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IMPLANTS FOR RECONSTRUCTIVE SURGERY BASED ON ELECTROSPUN POLY(3-HYDROXYBUTYRATE) FIBERS

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Nonwoven microfibrous materials based on poly(3-hydroxybutyrate) (PHB) were produced by electrospinning. The fiber supramolecular structure was investigated using DSC and EPR. It was shown that the PHB fiber consisted of perfect and less perfect crystallites with an amorphous phase that combined regions of different density. A model of an artificial implant for reconstructive surgery was developed based on a nonwoven fibrous PHB matrix with the required physicomechanical characteristics, high biocompatibility, and the optimal morphology for growth of cell cultures and increased implant regenerative capability.

Various materials and constructs based on biocompatible polymers are used in reconstructive surgery and tissue engineering to treat many injuries [1].

Electrospinning is currently widely used to produce nonwoven matrices. This method can produce fibrous materials with high surface-area-to-volume ratios that allow cells to migrate and proliferate freely in the three-dimensional matrix space, thereby giving the material a high level of iterative and reconstructive capabilities in living tissues [2].

Poly(hydroxyalkanoates) occupy a special place among biodegradable, biocompatible, and clot-resistant polymers. Poly(3-hydroxybutyrate) (PHB) is the most widely studied of them [3]. PHB is currently used to develop series of medical items for surgery (implants), dentistry, cardiac surgery, orthopedics, and other areas [4].

Implants are known to be capable of reconstructing damaged connective tissue and enabling it to fulfill its function. Synthetic polymers are especially interesting. However, synthetic polymers have different structures than human body tissues so that the risk of aseptic inflammation and rejection persists and can interfere with reconstruction of the damaged tissue. Polymers are typically deformable and can stretch and lose elasticity and resilience over time [5]. Therefore, implant materials must fulfill the functions of the damaged connective tissue and facilitate its regeneration. For example, artificial ligaments should facilitate reconstruction of the natural ligaments.

The supramolecular structure of the polymer in the fiber and the morphology of the nonwoven fibrous material affect considerably the biodegradation (bioresorption) kinetics and the absorption and diffusion parameters [6]. The goals of the present research were to study the supramolecular structure of PHM fibers and to design an artificial implant from them that was highly biocompatible, had the optimal structural features for cell culture growth, and was capable of improved regeneration for reconstructive surgery.

We used 3-hydroxybutyrate of molecular mass 460 kDa (Biomer, Germany) that was synthesized microbiologically. PHB fibers were produced by electrostatic spinning (electrospinning, ES) of a PHB solution (5%) in $CHCl_3$ [7]. Fibrous matrices were electrospun on a pilot laboratory installation at the Institute of Chemical Physics, RAS. ES was performed with solution dynamic viscosity 2-9 P; specific bulk electrical conductivity, ~10^{-3} $(\Omega \cdot m)^{-1}$,

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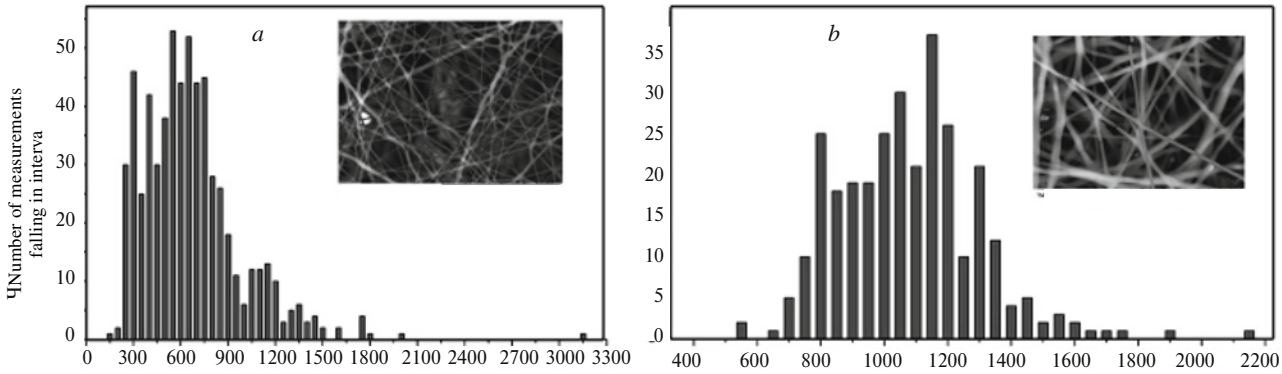

Fig. 1. Fiber diameter distribution histograms for PHB solution concentrations 5% (a) and 7% (b).

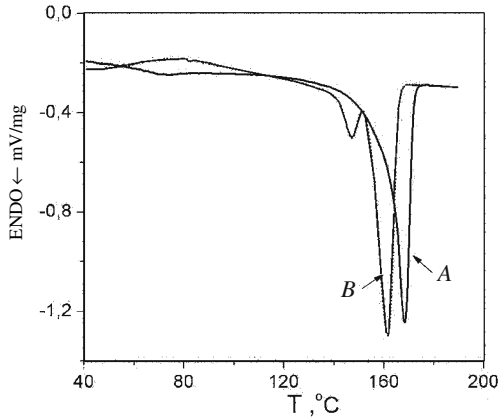

Fig. 2. Melting thermograms of PHB fibrous matrix: A) first and B) second temperature scans.

spinning-solution volume flow rate, (10-12)·10^{-5} g/s; electric field potential, 15 kV; and distance between electrodes, 18 cm. The spinning solution was prepared using chemically pure $CHCl_3$.

Biocompatibility of the polymer matrices was assessed using cell cultivation on their surfaces and mesenchymal stem cells (MSCs) of human adipocytes (Biolot, Russia). Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) with an elevated glucose content (4.5 g/L) that contained fetal bovine serum (FBS, 10%), penicillin (100 IU/mL), and streptomycin (100 μg/mL, Invitrogen, USA). Cells were incubated at 37°C in an atmosphere containing CO_2 (5%) that was exchanged three times per day. Fibroblasts were removed from the substrate using trypsin—versene solution (0.05 + 0.02% in phosphate-buffered saline) (Serva, Germany) and were counted using a Goryaev chamber. Eight samples of each matrix (5 × 5 mm) were placed into the wells of a 96-well plate. Cell suspension calculated for 5,000 cells per single matrix was placed onto each sample. The plates were incubated for 1, 3, 4, and 8 d.

Cell survival and proliferation were monitored using an assay based on the transformation of an insoluble tetrazolium salt into a soluble colored formazan salt by active cellular mitochondrial enzymes (XTT Cell Proliferation Kit, Biological Industries, Israel). Measurements were taken on a Zenyth 3100 Microplate Multimode Detector plate spectrophotometer (Anthos Labtec Instruments GmbH, Austria) at wavelength 450 nm vs. 620 nm. The number of living cells was determined from a standard calibration curve for the XTT assay [8]. Images of PHB fibers and their average diameter were obtained using optical and electron microscopy (MBI-6 optical microscope, Hitachi TM-1000 scanning electron microscope).

Bioresorbable implants for reconstructing damaged ligaments were complicated to design. This problem was solved by choosing PHB, one of the most biocompatible natural polymers. ES of ultrathin fiber was used successfully in the work to create a material for bioresorbable implants forming the original connective tissue. Nonwoven fibrous materials based on PHB were obtained by optimizing the parameters of the spinning solution and the ES process.

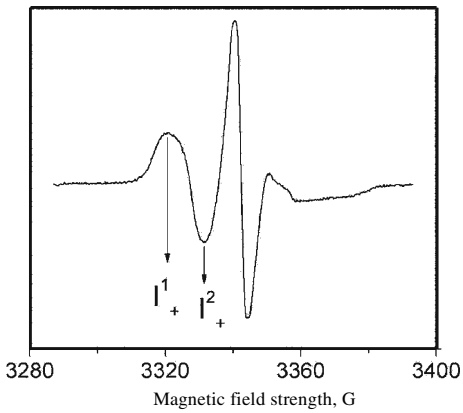

Fig. 3.

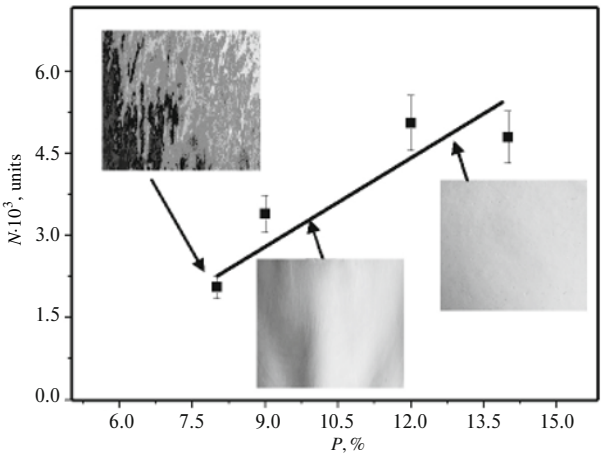

Fig. 4.

Fig. 3. EPR spectrum of TEMPO in PHB fibrous material at 25°C showing signs of superposition of two simpler spectra from radicals with different rotation correlation times in fast (I^1) and slow (I^2) modes.

Fig. 4. Number of cells (N) averaged over 8 d as a function of fiber packing density (P) in obtained matrices. Insets show optical photomicrographs of nonwoven matrices of the corresponding packing density.

SEM photomicrographs showed that a relatively small increase of the PHB concentration (from 5 to 7 mass%) in the spinning solution changed the fiber diameter distribution and the position of its maximum (Fig. 1). The requirements for fiber geometry can change depending on the application. Decreasing to the nano-sized level the diameter of biomedical fibrous materials for fabricating cell-engineering matrices decreased the adhesion of cells and reduced their growth rate [6].

A spinning solution of PHB (7%) in $CHCl_3$ was used as the basic formulation for solving our problem. This was related primarily to the good reproducibility of the physicomechanical characteristics and the geometry of fibers produced from it. Our previous research showed that fibers of diameter ~1 μm demonstrated high biocompatibility as compared with fibers of smaller diameter [8]. Therefore, subsequent investigations used fiber-matrices in which the maximum of the fiber diameter distribution was located near 1 μm.

The morphology of the bioresorbable implant affected biodegradation of the fibrous material *in vivo*. The supramolecular structure of the PHB fiber had the greatest effect on the process kinetics. Calorimetric studies using DSC were carried out to study the fiber crystalline phase. The main conclusion resulting from a quantitative evaluation of the melting thermograms of the PHB fibrous matrices was that PHB fibers contained a bimodal distribution of the crystalline phase. The material had more and less perfect crystalline regions, i.e., regions with different amounts of defects (Fig. 2). The thermogram obtained from the first temperature scan had a maximum near 169°C and a broad low-temperature shoulder. This shoulder indicated that the PHB fiber contained a less ordered (defective) or finer crystallite fraction. This hypothesis was checked by making a second scan in heating mode (thermogram B). A low-temperature peak near 150°C was clearly visible on the thermogram.

The presence of two crystallite fractions in the fiber structure could make the PHB amorphous intercrystalline phase heterogeneous, i.e., ordered (denser) and less ordered. EPR was used to study the structure of the amorphous phase.

The EPR spectrum of the TEMPO radical in a nonwoven matrix of ultrathin PHB fibers was a superposition of two individual spectra belonging to radicals with different correlation times, τ_1 and τ_2 (Fig. 3). Here, τ_1 characterized the segmental mobility in the denser amorphous regions; τ_2, in the lighter ones. The presence of two correlation times in the PHB fiber amorphous region indicated that the polymer intercrystalline regions had a heterophasic structure and agreed with the current model of a bimodal amorphous phase in partially crystallized polymers such as PHB, polylactide, and polyethyleneterephthalate [9].

Temperature dependences of the correlation time in traditional semi-logarithmic coordinates enabled the activation energy for radical rotational motion in the studied polymers to be determined. The calculations showed that the activation energy in PHB fibers was 42 kJ/mol.

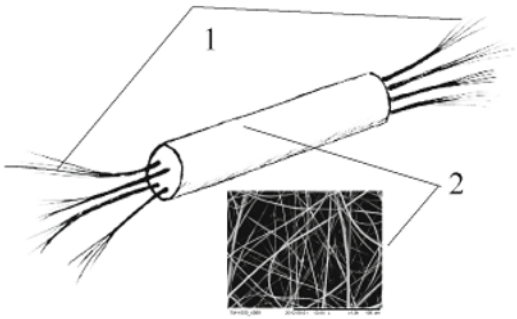

Fig. 5. Biopolymeric implant consisting of polyamide threads (1) acting as axial supports for the prosthesis with a layer of PHB fibrous nonwoven material (2) placed near them.

EPR spectra were analyzed quantitatively by mathematical processing of the intensity ratios of low-field peaks belonging to radicals with slower and faster motion, I_+^1 and I_+^2, respectively. The ratio of I_+^1 and I_+^2 in the fiber was 0.37. Thus, the effective correlation time corresponding to rotational motion of the radical in the fiber was $0.92 \cdot 10^{-9}$ s. This indicated that the radical rotated rather fast in the PHB fiber amorphous phase. The alternating regions of different ordering (density) that were observed in the PHB fiber amorphous phase should surely affect the kinetics of hydrolytic degradation of the fibers through the action of the physiologically active medium and the living cell growth dynamics.

Next, the growth kinetics of mesenchymal stem cells (MSCs) as a function of the packing density of the nonwoven material were studied. Figure 4 shows the average number of MSCs cultivated in the nonwoven PHB fibrous matrices as a function of the fiber packing density in the matrix. The results showed that the extent of cell growth in the matrices and the fiber packing density were directly proportional to a high degree of accuracy. It was found that fibers of diameter ~1 μm had structures that were most favorable for MSC growth because the number of linkages and weavings per unit volume was greater than those in fibers of smaller diameters. The rate of hydrolytic destruction of the nonwoven material in simulated physiological fluids was satisfactory.

A construct of an artificial bioresorbable implant based on nonwoven fibrous material was developed in the final stage of the present research. The implant was a scaffold of several load-bearing monofilaments coated with PHB bioresorbable fibrous material (Fig. 5). Both *in vitro* and *in vivo* tests of the fabricated implants were performed.

The biocompatibility of the implants was tested using human skin primary fibroblast culture. For this, a culture of human fibroblasts was inoculated onto material located in a culture dish containing DMEM with added (10%) FBS. The number of living cells in the materials was estimated after 1 d. Living cells were determined using simultaneous staining by Hoechst 33342 fluorescent nuclear stain (penetrating into living cells) and ethidium bromide (not penetrating into living cells). This enabled cell viability, the type of cell death (necrosis, apoptosis), and cell division to be determined from a microscopic analysis of the fluorescence from cell nuclei. The cells were alive 1 d after inoculation on the layer of ultrathin PHB fibers. The number of dead cells was <3%, like in the control culture vials.

Microscopic analysis of the chromatin distribution, which was characteristic of mitosis (metaphase, anaphase, telophase, cytokinesis), enabled cell mitotic activity in the implant to be estimated as 5.2 ± 0.7%, like in the control culture vials. The regenerative potential of the model implant modified by a layer of ultrathin PHB fibers was increased substantially according to the detachment of cells from the studied implants and their numbers as counted using a Goryaev chamber.

The effectiveness of reconstructing tendons and ligaments using our implants was studied on an experimental model of Achilles tendon damage in Wistar rats. Loss of this tendon leads to patient invalidism in clinical practice. The experimental Achilles tendon was severed from the gastrocnemius muscle to the heel protuberance. The tendon became totally defective after it was severed. The composite biopolymer implant shown in Fig. 5 was used to reconstruct the tendon in the experimental defect because of the primarily axial mechanical stresses experienced by the Achilles tendon during functional loading and its round anatomical cross section. It consisted of four braided polyamide threads (200 μm diameter) that were not bonded to each other and acted as axial support threads for the prosthesis near which the layer of PHB microfibers was situated.

The finished implant was set into the Achilles tendon defect and fixed in place by attaching the support threads to the gastrocnemius muscle and heel protuberance, maintaining the physiological tone of the former.

The implant fixed in the wound displayed a high degree of hydrophilicity and was soaked quickly and evenly by blood. The wound was stitched layer-by-layer using sutures. The Achilles tendon defect in the controls was replaced by four braided polyamide threads that were not bonded to each other. Their surfaces were coated with PHB fibrous material. The threads were attached by stitches to the heel protuberance and gastrocnemius muscle, maintaining the initial physiological tone of the former.

Animals in the test group demonstrated during the post-operative period that the substituted plastic tendon was adequate and used the repaired extremity actively and fully from the moment of waking from anesthesia.

Animals of both groups were withdrawn from the test after five weeks. During this time, the support threads in the controls lost tension because of natural gradual wear and tear on the stitches holding the threads. This led to contraction, deformation, and atrophy of the gastrocnemius muscle and was characteristic of the functional deficiency of the Achilles tendon plastic reconstruction.

In contrast to the controls, experimental test results by the fifth week showed strong connective tissue regeneration that attached the gastrocnemius muscle firmly to the heel protuberance before the support threads lost tension and a fully functional anatomical replacement of the excised Achilles tendon.

A macroscopic assessment of the obtained reconstructions showed that the structural and anatomical correspondence of the test Achilles tendon reconstruction was adequate, in contrast with the control, where a thin and elongated reconstruction formed after five weeks and did not reach the density and shape of the tendon.

The results showed that a fully functional reconstruction of the missing Achilles tendon and the formation of anatomically adequate tissue structures to replace the resorbable part of the implanted construct appeared if a layer of nonwoven PHB microfibrous material was inserted between and around the implant support threads. The shape and structure of the fibrous material simulated the reconstruction structure and helped it correspond more fully anatomically and mechanically to the natural tendon.

Thus, the artificial implant construct developed by us for reconstructive surgery of connective tissue enabled the surface area of the conductive implant to be increased multiple times. This increased substantially the absorption of reconstruction signal molecules, enhanced migration of cells throughout the whole implant volume, and increased the reconstructive potential of the implants [8].

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