

In Vitro Study of Tetracycline Penetration into the Enamel and Dentin of the Tooth by Fluorescence Spectroscopy

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Abstract. With the use of tetracycline in childhood, as well as with intrauterine or ectopic exposure before teething through the gums during mineralization or calcification of the teeth, the antibiotic binds to calcium ions in the teeth. This results in permanent staining of the teeth. In dentistry, the term "tetracycline teeth" even arose. During teething and exposure to light, calcium-bound tetracycline oxidizes, causing the enamel to change color from fluorescent yellow to brown. In this study, using fluorescence spectroscopy, it was found that tetracycline exhibits characteristic fluorescence peaks in the dentin and in the enamel of the teeth after exposure of the antibiotic solution to the extracted teeth *in vitro*. Thus, it was shown that in non-carious fully mineralized human dental tissues, tetracycline penetrates quite well into the dentin matrix and, to a much lesser extent, into the enamel matrix, retaining its fluorescence. © 2023 Journal of Biomedical Photonics & Engineering.

Keywords: enamel; dentin; human teeth; tetracycline hydrochloride; fluorescence spectroscopy; diffuse reflection spectroscopy.

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1 Introduction

Optical methods in clinical practice, which have been actively developed in recent years, are of great importance and great prospects both for the early diagnosis of various diseases and as methods of specific effective treatment [1, 2]. In dentistry, optical methods, in particular fluorescent, are used for aesthetic rehabilitation of teeth, diagnosis of caries, and detection of tartar in the prevention of periodontal disease, etc. [3]. The color of the teeth is determined by the absorption, scattering, and transmission of ambient light inside the tooth, the autofluorescence of the teeth is based on the presence of endogenous fluorophores found in the enamel and dentin [4]. Early diagnosis of caries by blue laser-induced autofluorescence is possible [5]. Occlusal,

secondary, and proximal dental caries were assessed using the Qraycam and Qraypen clinical systems based on laser-induced fluorescence [6].

A decrease in the intensity of fluorescence in the teeth was revealed, which effectively correlates with the loss of minerals inside the lesion, which can be used to effectively detection and monitor the smallest changes in demineralization / remineralization in early carious lesions without carious cavities [7].

Using fluorescence, it is possible to visualize the localization of pathogenic microorganisms of the oral cavity (*Candida albicans*, *Porphyromonas gingivalis*, *Streptococcus mutans*) and teeth, but the identification of each pathogen by this method is unlikely [8]. Registration of red fluorescence, which comes from porphyrin-induced metabolites produced by

microorganisms in the oral cavity, provides diagnostic of caries, dental plaque and tartar [9]. Raman spectrometry can be used to detect molecular structural changes responsible for laser-induced tooth fluorescence to improve the prediction of chemical whitening efficacy [10]. Autofluorescence imaging with 1% toluidin blue staining is effective as a method for diagnosing and detecting neoplasms of the oral mucosa [11]. Using wide-field multiwavelength fluorescence imaging and spectral analysis of the main components, various anatomical and morphological features of the periodontium, gum tissues, alveolar bone, cementum, and periodontal ligaments were determined [12].

Currently, new dental materials are being created for the restoration and restoration of dental tissue, which have similar fluorescent and the opalescence properties, along with shade and color [13, 14]. In dental composites and ceramics, fluorescence is achieved by incorporating materials containing rare earth luminescence centers to look more beautiful and lively [15]. However, many factors in the process of human life affect the color and fluorescence of teeth and restorative materials in different ways, for example, aging, temperature, whitening, bone grafting or bone augmentation, implant placement, taking various pharmaceuticals, and antibiotics, etc. [16]. The lack of knowledge about this matter makes more and intensive studies to be done. So, one of the biologically active drugs that actively accumulates in hard tissue is tetracycline. This broad-spectrum antibiotic may cause discoloration and hypoplasia of developing teeth in a growing child [17].

For identification and determination of tetracycline in aqueous solutions, an effective approach is a combination of adsorption pre-concentration followed by chromatographic [18] or luminescent detection [19]. An efficient method for the solid-phase luminescent determination of tetracycline using systems of chemically modified silica has been developed [20].

In recent years, organometallic scaffolds for the detection of antibiotics in food and the environment have been effectively studied. Using preliminary adsorption pre-concentration, sensitive responses to a number of tetracycline antibiotics (tetracycline, chlortetracycline and oxytetracycline) with detection limits of 0.28–0.30 μM have been obtained [21, 22].

A water-resistant carboxy-functionalized organometallic europium skeleton has shown effectiveness as a double reacting fluorescent sensor for tetracycline antibiotics (oxytetracycline, tetracycline, doxycycline) and dihydrogen phosphate detection [23]. This sensitive and selective tetracycline antibiotics sensing can be used to detect latent fingerprints on various surfaces. Since tetracycline is widely used in animal husbandry, antibiotic residues can be found in animal products. Fluorescence probing using Zn^{2+} as sensor probes detected tetracycline in a complex sample of chicken broth [24]. For rapid and specific detection of tetracycline residues in milk, copper-doped zinc sulfide

quantum dots were used as ratiometric fluorescent probes [25].

The use of antibiotics in young children is widespread and can lead to adverse dental health outcomes, including staining, developmental defects, and caries. Therefore, the development of simple and sensitive methods for the rapid screening of biologically active substances, for example, tetracycline in complex biological samples, is of great importance.

The review [26] described a dose-response relationship between exposure to tetracycline doses $>20 \text{ mg/kg}$ per day and tooth staining. Early childhood doxycycline exposure (at any dose) has not been associated with tooth staining, dental caries, or enamel defects. Further research is needed to determine the effect of antibiotics on oral tissues. The present study discusses whether tetracycline can be incorporated into the crystal lattice in fully mineralized enamel and dentin. To do this, tetracycline was qualitatively determined in the enamel and dentin of extracted human teeth *in vitro* using luminescent spectroscopy.

2 Materials and Methods

The material of the study was human teeth removed for orthodontic indications in a dental clinic. The penetration of tetracycline hydrochloride was studied on non-carious fully mineralized human teeth, namely dentin and enamel. For the study of enamel, a whole tooth was taken. To study the dentin, the teeth were cut with a diamond disk into longitudinal cuts. The samples were placed for 20 s in 35% phosphoric acid. To clean the surface from saw cut products, the samples were placed in a Techsonic UD100 SH-45 L ultrasonic bath with water for 10 min, and wiped with a brush dipped in ethanol. The samples were air dried for several days. The thickness of the sections (samples) of the teeth was measured with a micrometer at several points of the sample and averaged. The accuracy of each measurement is $\pm 10 \mu\text{m}$. The mean thickness of the sections was $0.52 \pm 0.08 \text{ mm}$ ($n = 3$, n is the number of samples). In total, three sections from different teeth and five whole teeth were examined in the experiment. The quality of cleaning from saw cut products and the study of morphology were carried out by scanning electron microscopy (SEM) on a Mira II LMU electron microscope (Tessan, Czech Republic) in the secondary electron recording mode (at an accelerating voltage of 30 kV). To do this, the samples were fixed on a special carbon substrate (carbon tape) and their surface was coated with gold. The diameter of the dentinal tubules was determined using scanning electron microscopy data.

As a fluorescence agent, we used a pharmaceutical preparation – tetracycline hydrochloride (manufacturer: Biochemist AO, Russia), with a concentration of tetracycline hydrochloride solution $C = 1.1 \cdot 10^{-4} \text{ mol/l}$. Working aqueous solutions of tetracycline hydrochloride were acidified by adding 1 drop of hydrochloric acid to $\text{pH} = 5$, since tetracycline solutions are unstable in alkaline and neutral media. Tetracycline can emit fluorescence when excited by ultraviolet light [27]. The

absorption spectrum of the obtained solution was studied on a two-beam spectrophotometer Shimadzu UV-2550 (Japan) in the range of 200–800 nm in a quartz cell with a thickness of 1 cm. The light source was a halogen lamp with radiation filtering in the investigated spectral range. All measurements were carried out at room temperature ($\sim 25^\circ\text{C}$) and normal atmospheric pressure. Diffuse reflectance and total transmittance spectra of tooth samples were recorded on the same spectrophotometer using a solid sample attachment. The conversion of diffuse reflectance spectra into absorption spectra was carried out using the Kubelka-Munk algorithm built into the software of the spectrophotometer. To study the penetration of the agent into the enamel and dentin of a human tooth, the test samples were placed in a Petri dish, with an aqueous solution of tetracycline hydrochloride. We previously determined the time of complete staining of *ex vivo* dentin samples of thickness 0.52 ± 0.08 mm as 5.0 ± 0.4 h [28]. Therefore, the samples were kept in the agent solution for 5 h. Before measurements, the samples were rinsed with distilled water to remove agent residues from the surface. The fluorimetric determination of tetracycline was carried out on an RF-5301PC spectrofluorimeter (SHIMADZU, Japan) using an attachment for solid samples with the following parameters: excitation light wavelength (λ_{ex}) was 376 nm, selectable spectral bandwidth of monochromators ($\Delta\lambda_{\text{ex/emission}}$) = 5/5 nm, scanning speed

was medium, spectral measurement range 220–800 nm with a step of 1.0 nm. A xenon lamp served as the light source. The scheme of the experiment is shown in Fig. 1. Photographs of human teeth samples were taken on a video densitometer “SORBFIL” (Russia) with an ultraviolet lamp with a maximum of emitting spectrum at 365 nm. Photos were taken on the smartphone “Iphone XS Max” (USA).

3 Results and Discussion

The human tooth consists of a root, a neck covered with cement, and a crown covered with enamel – the hardest tissue of the tooth. Cementum is a mineralized tissue consisting of calcified intercellular substance that covers the tooth in the area of the root and neck.

The morphology of enamel and dentin was studied using scanning electron microscopy (Fig. 2). Enamel is formed from prisms, which are located at different angles to the surface. Down the enamel is dentin (in the crown and root of the tooth). Enamel and dentin are mineralized connective tissue. A feature of dentin is the presence of dentinal tubules with a diameter of 2 to 5 μm , penetrating the entire thickness of the dentin and supporting cytoplasmic processes of odontoblasts – cells located on the periphery of the pulp. The number and diameter of the lumen of dentinal tubules are different in different parts of the dental organ [29, 30].

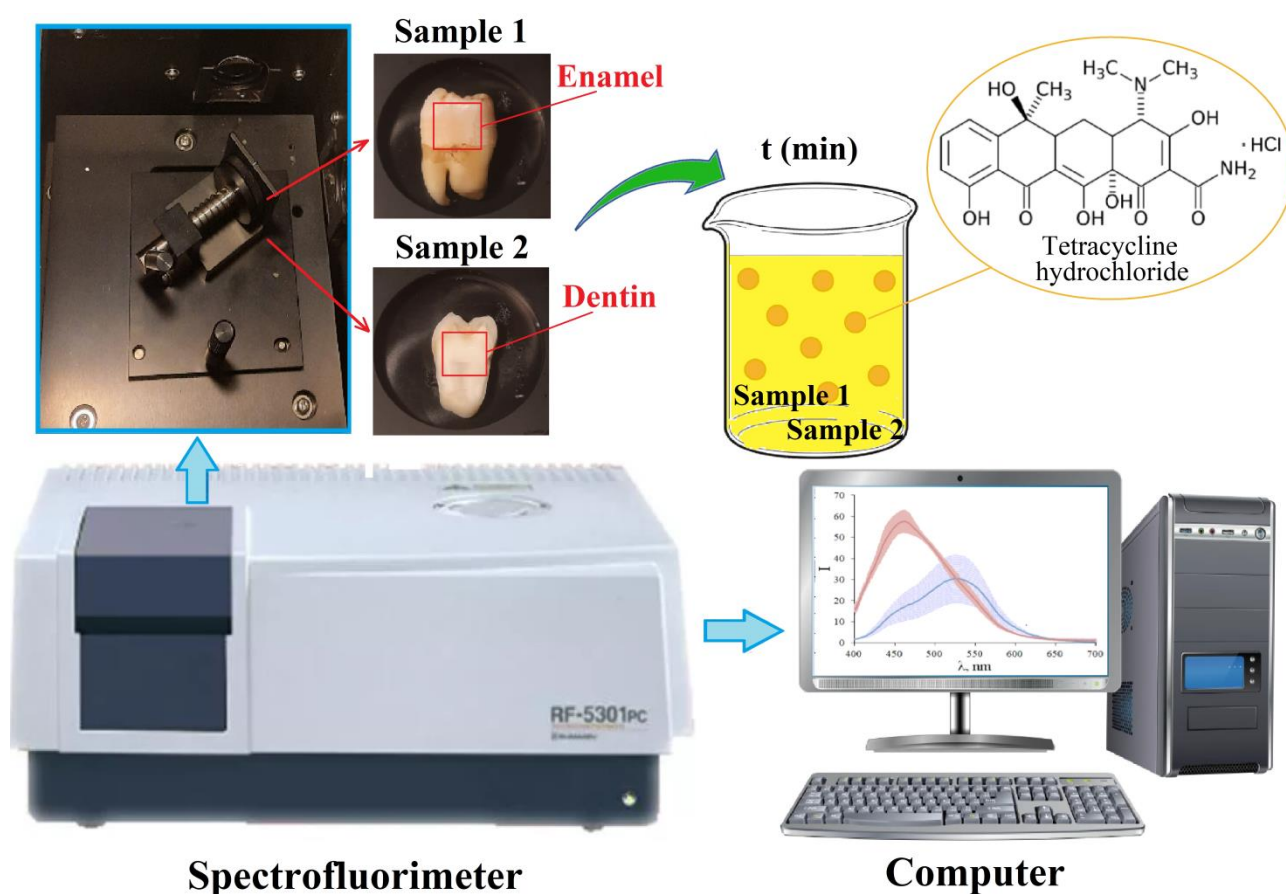


Fig. 1 Experimental scheme for measuring fluorescence spectra using an attachment for solid samples.

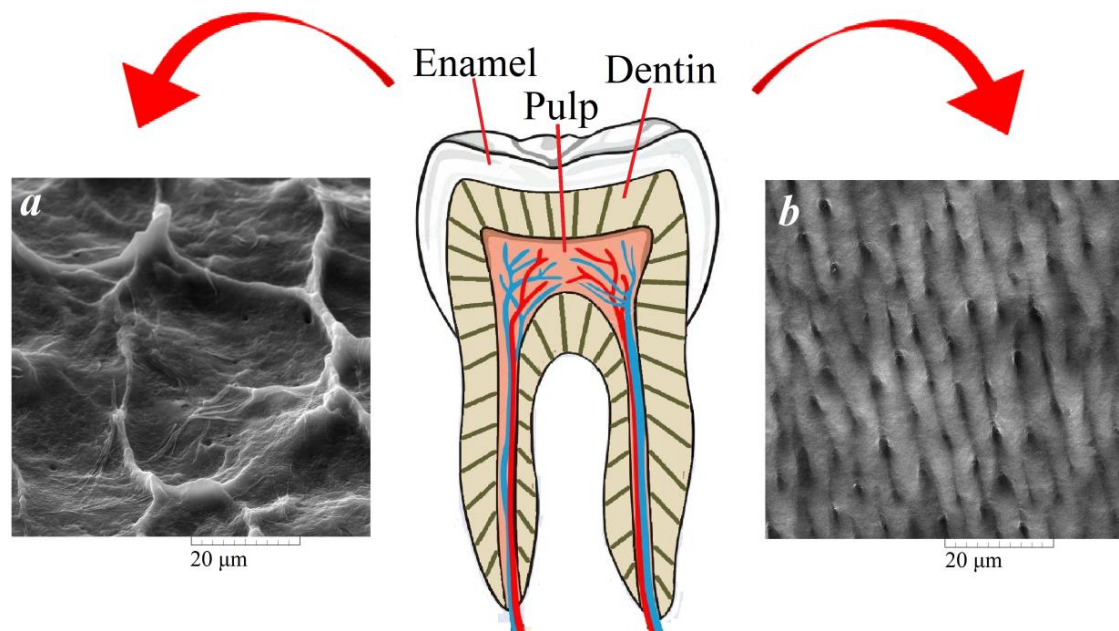


Fig. 2 Electronic micrographs (SEM) of a longitudinal section (sample) of a human tooth: (a) enamel (magnification $\times 5k$), (b) dentin (magnification $\times 5k$).

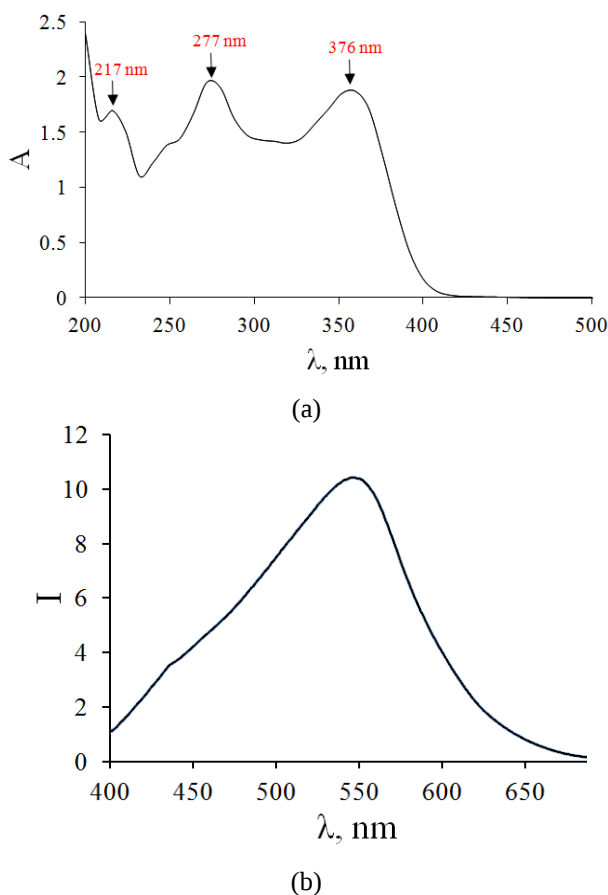


Fig. 3 Spectra of tetracycline aqueous solution: (a) absorption spectrum, (b) fluorescence spectrum at an excitation wavelength of 376 nm.

Regardless of how the light hits the outer surface of the tooth, pulp illumination is always effective due to the sinuous shape of the light guides, which are formed by

enamel prisms and structures of dentinal tubules. The waveguide effect is much more pronounced in dentin than in enamel [31].

From an optical point of view, dentinal tissue can be attributed to optically turbid media in which the shape of the diffuse reflectance and total transmittance spectra correlate over the entire studied range (200–800 nm). The optical properties of the tooth are mainly determined by dentin due to its bigger thickness than for enamel. Reflection of light by periodic structures of dental tissues provides information about those structures.

An absorption spectrum of tetracycline solution was obtained, which has characteristic maxima in the UV region of the spectrum at 220 nm, 277, and 376 nm (Fig. 3(a)). The fluorescence spectrum of the tetracycline solution has a pronounced peak at 550 nm at an excitation wavelength of 376 nm (Fig. 3(b)).

Endogenous chromophores determine measured diffuse reflectance and total transmittance spectra of tissues, from which absorption spectra can be reconstructed using the Kubelka-Munk algorithm. Chromophores in the dentin are protein-like molecules, amino acid residues, DNA, NADH-H, and partly collagen and elastin, causing the diffuse reflectance spectra to decrease at characteristic wavelengths. Starting from 400 nm, the spectra are determined mainly by scattering on the hydroxyapatite matrix [2, 32]. In enamel, tartar acts as a light absorber – hardened plaque, mineralized bacterial plaque (biofilm). It is formed on the surface of the teeth from food debris, dead microorganisms, calcium, phosphorus, and iron salts and determines the peak in the UV region of the enamel absorption spectrum.

The Kubelka-Munk reconstructed absorption spectra of dentin and enamel before and after tetracycline diffusion from aqueous solution are shown in Fig. 4.

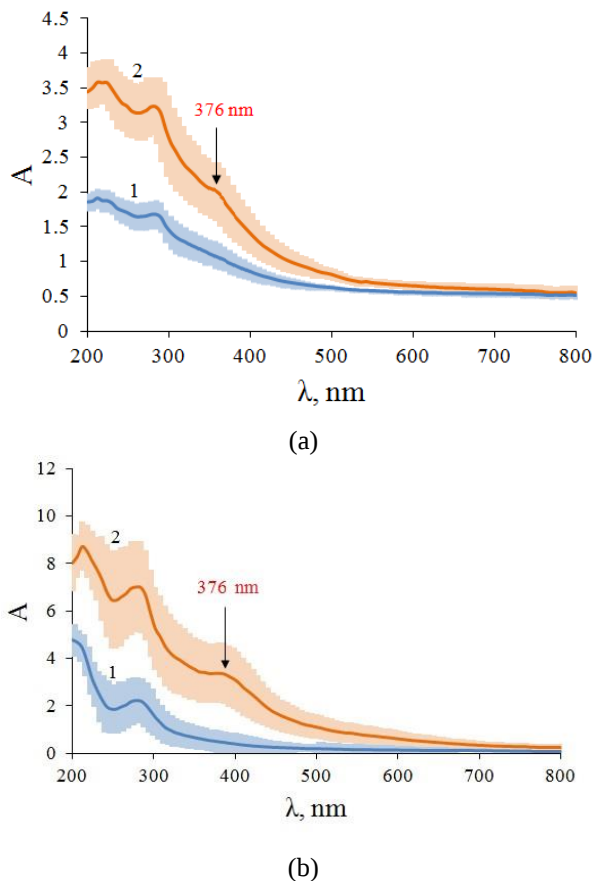


Fig. 4 Absorption spectra of dentin (a) and whole tooth with enamel (b) obtained from diffuse reflectance spectra reconstructed using the Kubelka-Munk algorithm: 1 – before tetracycline diffusion; 2 – after 5 h of tetracycline diffusion ($n = 3$).

In the process of diffusion of the agent, the spectra change with increasing the absorption in the UV region. After the penetration of tetracycline into dentin and enamel, the spectra show an increase in peaks at wavelengths characteristic for tetracycline. At 220 nm and 257 nm, the tetracycline bands coincide with the bands of the tooth endogenous chromophores; therefore, tetracycline identification in tissues was performed at 376 nm. After being in solution with the agent for 5 h, a peak is clearly detected in the absorption spectra at 376 nm, which indicates the presence of tetracycline in the samples (curve 2 in Fig. 4(a, b)).

The fluorescence spectra of the studied samples at an excitation wavelength of $\lambda = 376$ nm are shown in Fig. 5.

The original dentin fluorescence spectrum (Fig. 5(a)) has a peak at 460 nm with an intensity of up to 58 a.u., which is caused by endogenous chromophores with an absorption peak in the UV region. Tetracycline strongly absorbs in UV region and therefore partially does not transmit fluorescence at short wavelengths – self-absorption. The whole tooth with enamel fluorescence spectrum (Fig. 5(b)) has peaks at 413 nm, 437 nm, and 462 nm. Self-absorption in the whole tooth is greater than

for dentin (Fig. 5). Dentin samples are well impregnated with tetracycline through the dentinal tubules and transverse processes of these canals, when a whole tooth is examined, then diffusion into the enamel for 5 h is quite insignificant, therefore tetracycline works as a filter that attenuates fluorescence from all possible chromophores. We find a slight increase in fluorescence at 550 nm from 16 a.u. in the original enamel sample up to 20 a.u. in a sample with tetracycline.

Thus, we see that the penetration of tetracycline in fully mineralized adult teeth is significant in the dentin and less in the enamel, which is also well seen in the photographs of samples before and after staining, taken using a video densitometer (Fig. 6).

Fig. 6 shows samples of a human tooth, where various structural elements are indicated: 1 – enamel, 2 – dentin, 3 – cement. The original samples did not contain tetracycline in any of the regions (Fig. 6(a, b)). The photo of tooth sample (Fig. 6(c)) after being in the tetracycline aqueous solution for 5 h shows that the dentin was completely stained and began to have a yellowish tint, which is not visible on the enamel section.

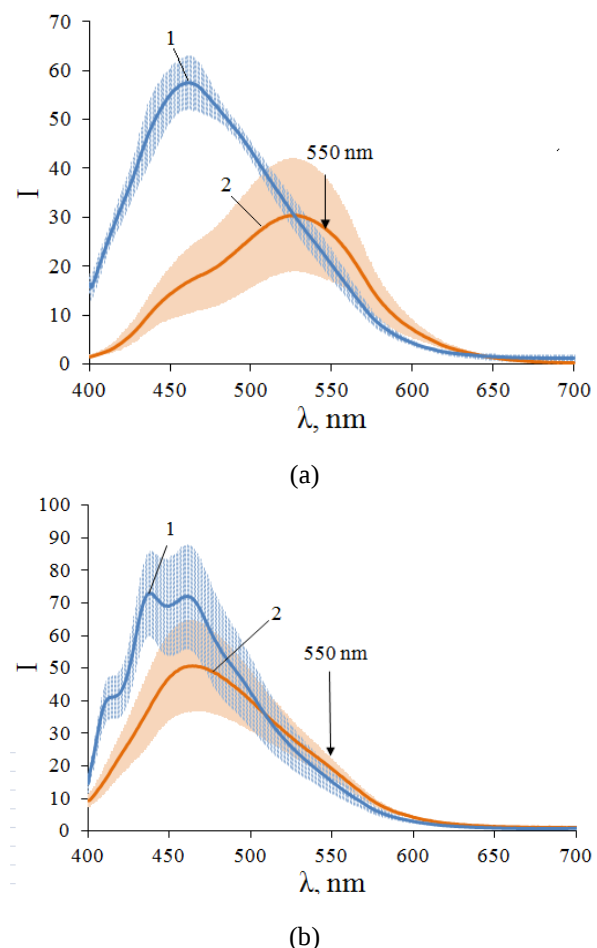


Fig. 5 Fluorescence spectra of dentin (a) and whole tooth with enamel (b): 1 – initial, 2 – after tetracycline diffusion from aqueous solution ($C = 1.1 \cdot 10^{-4}$ Mol/l) at excitation wavelength $\lambda = 376$ nm ($n = 3$).

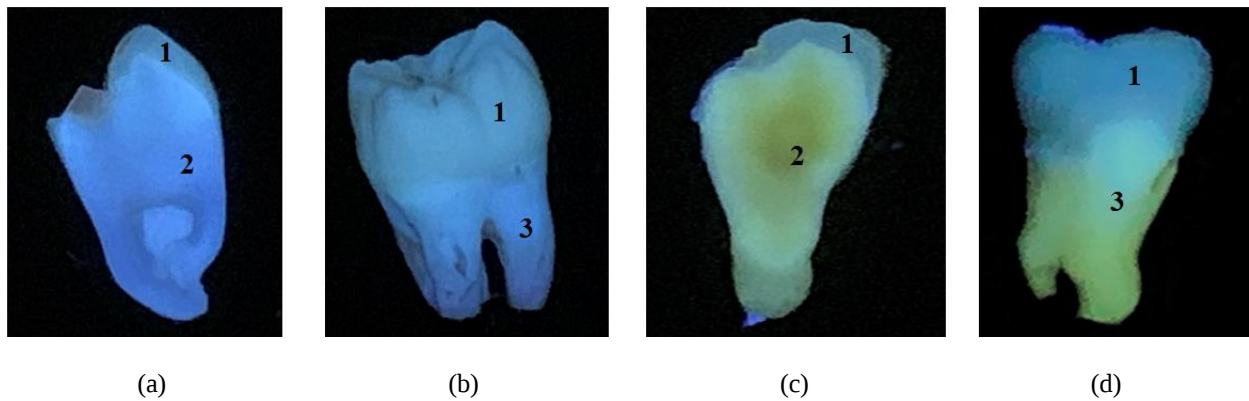


Fig. 6 Photographs of human tooth samples (1 – enamel, 2 – dentin, 3 – cement): (a) original tooth section, (b) original whole tooth, (c) – tooth section after tetracycline solution diffusion, and (d) whole tooth after tetracycline solution diffusion (emission spectrum at the 365 nm).

Table 1 Biophysical characteristics of human dentin samples.

Cut number	$D \times 10^6 \text{ cm}^2/\text{s}$	Thickness l , cm	Average diameter of dentinal tubules, μm	Density of the number of dentinal tubules, $1/\text{mm}^2$
1	5.25	0.05	1.98 ± 0.34	10023
2	6.97	0.06	1.50 ± 0.41	6790
3	7.25	0.05	3.08 ± 0.94	27000

Fig. 6(d) shows a whole human tooth that has been in tetracycline solution for 5 h. It can be seen that the cement of the tooth was intensively stained with the agent, and the changes in the enamel are visually hardly noticeable.

Dentin has high permeability due to the presence of a huge number of tubules that penetrate it. This is of clinical significance, causing a rapid response of the pulp to damage of dentin. The ground substance surrounding the tubules is more compacted (hypermineralized) and homogeneous than the substance in the spaces between them. In this regard, peritubular (around the tubules, that forming their walls) and intertubular dentin are distinguished.

During caries (demineralization) peritubular dentin undergoes intense destruction, which leads to the expansion of the tubules and an increase in their permeability.

The location of collagen fibers and their structure change in different parts of dentin. In this regard, two layers of dentin are distinguished: outermost, or mantle dentin and innermost, or peripulpal (circumpulpal) dentin. Mantle dentin smoothly transitions into peripulpal dentin, which is less mineralized and contains larger dentinal tubules than mantle dentin [33, 34]. The mantle dentin matrix is more mineralized than the peripulpal dentin matrix and contains relatively fewer collagen fibers [34, 35].

Indeed, composition of dentin has impact on molecular diffusion. In the present study, we received averaged data for both types of dentin - mantle and peripulpal. However, proposed technology can be applicable for differentiated studies, which will need

specially prepared samples and monitoring of their structure using electron microscopy. The diameters of the dentinal tubules were $0.794 \mu\text{m}$ and $1.0 \mu\text{m}$ for the first and second molars, respectively (measurements were taken at a distance of 35–65% from the walls of the pulp chamber). The tubule density was $17,997.594 \text{ tubules}/\text{mm}^2$ and $25,211.317 \text{ tubules}/\text{mm}^2$ for the first and second molars, respectively [36].

Peripulpal dentin forms the majority of the dentinal layer and consists mainly of a layer of immature predentin with a constant thickness of 15–20 μm and a layer of dentin with increasing mineralization, starting from the mineralization front to the mantle dentino-enamel junction, and thickness of the order of several hundred micrometers, which significantly exceeds the thickness highly mineralized mantle dentin, which is 15–30 μm .

The studied samples included both zones of dentin, peripulpal, and mantle, because as it follows from Table 1 average diameter of dentinal tubules and their density was quite different. The average for 3 samples diameter of dentinal tubules is $2.2 \mu\text{m}$ and the density of the number of dentinal tubules is approximately 14600 per mm^2 . Data for each sample are shown in Table 1.

The mean value of diffusion coefficient of tetracycline was obtained as $D = (6.49 \pm 1.12) \cdot 10^{-6} \text{ cm}^2/\text{s}$ [28]. The measured properties of each three dentinal cuts are presented in Table 1. Let us consider dentin as a two-layer tissue of mantle and peripulpal layers, then total diffusion coefficient can be calculated as following [37]:

$$D = (l_1 + l_2)^2 / [(l_1)^2/D_1 + (l_2)^2/D_2], \quad (1)$$

where l_1 and l_2 are thicknesses of layers, D_1 and D_2 are diffusion coefficients in these layers, respectively.

Accounting that the peripulpal dentin matrix is less mineralized and denser than the mantle dentin matrix, let us assume that the sample with the highest diffusion rate was cut mostly from the peripulpal layer closer to pulp: $D_1 = 7.25 \cdot 10^{-6} \text{ cm}^2/\text{s}$. The high density of dentinal tubules 27000 per mm^2 and their relatively large diameter 3.08 ± 0.94 also indicate that the sample belongs to the inner layer of dentin. At the same time, samples 1 and 2 can be attributed to the outer layer of dentin, consisting of a more mineralized part of peripapillary dentin and mantle dentin. To be specific, let us take sample 1, which diffusion coefficient is $D_2 = 5.25 \cdot 10^{-6} \text{ cm}^2/\text{s}$. Then, using the Eq. (1), it is possible to estimate the average diffusion coefficient for dentin sample, consisting of different layers. Assuming that the thickness of the layers is the same, it is possible to calculate the average diffusion coefficient of dentin sample of 0.1 cm thick, which include both layers of dentin, internal and external, as $D_1 = 7.25 \cdot 10^{-6} \text{ cm}^2/\text{s}$ and $D_2 = 5.25 \cdot 10^{-6} \text{ cm}^2/\text{s}$ at $l_1 = l_2 = 0.05 \text{ cm}$. Thus $D = (l_1 + l_2)^2 / [(l_1)^2/D_1 + (l_2)^2/D_2] = 1.22 \cdot 10^{-5} \text{ cm}^2/\text{s}$.

4 Conclusions

In this study tetracycline penetration into the enamel and dentin of the tooth was studied by fluorescence

spectroscopy. Preliminary the absorption and fluorescence spectra of a solution of tetracycline hydrochloride were obtained. This agent has been shown to fluoresce with a peak at 550 nm at an excitation wavelength of 376 nm. It has been shown that tetracycline penetrates little through the enamel as it relatively well impregnates dentin. In *in vitro* conditions tetracycline comes from the pulp into the dentin and stains it, changing the color of the tooth. Therefore, it is important to study diffusion of tetracycline hydrochloride in dentin. The cement of the tooth is also stained significantly. In *in vitro* conditions tetracycline enters cement through the surrounding blood vessels and partially through the pulp. The quantitative determination of tetracycline in the structural components of the tooth requires further study.

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Disclosures

The authors declare that they have no conflict of interest.

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