Biomedical Investigations of Biodegradable PHAs

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Summary: This work is a review of the results of biomedical studies of polymer devices (films, fibers, microparticles, 3D implants) made from resorbable PHAs synthesized by the bacterium *Wautersia* (*Ralstonia*) *eutropha* B5786, using the technology developed at the Institute of Biophysics of the Siberian Branch of the Russian Academy of Sciences. Two types of PHAs – polyhydroxybutyrate (PHB) and a hydroxybutyrate/hydroxyvalerate copolymer (PHB/PHV) – have been proven to be biocompatible *in vitro* in cultures of fibroblasts, endothelial cells, hepatocytes, and osteoblasts, and in short- and long-duration experiments on animals. Polymer films and membranes have been found to be usable as scaffolds for functioning cells and monofilament suture fibers – for stitching muscular-fascial wounds and in abdominal surgery. Ectopic bone formation assay and experiments with the model of segmental osteotomy showed that 3D PHB and PHB/HA implants can be used for reparative osteogenesis. The paper reports beneficial results of using polymers to repair bone defects in oral surgery.

Keywords: biodegradable polymers; PHA; biomedical investigations

Introduction

Production of environmentally friendly materials with useful properties is one of the key tasks of the present time. The development of materials that would contact with the internal environment of the living organism is of particular significance. Among biodegradable polymers used in medicine a special place is occupied by polyhydroxyalkanoates, PHAs – polymers of microbiological origin. Being thermoplastic, they are also highly biocompatible and are degraded in biological media to end products, carbon dioxide and water. These properties make PHAs promising materials for applications in various spheres, including medicine. The range of potential applications of PHAs in medicine is very wide, including fabrication of medical instruments and appliances and use in surgical reconstruction and transplant surgery.[1–9]

Investigations of microbiological synthesis of PHAs in Russia have various aspects of biotechnology of polymers. Researchers of the Institute of Microbiology of the Russian Academy of Sciences (RAS) headed by Academician G.A. Zavarzin isolated, investigated, and systematized a large number of new strains of chemolithotrophic hydrogen-oxidizing bacteria. They carried out a thorough investigation of their physiological features and structural-functional organization of the energy and anabolic systems, including the mechanisms and stoichiometry of P(3HB) synthesis. Academician E.N. Kondratieva of Moscow State University screened a collection of phototrophic bacteria to find producers of PHB and discovered the ability of some organisms to synthesize PHB. Researchers of the Institute of the A.N. Bakh Institute of Biochemistry RAS studied the protective function of PHB in processes of nitrogen fixation by free-living and symbiotic diazotrophic microorganisms. Among the investigated strains, the researchers selected an

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organism that could be used for commercial production of the PHB with high molecular mass, over 1 000 000 Da, *Azotobacter*. At present they are producing experimental specimens of PHB and conducting investigations of physicochemical and technological properties of the material.[10,11] Researchers of the Institute of Physiology and Biochemistry of Microorganisms RAS are comprehensively and systematically investigating methylotrophic microorganisms as an object for PHB biotechnology. Professor Y.A. Trotsenko's team has extensively investigated PHB synthesis by methylotrophic microorganisms assimilating methanol in different biochemical pathways. [12] The total yield of the polymer was about 50%, and some specimens contained 57.4% (mol) of hydroxyvalerate. The researchers of Trotsenko's team realized the laboratory technology of producing PHAs and began investigations of their properties.[13]

A large body of information about PHA synthesis by hydrogen bacteria and the properties of PHAs was gathered in the Institute of Biophysics (IBP) SB RAS.[14] Since the late 1970s, the researchers of the Institute have been investigating the physicochemical regulation of the functioning of chemolithotrophic microorganisms. Since 1990, they have been conducting comprehensive investigations of microbial synthesis of PHAs. A family of PHAs (polyhydroxybutyrate, PHB, polyhydroxybutyrate/polyhydroxyvalerate, PHB/PHV, polyhydroxybutyrate/polyhydroxyhexanoate - PHB/PHHx)[15] has been produced and it has been shown that the proportions of copolymers can be regulated by conditions of carbon nutrition. In collaboration with the L.V. Kirensky Institute of Physics SB RAS, the molecular structure and properties of the solid polymer, films and fibers, made from PHAs, were investigated, using high-resolution nucleo-magnetic resonance, electron- paramagnetic resonance and X-Ray.[14] Researchers of the Bakulev Institute of Cardio-Vascular Surgery of the Russian Ministry of Health started investigating resistance and strength of polymer films exposed to animal blood. Specialists of the Research Institute of Paper managed to produce composites of PHB and high-pressure polyethylene with various proportions of the components and to cast films from them; they showed the feasibility of using composites as paper coating. Researchers of the Department of Industrial Biotechnology at the D.I. Mendeleev Moscow Chemical-Technological Institute investigated the influence of PHB on the typical microflora of meat and dairy products. The three-month experiments did not reveal any adverse effect of the polymer films contacting microorganisms on growth and development of colonies of several dozen strains. Supported by the Russian Ministry of Education and the U.S. Civilian Research & Development Foundation (CRDF) the concept of environmentally friendly technologies by developing the new chemical-biological technology of producing PHA was realized, that would fit into the biospheric cycles. The revealed mechanisms of biosynthesis and the optimized regime of brown coals gasification were the basis for the scientific substantiation and development as well as for the first in the world practice realization of the technology of PHA production on synthesis gas.[18] In 2005, supported by the International Scientific and Technological Center (ISTC), the first Russian pilot production facility for fabricating PHAs with productivity tens of kg/year for medical applications made from them has been put into operation in IBP.[19]

Since the end of the 1990s, together with the Institute of Transplantology and artificial organs (ITAO) of the Russian Ministry of Health, specialists of IBP have been conducting integrated biomedical investigations of polyhydroxyalkanoates and experimental samples of PHA-items for medical applications.[20] Until the present time, in cooperation with such medical research bodies as the State Center for Investigating New Biomaterials at the ITAO, the Hematology Research Center of the Russian Academy of Medical Sciences, the Krasnoyarsk Dentistry Research Center,

the Krasnoyarsk State Medical Academy and others, we have carried out preclinical studies of the PHAs and have proven high biocompatibility of polymer membranes, films, sutures, and 3D constructs.

The purpose of this work is to present a review of the results of the biomedical studies of PHAs performed at the Institute of Biophysics SB RAS.

Material and Methods

PHA Samples

The material used in this study was PHAs synthesized by the bacterium *Wautersia (Ralstonia) eutropha* B5786. The material was prepared at the IBP using the technology that can yield polymers of various chemical structures: polyhydroxybutyrate (PHB), copolymers of hydroxybutyrate with macroinclusions of hydroxyvalerate (HV), hydroxyhexanoate (HHx), and microinclusions of hydroxyheptanoate (HHp) and hydroxyoctanoate (HO).[14–15] The PHA were extracted from bacterial biomass with chloroform (or dichloromethane) and precipitated with ethanol (or hexane). To detect biologically active components in the PHAs, a detailed analysis of the composition of the polymers was conducted. The chemical purity of the resulting specimens was estimated by conventional biochemical methods.[21] The presence of protein impurities was determined by the Kjeldal micro-method and carbohydrates by the anthranone method. Chromo-mass-spectrometry revealed long-chain fatty acids (FAs) in the tested PHAs. Their total concentration in the polymer ranged from tenths of mol% to 2–3 mol%, depending on the purification method. Of the long-chain hydroxy acids, β-OH-$C_{14:0}$ was detected, and it did not exceed 0.06 mol%. $C_{16:0}$ constituted was the largest proportion, up to 70%. It has been found out that the lipopolysaccharides of bacteria producing PHAs, which contain mostly long-chain hydroxy acids, can be the factor activating the hemostasis systems.[21] Thus, the technology of PHA purification must satisfy rather stringent specific requirements. The analysis of the hemocompatible properties of the PHAs purified by a specialized procedure, including the quantitative and morphological estimation of platelets adherent to the surface of polymer films, the plasma recalcification time, and the complement activation, suggested a conclusion that PHB and PHBV can be used in contact with blood. To obtain polymer samples for biomedical applications the extraction of PHA from biomass was conducted in several stages. All the organic solvents used in the procedure were initially distilled to remove impurities (reagents of Sigma, USA). In the first stage, to partially destroy the cell wall and attain a fuller extraction of lipids, the bacterial biomass was centrifuged (15 min, 6000 rpm), collected, and covered with ethanol, pH 10.5–11.0 (0.5–0.7 g KOH/L ethanol). The sample was boiled using backflow condenser for 30 min. Then the alcohol was removed, the biomass was covered with 86% ethanol and separated from alcohol by centrifuging. In the next stage the partly destroyed and defatted biomass was covered with chloroform and boiled for 30–40 min using a water bath with a backflow condenser. The sample was cooled and placed into a funnel to separate the chloroform extract of the polymer from the biomass. After separation of the phases, the polymer was precipitated by adding ethanol. The procedure of re-dissolution and further precipitation of polymers was repeated several times to prepare specimens that would not contain organic impurities of protein, carbohydrate or lipid nature, including long-chain fatty acids.

Dry biomass samples were subjected to methanolysis and the total polymer content of the biomass and monomer compositions were determined by the chromatography of methyl esters of fatty acids on a GCD-Plus gas chromatograph-mass spectrometer (''Hewlett Packard'', USA). The chemical structure of the extracted and precipitated polymer was analyzed in a similar manner, using the chromatograph-mass spectrometer. Monomers were identified by their retention time and mass spectra.

The molecular mass of the polymers were measured in an Ubbellohde viscometer, with capillary of diameter 0.34 mm, at 30 °C. Polymer solutions in chloroform were used, with the concentration of polymer ranging between 0.25 and 1.0 $g \cdot L^{-1}$. Temperature characteristics of the polymers were measured with a derivatograph MOM (Hungary), which simultaneously registered curves of differential thermal analysis (DTA) and performed thermogravimetry (TG) and derivative thermogravimetry (DTG). 0.1-mm thick PHA samples of various compositions were placed into platinum crucibles. The polymers were analyzed in the inert gas medium; the heating was conducted at a rate of 5 °C/min, from 20 to 300 °C. Melting points and temperatures for the onset of decomposition were determined as temperatures of heat absorption peaks of the corresponding endothermic effects. The error of derivatogram-based determination of endothermic effect temperatures was ±1 °C. The X-ray structure analysis was conducted using a graphite monochromator on a reflected beam D8 ADANCE X-ray spectrometer Bruker (Germany). To determine the degree of crystallinity (C_x), spectra were taken in a scan-step mode, with step 0.04 ^{0}C, exposure time 2 min, to measure intensity at point. The operating mode of the instrument was 40 kV × 40 μA.

Processing of PHAs into Biomedical Objects

The high-purity PHA samples (not containing organic impurities of protein, carbohydrate or lipid nature, and long-chain hydroxy acids, which can be the factor activating the hemostasis systems[21]), were used to prepare films, membranes, fibers, microparticles, and 3-dimensional solid and porous constructs.

Films were prepared by casting of PHB and PHB/PHV solutions in chloroform (5–10% w/v) on degreased glass and subsequent drying at room temperature for 2–3 days in a dust-free box. Porous films (membranes) with pore sizes from 50 to 200 μm were prepared using the leaching technique.[22] For this the polymer solution was mixed with crystals of sodium chloride in a weight ratio of 1:2 to yield a homogeneous mixture. The salt particle sizes in this instance were 50–200 μm. The PHB/salt or PHB/PHV/salt mixture was poured onto degreased glass. The dried films were placed into water and left to stay for 48 h to remove salt. Also, to perforate solid films CO_2-laser LCD-50W, ''Plazma'' (Russia), was used, with power from 3.0 to 30.0 W (unpublished data). Film thickness was measured with an MKO-25 micrometer (Russia). Segments of equal thickness were selected and disks of diameter 15 mm were cut out to be further used in the determination of properties. The following characteristics of film surfaces were calculated by measuring contact angles for water in air (θ, degrees): free surface energy (γ_S) (erg/cm^2), free interface energy (γ_{SL}) (erg/cm^2), and cohesive force (W_{SL}) (erg/cm^2).

Monofilament PHB and PHB/PHV fibers were produced using melt spinning with the help of single-screw extruder with round nozzle, diameter 1 mm, made by Brabender Co. (Germany). Optimal temperature modes for PHB and PHB/PHV copolymers spinning were 165–170 °C and 145–150 °C. To reach the maximal extension range, quality and mechanical properties of fibers, the optimal temperature was set between 80 and 100 °C.[23] The physicomechanical characteristics of the films and of the fibers were studied with the help of Instron 1122 (UK) testing device at room temperature; the initial length of samples were from 10 to 50 mm; rate of extension −100 mm/min.

Ultrafine fibers of diameter 2–3 μm were formed from PHB and PHB/PHV solutions with the method of electrostatic spinning.[24]

PHB-based microspheres were prepared by the solvent evaporation technique, using a triple emulsion (solution of PHB and polyethylene glycol - PEG40, with molecular mass 40 kDa-in dichloromethane with addition of gelatin solution).[25] The emulsion was continuously mixed mechanically, until the solvent was completely evaporated. Microspheres were collected by

centrifuging at 10 000 rpm, for 5 min, rinsed 7–8 times in distilled water, and freeze dried in an LS-500 lyophilic dryer (Russia). The size (number and volume weight mean diameter) and the size distribution of microspheres were determined with an optical particle sizer particle counter CASY TTC Scharle system GmbH (Germany).

Three dimensional polymer matrices from PHB were prepared by direct cold molding of the ground polymer under the pressure 127.2 kgf/cm^2.[26] The hybrid composite was prepared from a mixture of PHB and biological hydroxyapatite (HA), Polistom (Russia), which was subjected to mechanical-physical treatment. The flocculent-fibrous polymer extracted from the bacterial biomass was crushed. The grain-sizes of the resulting particles were: 0.50 mm –70.6%, 0.32 mm –23.4%, 0.20 mm –3.8%, and 0.14 mm –2.2%. The granulated polymer was mixed with HA and ground in an agate mortar in liquid nitrogen to uniform powder. Then, by cold molding 50 and 100 mg solid compact cylinders were made. To prepare porous constructions with the pore size 100–250 μm, salt leaching technique was used.

Microstructure of devices produced was investigated with the help of scanning electron microscopy, JEM-100C (Japan).

Biomedical Studies

Investigations of PHAs, producted in IBP, and PHA-devices for medical applications were conducted *in vitro* and *in vivo* in accordance with rules of estimation of biological safety of medical materials and devices, established now in Russian Federation, USA and EU.[27,28]

Investigation of Cytotoxity of PHAs *in Vitro*

Cytotoxicity of PHB and PHB/PHV films, containing from 4 to 30% (mol) of polyhydroxyvalerate was investigated *in vitro*.[22] The objects used were cultured cells: mouse fibroblast cell line NIH 3T3, which belongs to the least transformed cell lines and retains many features of normal diploid cells, and primary cultures of parenchymal cells (hepatocytes) and non-parenchymal (mostly endothelial) cells of the mouse liver. Hepatocytes were extracted by the two-stage (non-enzymatic and enzymatic) non-recirculation perfusion of the liver of anesthetized mice. Fibroblasts of line NIH 3T3 were cultured in DMEM supplemented with 10% FCS (RPA ''Vector'', Novosibirsk), 1.0 mM L-glutamine, 10 mM HEPES, and 100 μg/ml kanamycin sulfate (BDSL, UK). Hepatocytes were re-suspended in the medium that contained 5% of inactivated at 56 °C FCS, 0.1% glucose, 0.2% serum albumin for cell cultures, 0.5 μg/ml of insulin, 50 ng/ml of dexamethasone, 0.2 μg/mL of glucagons, 0.02 mM β-mercaptoethanol, 10 mM HEPES, and 100 μg/mL of kanamycin sulfate (Sigma, USA). Endothelial cells were re-suspended in Ham's F12 medium (BDSL, UK), containing 10% of FCS. The cell count increased after the cells were passaged two or three times using a 0.2% collagenase solution diluted with a 0.02% versene solution. All stages were conducted under sterile conditions. Cells were plated into vials (5 vials per test site) containing either PHA films or glass supports (control). Cells were cultured in a humidified atmosphere at 5% CO_2 and 37 °C. The duration of experiments ranged from 4 h to 3 days. To investigate the adhesion of cells on films and their morphology, samples were taken every hour; to determine protein concentration in the culture; cells were grown for 3 days and sampling was made once a day. To detect possible toxicity of PHB and PHB/PHV with 15 and 28 mol% HV, the authors investigated DNA synthesis in cell cultures grown on polymer films and compared it with control. The concentrations of both fibroblasts and endothelial cells were 30000 cells/mL, and that of hepatocytes was 50000 cells/mL. A radioisotope method was used. The culture medium was supplemented with ^{3}H-thymidine as a ^{3}H-labeled substrate. The reagent dose was 37 kBq/mL (92.5 GBq/mM). Cells were cultivated with ^{3}H-thymidine for 16 hours and sampled every 2 hours for radioactivity count using Beta-1 counter.[22]

Biocompatibility of 3D PHB/HA composites was investigated in the primary culture of osteoblasts. Stromal osteoblastic cells were obtained from the marrow of Wistar rats, cells of bone marrow were differentiated into osteoblasts using specific addings. Cell proliferation was measured at 10 days using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test (MTT). The number of living cells in the culture on the PHB/HA matrices were determined from the MTT absorbance standard curve. Alkaline phosphatase (ALP) activity was determined after the osteoblasts were cultured for 10 days with a Diagnostic kit 245 (Sigma, USA). The *p*-nitrophenol, which was produced in the presence of alkaline phosphatase (yellow in alkaline medium), was measured with a recording spectrophotometer Uvicon 943, Italy, at 405 nm. The absorbance was measured at 1 and 2 min and the slope of absorbance vs. time plot was used to calculate the ALP activity. ALP activity was expressed as μmol $min^{-1}cell^{-1}$.[26]

After sterilization, produced PHB-microspheres were tested for cytotoxicity towards the viability, growth, morphology and metabolism of NIH 3T3 fibroblasts.[29] MEM Elution-Test on Extracts was conducted with 100 mg of the dry microspheres (equals at least 120 cm^2), which were extracted at 37 °C for 24 h in 20 mL of Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum. As a positive control we used red technical rubber. This standard corresponds to the European analogue of the cytotoxic positive control – Para rubber. 60 cm^2 of polystyrene ''Greiner bio-one'' was used as a negative control. The maximum negative control was cells cultured in standard medium. An extract was prepared from the test material, which was then placed on mouse fibroblasts NIH 3T3 monolayers. The cells were examined for morphologic changes and cytolysis. After exposure to the extract for 72 h at 37 °C the medium was removed, leaving a film of medium in each well, and the cells were examined and scored microscopically for cytotoxic effects: cell viability, change of cellular morphology and cell doubling time. For cytological investigations, fixed and incompletely dried cells were Giemsa stained in a standard Jurr 65500 buffer at pH 6.8 and examined under a microscope. Initially, cell viability was estimated by live staining with Trypan blue (0.5% stain solution in 0.85% NaCl). Cell metabolic activity was measured at 3 days using MTT. The number of living cells in the culture was determined from the MTT absorbance standard curve. The mean OD570 value of the positive control was standardized as 100% inhibition. The mean OD570 value of a test sample was expressed as % of inhibition, resulting in a cytotoxicity grade.

Biomedical Studies *in Vivo*

A team of researchers of the IBP and of the ITAO carried out a series of investigations to compare biological compatibility of homogenous PHB and copolymers of PHB/PHV. Short- and long-term experiments were conducted with laboratory white mice, rats, and rabbits.[20] Experiments with the laboratory animals were conducted by the permission of bioethical commissions of ITAO and IBP, in accordance with directions accepted in RF,[30,31] EU, USA, and Japan.[32,33] Duration of the *in vivo* experiments was from 7 days, in the investigation of acute toxicity, to 6 months in the chronic toxicological experiment.

In the first stage of toxicological studies extracts from PHB and PHB/PHV containing 4 and 18% HV were applied to the skin of white mice and instilled into rabbits' eyes to determine whether the polymers produced an irritating and sensitizing action, in comparison with the physiologic saline. The formation of the antigen-antibody complex was determined using a standard method.

The preparations were observed under a microscope at a large magnification, counting the degranulated giant cells (the target cells) and the normal ones.[34] The acute toxic effect of PHB and PHB/PHV was investigated in a short-term 7-day experiment with white Balb/c mice. One intra-abdominal injection of extracts from PHB

and PHB/PHV containing 15% HV was made to each animal. The mice of the positive control received physiological saline, and the animals of the negative control remained intact. The estimated parameters were the general condition of the animals and their behavior and the mass of the body and internal organs; the internal organs were also examined macroscopically.[34] At the end of the experiment, before the animals were euthanasied, the phagocytic activity of macrophages in the peritoneal fluid was measured to determine immunotoxicity of the polymers.

The biological action of PHAs in the form of monofilament surgical sutures has been investigated in a 6-month chronic toxicological experiment on white Wistar rats.[35] PHB and PHB/PHV with 15 mol% of HV filaments of 2 metric dimension had the following properties: force at fracture – 7.1 and 9.4 N; tensile strength – 205 and 274 MPa; tensile modulus 3.75 and 3.13 GPa. Experiment was conducted on female Wistar rats with weight 180–200 g. The total number of animals was 96, 18 in each group: Group 1 – negative control (intact); Group 2 – positive control (surgical silk); Group 3 – reference biodegradable suture (catgut); Group 4 – test PHB/PHV suture; Group 5 – test PHB suture. The animals were ether-anesthetized under aseptic conditions. Longitudinal 2-cm skin and muscle incisions were made on the right femur. The muscle wound edges were closed with 3 sutures of the tested material, with total length 3.0–3.5 cm, and the skin edges were closed with silk sutures. Three animals of each group were killed by an overdose of ether at 1, 2, 4, 8, 16, and 24 weeks after the surgery. The rats' blood, internal organs, lymph nodes, and fragments of tissues surrounding the suture implants were isolated for analysis. The physiological and biochemical characteristics were studied to comprehensively assess the condition of the animals. Specimens of peripheral blood were analyzed for the blood cell concentrations, the erythrocyte sedimentation rate (ESR), and the hemoglobin content using the standard techniques. Blood for biochemical assays was sampled from the left ventricle of narcotized animals. All the assays were performed with clinical diagnostic kits; spectrophotometric measurements were taken with a recording Uvikon 943 spectrofluorimeter (Italy). Fragments of tissues surrounding the implants were excised out of the femur muscle, fixed with 10% formalin, embedded in paraffin, and 5 to 10 μm-thick microscopic sections were prepared from the paraffin blocks. To analyze the general tissue response and the processes of collagen fiber development, the sections were stained by the method of Van-Gieson with hematoxylin-eosin and with pycrofuchsin, respectively. Histomorphological investigation of inner organs was performed using conventional methods. A Carl Zeiss Image Analysis System was used for viewing microscopic images and analyzing morphometric characteristics of sections.

PHA fibers were also used as suture materials for the formation of inter-intestinal anastomoses in a 100 day experiment in dogs. Test animals were subjected to laparotomy and then side-to-side intestinal anastomosis was done between loops of small intestine using PHB monofilament fiber. Anastomosis was formed with a single layer, uninterrupted suture. All zones of anastomoses were histomorphologically analyzed.

Osteogenic potential of the PHB and PHB/HA (HA of Polistom, Russia) composites, seeded with cells was determined in ectopic (atypical) bone formation assay in female Wistar rats.[26] Bone marrow, extracted from rat femur ($15–20 \times 10^6$ cells) was aseptically placed onto every 3D matrix in complete culture medium (90% DMEM, 10% FCS, 80 mg/L gentamycin, 280 mg/L L-glutamine, Sigma). The samples were incubated for 60 min in a CO_2-incubator to allow the bone marrow cells to attach to the matrix. Subcutaneous implantation of composites was performed. After 45 days, the rats were euthanized with an overdose of diethyl ether; the tissues surrounding the implant were examined for inflammation, suppuration, and the implant adhesion to tissues and encapsulation. The implants

were extracted, decalcified, and fixed in 10% neutral formalin. For the histological examination of the newly formed tissue, longitudinal paraffin cuts were prepared, which were then stained with hematoxylin-eosin and examined using transmitted light microscopy.

Experiment with the model of segmental osteotomy was performed on Wistar rats.[36] The tested scaffolds were made of different materials: PHB, hybrid material PHB/HA, and PHB+rhBMP-2, recombinant human bone morphogenetic protein-2 (ProSpec-Tany TechnoGene Ltd, Israel), which was activated in acid medium and in sterile condition was applied on the porous implant, 6 μg/implant, according to Kokubo *et al*.[37] Dimensions of the tested scaffolds were 2.9 mm diameter and 1.3 mm height. For reference purposes Kollapol®, a hydroxyapatite/collagen composite material (Polistom) and bone allograft Bio-OSS (Geistlich Gmbh., Switzerland), of the same size, were used. These materials are currently in use in Russian clinics for filling bone defects. The rats were given volatile inhalation anesthetic and a bone defect 3 mm in diameter and 1.5 mm deep was made with an osteotome at the epiphysis of the tibia, under continuous cooling using physiological saline. Then the defect was filled with the sterile test implants. During the experiment (at 14 days, 1 month, 3 months) rats were euthanized with an overdose of diethyl ether. Sections of the bone tissue with the tested osteoplastic material were fixed in a 10% neutral formalin solution. The histological cuts were decalcified and stained with hematoxylin-eosin. Morphological examination was performed using an Axioskop 40 Pol., transmitted light polarizing microscope Karl Zeiss (Germany) equipped with an AxioCam MRc-5 digital camera.

Clinical Studies

PHA membranes were tested at the Krasnoyarsk Dental Research Center as materials for bone defect repair in human maxillofacial surgery.[38] PHB membranes were tested using the method of guided tissue regeneration in 22 patients aged 18 to 58 years. All surgical procedures were performed under standard anesthesia, using conventional techniques. Bone defects that resulted from removal of the cyst, were covered with polymeric membrane and left to heal under a blood clot (the first group of patients); other defects were filled with ground PHB and, once the material was impregnated with blood, also covered with the polymer membrane (the second experimental group). Clinical examination of the patients was performed using traditional procedures, taking into account results of X-ray visiographic tests and estimation of bone tissue mineral content (using a "Troffi-2000" computer program) and density by ultrasound echo-osteometry (measurements were done by the absolute technique using an "EOM-02" ultrasound echo-osteometer with the ultrasound oscillation frequency 140 kHz). The second direction of limited clinical investigations was using PHB membranes as material for repairing the front wall of maxillary sinus.[38] PHB membranes were used after surgical treatment of chronic inflammation of maxilar sinus (6 patients aged 25 to 36 years). The artificial defect created in the front wall of maxillary sinus to get a surgical access inside was repaired using PHB films. Due to its good strength and flexibility, the polymeric film clung tightly to the patient's tissues and adhered well to the wound surface. Wounds were clothed with soft auto-tissues. To monitor the reparative process, clinical and X-ray visiographic examination tests were used.

Results

PHA Samples

Polymers of various chemical structures were synthesized in the course of the investigation. Results of determining molecular masses (M_w), melting points (T_m), temperatures of degradation (T_d) and degrees of crystallinity (C_x) of PHAs having different chemical structures are listed in Table 1.

Table 1.
Chemical structure and properties of multi-component polymers of *Wautersia (Ralstonia) eutropha* B5786.

PHA composition, mol%						PHA properties		
HB	HV	HHx	HHp	HO	C_x, %	M_w kDa	T_m, °C	T_d, °C
11.22	88.46	0.13	0.16	0.04	49	380	146	210
36.01	63.64	0.12	0.21	0.02	51	420	150	213
41.94	56.30	0.71	0.98	0.06	48	260	153	230
52.25	47.33	0.09	0.33	ND	46	340	158	234
79.61	1.50	18.03	ND	0.85	53	480	155	253
81.81	3.69	13.76	ND	0.74	60	300	156	253
83.38	4.47	11.63	ND	0.52	62	540	157	257
90.63	2.29	6.52	ND	0.56	65	280	159	256
91.70	4.59	2.55	ND	1.17	74	370	163	265
99.76	0.24	tr.	ND	ND	74	420	168	268

HB- hydroxybutyrate; HV- hydroxyvalerate; HHx- hydroxyhexanoate; HHp- hydroxyheptanoate; HO- hydroxyoctanoate.

It was found that macroinclusions of HB and HHx affect PHA properties in a similar way: they somewhat reduce the temperatures of melting and the onset of thermal degradation and the degree of crystallinity, improving the processability of the material.[14,15] Thus, in our investigations we mostly used PHB/PHV and PHB samples.

Having investigated the dissolution and melting behavior of PHB and PHB/PHV (from 4 to 30 mol% of HV) and physicochemical properties of polymer solutions, gels, and melts, we prepared a series of various 2D and 3D matrices: flexible films and porous membranes, monofilament fibers, ultrathin fibers, microparticles, solid and porous 3D matrices, including composites with hydroxyapatite (Figure 1).

Polymer solutions of various densities (1–10%) were used to prepare thin flexible films with the following properties: thickness 0.05 to 0.4 ± 0.01 mm, strength 4.0 ± 0.28 kg/mm^2, Young's modulus 130 ± 28 kg/mm^2, elongation at break about 4%.[22] The surface properties of PHB and PHB/PHV membranes calculated from the measured water contact angles (θ) were similar: surface tension (γ) – 34.66–36.18 erg/cm^2; interface free energy – 6.35–7.00 erg/cm^2; and cohesive force (W_{SL}) – 100.81–102.63 erg/cm^2. To improve hydrophilic properties of polymer film matrices, we used laser processing. The advantage of laser processing over other techniques is targeted modification of the surface, which is not accompanied by either destruction of

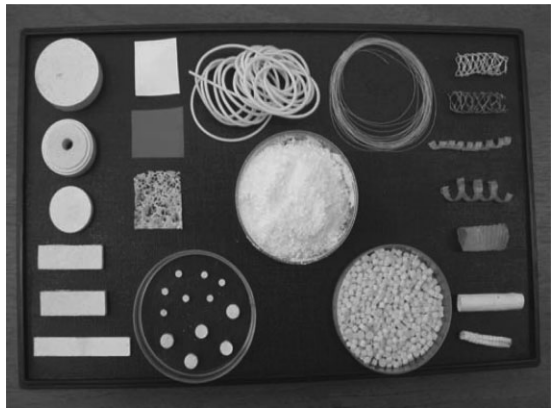

Figure 1.
Biomedical articles experimentally prepared at the Institute of Biophysics SB RAS, using PHA.

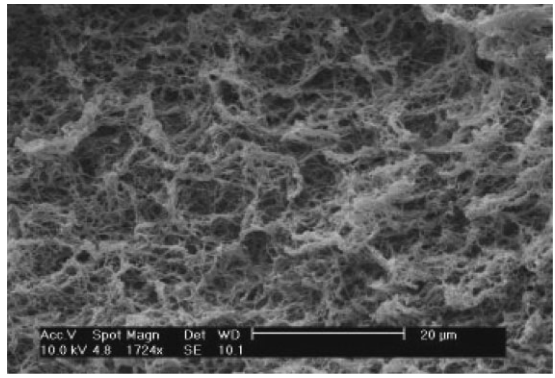

Figure 2.
Morphology of the porous film made from PHB/PHV.

the material or formation of toxic products. A series of films with modified surface properties ranging from pronounced roughness to perforations were prepared. The microstructure and properties of the surface were examined in relation to the parameters of the exposure, showing that at $P_{pulse} = 18.46$ W and $\tau = 1$ and 3 ms. The film surface had minimal meaning of water contact angles, WCA, 60° and 51°, respectively (unpublished data). Consequently, it was the most hydrophilic one compared to the untreated film, WCA 70°. In a typical procedure used to prepare a porous PHB and PHB/PHV films using the leaching method. Were reported fabrication of polymer films with pore size ranges of 50–200 mm and a porosity in the range 50–90% (Figure 2).

Monofilament fibers were produced using PHB and PHB/PHV copolymers with various inclusions of HV (from 10 to 21 mol %), they were melt-spun and extended to produce oriented monofilaments 0.17–0.20 mm in diameter with high mechanical characteristics (strength 306 MPa, elasticity modulus 3 GPa, elongation at break 24%).[23] Ultrafine fibers with diameters 2–3 μm were formed from PHB and PHB/PHV solutions. The best results for these two solutions PHB/PHV (6% and 10%) (Figure 3) were obtained at voltage 25 kW, distance between the needle and the target 25–30 cm, needle No. 20, and solution supply rate 1.0–1.5 mL/h.[24]

Morphology of microspheres did not significantly depend upon the preparation procedure (Figure 4). The obtained microspheres were of regular spherical shape and had a well-developed ''wrinkled'' porous surface; their diameters were significantly heterogeneous. The size distribution was

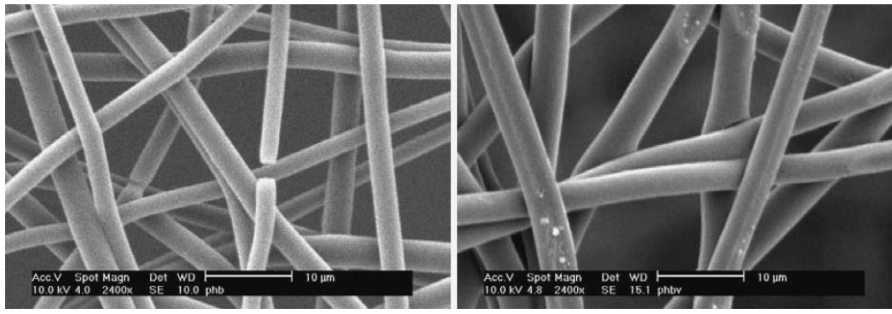

Figure 3.
Ultrafibers formed from the 6% and 10% PHB/PHV solutions at a higher magnification (Bar: 10 μm).

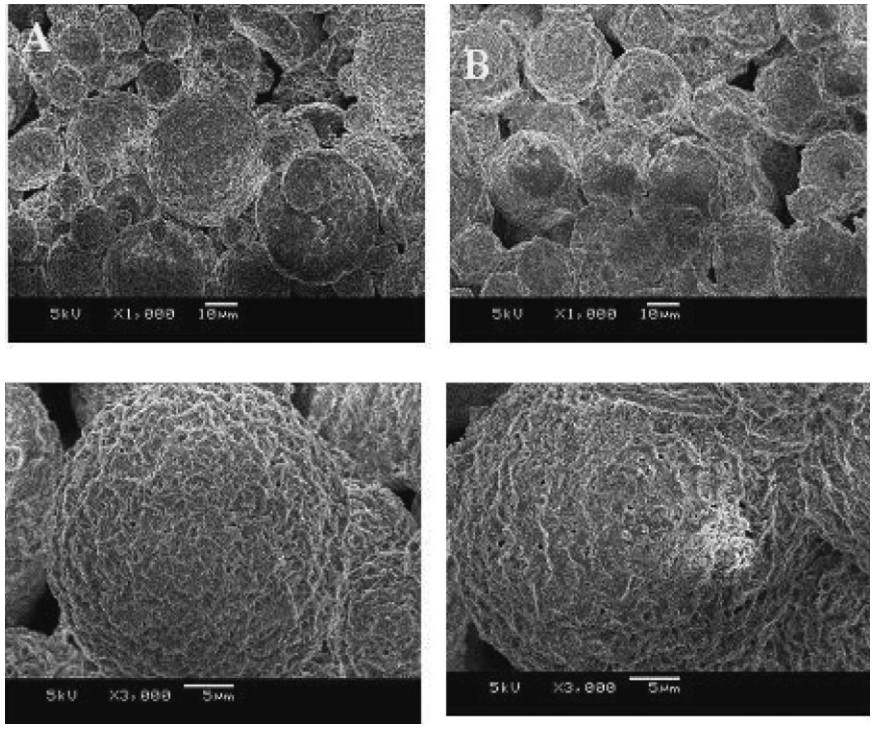

Figure 4.
Micrographs of polymeric microspheres prepared using PHB (A) and PHB/PHV (B). Bar: – 5 and 10 μm.

from 1.73 ± 0.22 to 35.57 ± 4.62 μm, with a mean microsphere diameter of 10 ± 0.23 μm.[25]

With the cold molding technique 3D matrices from PHB and its composite of HA were produced. The specimens contained from 10 to 50% (w/w) HA.[26] The compacted PHB/HA 50 mg specimens were 2.17 to 1.91 mm high and 5.70 ± 0.05 mm in diameter and had moisture absorption of 2.32 – 118.90%; porosity of 0.016 – 0.083 cm^3/g; density of 0.91 – 1.02 g/cm^3; interfacial angle of $68^0 01^I$ – $46^0 03^I$ (θ, degrees) depending on the HA content. Investigations of structure and physicochemical properties of these composites revealed that as the HA percentage in the composite increased, the surface wettability increased and so did the degree of crystallinity of the matrices (from 77% to 89%), but their thermal stability decreased; the temperature for the onset of decomposition decreased from 260 to 225 °C. To prepare porous matrices, crystals of sodium chloride was added to PHB and/or PHB/HA prior to molding. Then, the molded articles were boiled in the de-ionized water to remove the salt and dried in the salt.

Results of Biomedical Studies

Cytotoxicity of PHA-Devices *in Vitro*

The NIH 3T3 fibroblasts cultured on polymer films of any composition retained cell morphology characteristic of normal cells, like in control on glass supports. Live staining with Trypan blue demonstrated that 99.8 ± 0.2% of cells cultured on PHB and PHB/PHV films did not incorporate the stain, i.e. remained highly viable.[22] The cultivation of fibroblasts on polymer films for three days did not affect protein synthesis in the culture (Figure 5). The doubling time of the fibroblasts cultured on the polymer films of all types corresponded to the generation time of the control cells cultured on glass supports (25 ± 2 h). Since

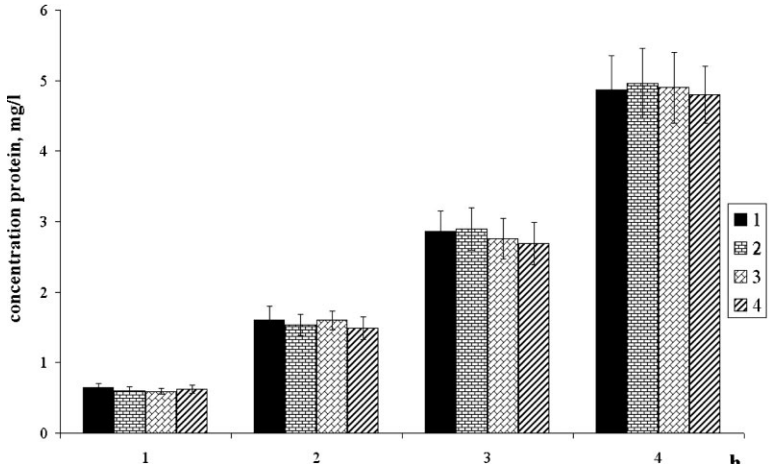

Figure 5.
Dynamics of protein synthesis in the fibroblasts line NIH 3T3 cultured on: 1- coverslips (control), 2- PHB, 3 and 4 – PHB/PHV with 15 and 28 mol% of HV films.

the template activity is one of the most important indications of cell viability, the authors have studied the dynamics of DNA synthesis in the cell culture. The ^{3}H-thymidine incorporation into the stimulated NIH 3T3 fibroblasts cultured on films of PHB and PHB/PHV was not reduced. Hence, direct contact of cells with a PHA of indicated composition did not result in the inhibition of DNA synthesis. The label incorporation into fibroblasts grown on polymers was similar to the incorporation observed in the control group (coverslips).

The proliferative activity of cells of mesenchymal origin (endothelial cells of the mouse liver), grown on polymer films, also remained unchanged. The template activity dynamics and intensity were similar in cells grown on the polymer films of both types/composition and in the control group. The next test object, mice hepatocytes, which feature high sensitivity to the effect of cytotoxic factors. Hepatocytes are known to proliferate *in vitro* only in the attached state. In preliminary experiments, when they were cultivated on a glass surface, which is not adhesive for these cells, the label incorporation was 2–3 times lower than the one observed when hepatocytes were cultivated on either collagen-treated glass supports or polymeric films. Thus, collagen-treated glass supports were used as control. The intensities of ^{3}H-thymidine incorporation into hepatocytes grown on polymer films of two types and on control were the same.[22] The results of investigation of cell morphology and protein and DNA synthesis in various animal cells on their direct contact with PHAs suggest that this material can be used to make matrices for *in vitro* functioning cells. None of the samples under investigation was cytotoxic. The properties of PHB and PHB/PHV films proved to be fundamentally similar. Attachment of the fibroblasts and osteoblasts to porous films was 18% higher than to initial ones.[39]

MTT assay demonstrated that the number of proliferating osteoblasts was evidently larger on all PHB/HA samples than on the PHB matrix.[26] The largest increase in the number of cells was registered on PHB/HA samples containing 10% and 20% of HA – 240 and 260×10^{6}/mL, respectively. On the composite samples containing larger HA fractions, 30 and 40%, the cell density was lower, but still significantly higher than the density of cells on the PHB matrix. ALP activity has been used as a marker for osteoblast differentiation. Compared with cultures on the PHB matrix, the ALP activity for the PHB/HA was significantly higher. The ALP activity of the cells growing on matrices containing low

levels of HA (10% and 20%) was the highest – 4.2 and 4.6 $mmol \cdot min^{-1} \cdot cell^{-1}$, respectively. The best parameters of growth and differentiation of bone marrow osteoblasts are registered on PHB/HA samples containing 10% and 20% HA.[26]

Investigations of the possible toxic effect of PHB microspheres are presented below. Evaluations of growth and metabolic parameters of the fibroblast cell culture in the treatment group and in the positive and negative control groups were conducted.[29] Mouse fibroblast cells cultured in the presence of extracts of polymeric (PHB) microsphere, similar to the negative control (polystyrene extract), retained the morphology of normal cells, like those grown in the control, on polystyrene. Cell viability test, performed by the method of live staining with Trypan blue, showed that $99.8 \pm 0.2\%$ of the cultured cells did not incorporate the dye, i.e. remained highly viable, in contrast to the positive control (rubber extract), in which most of the cells died. The doubling time of fibroblasts corresponded to the generation time of the cells cultured on standard medium and experimental culture were 25.1 ± 1.8 and 25 ± 2h; on negative and positive controls 24.9 ± 2.1 and 168 ± 21.3. The MTT test did not indicate any toxic effect of the polymer extract on the metabolic activity of fibroblasts, either. Having tested extracts of PHB-microspheres, no cytotoxic effects, as indicated by the absence of cell morphology changes, changes in cell viability and doubling time, and shifts in the cell proliferative activity were observed. These parameters were virtually the same as those of the negative control, polystyrene, and standard media. Statistically proven differences were detected only for industrial red rubber - the positive control.[29]

Results of Biomedical Studies *in Vivo*

No irritating effect in animals was observed with testing of extracts from PHB and PHB/PHV. The sites of skin applications exhibited no signs of irritating reactions, similar to the control. The extracts from the PHAs instilled into the conjunctival sac of the animals did not produce any irritating action on eye tissues. No immediate allergic reaction was observed, either.[34] In every experimental group of animals sensitized by the above method, degranulation of giant cells was less than 10% of the control. This suggests that the tested PHAs produced no sensitizing effects.[34] Throughout the 7-day toxicological experiment, the animals did not exhibit any intoxication reaction. The behavior of the experimental mice was similar to that of the controls. The body weight and the mass of internal organs of the experimental animals were similar to those of the control groups. The macroscopic examination of the internal organs of the animals in 7 days after the beginning of the experiment did not reveal any pathological changes. Concentrations of erythrocytes and leukocytes in the peripheral blood of the control and experimental mice were within physiological norm. No significant shifts were revealed in the leukocyte formula of the blood of the animals that received PHA extracts. The phagocytic activity of macrophages in the peritoneal fluid of animals in all the test groups was similar to the control. No immunotoxic effects were induced by either PHB or PHB/PHV.[34]

During the chronic experiments with rats all animals with implanted PHB/PHV or PHB threads were healthy and active.[35] They gained weight at a constant rate. No significant differences were detected between them and the control animals. The control and experimental groups were also similar in the relative weights of the inner organs. On macroscopic inspection of inner organs performed at specified intervals after the surgery, they had no detectable abnormalities. Morphological analysis of the composition of the peripheral blood and biochemical analysis of the venous blood did not reveal any significant differences between the control and experimental groups.[40] It was only once that a significant difference was observed. One week after the surgery, all operated animals had a mild leukocytosis. Later, all groups had normal values of blood parameters. Hence, the presence of hydroxyvalerate in

the polymer did not affect the state of the animals after the surgery and did not enhance the inflammation response.[35] Microscopic examination of the postoperative sutures with surrounding tissues demonstrated that PHB and PHB/PHV fibers provided reliable holding of the suture of the muscular-fascial incision in the animals throughout the period of observation. Wound healing in all animals of the test groups proceeded in the same way as in the positive control group (silk) and in the reference group (catgut), through primary stretching. None of the animals exhibited rejection of fibers, suture disjunction or any other adverse reaction. The tissue reaction to the surgical intervention and subsequent implantation of PHA fibers was generally similar to the typical reaction of the wound process and foreign-body invasion. This scheme includes the stages of traumatic inflammation, formation of connective tissue, and formation and rearrangement of the cicatrix. The reaction of tissues to polymeric implants is similar to their reaction to silk and is less pronounced than the reaction to biological suture material-catgut; it is expressed in a transient post-traumatic inflammation (up to 4 weeks) and the formation of a fibrous capsule which becomes 4–5 times thinner after 16 weeks, due to reverse development. Macrophages and giant cells of foreign bodies with a high activity of acid phosphatase are actively involved in this process. PHB and PHB/PHV sutures implanted intramuscularly for an extended period did not cause any acute vascular reaction at the site of implantation or any adverse events. No differences have been revealed in the tissue response to polymer sutures of the two types.[40,41]

PHA fibers were successfully used as suture materials for the formation of interintestinal anastomoses in dogs. Follow up observations for 100 days did not reveal failure of anastomoses. After the experiment morphological examination of the portion of anastomosis between the duodenum and the gall bladder did not show any negative changes and indicates that the regeneration process is nearing completion.

In ectopic bone formation assay, with 3D matrices of various composition, carrying bone marrow cells and implanted to rats subcutaneously in the abdominal area, it was proven that both PHB and PHB/HA constructs are biocompatible, have osteoconductive properties, and facilitate bone tissue formation.[26] Tested PHB and PHB/HA implants seeded with bone marrow cells were bioinert and did not cause any pronounced inflammatory or other negative reactions in the tissues surrounding the implants. In 45 days after the surgery, no inflammation of the adjacent tissues was registered at any of the implantation sites. Thin connective tissue capsules (not more than 55–80 μm thick) surrounded the implants as tissue response to foreign bodies; the capsules formed around different types of implants were of similar thickness. The areas of osteoid tissue of different size and shape, which formed both separate islets and sectors containing bone marrow parenchymatous (bone marrow) cells, stromal cells, and erythrocytes were attended. The base substance of osteoid tissue was stained homogeneously, mainly basophilically (indicating tissue immaturity), it was ''layered'', determined by the direction of collagen fibers and cells along them.[26]

Osteogenic potential of PHAs was confirmed in the experiment with the model of segmental osteotomy in rats.[36] Healing of bone defects in all animals occurred in phase characteristic of reparative osteogenesis, including posttraumatic changes in tissue elements, regeneration, and adaptive remodeling. In 14 days after bone defects were filled with PHB matrices, we registered proliferation of osteogenic cells, their transformation into osteoblasts, and formation of new bone tissue in the form of bone trabeculae, with capillary vessels, formed between them. At day 30 we registered the onset of biodegradation of the matrices and active formation of bone lamellae, which were rearranged into compact bone. That was confirmed by the presence of osteons with distinct cement lines. In some parts we

observed weakly pronounced growth of mesenchymal tissue around the implant with proliferation of osteoblasts and formation of osteoid (bone-similar tissue). By day 90, osteogenesis had been completed, bone tissue restructured, and mature compact bone with developed Haversian system formed.

With PHB/HA (80:20% mass) implanted into the bone defect, reparative osteogenesis occurred in much the same way as with the polymer implant.

Bone repair in the presence of rhBMP-2 occurred at a much higher rate than in other treatments: perichondral ossification was registered at day 14 and mature compact bone was actually formed in 30 days after implantation.

In animals whose bone defects were filled with Kollapan and Bio-OSS osteogenesis was less active. At day 14, in the region of the defect filled with Kollapan implants, numerous small bone trabeculae of newly formed lamellar bone were detected. After 1 month no implant material was detected on the cuts. The activity of osteogenic cells and ostoblasts was moderate, i.e. osteogenesis continued as bone restructuring and formation of compact bone began. At day 90, signs of Haversian systems being formed were noted. After 90 days of the implantation, similar to 1 month, the preparations mainly presented compact bone with a wide intramedullary canal filled with bone marrow. There were no bone trabeculae. The endosteum was lined with osteogenic cells. In that treatment of the experiment, morphological signs of bone repair were generally very weakly expressed.

The morphological investigations of polyhydroxybutyrate proper (the most common and the best studied polyhydroxyalkanoate) and combinations of this biopolymer with hydroxyapatite and/or morphogenetic protein suggest that this biomaterial has pronounced osteoblastic properties.

Clinical Investigations of PHA Membranes

Results of using PHB membranes in maxillofacial surgery in humans are positive.[38] During the postoperative period, after polymer membranes and polymer granulate were used to cover the cavities left after removal of cysts, patients did not complain of any medical problems; the wounds healed through primary intention at days 7–9. X-ray examination of the parts of the jaws subjected to surgery showed complete regeneration of bone tissue at the sites of defects filled with polymer granulate, using PHB films, in the average of 4–5 months after the surgery. Mineral content of the bone was similar to that of healthy bone tissue, averaging 2.26–0.02 mg/mm^3 in the mandible and 2.10–0.01 mg/mm^3 in the maxilla. Ultrasound measurements showed that bone tissue density was similar to that of the healthy bone tissue, averaging 3410-30 m/s in the mandible and 3280-25 m/s in the maxilla. When polymer films were used alone (without filling the defect with ground polymer), the process of reparative bone formation in bone defects was also normal, but lasted longer – 5 to 6 months.

In the cases, when PHB membranes were used to close the defect artificially created in the front wall to approach the maxillary sinus, operations were performed on 6 patients. The polymeric film clung tightly to the tissues of the patients and adhered well to the wound surface. During the postoperative period, patients did not complain of any serious medical problems; the wounds healed through primary intention at day 8. Clinical follow-up observations for 1.5 years proved that the treatment yielded lasting results: sensitivity in the zone of surgery was restored quickly and X-ray examination did not show any inflammatory processes in the operated sinus. Thus, PHB flexible films used to repair the front wall of the maxillary sinus provide a good support for soft tissues and prevent complications caused by growth of the scar tissue into the sinus.

Results of clinical investigations suggest a conclusion that PHA membranes are good candidates to be used as an artificial barrier in guided tissue regeneration in maxillofacial surgery; a particularly effec-

tive technique is to simultaneously use PHB membranes and polymer granulate to fill bone tissue defects.

Conclusion

Results of the investigations performed in cell cultures *in vitro*, on laboratory animals, and under clinical conditions allow the conclusion that PHAs are highly biocompatible and have good functional properties, thus suggesting their good potential for a wide range of biomedical applications. At the present time, a number of clinical institutions are conducting controlled clinical studies of PHAs as materials for surgical reconstruction and drug delivery and as scaffolds for cellular and tissue engineering. Researchers of the Institute of Transplantology and Artificial Organs of the RF Ministry of Health have completed clinical trials of polymer matrices and obtained the permission of the RF Ministry of Health to manufacture them and use as implants under clinical conditions. All PHA types synthesized at the Institute of Biophysics SB RAS and polymer items for biomedicine been registered and trademarked as Bioplastotan™[42] and Elasto-POB.[43]

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