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FTIR MICRO-SPECTROSCOPY APPLIED FOR STUDYING BIOLOGICAL MINERALIZATIONS

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Abstract: *Infrared micro-spectroscopy is a powerful tool, sensitive to chemical and structural inhomogeneity, phase impurities, isomorphic substitution, degree of structural disorder, crystal size and orientation. This method and various techniques for its application are very suitable for the study of biologically formed mineralized hard tissues which have a complex structure and specific crystal-chemical properties. The most highly mineralized tissues in vertebrates are the teeth, which are composed mainly of non-stoichiometric hydroxylapatite* $Ca_5(PO_4)_3(OH)_2$*. This paper presents the spectral differences between healthy mineralization of tooth enamel and dentin, which are important initial condition for studying changes that occur as a result of pathology, dental treatment or external factors. Also, the differences in the use of reflection and ATR microspectroscopy are considered. Examples of pathological mineralizations such as urinary stones are presented. This paper summarizes the advantages of the method and focuses on the information that can be extracted not only for the inhomogeneities in phase composition, but also for the structural characteristics of the most common mineralizations of biological origin.*

Keywords: *Infrared microscopy, biological mineralization, inhomogeneity, phase composition, structure*

INTRODUCTION

Biomineralization is the process by which living organisms form solid inorganic mineral. This process is widespread among various species. It has been known since the evolution of exoskeletons during the so called "Cambrian explosion" (500-600 milion years ago) and continues with the development of the skeleton and teeth in vertebrates and humans.

The major components building hard tissues are minerals, macromolecules and water, all playing important role in building complex structures with multifunctional properties. Minerals and composites produced by organisms have properties that usually surpass those of analogous synthetically manufactured materials with similar phase composition. Understanding and elucidating the mechanisms of biologically controlled biomineralization is important for materials engineering, where similar control over mineral growth and similar properties are highly desirable but not yet achieved.

The most highly mineralized tissues in vertebrates are the teeth. Dental hard tissues are heterogeneous structures composed of an inorganic mineral part, an organic part (residues of protein matrices) and water in various proportions (Elliott, 1997). The inorganic mineral part is composed of hydroxylapatite - $Ca_5(PO_4)_3(OH)_2$. As a result of different mechanism of formation and specific biological function that perform, the hard tissues vary considerably in composition, degree of crystallinity, crystal size, orientation, etc. Apatite that builds various hard tissues, varies in the size of the crystallites, degree of crystalinity and in the degree of isomorphic substitution by hydroxyl and carbonate groups occupying different structural positions.

Enamel is the most resistant, non-regenerative tissue, that is composed mainly of inorganic mineral hydroxylapatite (about 96 wt%) containing only traces of organic residues (LeGeros, 1984). Hydroxylapatite nanosized crystals are oriented and arranged in long, thin structures called rods that are also oriented with their extension vertical to the dentine-enamel junction. The main crystallographic *c*- axis of the crystals is parallel to the elongation of the larger prismatic bodies, and this orientation changes near the dentine.

Dentine is a hard, porous tissue, approximately 70 wt% inorganic carbonated apatite and 30 wt% organic matter and water. Carbonated apatite crystals are much smaller than those building

enamel, making dentine somewhat softer. Unlike enamel, dentine is living tissue with the ability for constant growth and repair.

Vibrational spectroscopy is sensitive to inhomogeneity of local atomic structure, degree of isomorphic substitution, type, relative quantity of additional phases and crystal orientation. A large number of studies with different methods have focused on the differences in apatite, building enamel, dentine and bone (Wopenka & Pasteris 2005, Young & Mackie 1980 and others). Raman spectroscopy has a better spatial resolution than infrared spectroscopy when using a microscope, but in the study of biological samples the measurment of Raman spectra is often hindered by the high level of photoluminescence, which significantly worsen the signal-to-noise ratio. Fourier transform infrared spectroscopy (FTIR) examines the direct absorption of light by atomic vibrations in the corresponding energy range. The method, especially when using infrared microscope, is sensitive and very useful for study inhomogeneous dental tissues as well as pathological mineralizations.

EXPOSITION

Sample preparation

Cross-sections from human teeth were cut using microtome (Leica 1600). The orientation of the slices was parallel and perpendicular to the elongation of the apatite rods building enamel. One side of the plates was polished subsequently with 3, 1 and 0.1 μm silica (SiO_2) polishing suspensions. Urinary and kidney stones were studied without sample preparation. Relatively oriented crystal aggregates were mounted on microscope glass slides.

Analitycal techniques

Infrared spectra were measured using Bruker FT-IR spectrometer Tensor 37 and IR microscope Hyperion 2000, equipped with MCT LN cooled detector. The spectra in reflection mode were collected using 15x Cassegrain objective in the range 600 - 5000 cm^{-1}. All the spectra were collected with spectral resolution of 4 cm^{-1}, averaged over 128 scans. The spectra were measured in backscattering (normal-incidence) geometry from a surface area of about 80-100 μm^2. The ATR (Attenuated Total Reflectance) spectra were measured using Ge-ATR x20 objective in the range 600 - 5000 cm^{-1}

RESULTS AND DISCUSSION

Dental apatite

The electron microscopic images of enamel and dentine are shown in Figure 1 a and b, respectively. The profiles of the dental cross-section on which the analyzes were performed are marked on the backscattering electron image image (Fig.1c).

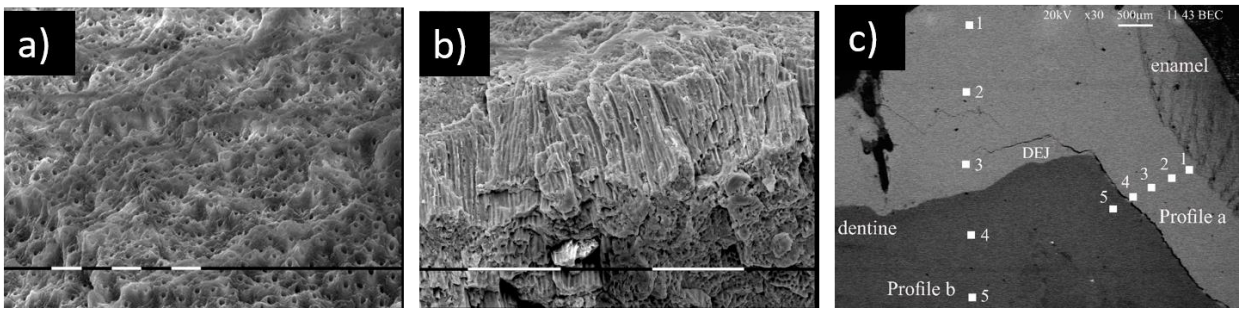

Fig. 1 SEM images of dentine (a), enamel (b) and cross section (c) analyzed along the profiles „a“ and „b“ (1-5).

Typical IR reflection spectra of untreated enamel along a profile from the surface to dentine are shown on Figure 2.

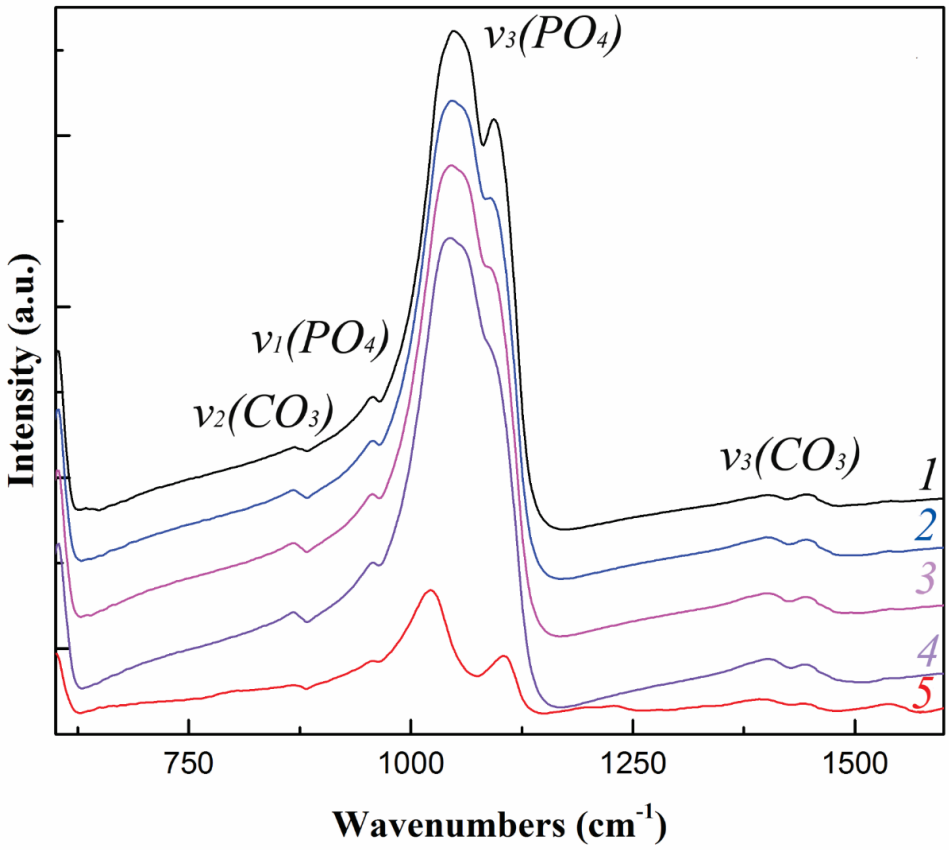

Fig. 2 Micro-infrared reflection spectra of dental cross-section collected along the profile „a" from the surface enamel (1) to dentine (5).

Most intensive peaks in the infrared (transmittance, absorption and reflection) spectra of enamel arise from the phosphate antisymmetric stretching mode ν_3 (PO_4) in the range between 1027 - 1092 cm^{-1} (Jegova et al., 2014; Titorenkova et al., 2019). This band is slightly shifted to lower wavenumbers for dentine. A weak IR reflection peak near 957 cm^{-1} results from the symmetric $\nu_1(PO_4)$ stretching in apatite. The weak peak near 870 cm^{-1} is generated by the out-of-plane ν_2 vibration of CO_3 impurities in apatite. Peaks in the range spectral range 1200-1680 cm^{-1} are due to organic compound. In the same range peaks between 1410-1540 cm^{-1} and near 1600 cm^{-1} occure due to antisymmetric $\nu_3(CO_3)$ stretching and ν_2 (H_2O) bending, respectively. Due to the difference in the protein/apatite molar ratio, the IR peaks related to organics vibrations are stronger in dentine than in enamel. Applying this approach to in-depth profile analysis, we found that the overall intensity of absorption decreases from the surface enamel in depth to dentine (Fig.2) (Vasilev et al., 2015). Also, the intensity ratio between the two most intensive peaks at 1050 and 1090 cm^{-1} changes gradually. These spectral features can be explained by the decrease in the size of the crystallites near the dentine-enamel junction (DEJ). Another significant spectral difference is that in dentine the peak at 633 cm^{-1}, which is due to the hydroxyl groups, is completely absent, however, there is an increase in carbonate bands in the 1400-1500 cm^{-1} spectral range. Therefore, from a spectral point of view, the apatite that forms dentine is not hydroxylapatite, but rather carbonate apatite.

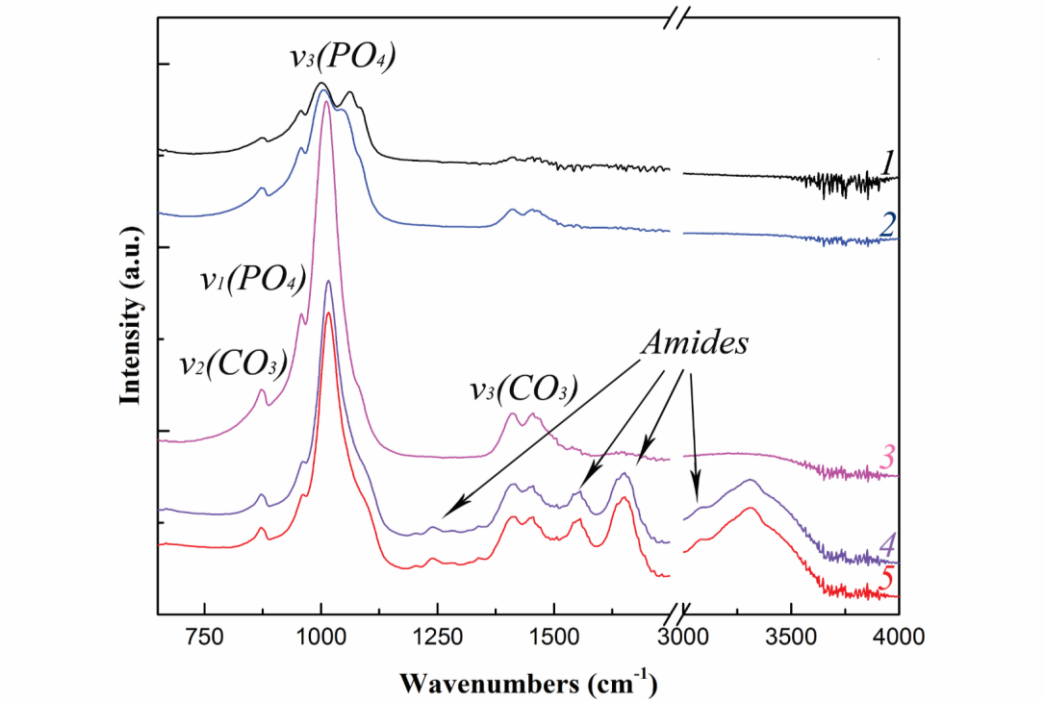

Fig. 3 Micro-infrared ATR spectra collected along the profile „b“ from the surface enamel (1) to dentine (4-5)

The spectra obtained by using micro attenuated total reflection (Fig.3) technique differ from those obtained in reflection mode (Fig.2). Because of the different mechanism of obtaining ATR spectrum, through so called evanescence wave, the intensity and position of phosphate peaks are changed and direct comparison with infrared reflection spectra cannot be done. As it is seen from Figure 3, intensity of the spectra collected from the most surface layer is less intensive as compared to the spectra near DEJ and dentine. This is due to different hardness of superficial and internal enamel. Therefore, when applying the same pressure with the ATR crystal, a different penetration depth is reached. The most intensive peaks of ν_3 (PO_4) mode are near 1010 and 1100 cm^{-1}. In the porouse dentine the ATR crystal penetrates in depth and the resulting spectra are more informative in terms of carbonate content and amide peaks. The characteristics amide vibrational modes associated with proteins are near 1640 cm^{-1} (Amide I), 1540 cm^{-1} (Amide II) and between 1200-1400 cm^{-1} (Amide III) (Jegova et al., 2014).

It is seen that the IR reflection spectrum of enamel (Fig. 2) gives relatively intensive spectra and well-resolved peaks of phosphate group, while ATR spectra of dentine (Fig. 3) are more intensive and reveal better results for the organic compound.

Pathological biological mineralizations

Micro-infrared spectroscopy can be successfully applied for non-destructive analysis of the phase composition of complex inhomogeneous pathological biomineralizations, such as kidney and urinary stones. Urinary stones are solid particles, aggregates or crystals of various chemical and phase composition - calcium phosphate, struvite, calcium oxalates, uric acid, cystine (Cloutier et al., 2015).

Example for complex inhomogeniuos mineralization is presented on Figure 4.

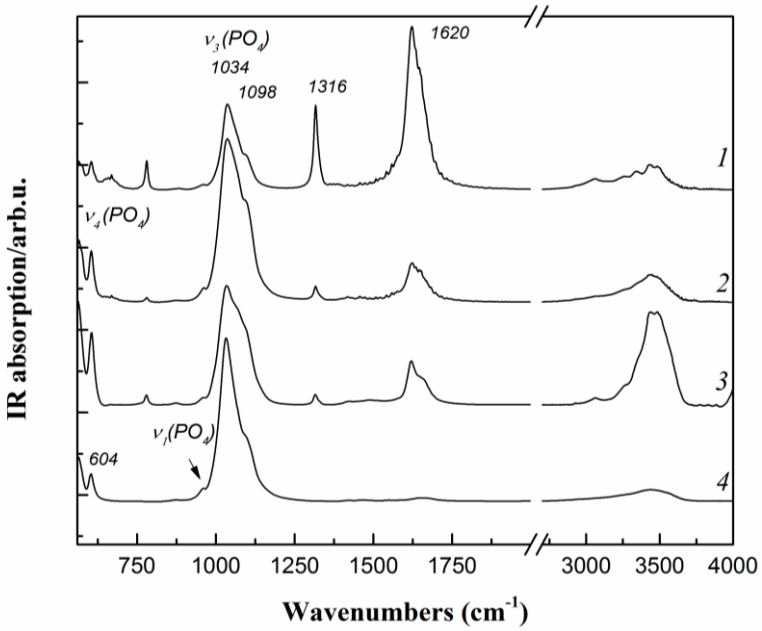

Fig. 4 Micro-infrared spectra of different areas (1-4) of urinary stone

The presented spectra collected from different areas of the same urinary agregate show that the central inner part (Fig.4-4) of the sample is composed of apatite. The position of the peaks and their assignment are presented in Table 1. The spectra from the other areas reveal a mixture of calcium phosphate and oxalates (Fig. 4-2,3). Calcium oxalates predominate on the surface which can be seen from the most intense bands near 1620 and 1320 cm^{-1}.

The study of inhomogeneities in the internal distribution of mineral phases and the sequence of crystallization over time will help to clarify the causes of the disease, diagnosis and subsequent treatment.

Table 1. Infrared peak`s position and assignment of the vibrational modes

Infrared peak position [cm^{-1}]	Vibration	Mineral
564 605 960 1030 1090	ν_4 (PO_4) ν_4 (PO_4) ν_1 (PO_4) ν_3 (PO_4) ν_3 (PO_4)	Apatite
3060-3490 1620 1660 1315 780	O-H water δ H-O-H ν_a(C=O) ν_s(C=O) δ(O-C=O)	Calcium oxalates

CONCLUSION

Infrared microspectroscopy is a powerful, non-destructive method for studying inhomogeneous in composition and structure complex biological mineralizations. Qualitative and quantitative chemical information is obtained in-situ from small areas down to 40 μm in size. It has been found that reflection and ATR microscpectroscopy are suitable for study mineralizations with high and low degree of crystallinity**.**

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