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# Developing Techniques of Acoustic Microscopy for Monitoring Processes of Osteogenesis in Regenerative Medicine

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**Abstract**—High-resolution ultrasonic imaging techniques are necessary for the non-invasive diagnostics of artificial cell-matrix systems. The results from experimental studies show these techniques are sensitive to variations in the elastic properties of biopolymer samples and can be used effectively to detect micro and macro voids and monitor processes of biodegradation in tissue-engineered constructs (TECs).

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## INTRODUCTION

In regenerative medicine, a tissue engineering construct is created by filling a biodegradable polymer matrix with a cell component [1]. There are several stages in monitoring the state of such a product: developing a biocompatible matrix, preparing the cell culture, combining these components into a tissue-engineered product, and ensuring the conditions for tissue or organ reconstruction. Assessing the state of implants and optimizing their bioengineering properties form the basis for their use in human surgery. Electron and optical microscopy are currently used to determine the volumetric microstructure of their matrices [2]; this requires the histological preparation of samples associated with violations of their integrity. Close attention is therefore being given to developing new noninvasive intravital ways of studying artificial tissue-engineered systems using high-frequency ultrasound [3, 4]. It has been shown that ultrasonic imaging can be used for tissue engineering. Due to its low intensity of radiation and short pulse duration (several mW $cm^{-2}$), high-frequency ultrasound (50–200 MHz) penetrates quite deeply into the volume of materials used in tissue engineering without damaging them [5–7].

Many works have been devoted to using acoustic microscopy to study cells, tissues, and even whole organs of humans and animals in normal condition, with pathology, and with age-related changes [8, 9]. However, there are still few works on using high-frequency ultrasound diagnostics in regenerative medicine [10–15].

The aim of this work was to perform an acoustic microscope study of the microstructure and elastic properties of a synthetic polymer bone matrix (implant) used to replace bone defects. This was done while creating a porous structure in a polymer bone implant and observing its biodegradation in a living system.

## EXPERIMENTAL

Our study included observing the microstructure of bone matrix samples made from biopolymer material (poly-3-hydroxybutyrate, PHB) at the developmental stage and during the regeneration of critical defects in rat skulls, using samples seeded with mesenchymal stem cells (MSCs). The procedure for manufacturing porous matrices was described in detail in [16–20].

Three types of porous matrices were made for our experiment. They were based on pure PHB filled with alginate gel, PHB–hydroxyapatite (HA) composite filled with alginate gel, and PHB–HA composite filled with alginate hydrogel and pre-seeded with MSCs.

A bone defect in the shape of a cylinder 8 mm in diameter and 1.5 mm thick was modeled in the center of the parietal bone of a rat skull. Computer tomography of the rat's head, modeling, and the 3D printing of forms for manufacturing bone implants were used to give the matrix the required shape [20].

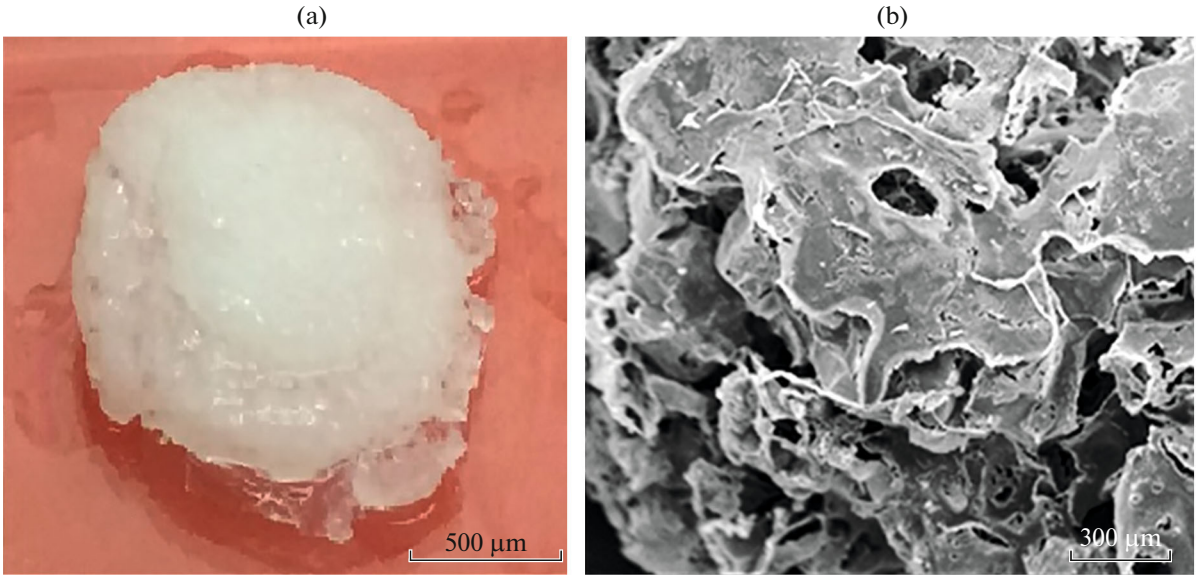


**Fig. 1.** Microstructure of a bone carcass sample made of biopolymer material (poly-3-hydroxybutyrate, PHB): (a) photo of a sample impregnated with sodium alginate; (b) scanning electron microscope image of the porous structure.

The morphology and structure of the resulting matrices were studied using a JSM-6380LA scanning electron microscope (SEM). This required sputtering the samples with gold for 15 min at a current of 15 mA.

The volumetric structure of the in vitro matrices was studied via pulsed acoustic microscopy before and after using them to regenerate bone tissue. Immersion acoustic lenses with an operating frequency of 50 and 100 MHz were used in our experiments. The pulse generator formed a short probe signal with durations of 30–40 ns and an amplitude of 30 V. The movement of the acoustic lens was controlled by precision motors with a step of 30 μm.

Acoustic images were acquired in the form of sections in the scanning plane (C-scan) or the depth sections (B-scan) traditional in medical practice. Dynamic focusing allowed us to obtain contrasting images over the depth of scanning (B/D-scans). The technique of bone tissue regeneration surgery was described in [20].

The porosity of the matrix was determined by weighing and measuring its volume. Allowing for the density of the biopolymer (1.25 g cm$^{-3}$), the porosity was calculated using the formula $P = (1 - m_1/m_2) \times 100\%$, where $m_1$ is the measured weight of the porous sample, and $m_2$ is the calculated weight of a monolithic sample without pores, the volume of which coincides with that of the porous sample. Acoustic microscopy and SEM revealed the presence of open pores on samples impregnated with alginate gel and stained with ink, respectively.

## RESULTS AND DISCUSSION

Images of PHB bone implant are shown in Fig. 1. Our study of polymer substrate samples via scanning electron microscopy (Fig. 1b) showed that the matrices had a three-dimensional porous structure with pore sizes of 23 ± 8 to 410 ± 75 μm. Micropore sizes of more than 300 μm are considered optimal for the penetration of nutrients throughout the material [21]. Staining revealed that the matrices had a communicating pore system. Matrix porosity was 93% on average.

Pulsed acoustic microscopy showed that the matrix had a highly porous microstructure (Fig. 2a). The maximum amplitude of echo signal *1* was characteristic of air cavities (Fig. 2b). The inhomogeneous distribution of acoustic contrast in the images testifies to the degree of impregnation of the porous samples with the liquid component (alginate). Echo signal *2* from the substrate in the B-scan of the sample (Fig. 2b) was due to the high degree of impregnation, making the 1.5-mm thick porous sample permeable to ultrasound ($f$ = 100 MHz).

The processes of bone tissue regeneration and implant biodegradation in a living system were studied via pulsed acoustic microscopy. Figure 3 shows acoustic images (C- and B/D-scans) of a rat skull one month after implantation. In one case, the implant in the form of a PHB-based polymer matrix was filled only with alginate hydrogel; in the other, the matrix was based on a PHB composite with HA, and MSCs were contained in the alginate hydrogel. The image in Fig. 3a clearly shows the area of the defect under the skull's surface; it has soft contours and is approximately 5 × 8 mm in size. The area of the defect could not be identified in Fig. 3b.

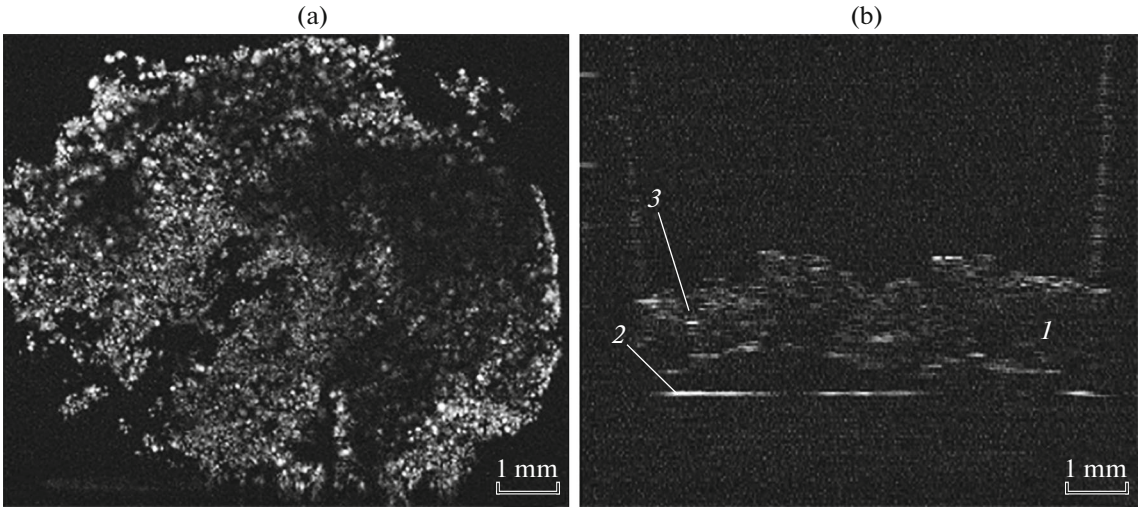


**Fig. 2.** Acoustic images of a sample of bone matrix from poly-3-hydroxybutyrate: (a) C-scan at a depth of 200–400 μm; (b) cross section of the sample (B/D-scan). (*1*) Sample; (*2*) substrate; (*3*) air bubble inside the alginate-impregnated structure.

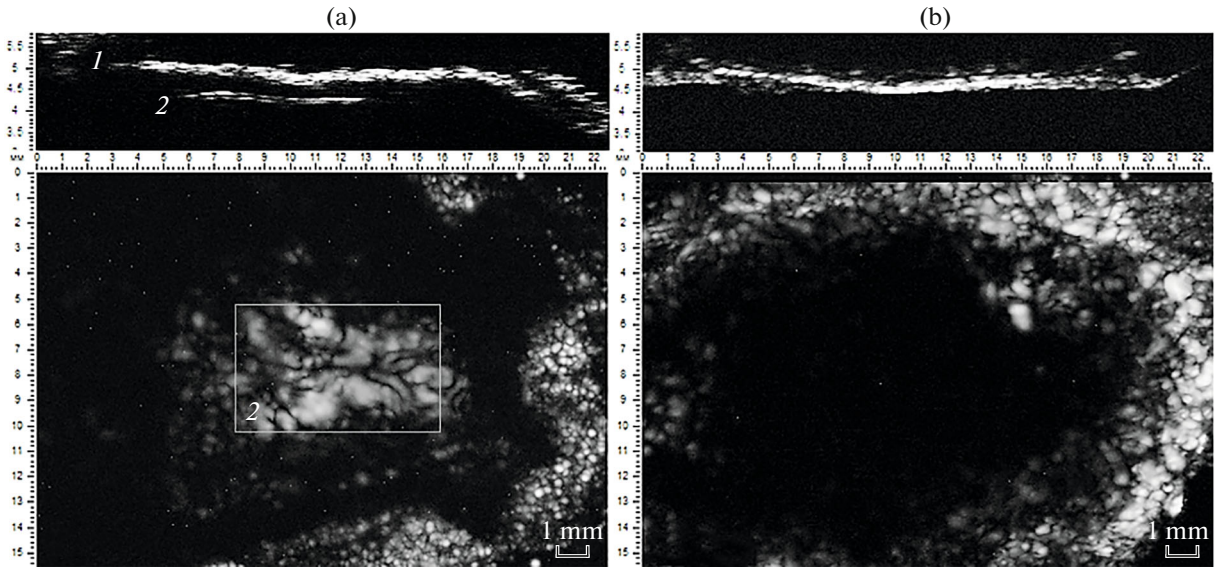


**Fig. 3.** Acoustic images (top, B/D-scans; bottom, C-scans) of implanted bone carcass samples of poly-3-hydroxybutyrate in a rat skull one month after surgery: (a) alginate-impregnated matrix; (b) matrix with alginate, hydroxyapatite particles, and mesenchymal stem cells. (*1*) surface of the skull; (*2*) area of the defect.

## CONCLUSIONS

We have presented our results from studying artificial tissue-engineered constructs via pulsed acoustic microscopy. Preliminary data on the possibility of using ultrasound imaging in regenerative medicine were obtained. Hybrid polymer 3D matrices of a given shape were studied experimentally, as were the microstructures of biocompatible poly-3-hydroxybutyrate and sodium alginate. Ultrasound imaging was used at the stage of creating a tissue-engineered construct and determining its therapeutic effectiveness on critical bone defects in laboratory rats.

It was shown that the matrix had a highly porous microstructure whose uniformity can be observed in acoustic images. A comparative analysis of acoustic images of three types of tissue-engineered constructs (PHB with alginate gel (AG), PHB/HA with AG, and PHB/HA with AG and MSCs), obtained during their implantation and the subsequent regeneration of bone tissue, showed that the cell culture contributed to a

pronounced stimulation of reparative osteogenesis, which is consistent with data from histological investigations.

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## COMPLIANCE WITH ETHICAL STANDARDS

### *Conflict of Interest*

The authors declare there was no conflict of interest.

### *Statement on the Welfare of Animals*

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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