at wavenumber  $1340\text{ cm}^{-1}$ , corresponding to the deformation of proteins and nucleic acids (DNA).

### Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Biographies for the authors are not available.