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Biomedical Studies of Polyhydroxyalkanoates

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The paper describes research of biomedical applications of experimental polymer devices prepared from polyhydroxyalkanoates (PHAs) in the Institute of Biophysics SB RAS and Siberian Federal University (Krasnoyarsk, Russia) between 2000 and 2012. The high-purity PHA specimens were investigated and then used to prepare surgical sutures, 2D and 3D dense and porous matrices, fully resorbable tubular stents, and polymer coatings. The polymer devices that differed in their shape and mass were introduced into muscles, bones, blood vessels, and internal organs in order to investigate the response of tissues with different structure to the implants. The studies showed that PHAs were highly biocompatible with different tissues and their implantation for extended periods of time did not induce adverse responses of the blood system, cells, tissues, or the entire organism. The authors proved that PHAs can be effectively used as prosthetic implants and bone-replacing implants.

Keywords: PHAs, biomedical studies, implants.

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Биомедицинские исследования полигидроксиалканоатов

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В статье представлены результаты исследований экспериментальных полимерных изделий, полученных из полигидроксиалканоатов (ПГА), выполненных в Институте биофизики СО РАН и Сибирском федеральном университете в период 2000-2012 гг. Из высокоочищенных ПГА разработаны и исследованы хирургические нити, резорбируемые трубчатые стенты, 2D и 3D плотные и пористые матриксы, полимерные покрытия. Полимерные изделия различной формы и массы были имплантированы в мышцы, костную ткань, внутривенно, во внутренние органы и исследован ответ тканей на различные имплантаты. Исследования показали, что ПГА обладают высокой биосовместимостью по отношению к различным тканям и их имплантация на длительные сроки не вызывает реактивных изменений со стороны клеток, тканей и крови. Авторы показали, что ПГА перспективны в качестве различных тканевых имплантатов и костнопластических материалов.

Ключевые слова: ПГА, биомедицинские исследования, имплантаты.

Introduction

Among the biodegradable polymers that have already been developed or are being developed now for various applications, including medical ones, are aliphatic polyesters, polyamides, segmented polyester urethanes, polymers of lactic and glycolic acids (polylactides and polyglycolactides), silicon, polyethylene terephthalate etc. At the present time, the most widely used biodegradable polymers are polyesters of monocarbon acids, polylactides (PLA) and polyglycolides (PGA), whose medical use was approved by the United States Food and Drug Administration – FDA – in 1970 (Martin and Williams, 2005). The second most popular type of biodegradable polymers is polyhydroxyalkanoates (PHAs), which are more thermoplastic than PLA and have a less significant effect on tissue pH, and whose *in vivo* resorption time is longer (Biopolymers, 2002; Williams and Martin, 2002; Volova, 2004; Sudesh, 2010; Chen, 2010;Volova et al., 2013a).

The Institute of Biophysics SB RAS was the first in Russia to start research of PHAs. Many years of systematic studies have produced fundamental results on the functioning of the PHA cell cycle, factors determining polymer chemical structure, and interactions between the structure of a PHA and its physicochemical properties. The study showed that by varying the composition of polyhydroxyalkanoates and their monomer fractions, one can produce materials and devices that would considerably differ in their basic properties. Subsequent studies were aimed at construction of various special devices, implants, and polymer constructs for tissue engineering and reconstructive medicine (Shishatskaya, 2008,

2009; Shishatskaya et al., 2006, 2008, 2012, 2014; Volova et al., 2008, 2013 a, b, c, 2014, 2016).

A study of biological compatibility of PHAs

One of the key features of the novel biomaterials must be their absolute harmlessness to a living organism and biocompatibility. The biomaterials intended for long-term contact with blood have to undergo particular scrutiny, because hemocompatibility is the most important aspect of biological compatibility of biomaterials.

Based on the processes of the synthesis of PHAs with different chemical compositions developed and implemented by the authors (Volova et al., 2008), a study was conducted to investigate *in vitro* and *in vivo* biological compatibility of matrices prepared from PHAs of different chemical compositions (Nikolaeva et al., 2011; Shishatskaya et al., 2012; Volova et al., 2013a). High-purity PHA specimens – a homopolymer of 3-hydroxybutyric acid P(3HB) and poly-3-hydroxybutyric-co-4-hydroxybutyric acids [P(3HB-*co*-4HB)], poly-3-hydroxybutyric-co-3-hydroxyvaleric acids [P(3HB-*co*-3HV)], and poly-3-hydroxybutyric-co-3-hydroxyhexanoic acids [P(3HB-*co*-3HHx)] copolymers were produced in the Institute of Biophysics SB RAS (Volova et al., 2008). Adhesive properties of matrix surface and the ability of the membranes to maintain cell proliferation potential were investigated in experiments with mouse fibroblast NIH 3T3 cells. The number of cells on the reference matrices (polylactide) was significantly lower than on all the PHA membranes throughout the observation period. As the differences in the number of cells proliferating on matrices prepared from different PHAs were insignificant, all PHAs tested in this study proved to be highly biocompatible and suitable for *in vitro* cell cultivation. This was also confirmed by examination of fibroblast morphology. In all phases of cultivation, the cells were viable and had a round star-like shape. The MTT assay results provided more evidence for high biocompatibility of the matrices prepared from all PHAs studied. Thus, experiments with fibroblast NIH 3T3 cells showed that cells can be successfully cultivated on matrices prepared from any of the PHAs tested in this study. There were no statistically significant differences between counts of viable cells on matrices prepared from PHAs of different chemical compositions, and this suggests that both the homopolymer of 3-hydroxybutyric acid and PHA copolymers, consisting of the 3-hydroxybutyrate monomer and one other monomer (4-hydroxybutyrate, 3-hydroxyvalerate, or 3-hydroxyhexanoate), are suitable materials to prepare constructions necessary for cell and medical technologies.

Then, biocompatibility of all types of PHA matrices was investigated in the experiment with animals. Response of the organism, blood reaction, local tissue reaction, and polymer biodegradation dynamics were studied by implanting polymer film matrices to sexually mature female Wistar rats for 6 months (Shishatskaya et al., 2012). The following results were obtained. All animals of 5 treatment groups, with 2D implants prepared from polymers of different chemical compositions, were healthy and active throughout the experiment, gaining weight uniformly. No significant differences were observed between treatment groups and control ones (intact rats and the positive control – rats with implanted 2D PLA matrices). Relative masses of internal organs of rats in the treatment groups were similar to those of the rats in the control groups. Macroscopic examinations of the rats' internal organs did not show any adverse changes in them. Analysis of the morphological composition of rats' peripheral blood in the control and treatment groups showed that these values were generally within the range of normal physiological values and were similar in the treatment and control groups.

At Day 30 post surgery, a thin fibrous capsule was formed around each matrix. No penetration of connective tissue into the implant was observed. No necroses, hematomas, lymphohistiocytic infiltration, or swelling were observed in the fibrous muscle tissue surrounding the implants. A small number of macrophages and fibroblasts were present on the inner side of the capsule, adjacent to the matrix. A few foreign body giant cells (FBGCs) were detected in the inner wall of the capsule. At this time point, the thinnest capsules (22.48±4.16 μm) were observed around the implants prepared from the P(3HB) homopolymer, and the thickest (42.36±3.43 μm) – around P(3HB-*co*-4HB) matrices. The densest and the best developed capsule surrounded the polylactide control matrix (56.75±4.5 μm). At Day 90 post surgery, no necroses, hematomas, lymphohistiocytic infiltration, or swelling were observed in the fibrous muscle tissue surrounding the implants. All fibrous capsules surrounding the matrices became thicker, but they were not coarse, and their thickness did not exceed 100 μm. A characteristic tissue response to the implantation of PHA matrices was an increase in the number of FBGCs and a considerable decrease in neutrophil-lymphocyte infiltration. The matrices were significantly destroyed. At Day 180 post surgery, the capsules surrounding PHA matrices were substantially thinner: the average thickness of the capsules surrounding PHA implants was 1.5-2.3 times smaller than at Day 90. The number of active macrophages in the tissues adjacent to the implants, however, remained high. There were macrophages "lying" on the surface of polymer fibers; some FBGCs had 10-12 nuclei. Active macrophages phagocytizing the polymer and FBGCs were also observed there, suggesting migration of matrix disintegration products. Almost all matrices, except the P(3HB)-based ones, were considerably destroyed and broken into pieces. P(3HB-*co*-3HHx), P(3HB-*co*-4HB), and PLA matrices were present as a few fragments of destroyed films.

This study showed that matrices prepared from poly(3-hydroxybutyrate), poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) were biocompatible and that tissue response to the subcutaneous implantation of these matrices was moderate. No significant differences were found in tissue responses to the implantation of 2D matrices prepared from these PHAs; P(3HB) did not cause a more pronounced tissue response than the copolymers tested in this study. These results are generally consistent with the data reported in rather few published studies that addressed similar subjects, which were performed on laboratory animals, using several PHAs of this kind.

The use of PHAs to enhance biocompatibility of mesh implants

Achievement of better outcomes of surgical interventions in abdominal surgery is impossible without using new materials. For instance, surgical treatment of the patients with postoperative ventral hernias of the anterior abdominal wall has been one of the challenges in abdominal surgery. Hernia formation is a complex condition, caused by imbalance between intra-abdominal pressure and resistivity of the abdominal wall. Surgery using local tissues does not ensure stable improvement. The employment of tension-free techniques in surgeries of the postoperative ventral hernias and the use of synthetic materials was a revolution in hernia repair. Meshes can be implanted in super-aponeurotic, sub-aponeurotic, and intramuscular positions. However, plastic surgery of the anterior abdominal wall involving the use of synthetic allografts is a complicated surgical procedure and may cause the development of postoperative

complications, both specific and nonspecific ones. Support meshes of a new generation are needed for hernia repair, which is one of the most common surgical operations, amounting to 10-15 % of all surgeries. More than 20 million herniotomies are performed in the world, with recurrent hernias appearing in 10-15 % (Fedorov et al., 2000). So-called barrier techniques are being developed to prevent surgical adhesions. Materials used to prepare such meshes should be able to prevent adhesions to internal organs, be resistant to infection, be mechanically strong, and tolerate long-term tension without deep scarring and encapsulation.

In vivo experiments were performed with meshes coated with P(3HB) by using the solvent evaporation. Results of implantation of the P(3HB) coated meshes were studied in experiments with laboratory animals (chinchilla rabbits) and compared to results of implantation of Esfil polypropylene meshes and Vypro II meshes (Johnson & Johnson, ETHICON, U.S.) consisting of equal proportions of polypropylene and polyglactin filaments (Markelova et al., 2012).

The degrees of pathomorphological changes in the tissues of animals in response to the implantation of meshes varied between groups. Implantation of meshes made of synthetic materials to rabbits of the reference groups induced a "body against transplant" inflammatory reaction; the immune response was manifested as vast tissue necrosis, pronounced $CD68^+$ macrophage infiltration, and formation of polynuclear foreign body giant cells. Implantation of Vypro II meshes resulted in a similar effect. The most pronounced necrosis and macrophage response were observed in the regions closest to the mesh implant. In the same regions we observed degradation of the extracellular matrix and separation of fibers and the presence of mast cell aggregates with signs of degranulation. The sites of destruction infiltrated by macrophages were surrounded by a bank of monocytoid and plasma cells, which formed fields and nest-like clusters. In the group of animals with P(3HB)-coated mesh implants, the inflammatory reaction was less pronounced. The morphology of the majority of inflammatory cells was that of small lymphocytes and plasma cells, which do not express CD68.

The morphometric investigation of tissue response to mesh implants included evaluations of several parameters such as the degree and extent of macrophage response, the presence of necrotic changes, and the degree of granulomatous reaction of tissues. The most pronounced $CD68^+$-cell infiltration of the tissues surrounding the implants was observed in the reference groups (Vypro II, Group 2, and Esfil, Group 1) and the least pronounced – in the group with P(3HB)-coated implants – Group 3. The activity of the macrophage response at 30 days was evaluated at 1.45±0.14, 1.9±0.33, and 1.12±0.11 points in Groups 1, 2, and 3, respectively. Then, in the reference groups the activity of the macrophage response increased, while in Group 3 it did not.

No granulomatous reaction was observed in the experiment with P(3HB)-coated mesh implants, while the implantation of composite meshes (Vypro II) induced granulomatous reaction in all animals ($p<0.05$ as compared to Group 3), and it did not tend to subside. No giant cell response was observed in Group 3 at any point of the experiment. The most intense giant cell response was revealed in Group 2 at Day 60 – 3.0±0.43 points. We recorded extensive necroses (usually with the perifocal granulomatous or macrophage reaction) in all animals of the reference groups, while in animals with P(3HB)-coated mesh implants, slight necrosis (1.0±0.18 %) was observed only at the beginning of the observation period (1 month). Then, this parameter remained almost unchanged – 1.3±0.32 % and 1.5±0.50 % (after three months). Tissues of the abdominal

wall were affected by fibrosis to a greater extent in the reference groups than in the group with P(3HB)-coated mesh implants, in which tissue response was less pronounced. A layer of fibrous tissue was formed at the implant site, but the adjacent tissue remained unaffected.

The study showed that strengthening of the abdominal wall occurred sooner and inflammation was less pronounced in rabbits with poly(3-hydroxybutyrate)-coated mesh implants.

Fully biodegradable biliary PHA stents

Among the greatest challenges facing medicine is treatment of the patients with obstructive jaundice. Achievements of the contemporary surgery improved results of the treatment, decreased complications and death rate (Kurzawinski et al., 1992). Introduction of instrumental drainage into practical medicine resulted in prevention of cholemia and damaging of the important organs and systems. In many cases, however, the situation remains unsatisfactory. The number of the patients with obstructive jaundice and cancer reaches 25-30 %. In 38-46 % of all patients the tumor blockades the common bile duct in the gate of the liver, and extends to the pancreas, stomach, gallbladder (Yamamoto et al., 1992).

PHAs are of great interest to reconstructive surgery because of their absolute biocompatibility, slow biodegradation, and good mechanical strength. Research in this area has become more intense in the last few years. It has been proven that PHAs of various chemical compositions, such as a polymer of hydroxybutyric acid [(P(3HB)] and copolymers of hydroxybutyric and hydroxyvaleric acids [P(3HB-*co*-P3HV)] can be successfully used to increase biocompatibility of vascular stents. Using polymer solutions, we constructed prototypes of fully resorbable tubular stents that differ in their diameter and length.

The purpose of the study was to find out whether PHAs were suitable for constructing biliary stents and test them in experiments with animals (Markelova et al., 2008). The stents were manufactured from P(3HB) in the Institute of Biophysics of Siberian Branch RAS. The diameter of stents ranged from 3.2 mm to 4.1 mm, the length – from 20 mm to 25 mm, and the wall thickness – from 0.08 mm to 0.1 mm. The initial mass of the stents ranged from 0.1039 g to 0.1333 g. Experiments were performed on 20 adult mongrel dogs, weighing 10-12 kg. The animals were divided into three equal groups: the negative control (intact animals); the positive control (animals with silicon stents); and the treatment group (animals with PHA stents).

All animals survived the operation and recovered from anesthesia. The sutures were removed at Day 14, the postoperative wound healed by first intention. From Day 5 on, the animals were fed as usual; there were no signs indicating a lack of anastomoses or a non-specific inflammatory reaction to the implant. At the end of Day 10, all animals were alive. After the opening of the common bile duct lumen, the following factors were taken into account: the common bile duct wall thickness, the presence of visible inflammatory changes, the state of the common bile duct mucous membrane, the reliability of the attachment of the stent to the common bile duct wall, the presence of defects in stent construction as a result of polymer biodegradation, the size of stent lumen, the presence of lumen diminution as a result of sludge and concrement sedimentation, and the thickness of the stent wall.

The autopsy detected no pathologies of abdominal cavity in the animals of the control group. Three animals of the 1st group had an insignificant amount of serous exudate in the abdominal cavity (up to 30-40 ml), and a moderate commissural process in the subhepatic spatium. The common bile duct was examined in the place

of stent implantation to check for infiltration, lumen expansion, and cicatrical changes. In two animals of this group, the stents had migrated towards the ampulla of Vater. The appearance of cholecystoduodenostomy suggested that 2 animals had anastomositis – hyperaemia, infiltration, and cicatrical deformation in the area of anastomosis and duodenum. The macroscopic examination of the liver showed that it had normal appearance. Two animals had moderate hepatomegaly. The opening of the common bile duct lumens of all animals showed that the common bile duct wall was thickened, sclerotized, and infiltrated. The silicon stent was easily extracted from the lumen. The common bile duct mucous membrane at the site of contact with the stent was of pale pink color; there were atrophied zones detected; and 3 animals had the stent lumens narrowed by 40-50 % as a result of sludge and bile component sedimentation. The stents were fragile and had sediments of salts and bile pigments. Cholecystoduodenostomy was obturated in 2 animals of this group. The other animals had sufficiently large, at least 10-mm, anastomosis lumens; a moderate inflammatory process was observed in the area of mucous membrane; the Vicryl sutures were visualized; they were substantial and insignificantly infiltrated.

After the withdrawal of the 2nd group of animals from the experiment, the autopsy did not reveal any exudates or cicatrical changes in the free abdominal cavity and subhepatic spatium. The common bile duct had normal appearance at the site of stent implantation; no expansion, inflammatory reaction or cicatrical process was visualized. All PHA stents stayed at their initial sites of implantation; no cases of migration were detected. All animals had substantial cholecystoduodenostomy, no inflammatory reaction or indication of anastomositis was detected. No macroscopic changes were revealed during the examination of liver and duodenum.

The common bile duct lumen remained in the place of stent implantation in all animals and had a normal size (0.4-0.5 mm); no deformations, strictures, cicatrical or inflammatory changes in the stent implantation region were observed. While extracting the stents, we registered a leaky adhesion to the common bile duct mucous membrane; a small effort was enough to extract the stents from the common bile duct lumen. The stents retained their initial physical properties; they were not subject to calcification processes; there was no narrowing of stent lumen, its diameter being 3.5±0.1 mm. Biodegradation did not cause any defects, except that the stent wall became thinner: the average wall thickness was 0.05-0.08 mm. No stent lumen narrowed areas were detected. The macroscopic examination of cholecystoduodenostomies through mucous membrane did not reveal any signs indicating inflammatory, infiltrative or cicatrical changes; all anastomoses were functioning. The average anastomosis lumen was 12.3±4.3 mm wide. No traces of PHA-based suture material were detected in the place of single-layer continuous anastomosis.

No pathological changes were detected in the course of morphological investigation of the samples of common bile duct, gallbladder, duodenum, and liver. All samples of the 1st group animals had signs of inflammatory cellular reaction and fibrosis. The common bile duct mucous membrane was atrophied, with necrotic patches. The liver had signs of cholestasis, beam and hepatocyte fractures. In the zone of anastomoses, too, there were signs of inflammatory cellular reaction, a large number of leucocytes and macrophages, and rough scar tissue. Morphological investigation of the common bile duct area of the 2nd group animals, with P(3HB) stents, did not reveal any pathological changes. Common bile duct was lined with a mucous membrane with high cylinder-shaped

epithelium; each cell was similar to the adjacent one. The lumen had traces of bile. Epithelium had microfibers. The interfacial cytoplasm of epithelial cells had granules. Epithelium formed numerous folds. Epithelium was located on the mucous lining with its loose connective tissue, and the whole mucous membrane lay on a layer of smooth muscle tissue, which was interleaved with connective tissue and elastic fibers. Behind the muscular layer there was a subserous membrane with its loose connective tissue, in which fat cell groups, arteries, veins, lymphatic vessels, and nerves were located. The serous membrane of common bile duct consisted of a thin layer of mesothelium. This morphological picture was within normal limits: no inflammatory cellular reaction or tissue proliferation was detected.

The histological picture of duodenum revealed fibers in the form of digitules out of mucous membrane layer covered with epithelium on the surface of mucous membrane. The fibers were wide and were covered with cylinder-shaped single-row epithelium with a few caliciform cells. The fiber stroma consisted of connective tissue – a prominence of mucous membrane layer – and was formed by loose and reticular connective tissue, smooth muscle fascicles, and blood vessels. Under the base of the fibers there were Lieberkuhn's glands in the form of tubules, lined like tectorial epithelium, which opened between the fibers into the intestine lumen (crypth openings). Under the mucous membrane layer there was a muscular layer – smooth muscle cells. Below there was a submucous layer, where among loose connective tissue there were Brunner's glands (which are present only in duodenum, like Lieberkuhn's glands). They had flat nuclei and weakly basophilic cytoplasm.

No pathological changes were revealed in the histological section of the liver of the 2nd group animals. At the anastomosis level, closer to the submucous layer, there was developing granulation tissue with vessels of capillaceous type, as well as fibroblasts, epithelial and plasmatic cells, lymphocytes, eosinophils, and a few leucocytes. This pattern complies with the anastomosis formation period (100 days) and indicates that the regeneration process is nearing completion. The study detected new vessels, smooth muscular cells, connective tissue, vessel sections, nerve cells, and a thin layer of mesothelium. No traces of suture material were detected in the specimen.

Thus, physiological, biochemical, and morphological examination of the treatment group animals, with PHA implants, did not reveal any indications of adverse effects of polymer stents and monofilament fibers on the tissues of bile ducts. These results suggest a conclusion that the use of PHA-based endobiliary stents in reconstructive surgery of bile ducts and as suture material is a promising technology that needs further investigation.

PHA potential
for bone tissue repair

PHAs are of great interest to orthopedics because of their absolute biocompatibility, slow biodegradation, and good mechanical strength. Studies reporting the use of PHA composites with calcium-phosphate materials are rather numerous. The addition of hydroxyapatite improves the properties of P(3HB), enhancing the strength of the material and making it similar to bone. Thus, most authors agree that PHA-based implants have pronounced osteoplastic properties, their *in vivo* degradation is slow and corresponds to the growth of new bone tissue, which gradually replaces the biomaterial, resulting in normal reparative osteogenesis.

In our study (Shishatskaya et al., 2006) we produced a composite of biodegradable P(3HB) (the most wide-spread and intensely investigated PHA) and hydroxyapatite [P(3HB)/HA]. The

compacted P(3HB)/HA 50-mg specimens were 2.17 to 1.91 mm thick and 5.70±0.05 mm in diameter, depending on the HA percentage – 10-50 %. Stromal osteoblastic cells were obtained from the marrow of Wistar rats. P(3HB)/HA (80:20, w/w) samples were sterilized and placed into Petri dishes. Cells of a bone marrow aspirate ($15\text{-}20 \times 10^6$ cells) were aseptically seeded onto a P(3HB)/HA matrix in complete culture medium (90 % RPMI-1640 medium, 10 % FBS, 80 mg/L gentamycin, 280 mg/L L-glutamine). The samples were incubated for 45 min in a CO_2 incubator to allow the bone marrow cells to attach to the matrix. The composite matrices inoculated with the osteoblast primary culture were used in the *in vivo* experiment. Osteoinductive properties of the P(3HB)/HA matrix were investigated in the ectopic bone formation assay. Volatile inhalation anesthetic (diethyl ether) was given to ten two-month-old Wistar rats and each animal underwent surgery. A midline skin incision of the abdomen was made, a subcutaneous pocket was created, and one hybrid implant per animal was inserted (ectopic bone formation assay). After 45 days, the rats were euthanized with an overdose of diethyl ether and the implants were extracted and investigated. On Day 45, the implants were covered with thin connective tissue capsules. On the side of the capsules adjacent to the implants, there was newly formed connective tissue with a large number of cell elements. The surface and the pores of the matrices were filled with connective tissue differing in the degree of development. The tissue consisted of mature fibroblasts and mono- and poly-nuclear macrophages; there were numerous capillaries between collagen fibers. Bone tissue formation was registered on very many sites adjacent to the implants and on their surface. We observed areas of osteoid tissue of different size and shape, which formed both separate islands and spots containing bone marrow parenchymatous cells, stromal cells, and erythrocytes. The base substance of osteoid tissue was stained homogeneously, mainly basophilically (indicating tissue immaturity), with some oxyphilia. The base substance of the tissue was "layered", determined by the direction of collagen fibers and cells along them. Bone cells were positioned more or less regularly; they were of oblong or round shape with oval or flat nuclei. The results show that the investigated PHB/HA implants loaded with bone marrow cells facilitate bone tissue formation *in vivo* (Shishatskaya et al., 2006). In ectopic bone formation assay it was proven that the hybrid P(3HB)/HA composites can function as scaffolds and that bone tissue develops on their surface and in pores. Thus, it was proven that the hybrid P(3HB)/HA composites studied are good candidates to be used for reconstruction of bone tissue.

Then we investigated osteoinductive properties of PHAs in experiments on laboratory animals, using models of segmental osteotomy (Shishatskaya et al., 2008). Three types of implant materials were investigated in the experiment: 1) poly-3-hydroxybutyrate [P(3HB)], 2) poly-3-hydroxybutyrate/hydroxyapatite hybrid material [P(3HB/HA)] containing 20 % (w/w) HA (Polist Company ®, Moscow) and 3) bone substitute material Bio-OSS® (control) (Geistlich, Switzerland) (used for reference purposes). Experiments were conducted on 36 adult female Wistar rats in accordance with the international and Russian ethical rules for laboratory animal care. The rats were kept in an animal facility and fed a standard diet in accordance with the directive on maintaining animals and experimenting on them. The rats were divided into 3 groups (12 rats in each group). Bone defects (1.5 to 3.0 mm in diameter and 1.0 to 3.5 mm in depth) were created in the sites that are known to provide the most representative data on bone formation in rats and enable correct evaluation of the effects of implants on the repair of the defect in the metaepiphysis

of the tibia. The initial area and the volume of P(3HB)-based implants were 23.56 mm^2 and 29.45 mm^3, respectively. Such parameters as the state of the animals, the support ability of the affected leg, and the state of the tissues at the site of implantation were monitored throughout the experiment. At Days 14, 30, 60, and 90 post surgery, the rats were euthanized with a lethal dose of anesthesia, bone sites were explanted and investigated histologically.

All animals responded adequately to surgery; no significant complications occurred in the postoperative period. All wounds healed by first intention. In 7-8 days after the surgery, the animals fully loaded the operated limb. No tissue infection or inflammatory reaction was observed at the implantation site. The effectiveness of osteogenesis employing implants was estimated using radiography. X-ray examination did not show any lysis zones or formation of fibrous capsules around the implants. The images showed that the employment of the P(3HB) and P(3HB)/HA implants resulted in more complete repair of the model defect of bone tissue than the employment of the commercial material Bio-Oss. At Day 60, for most animals of the treatment group, radiography showed regeneration of the cortical plate and medullary cavity; the bone structure was entirely normal. At Days 90, radiography showed regeneration of the anatomical structure of the bone in all rats. In the control group (Bio-Oss), at Days 90, the regenerating bone tissue still had nonuniform structure, the periosteum was thickened, and the medullary cavity was undetectable. Healing of bone defects in all animals occurred in phases characteristic of reparative osteogenesis, including posttraumatic changes in tissue elements, regeneration, and adaptive remodeling. There were some differences in reparative osteogenesis, depending on which implant had been used to fill the model defect. In animals whose bone defects were filled with Bio-OSS®, osteogenesis was less active.

Histological examination of the tissue sections at the site of the model defect also showed that implantation of P(3HB) gave better results than implantation of reference materials. In 30 days after bone defects were filled with P(3HB), bone lamellae were being rearranged into compact bone. That was confirmed by the presence of osteons with distinct cement lines. The P(3HB) implanted material was considerably reduced, with the area of polymer particles on histological sections decreased to about 60 % of its initial size. Growth of osteogenic tissue around the implants was observed in some sections. At the end of the experiment (90 days), new mature bone tissue was actively developing at the site of P(3HB) implantation; the tissue had lamellar structure; developing osteons were detected. The sections contained large particles of the degrading polymer, which were not incorporated in the osteogenic tissue and were grouped in clusters. The presence of the polymer at this time point suggests the low rate of its resorption. No inflammatory reaction or capsule formation was observed at the implant/native bone interface.

Reparative osteogenesis was somewhat different in the experiment with the P(3HB)/HA composite implanted to the bone defect. At Day 30, the amount of osteogenic tissue was noticeably larger, but the position of bone lamellae was chaotic; the Haversian system was less pronounced than in the experiment with pure P(3HB), at the same time point. The sections clearly exhibited particles of the composite material in osseous lacunas, with the red bone marrow and mesenchymal cells not integrated in the new bone tissue. Increased generation of haematopoietic cells was observed. The developing cortical bone contained large lacunas and was rather loose. Hence, its biomechanical properties must be much worse than those of the new bones in the

animals with P(3HB) implants. The resorption of the composite P(3HB)/HA matrix occurred at a lower rate than the P(3HB) resorption. By the end of the experiment, at Day 90, the cortical bone had been formed almost completely, with the Haversian system, osteocytes, and osteoclasts. In some regions of histological sections, the direction of bone lamellae of the newly formed tissue was rather chaotic than longitudinal. The sections still showed HA and P(3HB) particles. There were particles in which HA was separated from P(3HB) by a layer of mesenchymal cells.

In the experiment with Bio-OSS® implants, at Day 30, the sections mainly showed compact bone with distinct Haversian systems. There were very few trabeculae and the proliferation activity of osteogenic cells and osteoblasts was very weak. The periosteum was thickened due to the fibrous layer. The intramedullary cavity was wide and filled with bone marrow. At Day 90, similarly to Day 30, the sections mainly presented compact bone with a wide intramedullary cavity filled with bone marrow. There were no bone trabeculae. The endosteum was lined with osteogenic cells. The periosteum was fibrosed. In this experiment, morphological signs of bone repair were generally very weakly expressed. The amount of the new bone was lower than in the experiments with the implants based on P(3HB). Results of *in vivo* experiments with pure poly(3-hydroxybutyrate) and P(3HB) loaded with hydroxyapatite (HA), using the model of segmental osteotomy, showed that osteoplastic properties of P(3HB) were more pronounced than those of Bio-Oss; its *in vivo* degradation was slow and corresponded to the growth of new bone tissue, which gradually replaced the biomaterial, resulting in normal reparative osteogenesis. Thus, poly(3-hydroxybutyrate) and the composite materials consisting of P(3HB) and HA exhibit pronounced osteoplastic properties; their *in vivo* degradation is slow and corresponds to the growth of new bone tissue, resulting in normal reparative osteogenesis.

Important results have been obtained in a study of PHA constructs used to treat experimental osteomyelitis. We carried out a study to determine the effectiveness of PHAs loaded with antibiotics in suppressing staphylococcal infection in bone cavities (Markelova et al., 2012; Shishatskaya et al., 2014). The experiments were carried out using 4-5-month old male chinchilla rabbits; 60 rabbits were used. The rabbits were divided into 3 groups (20 rabbits in each group): 2 treatment groups and 1 control. Rabbits were anesthetized with ketamine-droperidol and an aperture 3 cm long was created through the anteromedial approach to the tibia. The muscles were bluntly separated and fixed. Osteotomy was performed through a 0.5-cm incision in the anterior surface of the metadiaphyseal region of the tibia; limited separation of periosteum was carried out; the medullary cavity was opened up and bone marrow was removed using a Volkmann's curette. A gauze wick with *Staphylococcus aureus* culture (10^9 cells) was placed into the wound. The postoperative wound was closed layer by layer. Formation of the model of experimental osteomyelitis took 1 month; the model was 100 % reproduced; primary chronic osteomyelitis was established. Then, treatment of bone defects was performed in the rabbits with chronic osteomyelitis. During the surgery, samples were collected to identify the causative agent and determine antibiotic sensitivity. Trepanation of the bone was performed to reach the apparently healthy bone; medullary cavities were opened up. The resulting trough-shaped bone cavity and the adjacent medullary cavities were thoroughly curetted using a Volkmann's spoon. After all nonviable tissues were removed, the cavity was washed with an antiseptic solution (plivasept, chlorhexidine solution). Counterincisions were made and irrigators for postoperative drainage

were placed at the surgery site. After that, the bone cavity was filled with bone substitute materials: demineralized autologous graft bone taken from the iliac crest (the control group) and experimental materials: P(3HB) and P(3HB)/ tienam composite (the treatment groups). During the postoperative period, the limbs were immobilized using plaster splints or orthoses. The state of the animals (appetite, locomotor activity, the state of the limbs that had been operated on) was monitored throughout the experiment. After termination at 30, 60, and 90 days postoperatively, bone specimens were retrieved and examined to estimate the state of the surrounding soft tissues, periosteum, cortical layer, and medullary cavity. Bacteriological plating on Chistovich medium (egg-yolk salt agar) was performed at the defect site. Results were evaluated at Days 2-4.

During the early postoperative period (up to 3 days), the rabbits remained sluggish, moved very little, did not load the affected limb. At Days 3-5, the rabbits recovered their appetite and locomotor activity, but they did not load the affected limb. At Days 5-12, 19 rabbits had soft tissue edema at the surgery site; 12 rabbits showed local dermal hyperemia. At Day 16.5±3.2, on average, the edema and hyperemia were reduced. The rabbits began to load the affected limbs at Day 6.22±1.72 post surgery. Surgical wounds healed at Day 11±1.32, on average. No animals died during the postoperative period. In the experiments with P(3HB) used as a filling material for the experimental osteomyelitis, we observed significantly more rapid healing of postoperative skin wounds and recovery of the support function of the affected limb than in the control group. In the treatment groups, healing of the surgical skin wound took 7.4±0.9 days, on average, after surgery and reduction of the edema and hyperemia – 8.75±0.96 days; while in the control group these processes took 9.9±1.7 and 11.7±1.8 days, respectively. Recovery of the support function of the affected limb was observed at Days 4.28±0.9 and 5.56±1.2 in the treatment and control groups, respectively. Radiography of the operated limbs revealed the following: at Day 30 after P(3HB) and P(3HB)/tienam were used to fill the bone defects, the projection image of the bone defect showed clear round areas with distinct boundaries and a cloudlike shadow in the center. At Day 90, radiography showed regeneration of the anatomical structure of the bone in all rabbits. In the control group, at Day 90, the regenerating bone tissue still had nonuniform structure, the periosteum was thickened, and the medullary cavity was undetectable. Results of microbiological investigation of the samples retrieved from the defect sites showed the following. The microbial profile of the control group was investigated by plating microbial associations at Days 30 and 90. These associations mainly contained *Staphilococcus aureus* (48.2 %) and associations of Gram-positive and Gram-negative anaerobic microorganisms and *E. coli*. In the P(3HB) treatment group, microbial associations were plated at Day 30; they mainly contained *Staphilococcus aureus* (44.1 %) and associations of Gram-positive and Gram-negative anaerobic microorganisms and *E. coli* (55.9 %). At Day 90, bacterioscopic examination of the samples retrieved from this group gave a negative result, i.e. the hydrophobic P(3HB) powder suppressed the infection. Bacterioscopic examination of samples of the material retrieved from the P(3HB)/tienam group did not show any growth of microorganisms at all time points.

Histological examination of tissue sections from the sites of model defects of the treatment groups with P(3HB) and P(3HB)/tienam filled defects showed that at Day 15, the center of the defect was filled with fibrous connective tissue with pronounced perifocal proliferation of capillary-like vessels and focal lymphohistocytic infiltration. Primitive osteogenesis, with the

formation of osteoid-like masses and bone trabeculae with architectural deformations and chaotic arrangement of osteocytes, was mainly observed around the new vessels. Osteogenesis regions showed a high degree of basophilia of cells and osseomucoid of the developing bone tissue. At this time point, in the control group the defect was filled by loose connective tissue and, partly, by granulation and fibro-reticular tissue and residual detritus. In the treatment groups, at Day 30, the defect became smaller and was filled by fibrous, bone, and cartilage tissues. Intercellular substance was arranged in concentric layers around the vessels of the new osteons; bone lamellae were being formed. Towards the periphery of the defect, the bone tissue was more mature and had an osteon-trabecular structure; the osteons were arranged irregularly; the Haversian canals had different widths and were hyperemic; no perivascular edema was observed. In the control group, the defect on the periphery was filled by immature bone tissue, with fibrous scar and cartilage tissue in the center. The new tissue and the surrounding bone tissue were weakly vascularized. The periosteum was thickened and infiltrated with macrophages. At Day 90, all histological sections of the treatment groups showed signs of complete recovery of the bone structure. The periosteum was completely formed and consisted of the outer and inner layers. The bone tissue showed different degrees of maturity and had a laminar structure; osteons were arranged irregularly; the number of mature fibrocytes and osteocytes increased. Interstitial substance became optically denser and homogenous. In the control group, at Day 90, the defect site was seen as the narrowing of the bone at the surgery site. Microscopic examination of the sections showed that the defect was filled by rather mature bone tissue, with some interlayers of cartilage tissue. Dense bone tissue with a few osteons prevailed; the intercellular substance was less homogenous and loose. The cells were mainly represented by mature fibroblasts and osteocytes; no basophilia of cells and intercellular substance was observed. Thus, the experiments showed that using P(3HB) to fill the bone defect complicated by chronic osteomyelitis considerably facilitated the curbing of inflammation, bone defect repair, and recovery of the functional properties of the affected limbs and was significantly more effective than using bone allograft. Repair processes in experimental cavities of rabbits' tibias filled with PHA resulted in anatomical and functional bone regeneration by Day 60 post surgery. The filling of the bone cavity with demineralized autologous bone graft did not result in the regeneration of the bone structure.

Filling of infected bone defects of long bones, which had been subjected to surgical debridement, with hydrophobic P(3HB) and P(3HB)/tienam resulted in quicker subsidence of infection, regeneration of bone defects, and recovery of the support ability of the affected limb than in the control group of animals (with defects filled with bone allograft). Biodegradable 3D implants and P(3HB)-based filling materials showed pronounced osteoplastic properties and degraded *in vivo* at a slow rate, enabling normal reparative osteogenesis. Based on these positive results, we were able to prepare suggestions for clinical trials of PHAs.

Conclusion

The technology of PHA synthesis has been certified; the hygienic conformance certificate has been issued for the Pilot Production Facility manufacturing polymers intended for medical use at the Institute of Biophysics SB RAS. Specifications of PHAs with different chemical structure intended for use as matrices for functioning cells and drugs and as surgical implants and a number of polymer devices have

been developed and registered in Rosstandart. The tradename "BIOPLASTOTAN" has been registered in the Russian Patent Office for PHAs and biomedical devices; a number of patents have been obtained for the medical devices designed by the authors. The scientific basis and experimental proofs have been obtained for the use of degradable biopolymers of microbial origin (polyhydroxyalkanoates, PHAs) in reconstructive medicine.

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