euspen's 22nd International Conference & Exhibition, Geneva, CH, May/June 2022
www.euspen.eu

Raman – linear vibrational spectroscopy as a tool to monitor decellularisation processes of devitalised allogeneic tissues in-line: A pilot study

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Abstract
In recent years, a large variety of approaches have emerged in the field of Raman spectroscopy, especially in the biomedical field. The non-contact and non-destructive measurement method is suitable for characterising and analysing tissues as well as cells and to detect molecular changes within the samples. As part of this scientific work, it was investigated whether the Raman spectroscopy can quantify the grade of decellularisation of allogeneic tissues based on bone tissue-specific components. For this purpose, the rinsing fluid, which was generated during the decellularisation process, was analysed to verify the suitability of an in-line Raman system to monitor the rinsing set up. Samples of the rinsing fluid were taken at various times and examined with a conventional Raman microscope by using a self-developed flow cell cuvette. The results were subsequently compared and validated with the deoxyribonucleic acid (DNA) content. Our results revealed a temporal increase of intensities of tissue-specific peaks in the measured Raman spectrum during the rinsing process followed by a decrease after 15 mins. Based on the results of the examinations, in which the decrease of the DNA content was measured, the potential of the method as a monitoring tool for the decellularisation process of allogeneic tissues could be shown. For a mobile in-line Raman setup, which is planned as a later application, further verifications and optimisations with regard to the measurement settings as well further comparative tests of individual bone tissue components, are necessary.

Keywords: Raman spectroscopy, in-line monitoring, decellularisation, allograft transplants

1. Introduction

1.1 Background

Tissue transplants may be essential if, for example, various defects or diseases such as tumours or congenital malformations occur. Sometimes the human body is not able to repair large defects on its own due to a large defect size or chronic wound healing disorders. The replacement materials (synthetic, allogeneic, xenogeneic) have advantages and disadvantages with regard to mechanical properties, biological tolerance, cost and availability due to the source [1]. In the case of bone grafts, considered in this study, the use of allografts, which for example reduces both the patient's comorbidity and the operating time, requires suitable pre-processing of the tissue. During this process the tissues are decellularised by means of applying different, mostly combined, mechanical and chemical as well as enzymatic protocols. Hereby, cells and cell residues that contain donor antigens have to be flushed out of the tissue without damaging the extracellular matrix (ECM) in order to prevent a severe immune reaction after implantation. The increasing demand for tissue replacement materials requires constant further development and optimisation of existing decellularisation (DC) methods [2]. Before a method can be used for clinical applications, the approach has to be checked for suitability and validated, for example, by applying histological analyses or residual DNA content measurements.

As a result of biological variance of each individual, their specifications such as age, gender and general health of the donor affect negatively the interpretation of the results. Optimisation in terms of time and digitalisation of the data to support the interpretation of the results in view of improved diagnostics is therefore difficult. In most cases, after the ex-vivo investigations, the tissues cannot be replaced back in an organism due to damage or destruction [2]. Modern analytical measurement methods can remedy these issues. Non-contact and non-destructive spectroscopic methods such as the Raman spectroscopy are capable of generating information about the chemical composition and properties of substances, structures and bondings, orientations and changes within [3]. In addition, since the end of the 1990s, research works have shown the potential for in-situ or in-line applications, in order to characterise substances and control and monitor processes. The aim of the present study was to prove if the Raman spectroscopy provides an alternative to the existing biological DC analyses. Furthermore, it was to examine whether the use of an in-line Raman setup is capable of monitoring the DC of allografts. Based on the results, a mobile Raman system will be developed to monitor DC processes in a novel rinsing chamber in-line.

1.2 Decellularisation of bone allografts

In Germany around 50 000 tissue grafts are used annually in regenerative medicine [4].

In particular, allogeneic bone grafts are used in a variety of surgical procedures including joint replacement, spinal fusions, oral and tumour surgeries. In clinical applications the decellularisation takes place based on different strongly chemical and physical DC methods, whereby cells and cellular residues are flushed out of the tissue to produce an immunologically safe scaffold. A successful DC of the bone graft is achieved when the Deoxyribonucleic acid (DNA) content is less than 50 ng/mg dry tissue weight. As part of the joint project HOGEMA, through high hydrostatic pressure (HHP), devitalised tissues were decellularised in the current rinsing chamber by using ultrasound and fluid jets (5]. To validate the grade of DC, biological analysis (e. g. histology, DNA content quantification), which involve many manual steps, are essential.

1.3 Raman spectroscopy

The Raman effect or Raman scattering is an inelastic scattering of light by molecules in gases, liquids or solids. In most cases the sample is irradiated by monochromatic light and the inelastic scattered light have lower or higher frequencies compared to the incident light. These frequency shifts (Raman shift) are caused by molecular vibration-rotation transitions and provide detailed information about the chemical structure as well as the molecular state and dynamics, known as biochemical fingerprint. The Raman effect is weak, only one of 10^6 photons is scattered, whereby fluorescence and heating of the sample can overlay the spectrum [6]. In the last decades since the experimental evidence of the Indian scientist C.V. Raman in 1928, the development of new lasers, charge-coupled device detectors and optical filters, Raman spectroscopy is successfully used in areas such as chemistry, geology, biology, pharmacy and medicine for a wide variety of applications. The non-contact and non-destructive method allows a quick analysis of molecules in micro-scaled samples without the need for time-consuming sample preparation, as compared to histochemical analyses. In addition, sample mapping of the spatial distribution of the molecules within the sample is possible [7,8]. Due to its advantages, Raman spectroscopy is also used for biomedical applications such as cell studies, analyses of tissues and organs by observing tissue-specific Raman bands of collagen, proteins and DNA. In bone tissues, which consists of 50 % minerals, 25 % water and 25 % organic matter, Raman spectroscopy is mainly used for the diagnosis of a range of human bone diseases such as osteoporosis, osteomalacia and osteogenesis imperfecta [4, 8].

1.4 In-line Raman spectroscopy

In-line Raman spectroscopy is mainly used for process engineering applications to observe chemical processes or in the pharmaceutical industry in the production of medicines [10]. In these applications the laser beam and scattered light are guided through an optical fibre into the process room using an optical probe. Another research approach for in-situ or in-line scopes is the use of flow cell cuvettes which can be integrated directly into the microscope. Post et al. [11] demonstrate the potential of Raman spectroscopy for real-time measurements for detecting various substances by using a sensor system consisting of a flow cell cuvette as well as a neural network. For the analysis of pharmaceuticals, microplastics and their additives, different concentrations, spectral masking of solvents and flow velocities in a flow cell were investigated. Nevertheless, the study showed the limitations of the method at lower concentrations and demonstrated the probability of detecting a particle. Hansson et al. [12] developed a flow cell that is characterised by reproducible Raman spectra with a high signal-to-noise ratio and low data acquisition time to record the smallest age-related changes in the spectral characteristics of the oxygenation status of blood. The flow cuvette, consisting of a quartz capillary tube, was fixed under a conventional microscope and made it possible to reproducibly examine the smallest quantities < 100 µl with an accuracy greater than 90 %. On the one hand, only a few publications show the potential of this method, but due to the complexity of this technique, especially for biomedical applications, further research is required. Besides, to the best of our knowledge, there is still no application for the special case of monitoring decellularisation processes of allografts in a rinsing system.

2. Methods

For the initial spectroscopic investigations, rinsing fluid samples were taken from a previously published rinsing chamber described in [5] and were examined for the tissue-specific Raman bands described in the literature. As rinsing fluid, sterile phosphate buffered saline was used (PBS, Sigma-Aldrich, St. Louis, USA). Here, the main component is water, which provides a clear Raman spectrum with peaks at 1665 cm^{-1} and 3605 cm^{-1} [7]. Table 1 lists the Raman bands that are assumed to allow conclusions to be drawn about molecules and thus the main components of bones such as phosphates (PO_4^{3-}), carbonates (CO_3^{2-}) and proteins (amides I and III). These Raman bands should be suitable for evaluating the rinsing process. As mentioned previously, essential factors such as the complete removal of cellular components while preserving the extracellular matrix, are decisive for a successful DC. Therefore, peaks were selected dominating the spectral region from 800 cm^{-1} to 900 cm^{-1}, that represent tyrosine ring breathing and O-P-O asymmetric stretching and are all indicative of the nucleic acid component of a cell. In addition, the bending and stretching modes of CH groups (1448 cm^{-1} and 2800 cm^{-1} to 3100 cm^{-1}), assigned to organic substances, were chosen.

Table 1 Spectral interpretation and Raman band location of relevant components in human bones.

Assignments	Raman band location (cm^{-1})	Reference
tyrosine ring and O-P-O asymmetric stretching (DNA/RNA)	833	[13]
phosphate group (PO_4^{3-})	858	[4, 13]
phosphate group ($PO4^{3-}$)	960	[4, 14-15]
phenylalanine	1002	[4, 14-15]
phosphate group (PO_4^{3-})	1073	[4, 14-15]
carbonate group (CO_3^{2-})	1073	[4, 14,15]
amide III	1257	[4, 14-15]
C-H bending	1448	[4, 14-15]
amide I	1665	[4, 13]
C-H stretching	2940	[4, 13]

For sample preparations, cancellous bone of femoral heads from patients, who had undergone a total hip joint replacement, were used (ethics approval number A 2010–0010). The femoral heads were cut into blocks with a side length of 5 mm using a diamond-coated saw and incubated at 4 °C in sterile phosphate buffered saline until HHP procedure. During the HHP process, the bone specimens were treated with 250 MPa for 20 mins in sterile PBS. During the subsequent DC process, bone samples were treated in pairs within the rinsing chamber for 20 mins, each with ultrasound (P = 200 W, f = 40 kHz, 0.5 cycle, 100 % amplitude) and fluid jet ($\dot{V}$ = 30 ml/s, f = 3 Hz), and with exchange of the rinsing fluids every 5 mins. For the in-line measurements, a flow cell cuvette was developed and manufactured additively using stereolithography, shown Fig. 1.

Due to the accurate manufacturing process, a flow cell with an internal diameter of 0.5 mm could be manufactured very precise(±25µm).

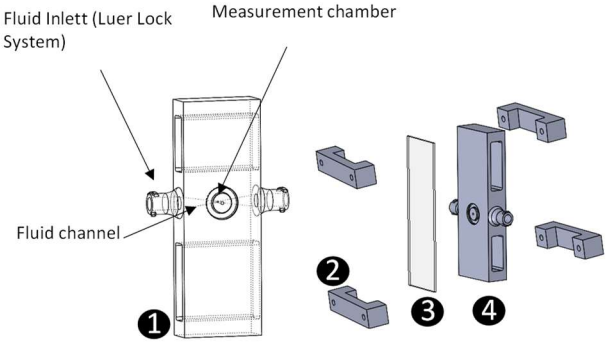

Fig. 1 Self-developed flow cell cuvette made of a photopolymer (clear resin, formlabs) by using stereolithography process (Form 3B, formlabs). 1) CAD data of the cuvette with: fluid channel, inlet and outlet for the fluid as well as a measurement chamber. 2) holder for fixation. 3) microscope glass. 4) cuvette with fluid adapters.

The printed cuvette is equipped with fluid connections, an internal channel with a diameter of 3 mm, as well an opening for the beam inlet and outlet, which is covered by a microscope glass and a seal.

The flow cell cuvette was fixed in a Raman microscope (inVia, Renishaw plc, England) and was connected to a peristaltic pump ($\dot{V}$ = 400 ml/min) with a control system (Raspberry PI 4B, python). Background correction and automatic peak search of the generated raw spectra were then processed with the native software of the microscope (Wire SW, Renishaw plc, England). Peaks which were not found by the software due to low intensities were added manually for the analysis if necessary. To validate the results from the spectroscopic examination, the residual DNA contents of the HHP-treated samples and those samples with a subsequent rinsing treatment were determined. For the DNA content quantification the previously stored samples (-80 °C) were analysed with a Zymo Research DNA MidiPrep Kit (Zymo Research, Freiburg, Germany), that followed the manuafacturer instructions. The total DNA content was measured at a wavelength of 2460/280 nm using a Tecan Infinite Reader.

3. Results and discussion

Fig. 2 (a) shows the Raman spectrum of the HHP-treated human bone specimens and the spectrum of the specimen after the rinsing process together with the assignments of the Raman bands.

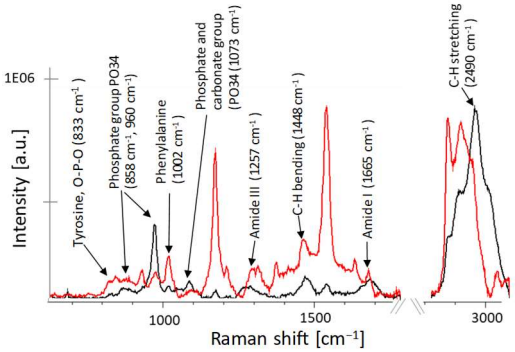

Fig. 2 Raman Spectra of human HHP-treated bone (red) as well as additionally decellularised bone (black) with tissue-specific peaks. Obtained with 532 nm laser excitation, laser power 0.05 mW, exposure time 0.01 s and 300 accumulations. The two obvious peaks for phenylalanine, tyrosine (C-C, C-N stretching, 1156 cm^{-1} and 1517 cm^{-1}) are not decisive for the analysis and were not considered.

Raman peaks at 858 cm^{-1} and 960 cm^{-1} are characteristic for the oscillation modes of phosphate and Raman peaks at 1073 cm^{-1} for the carbonate group. The band positions have changed slightly. The protein-related amide I + III Raman bands at 1257 cm^{-1} as well as 1665 cm^{-1} show substantial changes. The CH deformations in areas 1488 cm^{-1} and 2940 cm^{-1} (wagging), which are assigned for proteins and DNA, were reduced after the rinsing treatment, but also with a clearer spectra correspond to the literature. The changes in signature of the Raman spectrum around 2940 cm^{-1}, due to the previous high pressure treatment in which cells in the tissue were destroyed, disappears after the DC process.

During the measurements with the flow cell cuvette at various times, all chosen components could be detected. The results were analysed taking into account the individual intensities that were recorded with the same measurement settings, as well as the intensities of the treated and untreated samples (Fig. 3). In the case of the bone samples, as shown in the previous spectrum, the phosphate and carbonate groups were only slightly reduced compared to the protein-related amide groups.

It was assumed that the substantial reduction in the range of 833 cm^{-1} is related to the removal of cells and cell residues that contain DNA that have been flushed out. Furthermore, there was an increase in intensities at 5-15 mins for all components examined, before lower intensities could be measured after 20 mins. The decrease of the intensities can be traced back to the changed quantities of components in the rinsing liquids.

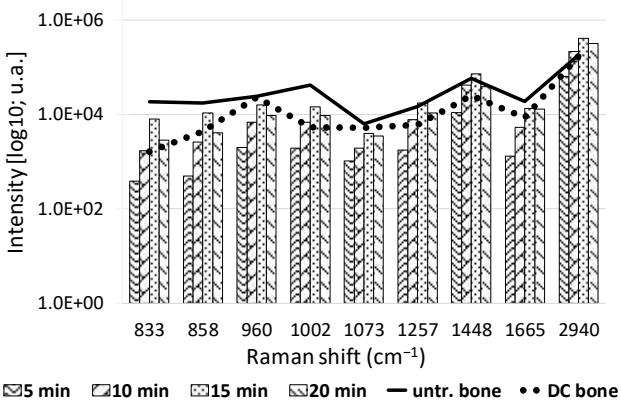

Fig. 3 Time dependence of Raman intensities of the individual components of bones measured with the flow cell cuvette (5, 10, 15 and 20 mins). All measurements were carried out with the same measurement settings and with a sample number of n = 5 (532 nm laser excitation, 50mW laser power, exposure time 0.1 s and 300 accumulations, $\dot{V}$ = 400 ml/min).

In Fig. 4, the Raman spectra of a measurement series of 5-20 mins are shown as an example, with the other four series giving the similar results. Particularly in the areas around 1448 cm^{-1} and 2940 cm^{-1}, which are characteristic for C-H bending and stretching modes, the greatest time-dependent difference in the intensities can be detected. A temporary increase up to a point (15 mins) during the rinsing treatment was followed by a decrease. With regard to Fig. 1, due to changes in signature of the spectra in the area of 2940 cm^{-1}, it can be assumed that the destroyed cells and cell debris were flushed out by the rinsing process.

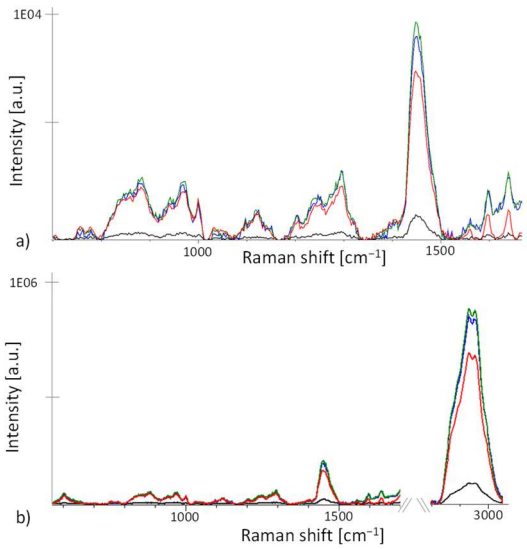

Fig. 4 Raman spectra from 600 cm^{-1} to 1700 cm^{-1} (a) and 600 cm^{-1} to 3100 cm^{-1} (b) with a series of measurements with the examined rinsing liquids 5 mins (black), 10 mins (blue), 15 mins (green) and 20 mins (red). The Raman bands at 1448 cm^{-1} and 2940 cm^{-1}, which are characteristic of the bending and stretching modes, show the greatest time-dependent difference in the intensities.

However, this assumption still needs to be investigated in further studies. The results from the DNA quantification of the decellularised tissue specimens show a reduction of the content up to 5 ng/mg tissue weight (Fig.5). Whether the DNA quantities can be assigned to the detected amount in the rinsing fluid in the ranges of 833 cm^{-1} and 2940 cm^{-1}, have to be evidenced by further investigations.

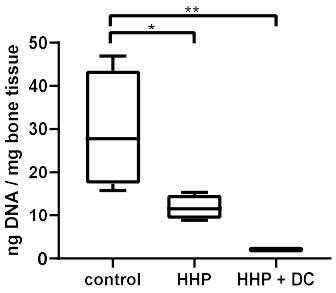

Fig. 5 Total DNA content of human bone w/o application (control), after HHP application (HHP) and treatment with HHP and rinsing chamber (HHP + DC). Statistical analysis was performed with One-Way-ANOVA (n=4). *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

4. Conclusion

In this pilot study it was investigated whether the Raman spectroscopy is suitable for the in-line monitoring of the decellularisation process of devitalised allografts in a rinsing chamber. For this purpose, fluid samples were taken at different time intervals during the rinsing process. Hereby, bone specific tissue components such as phosphates and carbonates, amide I and III as well C-H vibrations were examined by applying Raman spectroscopy by using a flow cell cuvette. The results, which were compared with HHP-treated and additional decellularised bone samples as well as by a DNA content quantification, confirmed substantial time-dependent differences during the rinsing process, especially in areas that are characteristic for DNA and in the location assigned of amide and C-H groups. The results presented show that Raman spectroscopy could provide a method to quantify the decellularisation processes of bone tissues. In addition, an in-line Raman setup as a process monitoring tool for this application, is conceivable. Nevertheless, our preliminary Raman approach requires modifications and optimisations with regard to the setup and measurement settings as well as further comparative tests of individual bone tissue components.

5. Funding

This work was part of the joint project “HOGEMA”, which is supported by the European Social Fund (ESF), reference: ESF/14-BM-A55-0017/18, and the Ministry of Education, Science and Culture of Mecklenburg-Vorpommern, Germany.

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