APPLICATION OF PULSED ACOUSTIC MICROSCOPY FOR STUDYING CHITOSAN-BASED BIOCOMPOSITE SPONGES

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Abstract. We proposed a method for local assessment of acoustic properties in spongy polymer samples, based on visualizing the microstructure, measuring attenuation, and sound velocity. This approach is relevant to the study of biopolymer composite materials and their structures, as it allows for in situ observation of structural degradation under external influences, as well as investigation of the evolution of native tissue replacement, if necessary.

Keywords: *acoustic microscopy, spongy materials, biocomposites, chitosan, Lobosphaera*

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INTRODUCTION

Environmental pollution due to the ingress of heavy metals, dye molecules, pesticides, herbicides, antibiotic residues and other pharmaceuticals into water can lead to various dangerous diseases [1]. Currently, new methods of water purification are being developed for water treatment [2], including environmentally safe porous materials based on the natural polymer chitosan with unique adsorption properties [1, 3-6]. This class of materials has attracted considerable attention due to their non-toxicity, biocompatibility and biodegradability. The most important functional property of sponge composites is their porous architecture, which increases the sorbing surface area and provides free diffusion through the micropore system. Immobilization of microorganisms, including microalgae (MA) capable of biodegradation of hazardous pollutants [7], on chitosan composites allows increasing the efficiency of wastewater treatment process. To increase the functional properties, efficiency and cost-effectiveness, a variety of natural fillers are introduced into the sorbent structure [8-10], which affects the mechanical and sorption characteristics of the final product. Since the developed composite with immobilized CF cells should maintain its integrity during prolonged incubation in aqueous medium, the study of its physicochemical and mechanical properties is of paramount importance.

The most effective nondestructive method for studying the bulk microstructure and elastic-mechanical properties of biopolymer composites is scanning impulse acoustic microscopy (SIAM). The method has been successfully applied in materials science for quite a long time to study the structure and elastic properties of inhomogeneous media [11-18]. The non-invasiveness of the method is particularly

valuable for biomedical research [18, 19], as it allows us to observe the dynamics of biobjects in vivo and analyze the influence of external influences on the evolution of tissue-engineered or composite structures [20-23]. Ultrasonic contact is performed using immersion fluid, which provides advantages in providing the necessary conditions for preserving intact biosamples.

In this work, the SIAM method was used to study porous sponge samples based on chitosan cross-linked with glutaric aldehyde, with functional filler from mycelium and CF cells immobilized on them. In addition to acoustic imaging and observation of the microstructure of the porous sponge material, sound velocities and attenuation in the samples intact and directly under load were investigated. This approach allows us to evaluate the dynamics of evolution of composite samples with CF cells immobilized on them, depending on the concentration of functional additives in the composite.

METHODS

High-resolution acoustic imaging systems use focused ultrasound pulses. Volumetric visualization is performed by short pulses of 1-2 periods at the fundamental frequency of 50-200 MHz [13]. Focusing of the ultrasound probing beam is determined by its angular aperture [25]. For volumetric ultrasound imaging, lenses with a narrow aperture are used, which provides an optimal ratio of the length and diameter of the focal constriction, minimizes aberrations, focus deformation inside the sample, and avoids excitation of surface elastic waves. In this work, a scanning acoustic microscope developed at IBCHF RAS was used [13]. The nominal frequency of the focusing transducer corresponded to 50 MHz. The echo signal reflected from the substrate-

object boundary was digitized by an analog-to-digital converter with a sampling frequency of 500 MHz. The voltage at the input of the acoustic transducer corresponded to 20 V. The maximum amplitude of the recorded echo-signal was determined by its envelope. In watered samples, the microstructure was visualized by an acoustic lens with an aperture angle of 30 .°

Quantification of sound velocity and attenuation, as well as measurement of sample thickness, was performed with an acoustic lens with an aperture angle of 8° . For the 50 MHz lens, the diameter of the focal region was 0.13 mm and its length was 6 mm. In this case, the wavefront inside the focal region appears to be close to flat. In an isotropic sample, longitudinal elastic waves propagate along the direction of the axis of the probing beam. The caustics of the ultrasonic beam are transformed weakly during the transition from the contact medium (water) to the sample (watered chitosan sponge), due to the insignificant difference in the acoustic impedances of the contacting media.

Fig. 1 shows an acoustic cell for sound velocity and attenuation measurements, which also allows the measurement of acoustic parameters for loaded samples. The loading and the flat parallelism of the boundaries are ensured by using a thin plate made of polystyrene, which is as transparent as possible to ultrasound: its acoustic impedance is close to immersion (water). Intact samples are measured without the loading plate.

The received acoustic signals(t) from the lower boundary of the acoustic cell between the sample and the substrate is described by the expression:

$$s(t) = A \cdot R \cdot \exp(-i\omega)\left(t - \frac{2d}{c}\right) \cdot \exp\left(-2d \cdot \alpha\right) \qquad (1)$$

where A is the transmitting characteristic of the measuring system;d is the distance inside the cell; c is the speed of sound,α is the attenuation coefficient in the biocomposite sample. The amplitude coefficient R is determined by the efficiency of reflection and passage of the probing ultrasonic pulse in the layers of the experimental cell and depends on the ratio of acoustic impedances of the contacting media [26,27]. From expression (1) we can see that the sound velocity in the sample is determined by the passage time of the echo pulse of longitudinal waves in the sample$\Delta t = \frac{2d}{c}$ (Fig. 1). The accuracy of delay measurement by the echo-pulse method depends on the choice of reference points within the measured signals. It should be taken into account that the shape of echo pulses can be deformed when the interface is defocused [28, 29], and also as a result of the dependence of the attenuation coefficient on the frequency of the used sound. In our case, the half sampling frequency (250 MHz) is several times higher than the maximum frequency in the signal spectrum, which allows us to select more accurately the reference points within the echo pulses to increase the signal resolution.

The attenuation coefficient of ultrasound in a composite sample is determined by the ratio of the amplitudes of the signals recorded from the bottom of the acoustic cell with and without the sample: $\alpha = \frac{1}{2d} \cdot 20 \cdot \lg \frac{s}{s_0}$ [26] (Fig. 1a). In this case, the focus region is positioned at the lower boundary of the sample with the substrate, the length of the focus exceeds the thickness of the sample, and its structural inhomogeneities are small relative to its diameter.

MATERIALS

The composite was made from chitosan (M_w= 500kDa, Bioprogress, Russia) in an aqueous solution of acetic acid (Component-Reactiv LLC, Russia) and crosslinking with glutaric aldehyde (50% aqueous solution, Hubei Jinghong Chemical Co. Ltd., China). Mushroom mycelium of oyster mushrooms was used as filler. Immersion bidistilled water was obtained on a Millipore Milli-Q Synthesis apparatus (Merck, Germany).

The initial sponge samples were prepared from a 2% (wt.) solution of chitosan in a 2% (wt.) aqueous solution of acetic acid (AA). For this purpose, 1.96 g of UC and 2 g of chitosan were added to 96 g of bidistilled water and stirred on a Heidolph MR Hei-Tec magnetic stirrer for 48 h at room temperature.

Aqueous acid suspension of mycelium was prepared by adding 2 g of dry powder to 98 ml of 2% (wt.) acetic acid solution. To obtain sorbents, a solution of chitosan and ground filler biomass was combined in the proportions of 1:1, 1:3, 3:1 and stirred for 1 h. The mixture was placed in the cells of a 24-well plate, frozen in a freezer for 24 h at -24 ℃, and freeze-dried on a Martin Christ Alpha 2-4LSC (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for 48 h at a vacuum depth of 0.25 Mbar, followed by 2 h at a vacuum depth of 0.010 Mbar (condenser temperature -75 ℃). Mycelium dispersion in chitosan solution had low suspension stability, which led to sedimentation of the dispersed phase in the process of suspension freezing and formation of sorbents with a gradient of filler concentration from the lower surface of the sponge to the upper one. Materials without filler were obtained as control samples according to the described methodology.

The algal monoculture of the microalgae (MA) *Lobosphaera* IPPAS C-2047 (hereinafter in the text *Lobosphaera*) with associated heterotrophic bacteria was used. *Lobosphaera* cells were immobilized on sorbents and cultured as described previously [30] for 30 days.

Mechanical tests of swollen sorbents were carried out on an Instron 5965 universal tensile machine (Instron, USA) under uniaxial compression at a constant strain rate of 50% per minute at room temperature. Pressure was applied using a 25 mm diameter cylindrical plate. The measurements were carried out in air on swollen samples.

RESULTS AND DISCUSSION

To analyze the filler concentration gradient, longitudinal slices of 1 mm thick sorbents including all layers were made. A higher concentration of immobilized *Lobosphaera* cells and associated bacteria was observed in the upper part of the samples, on the opposite side of the mycelium cluster. Three heterogeneous regions were identified on the slice: the upper part with MB, the middle homogeneous (most of the sample volume), and the lower part with mycelium.

When measuring the mechanical properties of the composite, uniaxial compression was used, the sample was compressed as a whole, it was impossible to take into account the difference in the mechanical properties of individual zones. High-resolution acoustic microscopy allows us to successfully differentiate these areas and determine their elastic properties by a non-contact method, as well as visualize their microstructure (Fig. 2).

Table 1 shows the quantitative data for the sample of 50% mycelium + 50% chitosan. The sound velocity and attenuation were found to vary in the range of 1496 - 1499 m-$s^{-1}$and 0.85 - 2.3 dB-mm^{-1}, respectively. At the same time, the central region (zone 2) occupies a larger volume, is the most homogeneous and determines the mechanical properties of the composite as a whole. This area was used for further ultrasonic studies.

Quantitative estimates of elastic-mechanical characteristics were obtained for composites with different proportions of filler in the matrix: 25, 50, 75 wt% of micelle. Fig. 3 shows the echo impulses of longitudinal waves reflected from the lower boundary "sample-substrate". For comparison, the pulse reflected from the substrate in immersion (water) without sample is shown. It is well seen that the pulse delay time increases, and the sound velocity decreases accordingly, with increasing amount of filler (Fig. 3a). It is found that upon swelling, the structure of the composite containing 75% filler approaches hydrogel in terms of acoustic properties. The increase in the echo amplitude with increasing filler concentration also indicates that the sponge walls become acoustically more transparent. Presumably, thin fibers of mycelium integrate into the sponge matrix, increase its hydrophilicity and contribute to additional swelling. The obtained values of sound velocity and attenuation in samples of different concentrations are presented in Table 2.

Analysis of the spectrum of the obtained echo impulses shows that the width of the spectrum correlates with the amount of filler in chitosan: with increasing concentration the spectrum becomes wider and shifts towards higher frequencies (Table 2).

Compression tests of sponge specimens performed by the classical method using a universal tensile testing machine showed that the presence of mycelium additive significantly affects the mechanical characteristics of the composite (Fig. 4). The measured Young's modulus for the sample with 50% filler content was 1.6 ± 0.2 kPa compared to 9.3 ± 3.0 kPa for pure chitosan. Since sorbents of this type will be used for immobilization of MB and subsequent purification of water bodies, it seems reasonable to investigate the changes in mechanical properties of the samples during prolonged incubation (30 days). It was obtained that after incubation the elastic modulus decreased 4 times (0.4 ± 0.1 kPa). Figure 4b shows the mechanical stress values for 45% strain of the composites. The results indicate that the composite after biodegradation in the presence of immobilized MBs at the same strain requires less stress (0.17± 0.02 kPa) compared to pure chitosan (1.30 ± 0.1 kPa) and intact composite (0.27 ± 0.06 kPa).

Under load, sound velocity and attenuation values were also measured in samples with different mycelium concentrations after biodegradation. The sound velocity and attenuation values were found to increase with increasing applied load. Unlike more brittle samples with low filler content (25 and 50%) for the composite with high content (75%) of mycelium, attenuation almost does not change with increasing load, which may be due to the high water content of its structure.

CONCLUSION

Thus, we present acoustic images of the structure of highly porous chitosan-based composite samples with different content of mushroom mycelium filler, and

describe the methodology of sound velocity and attenuation measurements for such samples using SIAM method, including under load in immersion.

Mechanical tests allowed to establish the influence of filler concentration on the elastic-mechanical characteristics of chitosan sponge matrix. A significant decrease in the elastic modulus of the composite in the presence of filler is probably due to the different degree of swelling of chitosan and mycelium. This leads to the appearance of additional stresses on the pore walls, resulting in their accelerated loss of stability and irreversible deformation of the sample at lower loads.

Acoustic microscopy made it possible to determine the heterogeneity of sponge composites with filler, to identify the homogeneous zone and to measure the acoustic properties of individual fractions in the composite volume. It also revealed a decrease in the mechanical properties of the sorbent as a result of biodegradation during long-term incubation (30 days) in the presence of MB and associated heterotrophic bacteria. The results showed high sensitivity of microacoustic measurements to changes in elastic properties of under the influence of external factors such as: the presence of bioactive component, hydrolysis and biodegradation, pressure, mechanical loads. The obtained data and the proposed methods in the future will serve as a basis for assessing the type and amount of adsorbed pollutants, which is relevant for research in the field of green technologies.

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FIGURE CAPTIONS

Fig. 1. Schematic and acoustic image of the experimental acoustic cell: 1 and 4 - polystyrene plate, 2 - sample, 3 - sample medium, 5 - transmitter, P - pressure on the sample.

Fig. 2. Images of the structure of the biocomposite based on chitosan sponge with mycelium (50%+50%) and algae: photo of the surface (a), acoustic image of the surface (b), distribution of the signal amplitude passed through a 1 mm thick sample (c). 1 - zone of maximum algae concentration, 2 - homogeneous central part of the sponge, 3 - inhomogeneous zone with macroinclusions of mycelium.

Fig. 3. Acoustic measurements of chitosan sponge with different amounts of filler (mycelium) - 25, 50 and 75%: echo impulses from the lower boundary "sample-substrate" (a), corresponding spectra of the received signals (b).

Fig. 4. Mechanical characteristics of the composite with 50% filler content: Young's modulus in uniaxial compression (a), stress at 45% strain (b).

Table 1. Local acoustic parameters of chitosan-based composite with mycelium (50%+50%) and algae filler.

Observation area	Speed of sound, m/s	Attenuation, dB/mm
1. algae and bacteria	1498 ±0.6	1.50 ±0.13
2. homogeneous sponge with mycelium and algae	1496 ±0.3	0.85 ±0.08
3. large mycelial inclusions	1499 ±0.6	2.30 ±0.2

Table 2. Parameters of the received acoustic signal for chitosan-based composite with mycelium and algae filler.

Amount of filler	Speed, m/s	Attenuation, dB/mm	Spectrum maximum, MHz	BandwidthΔ f at -3 dB level, MHz
25%	1498 ±0.6	0.94 ±0.06	18.5 ±0.1	16.1 ±0.1
50%	1496 ±1.0	0.85 ±0.06	19.5 ±0.1	16.4 ±0.1
75%	1494 ±0.6	0.60 ±0.06	21.5 ±0.1	18.1 ±0.1
water	1490 ±0.5	0.01 ±0.01	22.4 ±0.1	21.8 ±0.1

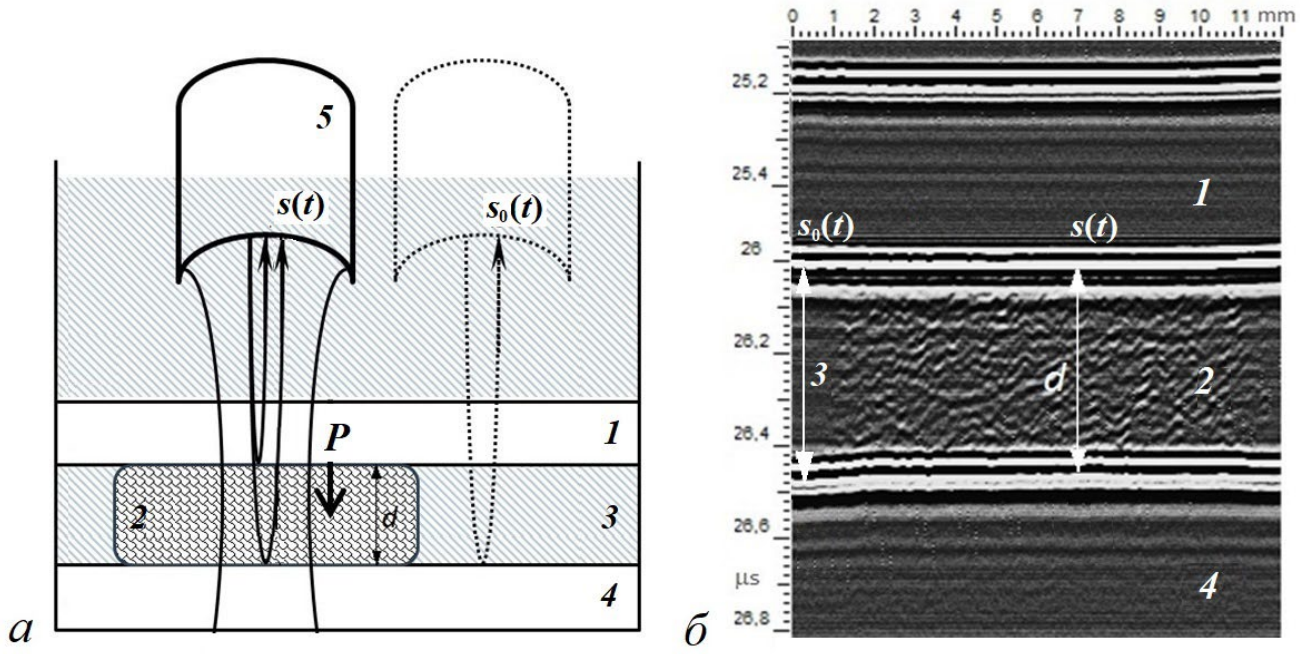

Fig. 1.

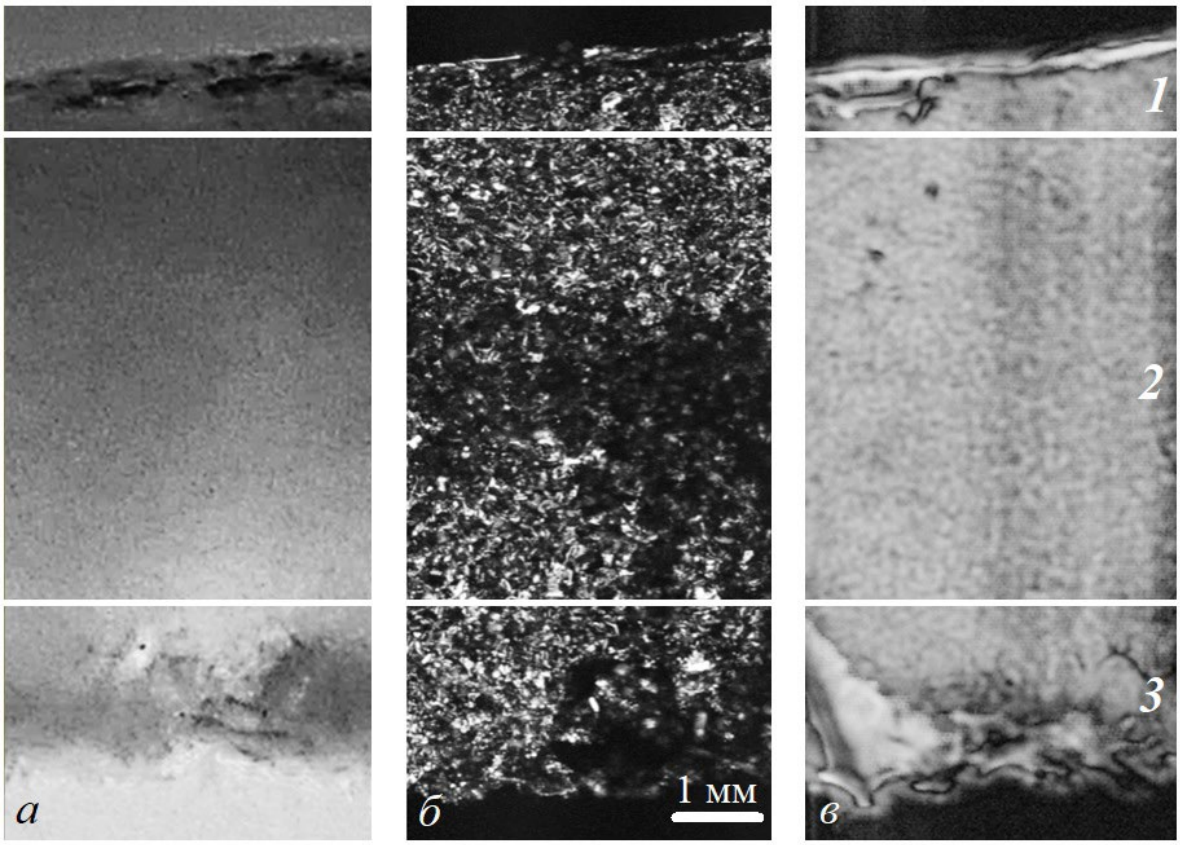

Fig. 2.

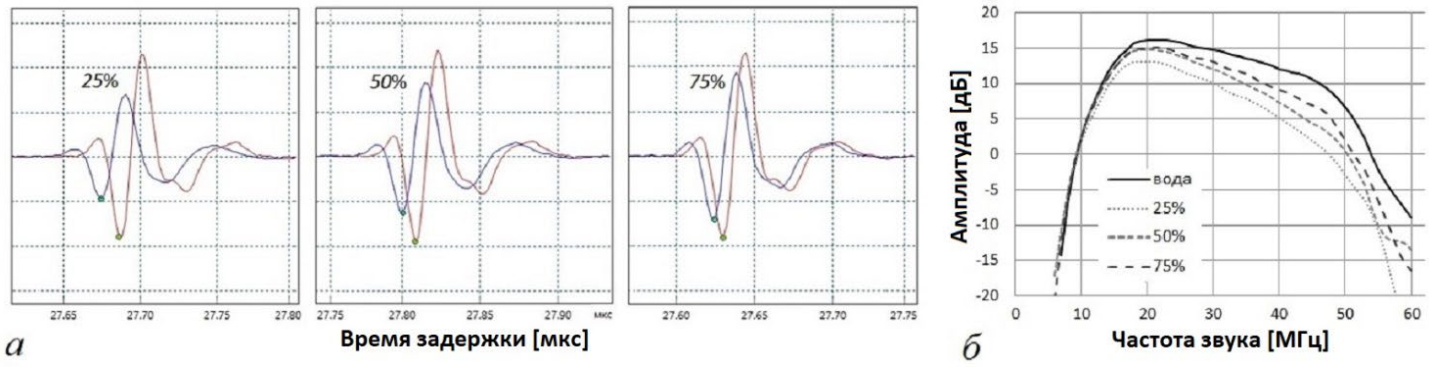

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Fig. 3.

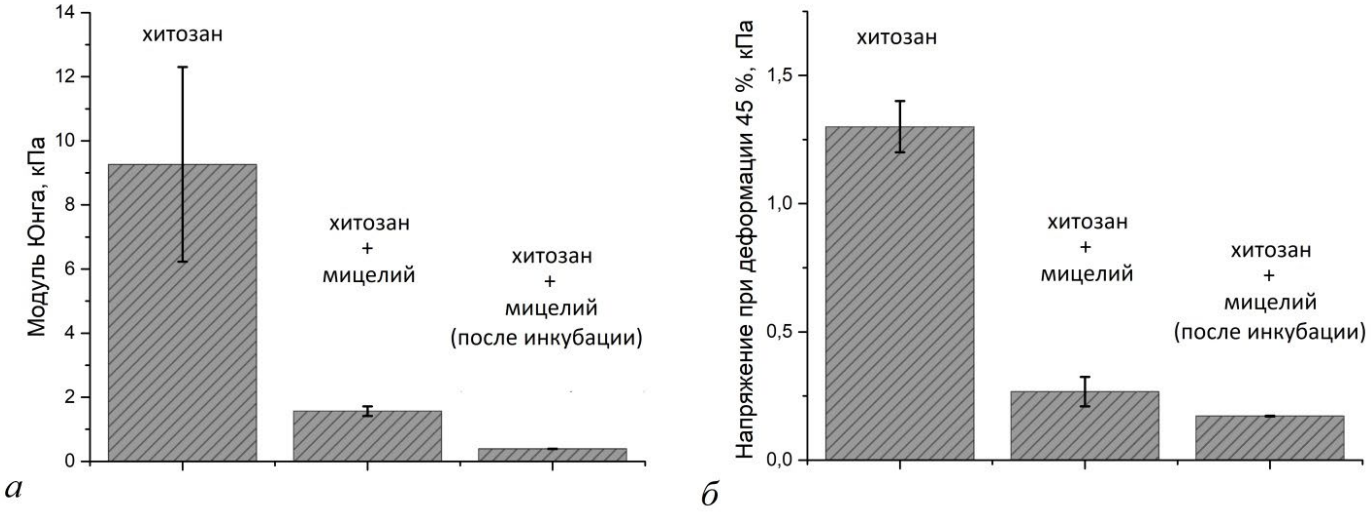

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Fig. 4.