

Study of the Effect of Formaldehyde Fixation on Collagen Optical Properties in the Terahertz Frequency Range

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Abstract. The study investigated the effect of collagen fixation with formaldehyde on optical properties in the THz frequency range. The spectral characteristics transformation of protein solutions occurs due to hydrogen bonds and intermolecular interactions among the various components of the solution. Changes of the optical characteristics of collagen solutions with distilled water, 10% formalin, and 40% formalin in the frequency range from 0.2 THz to 1 THz were studied. © 2024 Journal of Biomedical Photonics & Engineering.

Keywords: terahertz spectroscopy; collagen; formalin.

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1 Introduction

Collagen is the main component of the extracellular matrix, which is involved in cell migration and differentiation and accumulates in tissues during oncological processes. The presence of oncological tumors is confirmed by histological examination of tissue biopsy. Histological preparations are usually analyzed using conventional optical microscopy, which does not use information on the molecular composition of the tissue. Combination optical microscopy with spectroscopy can provide more comprehensive information for making a medical decision. However, it is important to understand how the process of formalin fixation during the histological preparation affects the spectral properties of collagen in the tissue. Collagen is the most common protein in mammals (more than 30%). It is a filamentous molecule consisting of three-stranded polypeptides forming a helix. The molecular weight of collagen is about 300 kDa [1].

In histology, during fixation and processing stage, it is essential to minimize post-surgical tissue alterations [2, 3]. Biopsy fixation is the critical initial step to preserve cellular and extracellular material [4]. Aqueous formaldehyde solutions (formalin) are the most commonly employed fixatives [5, 6]. In practice, a neutral solution of 10% formalin is used as a universal fixative.

The treatment of collagen structures with fixing and preservative substances has been demonstrated to enhance collagen resistance to degradation by collagenolytic enzymes, reduces its solubility, and

also increases the temperature of hydrothermal contraction [6–8]. Formalin fixation displaces water molecules and introduces new intermolecular interactions between the sample and formalin [9]. Formaldehyde also reversibly forms methanediol, also known as formaldehyde monohydrate or methylene glycol, in the presence of water. Methylene glycol reacts with several side chains of proteins and forms reactive hydroxymethylene groups ($-\text{CH}_2-\text{OH}$). A characteristic reaction for formalin fixatives is the formation of intra- and intermolecular methylene cross-links between nucleic acids, as well as between amino acids and nucleic acids [10–14]. Cross-links can form only in the presence of uncharged amino groups, which is possible in neutral solutions.

In the terahertz (THz) spectral range, there are strong absorption lines of many biopolymers, including collagen. Collagen THz absorption spectrum is sensitive to its spatial structure and environment [15–16]. The strong absorption in the THz range of water solutions caused by collective fluctuations in the structure of the network of hydrogen bonds in water makes it possible to control changes in protein conformation in solutions [17]. Moreover, the vibrational, translational and intermolecular collective motions of hydrated water also corresponds to low-frequency THz modes (Fig. 1) [18].

As protein structures break down during unfolding, hydrophobic moieties (amino acid residues), which are otherwise hidden within the native structure are exposed, it influences on the absorption spectrum in the corresponding spectral range.

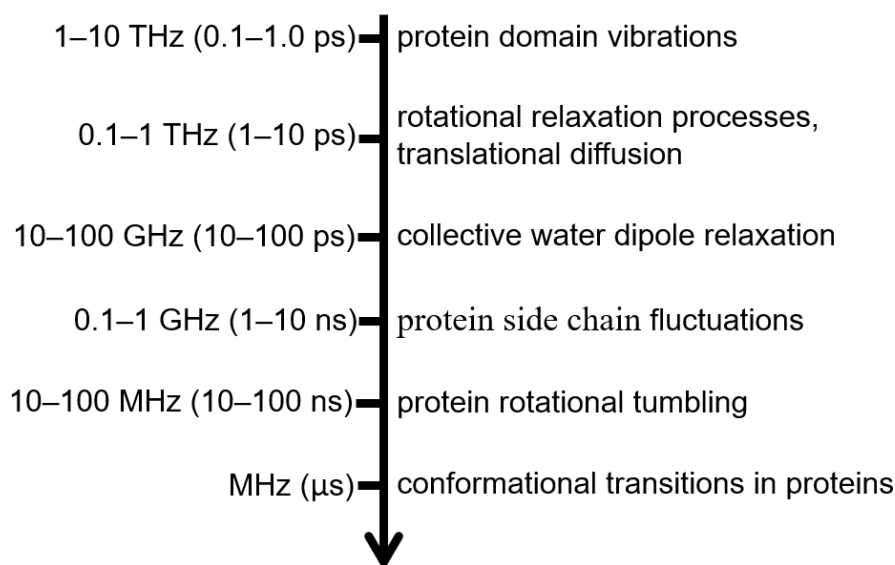


Fig. 1 Hierarchy of time scales for protein motions.

Therefore, analyzing protein THz absorption offers a direct means of assessing structural changes. Havenith et al. investigated the dynamics of protein hydration using THz spectroscopy [19–21]. They established that hydrophobic and hydrophilic protein residues exhibit different THz absorption when interacting with water molecules [19].

The addition of protein molecules to water leads to disruption of the network structure of water hydrogen bonds and the formation of hydrogen bonds between the protein and water molecules. The process of hydration involves not only water molecules directly associated with protein molecules in the primary layer, but also those outside the latter. Therefore, it can be expected significant changes in the collective oscillations of the network structure of hydrogen bonds of water molecules and, consequently, in the THz absorption spectra of protein solutions [22–25]. Manna et al. studied in detail the dependence of the THz absorption of aqueous buffer solutions of the human serum albumin (HSA) protein (up to 2.6 mM protein concentration) on its concentration [23]. The absorption of the solution in the THz range was shown to increase initially, following a clearly linear behavior up to a concentration of $\sim 6 \times 10^{-4} \text{ mol dm}^{-3}$, but then gradually decreases with a further increase in the HSA concentration. Such a nonlinear behavior of the change in the absorption of protein solutions in the THz range with increasing protein concentration is explained by the formation of protein aggregates. The critical concentration of collagen aggregation in an aqueous solution was 0.5 mg/ml [26]. R. K. Mitra and D. K. Palit supposed that protein solutions should be considered as three-component systems, where one component is the protein itself, and the other two are water molecules in the hydration layer and bulk water molecules [27].

In the study, we assessed the effect of collagen fixation with formaldehyde on collagen optical properties in the THz frequency range.

2 Materials and Methods

2.1 Sample Preparation

Immersion formalin fixation, a chemical process, significantly alters the molecular structure. This treatment prevents tissue degradation by inhibiting cellular enzymes and decay processes, preserving the vital structure for histological analysis. The principle of fixing liquids is based on the rapid death of cells and protein coagulation. During formalin fixation, collagen can successively lose its quaternary, tertiary and even secondary structure, while the primary structure (amino acid composition) remains intact. Therefore, to describe the process of tissue fixation with formalin when preparing a histological preparation, we will consider a mixture of collagen and water as a simplified model of biological tissue. In this case, it is possible to consider, for example, only hydrolyzed collagen.

Collagen solutions in distilled water, 40% formalin and in 10% formalin were prepared as follows. Dry Collagen Type 1 (Doctor's Best, USA) was weighed on an analytical balance (accuracy 0.001 g) and dissolved in 0.5 ml liquid (i.e. 0.5 ml water, 10% or 40% formalin). Thus, 10 solutions were prepared with a collagen content of 0.026, 0.05, 0.08, 0.125, 0.166, 0.214, 0.27, 0.33, 0.409, 0.5 g, respectively. The amount of components added to the solution and the resulting ratios of the concentrations of collagen (COL), free water (H_2O) and formaldehyde (F) in the prepared solutions of collagen with formalin are shown in Table 1. Sample preparation for dissolving collagen with formaldehyde was carried out as follows: collagen was weighed on an analytical balance GR-120 (AND, Japan) ($d = 0.0001 \text{ g}$) and placed in disposable IMEC tubes (volume 2 ml). Formalin was added to each test tube using an ECOHIM single-channel variable volume pipette (ECROSKHIM Co., Ltd, Russia) (100–1000 μl).

Table 1 Percentage ratio of components of collagen solutions with formalin.

Mass of components, g			Content in solution, %		
	COL	H ₂ O		F	COL
10% formalin	0.026		84.4	10.3	5.3
	0.050		80.4	9.8	9.8
	0.080		75.9	9.3	14.8
	0.125		70.1	8.5	21.4
	0.166	0.410	65.5	8.0	26.5
	0.214		60.8	7.4	31.8
	0.270		56.2	6.8	37.0
	0.330		51.9	6.3	41.8
	0.409		47.2	5.8	47.1
	0.500		42.7	5.2	52.1
40% formalin	0.026		57.6	37.0	5.3
	0.050		54.9	35.3	9.8
	0.080		51.9	33.3	14.8
	0.125		47.9	30.8	21.4
	0.166	0.280	44.7	28.8	26.5
	0.214		41.5	26.7	31.8
	0.270		38.4	24.7	37.0
	0.330		35.4	22.8	41.8
	0.409		32.2	20.7	47.1
	0.500		29.2	18.8	52.1

Mixing of hydrolyzed collagen (in dry form) and formalin was carried out using a Vortex V-1 plus (Biosan, Latvia) at a speed of 2000 rpm until a homogeneous solution was obtained. Ten solutions were prepared for each solvent (distilled water and formalin), with varying concentrations of hydrolyzed collagen ranging from 5 % to 50 % of the total solution volume.

2.2 THz Spectra Acquisition and Analysis

For each individual components of the solutions: dry collagen, distilled water, 10% formalin and 40% formaldehyde, 6 spectra in THz region were obtained. Additionally, 6 spectra were acquired for each collagen concentration (see Section 2.1), representing 60 spectra of collagen solutions with distilled water (6 spectra for each of the 10 concentrations of collagen added to the solution), 60 spectra for solutions with 10% formalin and 60 spectra for solutions with 40% formalin.

Measurements of the absorption and refraction spectra of collagen solutions were carried out using a THz-TDS spectrometer T-spec from EXPLA in transmission mode (Fig. 2) in the frequency range from 0.2 to 1 THz.

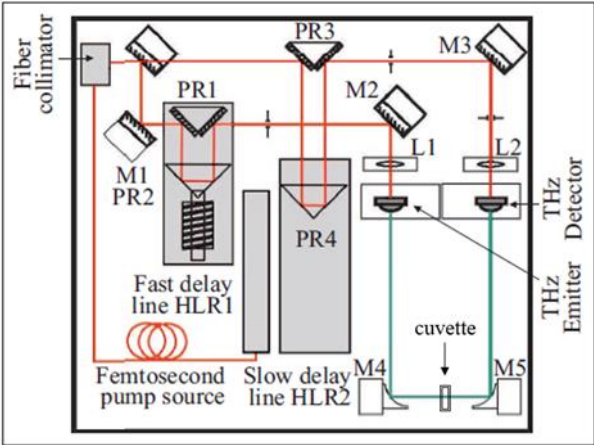


Fig. 2 Scheme of the experimental setup. M1–M5: mirrors; HLR1, HLR2: hollow retro-reflectors; PR1– PR4: prisms; L1, L2: lens.

The studied samples were placed in plastic cuvettes with a thickness of 0.5 mm [28], providing sufficient transparency in the THz spectral range. Scanning with a fast delay line at a frequency of 10 Hz forms a wave front of the electric field of THz radiation (Fig. 3a). The Fourier transform allows us to reconstruct the

dependence of the amplitude of the recorded signal on the frequency, and gives a spectral representation of the THz radiation (Fig. 3b). The obtained values of the signal amplitude allow us to calculate the intensities of the reference I_0 and the signals transmitted through the sample I . Relative transmittance is a measure of how much radiation is attenuated by the material at a given frequency. The intensity of radiation transmitted through the sample (Fig. 3a) can be described using the Bouguer-Lambert-Beer law

$$I = I_0 e^{-\alpha d}.$$

Here I_0 is the intensity of the signal generated by the THz emitter, I is the intensity of the signal transmitted through a medium of thickness d , α is the absorption coefficient

$$\alpha = -\frac{1}{d} \ln \left(\frac{I}{I_0} \right).$$

The refractive index through the phase difference has the following form:

$$n = 1 + \frac{c}{\omega d} (\varphi_{\text{samp}} - \varphi_{\text{ref}}),$$

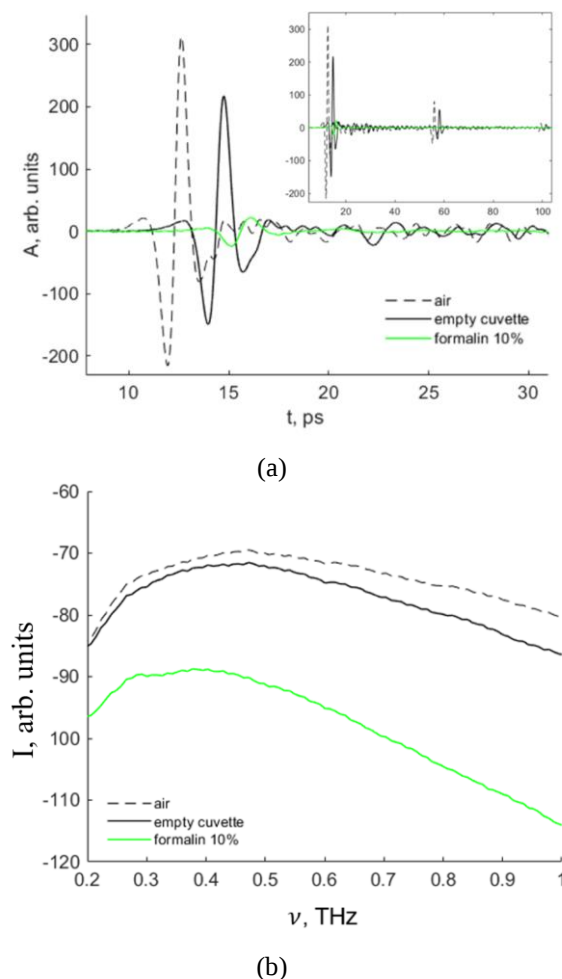


Fig. 3 (a) Incident time-domain THz pulse and (b) frequency domain signal following Fourier transform.

where c is the speed of light, ω is the frequency, d is the thickness of the sample, φ_{samp} and φ_{ref} are the phases of the signal transmitted through the sample and in the absence of the sample, respectively.

The study of solutions was carried out as follows: an empty cuvette was initially placed in the path of the THz radiation beam, as shown in Fig. 2. The THz beam diameter at the focal point was $\sim 3.5\text{--}4$ mm. The cuvette was placed in the THz spectrometer to ensure the beam passed directly through its center. The THz radiation was focused onto the sample using parabolic mirrors. Then, the reference intensity $I_{\text{ref}}(\nu)$ of THz wave passed through an empty cuvette, was recorded (Fig. 3b, black solid line). Next, immediately after mixing the solution, the test sample was placed into the cuvette using a Lempipet dispenser. The solution was poured evenly along the wall of the cuvette, avoiding the formation of bubbles. Measurement of samples began 5 min after their preparation. The cuvette with the sample was scanned in 2D space with a step of 0.1 mm vertically and horizontally and THz spectra were measured several times in every spatial point. Each intensity $I_{\text{samp}}(\nu)$ was averaged over 128 time intervals to obtain a better signal-to-noise ratio. All measurements were carried out at room temperature, 21 ± 1 °C.

3 Result and Discussion

The averaged THz absorption and refraction spectra of hydrolyzed collagen and the solvents are presented in Fig. 4. The THz spectrum of dry collagen (red line, Fig. 4) shows a sharp rise in absorption in the frequency range from 0.2 to 0.3 THz, followed by a steady increase in the absorption coefficient with increasing THz frequency.

There are no obvious peaks in this spectral range. The THz spectra of distilled water (blue line, Fig. 4) and 10% formalin (green line, Fig. 4) have a similar shape, characterized by a gradual increase in absorption with increasing frequency, with an increase in the presence of dissolved formalin in water (black line, Fig. 4) in absorption becomes less. The refractive index (average in the studied THz frequency range) for hydrolyzed collagen was 1.84 ± 0.06 , for distilled water was 1.97 ± 0.02 , for 10% and 40% formalin were 1.92 ± 0.02 and 1.82 ± 0.03 , respectively.

When collagen was added to 10% formalin, the THz absorption spectra of solutions did not have characteristic peaks; for all concentrations of dry collagen in solutions, there was a tendency for absorption to increase with increasing frequency (Fig. 5). As the collagen content of collagen solutions with 10% formalin increases, the total absorption decreases.

Water intermolecular hydrogen bonds vibrations exceed the intramolecular vibrations of the solute, thereby we observed a decrease in the absorption of solutions in the THz range with increasing collagen content. Considering the refractive index spectra for collagen solutions with 10% formalin, it was noted that as the THz frequency increases, the values decrease.

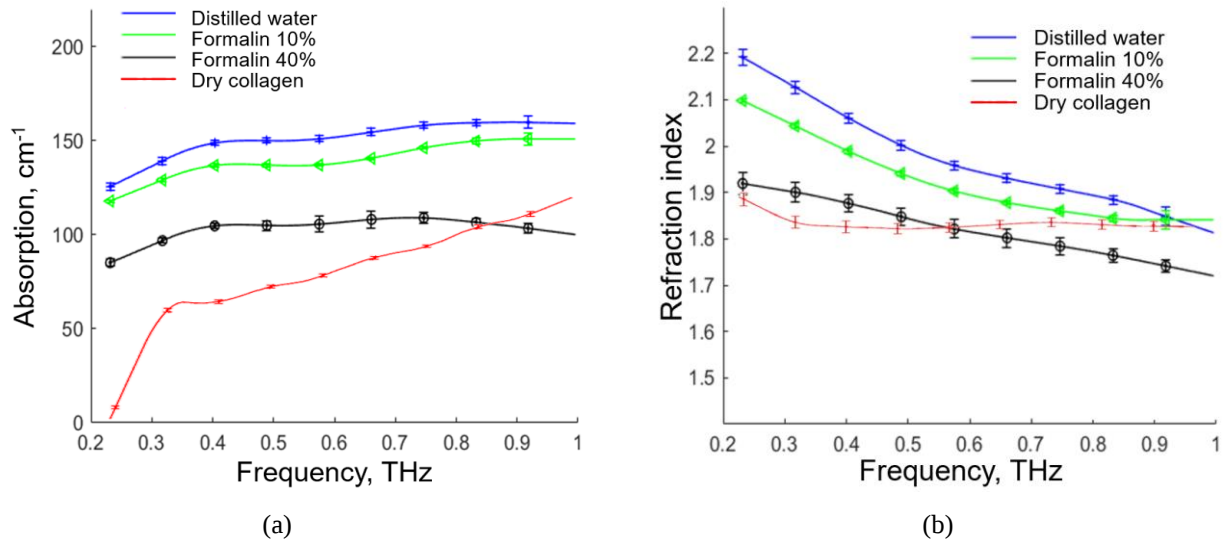


Fig. 4 (a) Absorption and (b) refraction spectra of dry collagen, 10% and 40% formalin and distilled water.

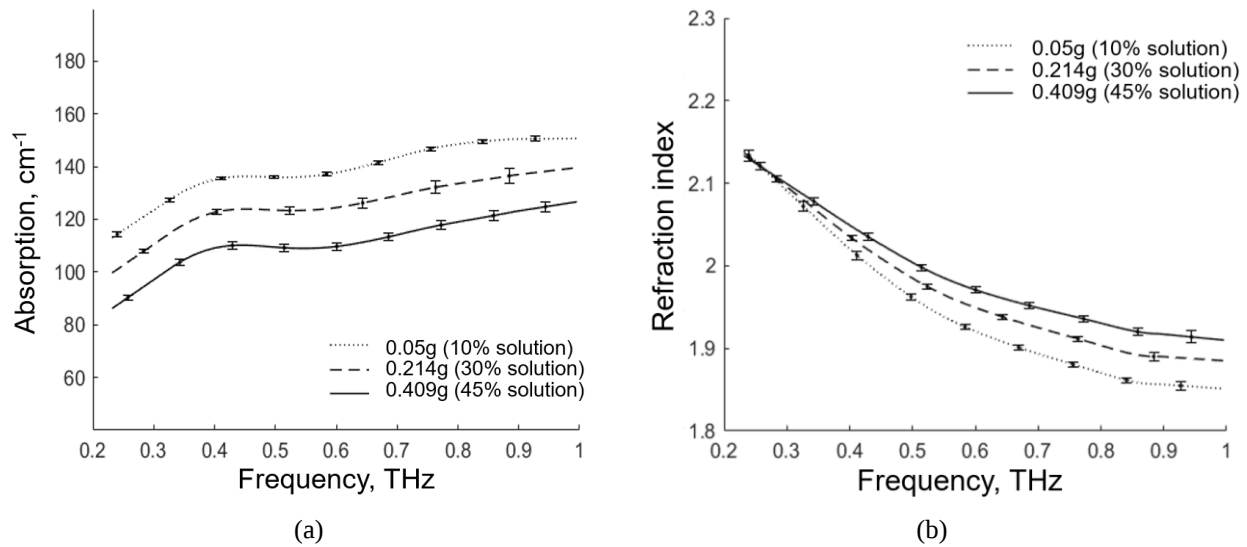


Fig. 5 THz (a) absorption and (b) refraction spectra of collagen in 10% formalin.

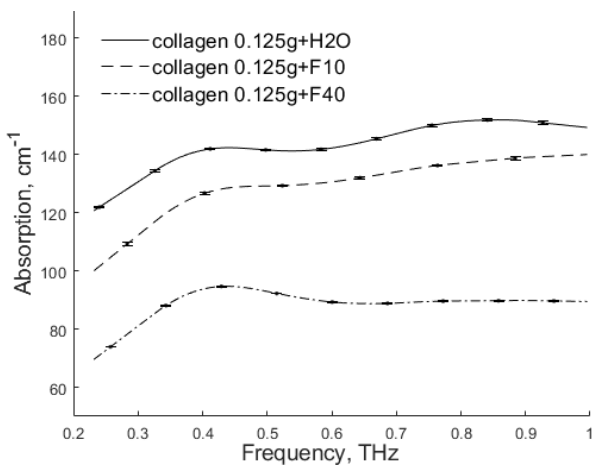


Fig. 6 THz absorption spectra of collagen of 0.125 g weight in water solution (solid line), 10% formalin (dashed line) and 40% formalin (dash-dot line).

The absorption spectra of collagen in an aqueous solution, in 10% formalin and in 40% formalin were examined to assess whether fixation with formaldehyde affects the optical properties of the solution in the THz frequency range. The absorption spectra of solutions containing 0.125 g of collagen dissolved in solvent are shown in Fig. 6. The dependences of the absorption of the studied solutions on the concentration of the components added to the solution (collagen, water and formalin) in percentage terms are shown in Fig. 7.

Since water is replaced by formaldehyde during the fixation process, a corresponding decrease in the absorption coefficient is expected, as shown in Fig. 8. Before fixation (collagen solution in distilled water, Fig. 8a), depending on the mass fraction of collagen added to the test solution, the absorption coefficient was $\sim 140 \text{ cm}^{-1}$ at frequencies from 0.6 to 1 THz, and decreased to values of $\sim 90 \text{ cm}^{-1}$ at frequencies 0.2 to 0.3 THz. When collagen was fixed with 10% formaldehyde (Fig. 8b), the absorption coefficient

decreased to values of $\sim 80 \text{ cm}^{-1}$ with a mass of collagen added to the solution equal to 0.5 g. With an increase in the percentage of formaldehyde content in the solution (Fig. 8c), it was shown that in the frequency range from 0.35 to 0.5 THz, the absorption coefficient remained practically unchanged at a collagen mass fraction from 0.08 to 0.409 g.

Using the distributions shown in Fig. 8, it is possible to evaluate the molecular interactions of formalin with collagen in the THz region of the spectrum in the frequency range from 0.2 to 1 THz. Depending on the

proportion of collagen content, the results obtained can be interpreted for different types of tissues and organs.

For a quantitative comparison of spectral profiles, we used the curve proximity factor (CPF) S , similar to the Pearson's correlation coefficient, to compare curves of collagen solutions in distilled water and in formalin [29]:

$$S = \frac{\sum_i |X_i - Y_i|}{\frac{1}{2} \sum_i |X_i + Y_i|},$$

where X_i , Y_i are the absorption coefficients of collagen solutions in distilled water and formalin, respectively.

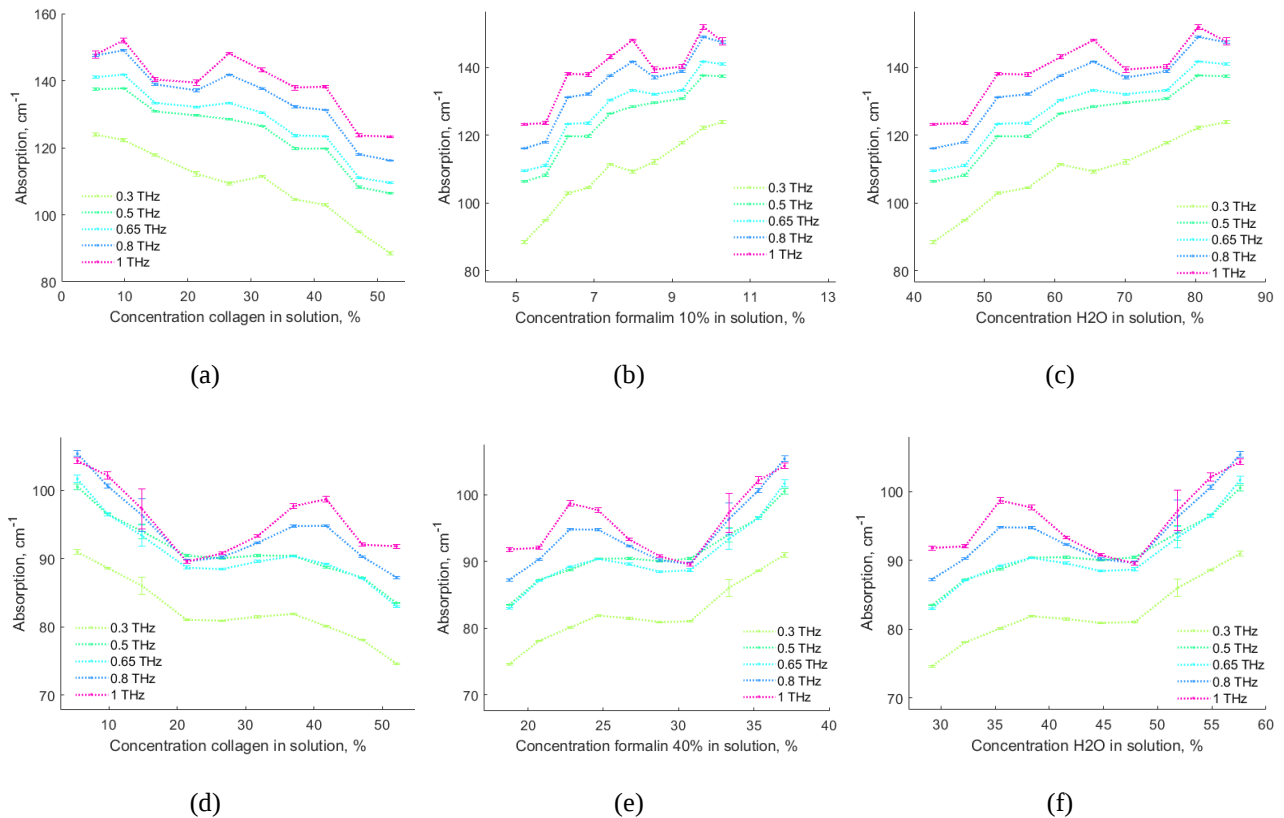


Fig. 7 Dependencies of the absorption of the studied solutions on the concentration of the components added to the solution. Upper images: the content of (a) collagen, (b) formalin and (c) water in a solution with 10% formalin; lower images: the content of (g) collagen, (d) formalin and (e) water in a solution with 40% formalin.

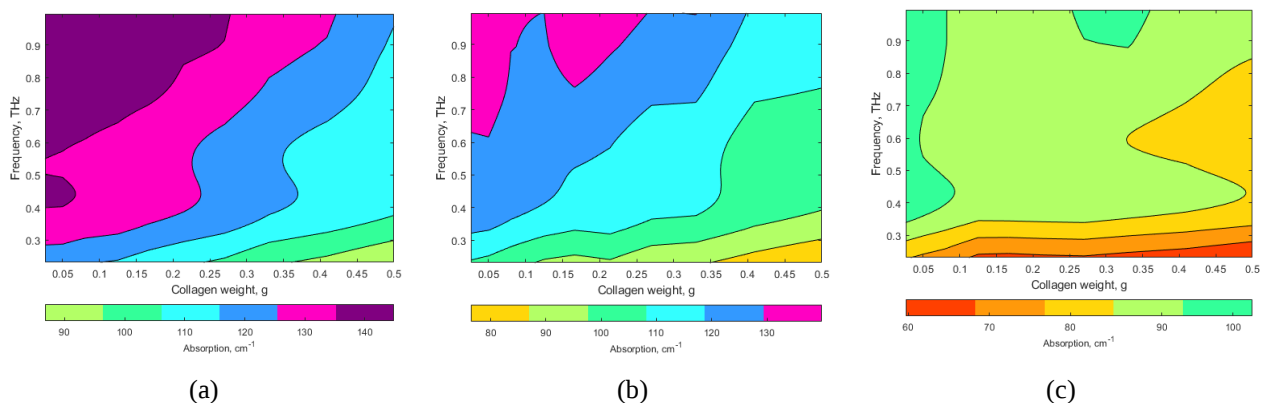


Fig. 8 Distribution of absorption coefficients depending on the mass fraction of collagen added to the solution in (a) aqueous solutions, (b) collagen solutions with 10% formaldehyde, (c) collagen solutions with 40% formaldehyde in the THz frequency range.

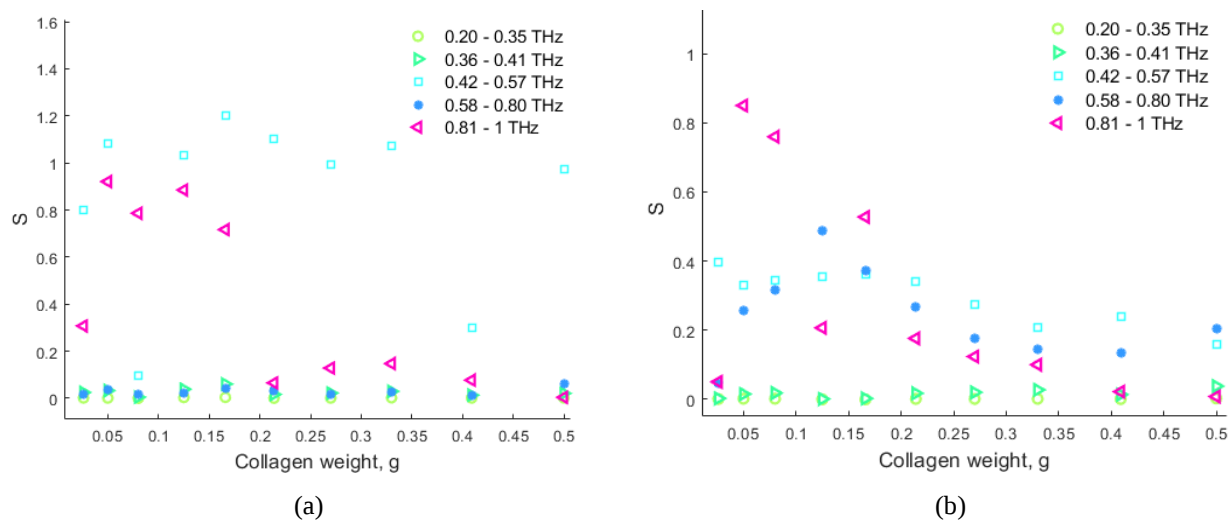


Fig. 9 CPF value (a) for collagen solutions in 10% formalin and (b) for solutions in 40% formalin.

Table 2 CPF values for the studied samples.

Frequency, THz		Collagen weight, g									
		0.026	0.05	0.08	0.125	0.166	0.214	0.27	0.33	0.409	0.5
10% formalin	0.20–0.35	0.0013	0.0013	0.0002	0.0036	0.0041	0.0007	0.0014	0.0024	0.0013	0.0018
	0.36–0.41	0.0253	0.0330	0.0051	0.0385	0.0606	0.0167	0.0223	0.0295	0.0137	0.0211
	0.42–0.57	0.8009	1.0817	0.0954	1.0334	1.2041	1.1014	0.9946	1.0742	0.3007	0.9745
	0.58–0.80	0.0190	0.0386	0.0162	0.0222	0.0426	0.0333	0.0194	0.0292	0.0116	0.0610
	0.81–1	0.3075	0.9210	0.7865	0.8853	0.7170	0.0655	0.1285	0.1476	0.0770	0.0041
40% formalin	0.20–0.35	0.0009	0.0020	0.0020	0.0002	0.0001	0.0015	0.0013	0.0016	0.0009	0.0028
	0.36–0.41	0.0030	0.0154	0.0184	0.0012	0.0028	0.0176	0.0202	0.0276	0.0140	0.0382
	0.42–0.57	0.3968	0.3288	0.3438	0.3548	0.3636	0.3401	0.2743	0.2064	0.2402	0.1589
	0.58–0.80	0.0475	0.2587	0.3163	0.4865	0.3723	0.2664	0.1759	0.1466	0.1343	0.2049
	0.81–1	0.0507	0.8500	0.7598	0.2070	0.5277	0.1766	0.1238	0.1003	0.0224	0.0083

The lower the CPF value, the closer the collagen solutions in formalin are to the collagen solutions in distilled water (Fig. 9a for solutions in 10% formalin, Fig. 9b for solutions in 40% formalin). CPF values for all groups are shown in Table 2.

As shown in Fig. 8, the CPF value in the low frequency range (0.2–0.4 THz) is small regardless of the concentration of collagen in solutions. Therefore, in this range, collagen fixation in both 10% formalin and 40% formalin, depending on the percentage of formaldehyde does not change their optical characteristics compared to solutions of collagen with distilled water. A similar situation can be observed in the frequency range from 0.8 to 1 THz for solutions with a collagen content of more than 0.2 g.

The dependence of the absorption coefficient on the amount of collagen content in the solutions was analyzed (Fig. 10). It has been shown that with frequency increasing, nonlinear changes in absorption coefficients

are observed for solutions of hydrolyzed collagen with 10% formaldehyde. At frequencies ranging from 0.8 THz (Fig. 10) and higher, it can be observed that the absorption coefficient values increase with increasing protein concentration in the range from 10% to 20%, and then decrease almost monotonically. The nonlinear behavior is associated with protein aggregation [23].

In order to evaluate the dependencies of absorption coefficients on protein concentration in solutions, absorption spectra of collagen solutions with distilled water and collagen solutions with 40% formalin were analyzed. When collagen interacts with water (Fig. 10b), linear dependence absorption coefficients on protein concentration in solutions is observed in the 0.2–0.8 THz interval, however, at a frequency of 1 THz, the absorption of aqueous solutions containing collagen from 0.026 g to 0.166 g is less than for the same at a frequency of 0.8 THz.

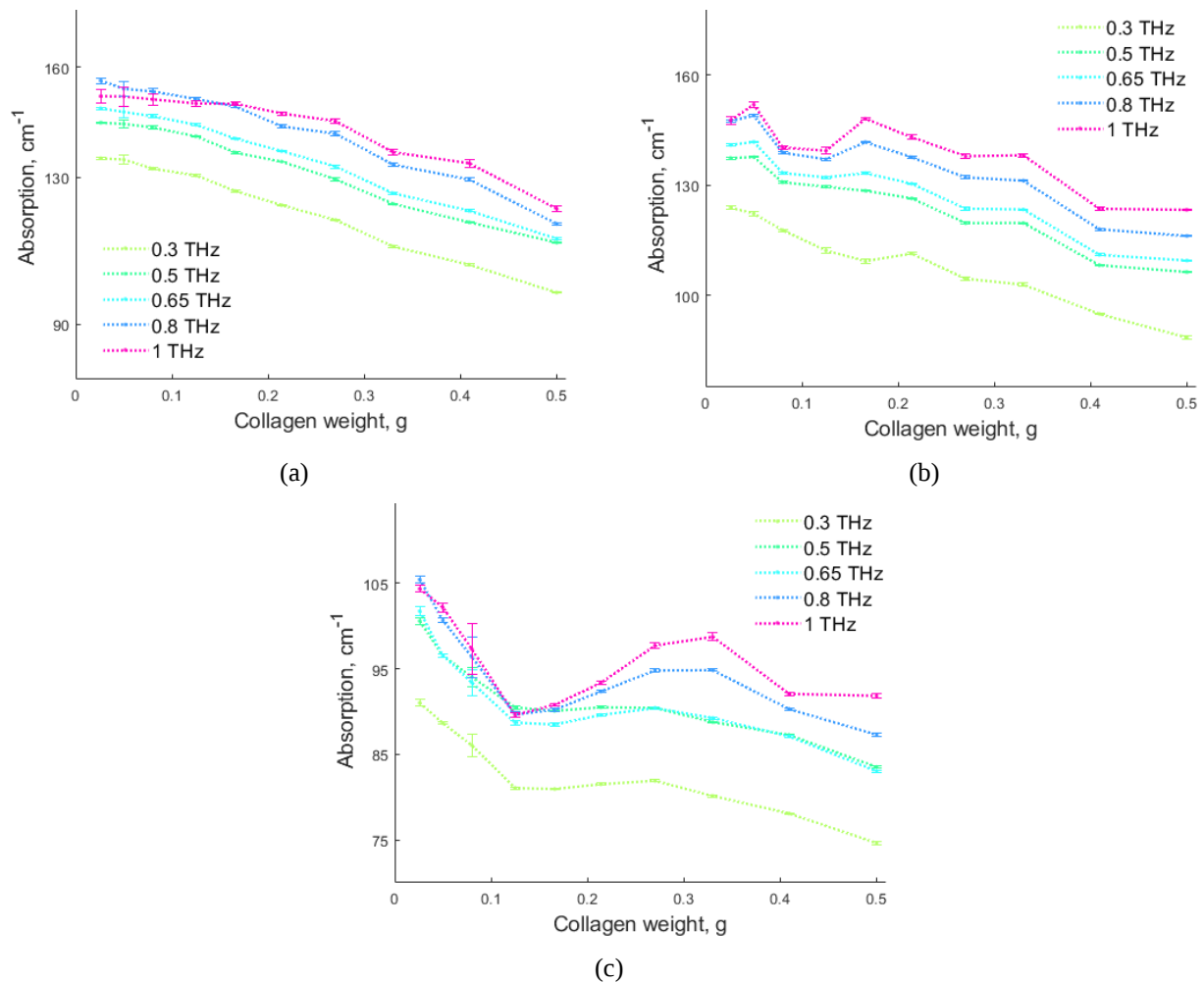
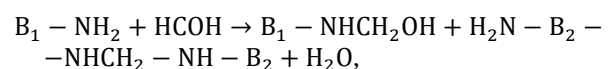


Fig. 10 Dependences of absorption coefficients on collagen content in (a) collagen aqueous solutions, (b) solutions of collagen with 10% formalin, and (c) solutions of collagen with 40% formalin.

The proportion of water in 40% formalin is significantly lower than in 10% formalin. This fact allows us to evaluate the interaction of collagen with formaldehyde in conditions of a small amount of water. As illustrated in Fig. 10c, the absorption spectra of collagen-40% formalin mixtures exhibit a nonlinear relationship at all frequencies between absorption coefficients and protein concentration. This nonlinearity is likely attributed to the decreased water content and increased formaldehyde concentration within the mixture.

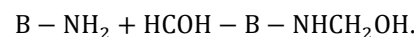
The first stage of the interaction of formaldehyde with collagen usually consists of the methylol derivatives formation. Formaldehyde, being a reactive electrophilic substance, easily reacts with various functional groups of biological macromolecules, forming cross-links. Studies indicate that the most common type of cross-link formed by formaldehyde in collagen is between the nitrogen atom at the end of the lysine side chain and the nitrogen atom of the peptide bond, and the number of such cross-links increases over time, which can reach 48 h and more then [5, 30]. The hydroxyl group formed exhibits substantial reactivity and can condense with any atomic group containing active oxygen. This reaction results in the formation of a methylene bridge between amino

acids. The interaction between formaldehyde and collagen can be represented by the following scheme:



where B_1 and B_2 are polypeptide chains of collagen.

Collagen treated with formaldehyde contains free methylol groups:



For this reason, the formation of additional cross-links in the protein structure continues after processing is completed. At the same time, further polymerization of formaldehyde on the fiber occurs. Also, when treated with formaldehyde, irreversible changes occur as a result of an increase in the number of C–N and C–C bonds.

The use of formalin in histological practice is due to the need to preserve the intravital structure of cells. However, it is essential to determine the optimal formaldehyde concentration that ensures adequate tissue stabilization without compromising the molecular integrity of the samples.

4 Conclusion

In the present study, we assessed the effect of collagen fixation with formaldehyde on optical properties in the THz frequency range. Protein solutions spectral characteristics transformation occurs due to hydrogen bonds and intermolecular interactions among the various components of the solution. The optical properties of real solutions differ significantly from the properties of a model ideal solution. In real solutions, deviations from the linear dependence of the absorption of the solution on the concentration of the solute were observed. To determine the nature of the interaction among the components in solutions of collagen with distilled water, collagen with 10% formalin, and collagen with 40% formalin, spectral characteristics (absorption coefficient and refractive index) of each individual component were measured in the frequency range from 0.2 THz to 1 THz.

Changes in the optical characteristics of collagen solutions with distilled water, collagen solutions with 10% formalin, and collagen solutions with 40% formalin in the frequency range from 0.2 THz to 1 THz were studied. It has been shown that in the frequency range from 0.2 THz to 1 THz, the refractive indices of collagen

solutions decrease with frequency increasing. It has been shown that in the frequency range from 0.2 THz to 0.35 THz the optical properties of the solution do not change.

A nonlinear dependence of the absorption coefficient of solutions of formalin with collagen on the content of the latter was established. Nonlinearity is significantly manifested in the interaction of collagen with 10% formalin in the frequency range from 0.8 THz to 1 THz and collagen with 40% formalin at frequencies from 0.2 THz to 1 THz. Nonlinear changes in the absorption of collagen solutions with formaldehyde are characterized by the appearance of another component in the solutions: water associated with collagen.

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Disclosures

The authors declare that they have no conflict of interest.

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