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Application of Raman Spectroscopy to Study the Mineralization of Bone Regenerates

Anzhela Valerievna Tedeeva, Ahmed Ruslanovich Sataev*, Saudi Timurlanovna Batraeva, Tamara Nugzarievna Gabitaeva, Napisat Nutsalovna Magomedsaugitova, Ani Arkadievna Azatyan

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Abstract

Assessment of the state of bone tissue during biopsy examination of the material of patients with degenerative or traumatic diseases is a very urgent task of modern medicine. Such an assessment makes it possible to characterize the regenerative processes at the site of the bone defect and to choose the most effective treatment strategies. Currently, in the study of bone tissue, methods of X-ray examination and survey histological staining are used. Such methods do not allow a highly sensitive analysis of individual components of the cellular substance. One approach to solving this problem can be the use of Raman spectroscopy. In contrast to infrared spectroscopy, Raman spectroscopy records in the optical range, which ensures high sensitivity and ease of use. Raman spectroscopy is a non-contact, non-destructive testing method suitable for working with substances in any phase. In this scientific work, a study of the distribution of hydroxyapatite in a section of the regenerative material of the parietal bone of the rat skull is carried out. As a result, it was shown that the Raman spectroscopy method can be used to determine the characteristics of the formation, mineralization, and maturation of bone tissue, to identify loci with maximum and minimum mineralization.

Keywords: Raman spectroscopy, Bone tissue, Hydroxyapatite, Regeneration

Anzhela Valerievna Tedeeva,
Department of Therapy, Faculty of Dentistry of North Ossetian State University named after K.L. Khetagurov, Vladikavkaz, Republic of North Ossetia-Alania, Russia.

Ahmed Ruslanovich Sataev*, Tamara Nugzarievna Gabitaeva
Department of Therapy, Faculty of Medicine of North Ossetian State Medical Academy, Vladikavkaz, Republic of North Ossetia-Alania, Russia.

Saudi Timurlanovna Batraeva
Department of Therapy, Faculty of Medicine of Ingush State University, Magas, Republic of Ingushetia, Russia.

Napisat Nutsalovna Magomedsaugitova, Ani Arkadievna Azatyan
Department of Therapy, Faculty of Pediatrics of Stavropol State Medical University, Stavropol, Russia.

***E-mail:** bucky99@yandex.ru

Introduction

At present, in the study of bone tissue, the emphasis is mainly on the structural analysis of the components of the regeneration using methods such as X-ray examination and survey histological staining (Domenyuk *et al*., 2018; Kimoto *et al*., 2021). Such approaches make it possible to assess the qualitative composition of the tissue under study, but do not make it possible to conduct a highly sensitive analysis of individual components of the cellular substance (Eldzharov *et al*., 2021). One approach to solving this problem can be the use of Raman spectroscopy. The method is based on the registration of Raman scattering of light (Raman effect) - an inelastic scattering of optical radiation on the molecules of a substance (solid, liquid, or gaseous), accompanied by a change in the radiation frequency (Unal *et al*., 2021; Fosca *et al*., 2022). The Raman spectra are specific for each molecular compound, which can be used to detect and identify molecules in various mixtures (Lunin *et al*., 2016).

Raman spectroscopy has several advantages over other analytical methods: unlike infrared spectroscopy, registration is carried out in the optical range, which ensures high sensitivity and ease of use (Blinov *et al*., 2021). Raman spectroscopy is a method of non-contact, non-destructive testing, suitable for working with substances in any phase, does not require special sample preparation, and analysis is quite fast (from seconds to minutes) (Hosu *et al*., 2019). Modern Raman spectrometers provide the possibility of constructing maps of the distribution of molecular components over the surface of the samples under study (Goodyear & Aspden, 2012). In this case, the spectrometer is combined with an optical microscope.

Raman spectroscopy has a variety of applications in biology and medicine. It is successfully used in histology for cytodiagnostics of surgical material (Castoldi *et al*., 2020; Blinov *et al*., 2022). Raman spectroscopy has also found its place in morphological studies in osteology and regenerative medicine (Maisigov *et al*., 2021). Thus, it was used to study the differentiation of osteoblasts, to study the distribution of proteins and lipids at different stages of oocyte division (Butler *et al*., 2016; Esmonde-White *et al*., 2022). Some studies have shown that Raman spectroscopy can be used to monitor bone biochemistry in the study of diseases such as osteoporosis (Khalid *et al*., 2018; AlHumaidi *et al*., 2022). At the

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same time, tests were carried out on drugs developed to combat this disease (Maher *et al*., 2011).

The objectives of this scientific article were to study the process of bone tissue mineralization by Raman spectroscopy and to assess the potential of using modern Raman spectroscopy to determine the characteristics of bone tissue formation and maturation (Deana *et al*., 2022).

Materials and Methods

To study the structure and mineralization of the newly formed bone tissue, bone tissue samples of the parietal bones of the skull in rats (n=10) were used. Under combined anesthesia (Zoletil/Rometar), trepanation of the parietal bones of the skull was performed using a 5 mm trephine with preservation of the dura mater.

Tissue samples were removed 30 days after injury and fixed in 4% neutral formalin (BioOptica, Italy) for 24–72 hours. The samples were then washed, dehydrated, and embedded in methyl methacrylate. First, primary sections 200 μm thick were made from the obtained blocks, and then secondary sections 40–50 μm thick were obtained from them by grinding. The cut thickness was controlled with a standard drum-type mechanical micrometer.

The Raman spectra were recorded on a Nicolet Almega XR spectrometer (Thermo Scientific, USA) using the 785 nm line of a semiconductor laser as excitation. For microscopic observation, an MPlan N 10x/0.25 BD objective was used, for which the irradiation area is 3 μm. The studied frequency range was 200–2000 cm^{-1}, the spectral resolution was 4.5 cm^{-1}.

Mapping was used to study and characterize the composition of the cut. A two-dimensional array of spectra was recorded over the cut surface at points with a given step. The analysis of bone tissue was carried out according to the spectrum of their main mineral component, hydroxyapatite. To select the analytical band, the spectra of hydroxyapatite and polymethylmethacrylate were preliminarily recorded, since the latter was used as an embedding medium for obtaining tissue microsections (Böhme *et al*., 2022).

Results and Discussion

The Raman spectra of the studied sample, polymethylmethacrylate, and hydroxyapatite are shown in **Figure 1**. A strong hydroxyapatite band, associated with symmetrical vibrations of the phosphate group, centered at about 960 cm^{-1}, present in the spectrum of the sample, is due to the presence of hydroxyapatite in bone tissue. This band is weakly masked by polymethylmethacrylate lines. It was chosen for analysis and was used to map the distribution of hydroxyapatite.

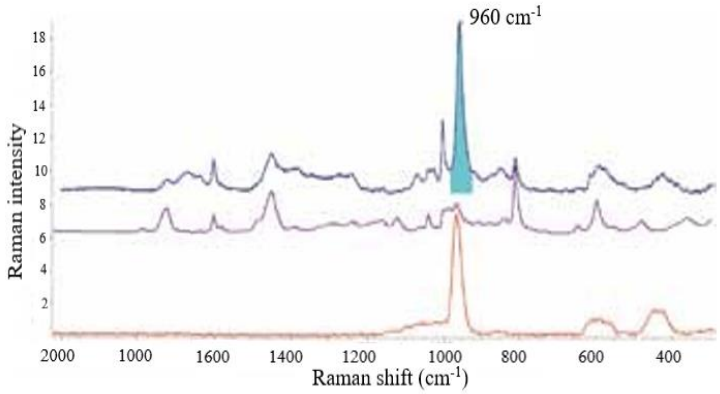
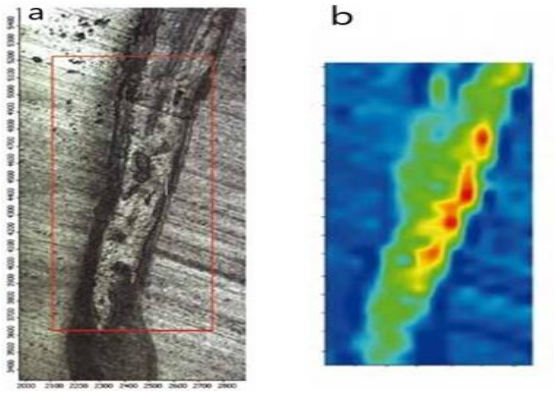


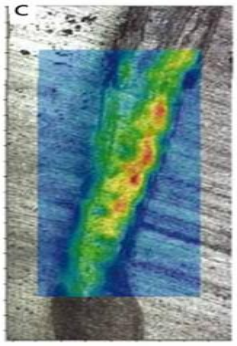
Figure 1. Raman spectra of the studied sample.

The upper graph is the spectrum of the sample, the middle graph is the spectrum of polymethylmethacrylate, and the lower graph is the spectrum of hydroxyapatite. The band corresponding to the peak of hydroxyapatite in the spectrum of the sample is highlighted in blue.

Figure 2 shows a micrograph of a sample section, a map of the distribution of hydroxyapatite, and a hybrid map of the combination of the distribution map with the photograph. The area under study, on which the distribution map was built, is marked in **Figure 2a**. Its size was 650 by 1600 μm^2. The spectra were recorded with a step of 50μm. The diameter of the probing radiation beam was 1μm. The obtained results correspond to the results of Shah (Shah, 2021).



a) b)



c)

Figure 2. Micrograph of a sample section with a selected area, which was used to build a distribution map (a), a map of the distribution of hydroxyapatite (b), and a hybrid map (c)

The map shows the intensity of the selected hydroxyapatite band, which is directly proportional to its amount in the spectrum registration area and, accordingly, is proportional to the bone tissue density. The area where hydroxyapatite is absent is colored blue. As the intensity of the hydroxyapatite band increases, the color changes to red.

Graphs of the relative amount of hydroxyapatite along the three lines selected on the map are shown in **Figure 3**. To quantify the distribution of hydroxyapatite, the figure shows a color scale that shows the correspondence of the color on the map and the intensity of the hydroxyapatite band in the spectrum.

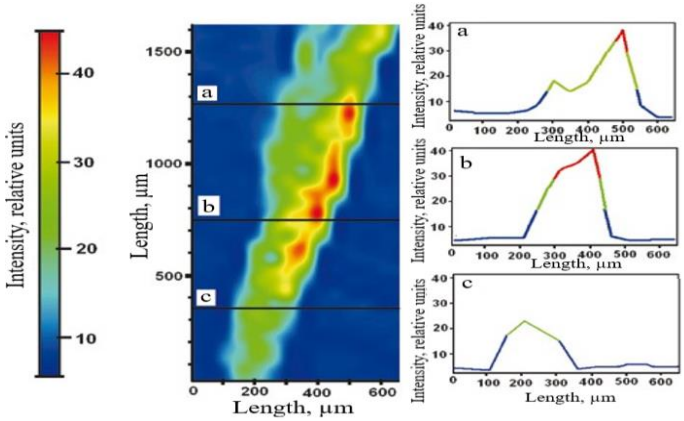


Figure 3. Color scale (left) and graphs of the relative amount of hydroxyapatite along three selected lines in the sample area under study

Thus, for each point of the surface under study, quantitative values of the presence of the selected molecular component can be obtained. A complete three-dimensional model of the distribution of hydroxyapatite in the study area of the sample is shown in **Figure 4**.

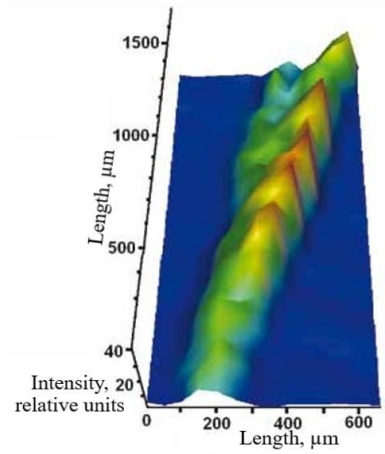


Figure 4. Three-dimensional model of the distribution of hydroxyapatite over the sample cut surface

Thus, the research results showed that Raman spectroscopy can be applied to study the mineralization of bone regeneration. This is especially important at the current stage of the development of medicine, including dentistry. With the further development of this direction, the combination of Raman spectroscopy with other methods of bone tissue identification, for example, microcomputer tomography, seems promising (Bartoš *et al*., 2018; Peña Fernández *et al*., 2018; Nagdalian *et al*., 2021). With a combination of several methods, it is also possible to connect 3D technologies, which will allow further detailed diagnostics and modeling of decision-making processes, as well as making recommendations of a preventive and hygienic nature.

Conclusion

Raman spectroscopy was used to study the distribution of hydroxyapatite in a section of regenerative material from the parietal bone of a rat skull.

As a result, it was shown that the method of combined scattering spectroscopy can be successfully used to determine the characteristics of the formation, mineralization, and maturation of bone tissue, to identify loci with maximum and minimum mineralization. The method proposed and used by us, in

combination with methods for determining the rate of bone tissue formation, can not only show the static characteristics of mineralization in bone regeneration but also determine the time intervals of mineralization with high accuracy. density is carried out on a histological preparation. Subsequently, the data obtained by Raman microscopy can be compared with histological and morphometric data and characteristics of bone regeneration, depending on experimental and clinical tasks.

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Conflict of interest: None

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Ethics statement: The protocol for experiments with laboratory animals complied with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

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