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In vitro terahertz monitoring of muscle tissue dehydration under the action of hyperosmotic agents

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Abstract. **Dehydration of muscle tissue *in vitro* under the action of biologically compatible hyperosmotic agents is studied using a laser terahertz spectrometer in the frequency range from 0.25 to 2.5 THz. Broadband terahertz absorption and reflection spectra of the bovine skeletal muscle tissue were obtained under the action of glycerol, polyethylene glycol with the molecular weight 600 (PEG-600), and propylene glycol. The presented results are proposed for application in developing the methods of image contrast enhancement and increasing the depth of biological tissue probing with terahertz radiation.**

Keywords: *pulsed terahertz spectroscopy, noninvasive dehydration, optical clearing of biotissues, hyperosmotic agents.*

1. Introduction

The terahertz frequency range belongs to far IR optical range and is adjacent to the microwave frequency range (1 THz → 1 ps → 300 μm → 33 cm^{-1} → 4.1 meV → 47.6 K). Although this range is poorly studied, the invention of THz broadband sources of radiation based on femtosecond lasers facilitated the progress in such fields of terahertz studies as pulsed terahertz spectroscopy (PTS), where the terahertz radiation demonstrated its high potentialities in various applications, including spectroscopy [1] and medicine [2, 3]. The main advantages of the terahertz range for the medical and biological studies are the small frequency dispersion of biological tissues and liquids in this range, the existence of specific spectral features in a majority of simple biomolecules in the crystalline phase, and the low scattering of radiation by inhomogeneities smaller than 10 μm. The use of ultrashort pulses allows for the investigation of a wide range of frequencies in one measurement; one can also attain high temporal resolution and extract information on the phase and, therefore, the refractive index.

Here we present a brief review of methods for studying biological tissues with terahertz radiation. We demonstrate the main reasons of the small probing depth and low contrast of terahertz images of pathological inhomogeneities in biotissues and make experimentally confirmed proposals on increasing the probing depth and additional image contrasting.

2. Review of literature and setting of the problem

The PTS technique is applicable to the spectroscopy of liquids [4] and the study of biological tissues due to its sensitivity to the concentration and condition of water [5]. The probing depth is limited by the terahertz radiation absorption. In medicine the PTS technique demonstrated good capabilities in the study of cancer tumours [6] and revealing of the depth and degree of skin burns [7, 8].

Using the PTS methods the absorption spectra of various liquids, including biological ones, were measured [9–11]. The absorption of terahertz radiation in liquids is caused by electric dipoles, both originally present in the medium and induced by the electric field of external radiation. For this reason the absorption of terahertz radiation in polar liquids is much stronger than in nonpolar ones.

In earlier published papers, in addition to the spectra of individual substances, the spectra of multicomponent biological tissues were investigated. The authors of Ref. [12] presented the results of the studies of healthy and pathologic stomach cells and demonstrated the difference in their absorption spectra. The authors of Ref. [13] reported the absorption spectra of various tissues in the range from 0.5 to 2 THz. Attempts were also made to create a statistical model for optimising the data processing aimed at classification of tissues with respect to such parameters as the absorption coefficient and refractive index [14, 15].

The specific properties of substances and tissues in the terahertz range imply the possibility of terahertz visualisation with the contrast, sufficient for tissue differentiation [16, 17]. First of all, this specificity is determined by the water content in the tissues and its properties. At the same time the studies of the effect of terahertz radiation on the tissues are being carried out. Thus, the data presented in Ref. [18] allow for the

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conclusion that under the action of THz radiation the damage of DNA in the skin structures is possible. However, in the same paper it was noted that the observations revealed protein modifications responsible for regulation and suppression of tumour growth, which is an evidence of the 'repair' process in DNA molecules. The authors of [19] report possible heating of water and, therefore, potential heating of tissues, with terahertz radiation. At the same time, the sources of radiation commonly used for medical purposes have the power, unable to injure living organisms. The topic of safety in the medical applications of terahertz radiation is a subject of separate study [20].

Currently it is already shown that the terahertz radiation is promising for medical diagnostics [21] and imaging of pathologic tissues, in particular, for early diagnostics of skin cancer [22, 23]. The affected areas are hard to assess by sight, since 85% of cancer cells lie in the epithelium under the skin surface. Terahertz waves sufficiently well penetrate into the upper layers of the skin through the dehydrated dead cells of stratum corneum and make it possible to control the development of malignant processes at the earliest stages. As compared to healthy cells, the cancerous cells contain more water [24], which intensively absorbs the radiation in the range of frequencies from 100 GHz to 3 THz. The spatial distribution of terahertz radiation intensity allows for complete mapping of the affected zone. In this connection, various research groups have proposed a number of imaging techniques, including the refection imaging [25], the continuous transmission imaging [26], the fibre scanning near-field microscopy [27, 28], and the molecular imaging [29, 30]. Among other reasons, the progress in this research field is caused by its high efficiency. Thus, in Ref. [31] using terahertz imaging the boundaries of a breast tumour were identified, in Ref. [32] the possibility of differentiation of healthy and pathologic tissues was demonstrated, and in Ref. [25] the successful assessment of location, boundaries and size of metastases with the characteristic size as small as 3 mm in the lymph nodes was reported.

The properties of protein aqueous solutions in the terahertz range are a subject of extensive studies [33, 34]. Models of interaction between the protein molecules and water were proposed [35]. The key feature is the ability of proteins to 'align' the layers of water molecules, immediately adjacent to the protein molecule, and also to reduce the mobility of water molecules and their ability to form hydrogen bonds, thus giving rise to changes in the terahertz absorption spectra, as compared to the absorption spectra of 'pure' water [36–38]. Because of this difference, the notions of free and bound water are commonly used (in some cases the term 'bulk' water is also used, but the properties of the latter are close to those of 'free' water). The degree of tissue hydration and the correlation between the optical parameters and the water content in tissues are being studied [39, 40]. Thus, the sensitivity of terahertz electromagnetic waves to the presence of water molecules allows this radiation to be used in cancer diagnostics [41–43]. Together with other structural changes of the tissue, the growth of water content leads to the increase in the absorption coefficient and reduction of reflection in tissues with tumours at terahertz frequencies [24]. However, in spite of different water ratio in healthy and pathologic tissues the implementation of imaging and diagnostics of tumours is hampered by just the significant water content and strong absorption of terahertz radiation in tissues, since the absorption of radiation in the upper layers of the tissue does not allow information to be extracted from deeper layers, in which the tumour growth occurs. This fact stimulates the development of various methods of contrast enhancement in the images, obtained by terahertz visualisation.

In Ref. [44] it was proposed to combine polarisation-sensitive methods of imaging in optical and terahertz ranges for more precise diagnostics of non-melanoma skin cancer (NMSC). As a result, it was shown that the images, produced in crossed polarisers in the terahertz range, allow the tumour location to be correctly assessed, while in the optical range the difference of images in crossed and parallel polarisers is good to reflect the specific features of morphology. The terahertz images in crossed polarisers demonstrated lower reflectance of the skin with a tumour as compared to the healthy skin. Combining optical and terahertz imaging methods provides a promising approach in the intraoperational diagnostics of NMSC. With all advantages of such diagnostics, the simultaneous exploitation of the methods mentioned requires unique instrumentation, which makes the overall use of such investigations hardly possible.

In addition, many research groups have shown the possibility to enhance the image contrast by tissue dehydration. In Ref. [45] it was proposed to use deep dehydration of biotissue by lyophilisation via freezing. The lyophilisation provides essential loss of water and offers a possibility to keep the parameters of the studied biotissue unchanged during the investigation, i.e., to provide good reproducibility of results. This method makes it possible to essentially reduce the terahertz signal absorption and, therefore, to increase the reflected signal. The method drawbacks are obvious, since it does not allow for *in vivo* investigations. Moreover, deep and prolonged freezing of the tissue essentially changes its structure. The dehydration of a biotissue can be implemented thermally (using a hairdryer), in fact, by mere drying [46]. This method suffers from the same drawbacks as the previous one. In Ref. [47] it was proposed to solve the contrast enhancement problem basing on the contrast-agent-enabled terahertz imaging (CATHI). The key idea of this method is using gold nanorods (GNRs) to enhance the contrast. The GNRs are labels of cancerous cells and heat the water in them under the action of IR radiation. Such method allows for enhancement of the reflected terahertz signal specifically from those parts of the tissue, where the cancerous cells are concentrated.

The reduced ability to bind water molecules by the proteins of damaged tissues allows for the conclusion that the ratio of free-to-bound water in tumour tissues is changed in favour of free water [48]. Thus a new opportunity arises to distinguish between healthy and pathologic cells not only by the total water content, but also by the ratio of free-to-bound water. In Ref. [37] among other results it was shown that when a protein is removed from the solution, the bound water is also removed with it, and in Ref. [49] the differences between the healthy and pathologic tissue due to different water content were detected even after treating the samples with formalin. These facts verify the existence of strong coupling between the protein molecule and the adjacent water molecules. All these observations allow for the assumption that the temporary removal of water from a tissue by means of dehydrating agents [i.e., optical clearing agents (OCA), immersion liquids] offers a possibility to change the water content in tumours and thus to enhance the image contrast not only due to the reduction of the total water content in the tissue, but also due to the change of the free-to-bound water ratio. This will allow for classification of tissues with respect to the content of free and bound water and, in particular, diagnosing cancer tumours.

Obviously, the application of dehydration methods *in vivo* requires the appropriate changes in the tissues to be temporary and reversible. In the present paper we propose to perform dehydration by means of hyperosmotic agents, the use of which is based on their ability to form a flow of free water directed outside from the tissue, as well as to penetrate into the tissue and temporarily substitute the free water. The promising agents of this kind are glycerol, polyethylene glycol, and glucose solution [50]. These agents are not toxic and, as shown below, do not introduce essential errors in the measurements carried out. Besides, they possess high enough diffusion coefficients in the tissues, which is necessary for successful application of these substances in the process of human tissue dehydration under clinical conditions [50–57]. The monitoring of the water content is conveniently performed with the terahertz radiation (because of its sensitivity to the presence of water) by recording the absorption and reflection spectra.

The aim of the present paper is to use the PTS method and the laser terahertz spectrometer for the study of the influence of dehydrating agents on the efficiency of propagation and reflection of terahertz radiation in tissues. The object of study was the muscle tissue, chosen as a model of fibrous soft tissue with high content of free water.

3. Materials and methods

In this work we used the pulsed terahertz radiation, generated by conversion of ultrashort pulses of laser radiation at the surface of a semiconductor. In this case the generation of THz radiation is caused by a spike of time-dependent electron–hole photoconductivity of the semiconductor (GaAs) in response to light pulses and a burst of photocurrent caused by the internal electric field. The detection of the terahertz pulse (THP) was implemented in the electrooptic crystal (ZnTe). A key unit of the terahertz setup was the terahertz spectrometer the schematic diagram of which is presented in Fig. 1 [52]. The setup allowed temporal profiles of reflected or transmitted signals from the sample to be recorded after the interaction with biotissues and then, after the Fourier transform, absorption and refraction spectra of the studied object to be calculated.

As clearing agents we used polyethylene glycol with the molecular weight 600 (PEG-600), dehydrated glycerol (99.9%), and propylene glycol (PG) (99.9%). Bovine muscle tissue samples were chosen as the object of study. The sample thickness before and after the experiment was measured with a micrometre gauge; for measurements the sample was placed between two slides. The measurements were carried out at several points of the sample; the error of each measurement amounted to ±50 μm.

The measurements were carried out at the temperature +21°C both in fresh samples and in those kept in a household freezer at the temperature –18°C for 3–72 hours.

3.1. Measurement of the absorption spectra

In the measurements of the absorption spectra the biotissue sample was placed on a metallic mount with windows (Fig. 1, the transmission configuration) and clamped between two polystyrene plates, each 750 μm thick. A spacer was placed in the cuvette to fix the thickness. For measuring spectra of liquids the spacer thickness was taken to be 0.2 mm, and for measuring the tissue spectra the spacers had the thickness 0.2 and 0.5 mm.

Before starting the measurements of the biotissue sample, the background spectrum was recorded, against which the sample spectrum was then normalised. The background spectrum was formed by the empty cuvette and included individual specific features of the optical scheme of the used experimental setup. Then the biotissue sample to be studied was placed in the cuvette in such a way, that the measurement window area was completely covered, the size of the window being smaller than the diameter of the focused terahertz beam. After recording the full series of spectra from a single sample the cuvette was carefully cleaned with the isopropyl alcohol to remove the remains of the studied substance from the measurement window.

First the spectrum of intact sample was recorded, then the cuvette was disassembled and the traces of water were removed from both sides of the sample by means of a paper napkin. Then on one side of the sample (always the same one) the immersion agent was applied in the volume of 200 μL, the

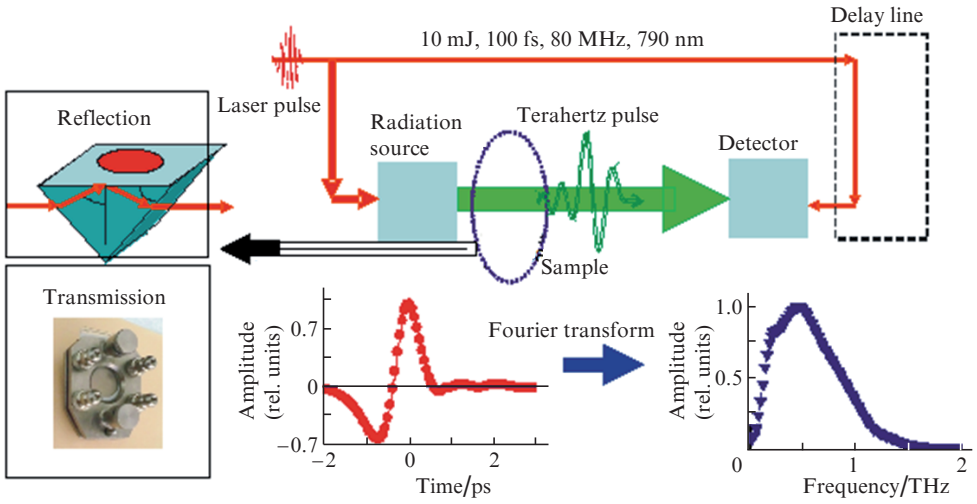

Figure 1. Schematic diagram of the terahertz spectrometer. In the reflection configuration we used a silicon prism, in the transmission configuration – a metallic holder with polystyrene windows.

cover glass was placed over it and in this arrangement the tissue was kept during 1–5 min. Then again using the paper napkin the excess amount of the agent was removed and the sample was placed into the cuvette for measurements. Each measurement lasted nearly 3 min.

Based on the experimental absorption spectra and the data on the sample thickness, the spectra of the absorption coefficient and refractive index under the action of hyperosmotic immersion agents were calculated [14, 52]. As shown below, for the experiments in the reflection configuration, the general tendency is always the same. For this reason and also with the similarity of the absorption spectra of the agents taken into account, in the transmission experiments only glycerol was used.

Experimentally only one sample was studied with each agent, that is why no statistical errors are presented in the spectra. Nevertheless, a notion of instrumental error can be introduced that includes the following components: the signal-to-noise ratio (detector noise), the reproducibility of the reference signal (source noise), and the precision of measuring the sample thickness (in the case of operation with a sample).

The distribution of such errors is presented by the example of the absorption spectra of the agents.

3.2. Measurement of the reflection spectra

The configuration for measuring the absorption allows for direct calculation of the absorption coefficient and refractive index, but in this case there are two essential drawbacks, namely, the strong attenuation of the informative signal and the necessity to work *in vitro*. The setup configuration specially intended for measuring the reflection [52] is free of these drawbacks.

The reflected signal was measured using the silicon prism, on which the studied sample was placed. A drop (or a tissue sample) was kept on the surface by the gravity force and the surface tension; the drop thickness (0.2–1 mm) was much greater than the penetration depth of the vertically polarised terahertz field into the sample (nearly 0.1 mm, depending on the frequency). The contact between the sample and the prism was monitored by tracing the shape of the terahertz pulse, namely, if an air gap appeared, the pulse peak became double-split. This was caused by the pulse re-reflection in the air gap. The scheme of propagation of the probing beam of terahertz radiation through the prism is presented in Fig. 1. The obtained data were normalised for the signal in the absence of the sample.

4. Results and discussion

Figures 2–4 present the experimental data that were obtained in the absorption configuration for the spectral and kinetic dependences of the absorption coefficient and refractive index of immersion agents, as well as of the bovine muscle tissue under the impact of glycerol.

With time the decrease in both the absorption coefficient and the refractive index of the biotissue samples was observed, which can be explained by the reduction of water content and the increase in the agent content in the tissue, since the agents have lower values of these parameters, than water. Hence, for the model muscle tissue it is definitely shown that one can reduce the absorption of radiation in the range 0.25–2.5 THz when it propagates through a biological tissue under the application of hyperosmotic agents. Depending on the radiation frequency, the absorption coefficient decreased by 3–5

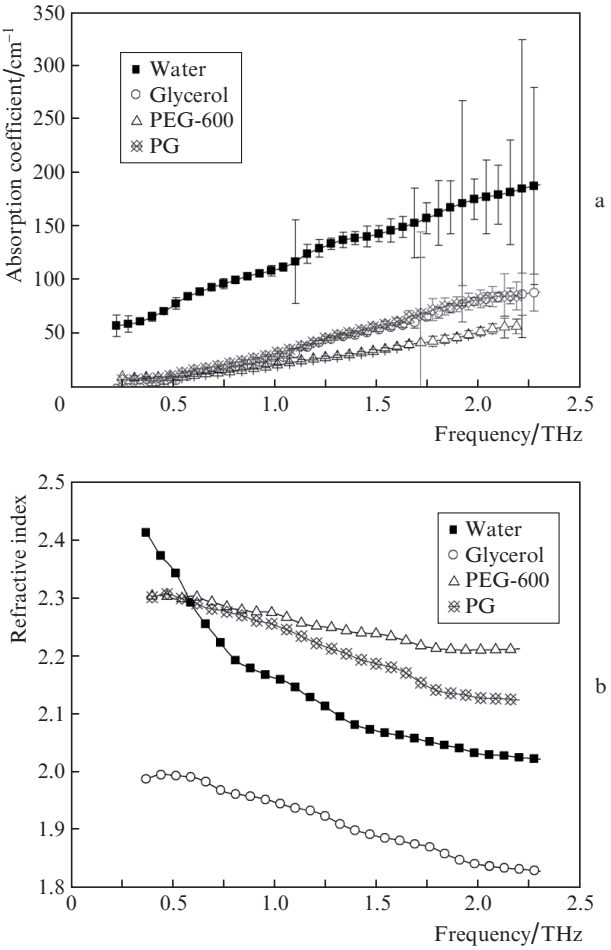

Figure 2. Spectral dependences of the absorption coefficient (a) and refractive index (b) of distilled water, glycerol, PEG-600, and PG.

times, the necessary agent application time being ~8–10 min. During this time interval the maximal impact of the agent was achieved, which agrees with the data of Refs [53–56].

Figure 5 shows the amplitude and phase spectra, measured using the geometry of attenuated total reflection (ATR) for distilled water and immersion liquids used.

Figures 6 and 7 present the ATR spectra for samples of normal tissue with dehydrating liquids applied. The agent was applied in the excessive volume from above to the tissue layer, and a series of spectra were sequentially recorded without any change in the sample configuration. The kinetic curves were used to determine the diffusion rate of different immersion liquids. As seen from Fig. 6, in the absence of agents the signal from the tissue was practically unchanged, while after the application of an agent the growth of the signal is noticeable (Fig. 7).

To obtain the data presented in Figs 8, 9 we measured the maximal amplitude of the pulse versus time without scanning the delay line, rather than the pulse temporal profile (Fig. 1). In this case the signal amplitude corresponds to the reflection coefficient, averaged over the spectrum, and the data on the frequency dependence are lost. However, in this case the possibility of getting fast and detailed information about the dehydration dynamics arises. As seen from the figures, after some time the curves become saturated, the moment of saturation occurs in 20–60 min, depending on the used agent and the sample thickness.

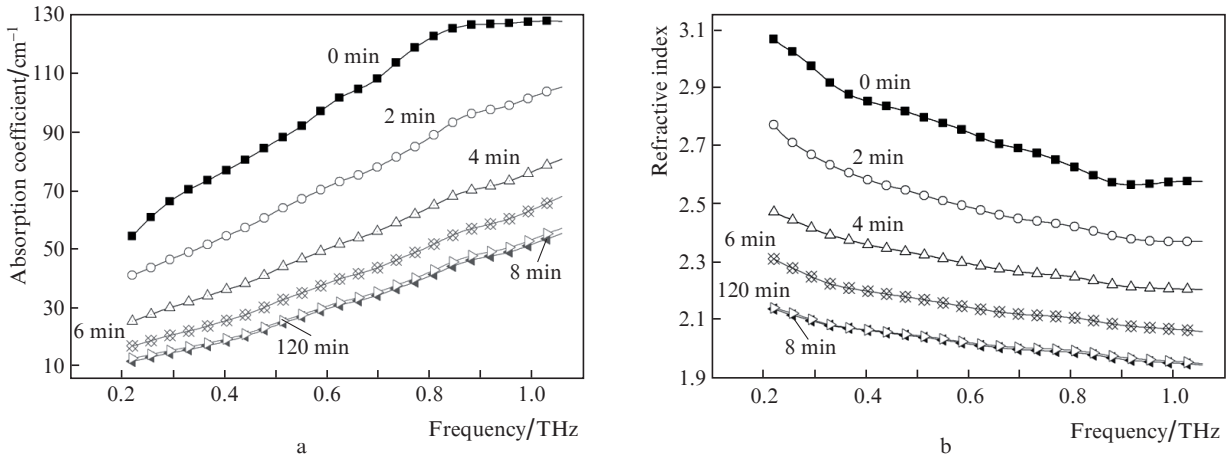

Figure 3. Spectral dependences of the absorption coefficient (a) and refractive index (b) of the bovine muscle tissue treated with glycerol. The time corresponds to the cumulative time of holding the sample with the agent applied.

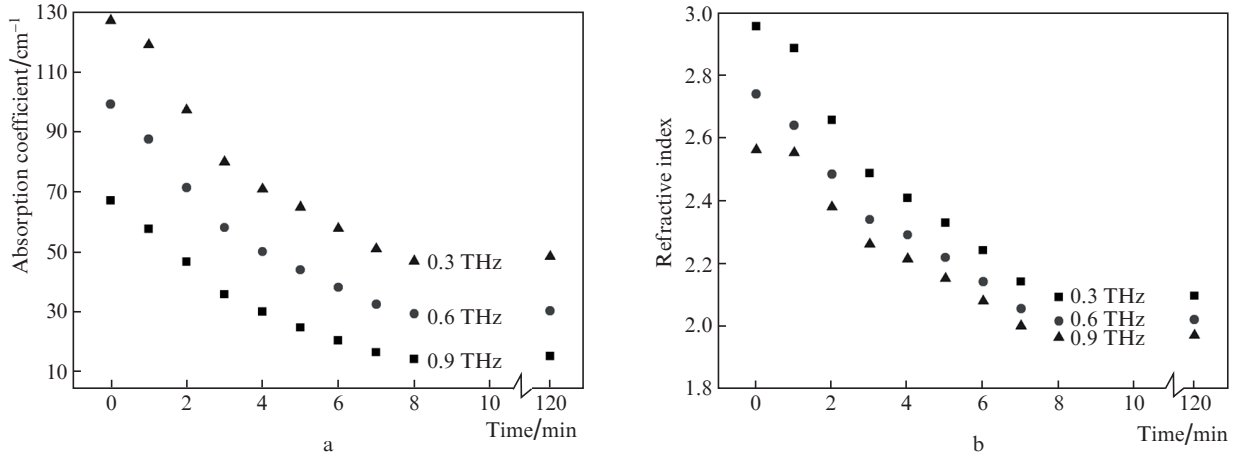

Figure 4. Kinetics of variation of the absorption coefficient (a) and refractive index (b) of the bovine muscle tissue treated with glycerol at the frequencies 0.3, 0.6, and 0.9 THz. The time corresponds to that of cumulative holding of the sample with the agent applied. The character of variation of the absorption coefficient and refractive index reflects the variation of free water content.

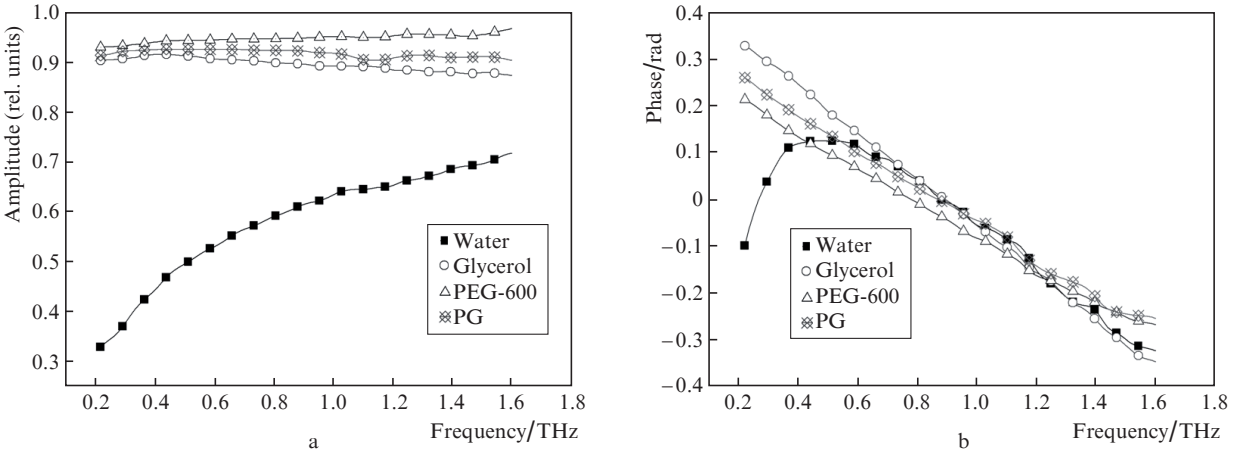

Figure 5. ATR spectrum of the amplitude (a) and phase (b) of distilled water and immersion liquids used in the paper.

The study was aimed at the investigation of spectral features and collecting a number of spectra in the range 0.2–1.6 THz, sufficient for estimating the suitability of using hyperosmotic agents for temporary and reversible dehydration of tissues.

The averaged diffusion coefficient D for the flows of dehydrating agent molecules and interstitial water was calculated using the following formula, valid in the case of the agent diffusion from one side of a planar layered sample [50]:

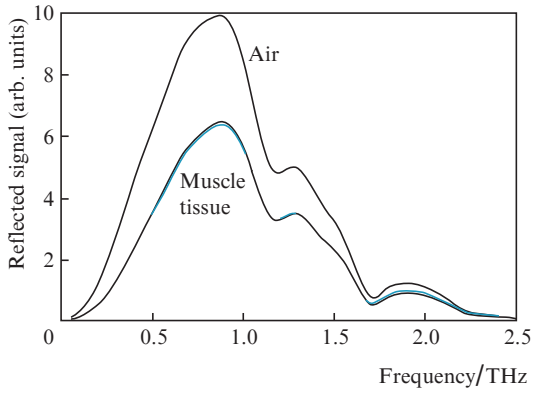

Figure 6. Spectrum of the terahertz signal, reflected from air and bovine muscle tissue (during 9 min) without treating with an optical clearing agent.

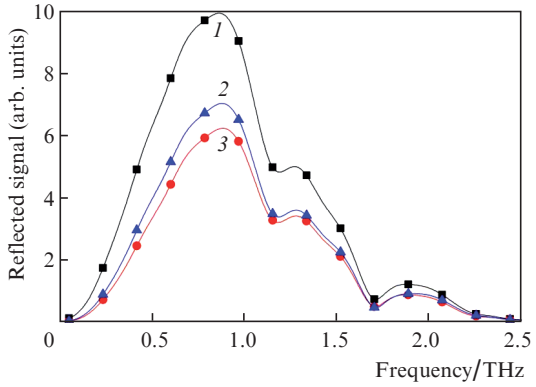

Figure 7. Spectrum of the terahertz signal, reflected from air (*1*) and bovine muscle tissue (*2*, *3*) under the action of PG during 3 minutes (*3*) and 90 minutes (*2*). The signal from the tissue gradually increases with time, which indicates the water loss in the interface region between the prism and the sample and the increase in the PG content.

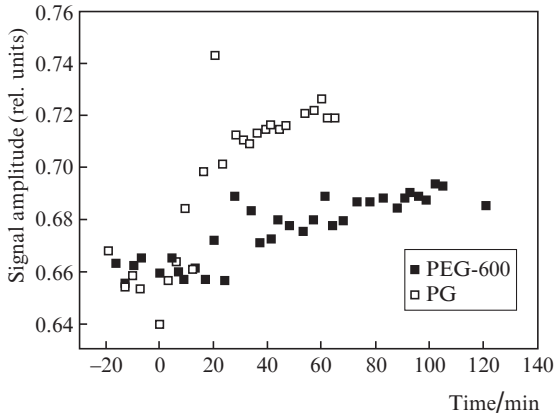

Figure 8. Kinetics of the signal reflected from the sample of muscle tissue under the action of PEG-600 and PG (the sample thickness 1.25 and 0.6 mm, respectively; the amount of the applied agent is 50 μL in both cases).

$$D = d^2/t, \qquad (1)$$

where d is the sample thickness, and t is the characteristic diffusion time for the molecular flows. The square-law dependence of the diffusion time on the thickness is confirmed by the data of Fig. 9, where for the thickness values $d_1 = 0.75$ mm and $d_2 = 1$ mm the diffusion times are $t_1 = 40$ min and $t_2 = 70$ min, respectively. Hence, this parameter allows the rate of the agent action, resulting in tissue dehydration, to be calculated. The averaged diffusion coefficients D, calculated using Eqn (1) for PG, PEG, and glycerol, acting on the muscle tissue, were equal to 2.0×10^{-6}, 3.2×10^{-6}, and 2.9×10^{-6} cm^2 s^{-1}, respectively. Thus, one can say that glycerol and PEG-600 diffuse into the tissue faster and, therefore, the time of the tissue clearing is smaller. For comparison, the diffusion coefficients of glycerol in water (at the temperature 15 °C and small glycerol concentration) and water in water (at the temperature 20 °C) are $D_{glyc} = 7.2 \times 10^{-6}$ cm^2 s^{-1} [58] and $D_{H_2O} = 8.9 \times 10^{-6}$ cm^2 s^{-1} [59]. Since soft biological tissues contain a considerable amount of water (upon the average, 75%), the diffusion of glycerol molecules in water is a good model for the diffusion of these molecules in soft tissues. The reduction of the diffusion rate by nearly 2–3 times can be explained by diffusion hampering, caused by the interaction with the organic matrix and the limited penetration through biological membranes in tissue cells.

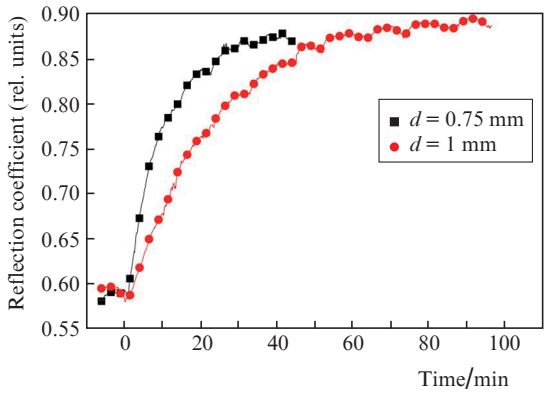

Figure 9. Kinetics of the reflected signal averaged over the spectrum from the sample of muscle tissue upon application of glycerol. The tissue layer thickness is 0.75 and 1 mm, the amount of the applied agent is 50 μL in both cases.

5. Conclusions

The presented results of the experimental study of the tissue absorption and reflection spectra lead to the following conclusions.

1. Using the muscle tissue model we definitely established the possibility to reduce the absorption of radiation in the range 0.25–1.6 THz, propagating through a biological tissue, by applying hyperosmotic agents, which allows for an essential increase in the depth of tissue probing with such radiation.

2. Depending on the radiation frequency within the range 0.25–1.6 THz, the decrease in the absorption coefficient amounts to 60%, the required application time being 8–10 min, which is acceptable for using this technology in medical practice.

3. It is important that the biotissue refractive index is also reduced and becomes less dependent on the radiation frequency, which facilitates deeper penetration of the terahertz radiation into the tissue at the expense of smaller Fresnel reflection at the layers inside the tissue.

4. From additional measurements it is known that the clearing time for healthy and pathologic regions of the tissue differs by 20%–40% (the pathologic tissue is cleared faster). Therefore, by choosing the time for imaging to be a half of the clearing saturation time for the healthy tissue, we will get considerable contrast improvement for selecting the region with pathology in the terahertz image.

From the obtained data on the agent study one can draw several conclusions. First, the reflection coefficients of the agents essentially differ from that of water. However, the reflection spectra of the agents differ from each other insignificantly: the mean value of the reflection coefficient amounts to 0.917 for glycerol, 0.948 for propylene glycol, and 0.917 for PEG-600. For water the mean value of the reflection coefficient equals 0.582. Second, in the plot of the signal phase versus frequency a characteristic bend is seen in the low-frequency region, which is typical for water and can be a potential marker of water content in tissue. The increase in the reflected signal amplitude amounted to 45% for glycerol, 12.5% for PG, and 6% for PEG-600, compared to the beginning of the experiment. Therefore, with the diffusion coefficient data taken into account, one can conclude that the most preferable clearing agent is glycerol.

Thus, it is possible to state that all chosen agents have spectral characteristics and hyperosmotic properties, satisfying the requirements for their use for tissue dehydration in terahertz visualisation and spectroscopy.

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