In vivo study of 2D PHA matrices of different chemical compositions: tissue reactions and biodegradations

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Matrices based on resorbable polyhydroxyalkanoates (PHAs) of five types {a homopolymer of 3-hydroxybutyric acid, copolymers of 3-hydroxybutyric and 4-hydroxybutyric acids [P(3HB/4HB)], 3-hydroxybutyric and 3-hydroxyvaleric acids [P(3HB/3HV)], 3-hydroxybutyric and 3-hydroxyhexanoic acids [P(3HB/3HHx)]} have been constructed and characterised. No significant differences have been found in tissue response to implantation of these PHAs. Non-coarse fibrous capsules that formed around PHA matrices reached their maximum thickness (60–90 μm) 90 days after implantation; by day 180, the average thickness of the capsules had decreased by 1·5–2·3 times. The number of foreign body giant cells, resorbing PHAs, remained high. *In vivo* biodegradation behaviour of polymer matrices is related to the chemical composition of the PHA. Matrices prepared from copolymers P(3HB/4HB) and P(3HB/3HHx) exhibited the fastest degradation rates. P3HB/3HV matrices were degraded more slowly, and P3HB matrices were the most durable. In the PHA matrices that were degraded more slowly, giant cell reaction developed later.

Keywords: PHA, Polyhydroxyalkanoates, Biocompatibility, Implantation, Tissue response, Biodegradation

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Introduction

Development and investigation of novel biocompatible materials for modern reconstructive technologies in biomedicine is among the key issues to be addressed by biotechnology. Preclinical studies of new materials involve long duration complex experiments on animals, whose purpose is to evaluate the biocompatibility of these materials and to investigate their interactions with the organism. Factors determining response to implantation include not only the chemical structure of the material and the degree of purity of the specimen but also the shape of the implant, the process used to prepare it and the implantation site. Although recent years have seen significant achievements in biotechnology, no materials that would be completely biocompatible with living organisms have been developed. The main reasons why biodegradable polymer materials, which are in great demand, are not yet widely used are that their variety is limited and that no way has been found yet to control their functioning and degradation in living organisms.[1–4]

The discovery of polyhydroxyalkanoates (PHAs) (microbial polyesters) was a major event for the biotechnology of novel materials that gave an impetus to PHA related studies. Polyhydroxyalkanoates are thermoplastic, biodegradable and biocompatible polymers. Their potential application areas can include surgical reconstruction, cellular and tissue engineering, and organ transplantation.[5–8] Although many PHAs have been identified, only few of them are being actively studied. A homopolymer of 3-hydroxybutyric acid (P3HB) is the most widespread and best characterised PHA. It is a natural product of metabolism of higher organisms and, as proven in numerous studies, exhibits high biocompatibility and can be used for various applications. Being highly crystalline, however, P3HB is difficult to process, and the resulting products are not durable enough; moreover, as P3HB is bioresorbed *in vivo* quite slowly, i.e. it stays in the organism for long periods of time, it may cause tissue inflammation in the implantation site.[9–10] Copolymers of 3-hydroxybutyric acid with other monomers of fatty acids are more readily processable, and their properties can be varied by changing the monomers and their proportions in the polymer. Copolymers of 3-hydroxybutyric and 3-hydroxyvaleric acids (P3HB/3HV), which are less crystalline than P3HB, are also being actively studied. These polymers are highly biocompatible and are degraded *in vivo* somewhat faster than P3HB, but items prepared from these PHAs remain stable for months (1–2 years or even more).[11–12]

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PHAs that have recently attracted the attention of researchers are elastomeric copolymers of 3-hydroxybutyric and 4-hydroxybutyric acids, which are also natural products of metabolism of higher organisms, with degradation rates considerably higher than those of P3HB and P3HB/3HV.[13] In recent years, advances in biosynthesis of medium chain length copolymer PHAs containing 3-hydroxyhexanoate monomers have aroused interest in this PHA type.[14] Consequences of implantation of copolymer PHAs have, however, been scantily reported in the available literature. The published data do not provide sufficient information either on the interactions between copolymer PHAs and tissues or on their *in vivo* bioresorption behaviour.

The purpose of this study was to *in vitro* and *in vivo* investigate matrices prepared from PHAs of different chemical compositions.

Experimental

Polyhydroxyalkanoate specimens

High purity PHA specimens, a homopolymer of 3-hydroxybutyric acid (P3HB) and 3-hydroxybutyric/4-hydroxybutyric acid (P3HB/4HB), 3-hydroxybutyric/3-hydroxyvaleric acid (P3HB/3HV) and 3-hydroxybutyric/3-hydroxyhexanoic acid (P3HB/3HHx) copolymers, were produced in the Institute of Biophysics SB RAS.[15] The PHAs were synthesised by the strain *R. eutropha* B5786, registered in the Russian Collection of Industrial Microorganisms

Matrices were prepared from high purity PHA specimens that did not contain any organic impurities such as long chain o-hydroxy acids, components of lipopolysaccharide cell walls of Gram negative PHA producing strains, including *R. eutropha* B5786, which, as shown previously, can activate enzyme systems of the blood when present at a concentration of 0·01 mol.-%.[8]

Analytical methods

The PHA specimens were subjected to methanolysis, and their chemical structure was analysed by determining fatty acid methyl esters with a GCD plus gas chromatograph mass spectrometer (Hewlett Packard, USA). X-ray structure analysis and crystallinity determination of PHA specimens were performed using a D8 ADVANCE X-ray spectrometer (Bruker, Germany) (graphite monochromator on a reflected beam). Thermal properties of biopolymers were examined using differential scanning calorimetry (DSC) with a NETZSCH analyser (Germany). Melting point and thermal degradation temperature were determined from DSC curves at a given heating rate. Molecular weight and molecular weight distribution of PHAs were examined using a gel permeation chromatograph (Waters Breeze System, Waters, USA) relative to reference polystyrenes from Fluka (Switzerland, Germany). The calculated parameters included the weight average molecular weight M_w, the number average molecular weight M_n and polydispersity ($PD = M_w/M_n$).

Preparation and characterisation of polymer matrices

Matrices were prepared by casting a 1·5% polymer solution in trichloromethane heated to 35°C onto degreased Petri dishes that had been preliminarily heated to the same temperature. Produced polymer films with thickness 0·05 mm were cut out to matrices of size 10×10. The matrices were sterilised using H_2O_2 plasma in a Sterrad NX sterilisation system (Johnson & Johnson, USA). Polylactide (PLA) (Sigma) was used as controls.

The microstructure of the surface of the matrices was analysed using scanning electron microscopy (Phillips SEM 525M, JUC LIN SB RAS, Irkutsk). The surface properties such as surface free energy γ_S, interfacial free energy γ_{SL} and cohesive forces W_{SL} (erg cm^{-2}) were calculated based on the measured water contact angles using de Gennes equations.[16]

The roughness of the matrix surface was determined using atomic force microscopy in a semicontact mode (SmartSPM, 'AIST-NT', Zelenograd, Russia). Average roughness R_a and root mean squared roughness $R_q$were calculated based on 10 points, as the arithmetic average of the absolute values of the vertical deviations of the five highest peaks and lowest valleys from the mean line of the profile of the 2×2 μm surface, using conventional equations.[17]

Animal model

Response of the organism, blood reaction, local tissue reaction and polymer biodegradation dynamics were studied by implanting polymer matrices to sexually mature female Wistar rats (180–200 g each) for 6 months; the rats were obtained from the animal facility at the Institute of Cytology and Genetics SB RAS. The total number of the rats was 105, 15 per group. There were five experimental groups (with matrices prepared from PHAs of different chemical compositions) and two control groups [1, intact animals; 2, the positive control, with PLA (Sigma) matrices]. Matrices of size $10 \times 10 \times 0{\cdot}05$ mm were implanted subcutaneously. Experimental animals were anesthetised with a volatile anesthetic (diethyl ether); a 2 cm long dorsal skin incision was made; a 1 cm deep subcutaneous pocket was formed on the right of the incision, using a blunt instrument. The skin was detached from subcutaneous fat and the dorsal fascia, and three implants were inserted into the pocket; the incision was closed with silk suture using a three-stitch interrupted pattern.

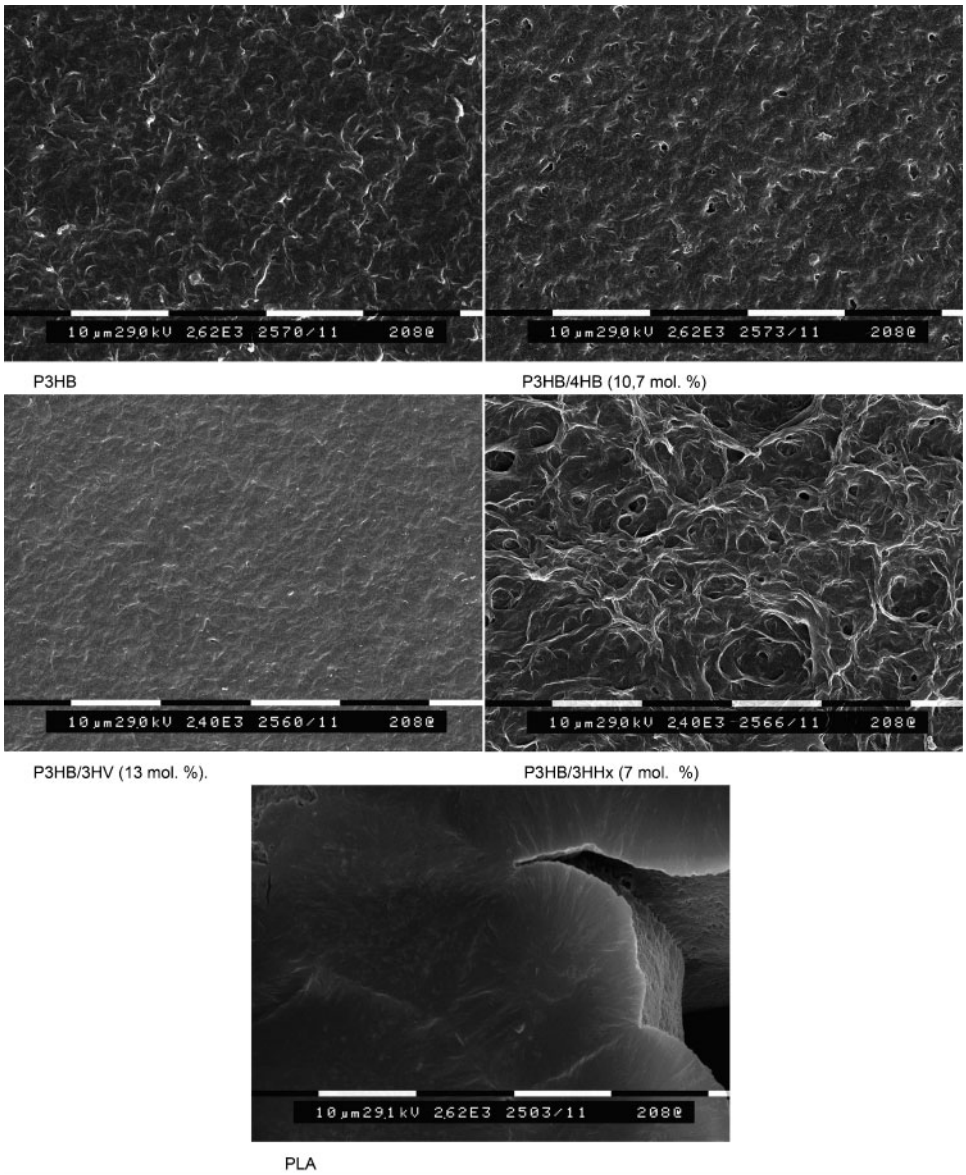
Observations of rats after surgery

At days 10, 30, 60, 90 and 180 of the experiment, three animals of each group were euthanised via overdose of ether anesthesia; their blood samples and implants with fragments of surrounding tissues were collected and analysed. Peripheral blood drawn from the tail vein was analysed using conventional techniques to determine erythrocyte sedimentation rate, hemoglobin and blood cells.

The blood serum of the rats was used to measure alkaline and acid phosphomonoesterases (AlP and AcP) using Gomori's method with a Uvikon 943 spectrophotometer (Italy); AcP measurements were performed with a Biozyme ACP/ACP-P reagent kit (Biozyme, Germany) and AlP with a Klini Test-SF AMP (Ekoservis, St. Petersburg) by a kinetic method.

Sectioning

Fragments of tissues surrounding the implants were excised out of the femur muscle, fixed with 10% formalin



1 SEM images of matrices prepared from polymers of different chemical compositions; magnification ×400 (bar=40 µm)

and embedded in paraffin, and 4–6 µm thick microscopic sections were prepared from the paraffin blocks. To analyse the general tissue response and the processes of collagen fiber development, the sections were stained according to van Gieson's method with hematoxylin–eosin and with pyrofuchsin, respectively. A Carl Zeiss Image Analysis System (Germany) was used for viewing microscopic images and analysing morphometric characteristics of sections. The estimated parameters were the length and intensity of inflammation, the dynamics of formation and thickness of the fibrous capsule, and its cellular composition. Cells in the sections were counted in 15 fields of view.

Matrix biodegradation

Residual polymer in tissues and parameters characterising changes in the PHA during degradation were measured at different time points of the experiment. Molecular mass and molecular mass distribution of PHAs were examined using a gel permeation chromatograph (Waters Breeze System, USA) relative to reference polystyrenes from Sigma (USA). The determined parameters were the number average molecular mass M_n, the weight average molecular mass of the polymer M_w and PD, which provides an estimate of the proportions of fragments with different polymerisation abilities in the polymer.

Statistics

Statistical analysis of the results was performed by conventional methods, using the standard software package of Microsoft Excel. Arithmetic means and standard deviations were found. The statistical significance of results was determined using Student's t test (significance level: $p \leq 0{\cdot}05$).

Results

Characterisation of two-dimensional matrices

Differences in basic physical properties of the tested polymers influenced the characteristics of the prepared matrices (Table 1). Images (SEM) of matrices prepared from PHAs differing in chemical composition and basic physicochemical properties showed certain differences of the surface microstructure (Fig. 1). The surfaces of matrices prepared from the homopolymer P3HB were

the smoothest, and no pores were detected. On the surface of the specimen prepared from the P(3HB/4HB) copolymer, there were numerous pores of diameter ~1 μm. The surface of the membranes prepared from P(3HB/3HV) was smoother and more homogeneous. The matrices prepared from P(3HB/3HHx) had the most uneven surface, with numerous pores of diameters varying from 0·5 to 5·0 μm. The surface of the matrices prepared from PLA had no pores and consisted of spherical layers.

Hydrophilic/hydrophobic balance of the surface is one of the main parameters that indirectly characterise biological compatibility.[18] This balance is expressed as water contact angle. Measurements of water contact angles provide a basis for calculating such significant parameters of the surface as cohesive forces, surface tension and interfacial free energy. The highest value of contact angle was recorded for PLA matrices followed by P3HB matrices. Contact angle values of the specimens prepared from copolymer PHAs of three types were much lower (Table 1). Surface energy is another major parameter that can influence the behaviour of cells. Results of this study showed that samples prepared from PLA and poly-3-hydroxybutyrate, which were the least hydrophilic of the tested matrices, had the lowest values of surface tension and cohesive forces (Table 1). For the copolymer matrices, the corresponding values were higher. Investigation of the roughness of PHA matrices surfaces yielded the following results: the root mean squared roughness R_q of PLA matrices was 241·629 nm, twice as high as the corresponding parameter of the matrices prepared from any of the tested PHA. The R_q values were similar for the homopolymer P3HB and for the copolymers of 3-hydroxybutyrate with 4HB, 3HVm and 3HHx, falling within the 109–113 nm range.

Effects of PHA implantation and tissue response

All animals of five experimental 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 experimental groups and control ones (intact rats and the positive control, rats with implanted PLA matrices). Relative masses of internal organs of experimental rats 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. There were no significant differences in these parameters between experimental groups of rats with implants prepared from PHAs of different chemical compositions, i.e. the presence of other monomers (4-hydroxybutyrate, 3-hydroxyvalerate and 3-hydroxyhexanoate) in addition to 3-hydroxybutyrate did not cause either an inflammatory reaction of the organism or a change in the blood counts.

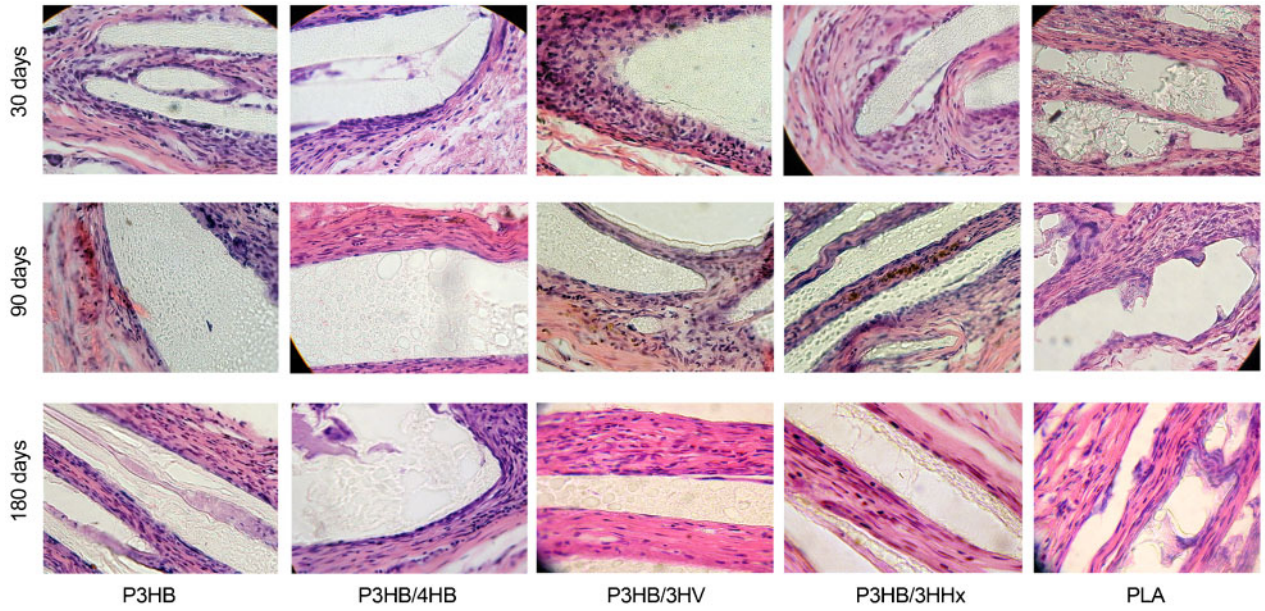
All matrices stayed at their implantation sites, and each was surrounded by a thin fibrous capsule. No necroses, hematomas, granulomas or pronounced swelling was observed in the fibrous muscle tissue surrounding the implants. Tissue behaviour after the surgery and subsequent implantation of 2D polymer matrices were generally typical of the wound healing process and response to the invasion by a foreign body and included such stages as traumatic inflammation, *de novo* formation of connective tissue, and scar formation and reorganisation.[19–21]

At day 10 after surgery, microscopy of tissues around the PHA implants of all types did not show any swelling of these tissues. The implants were surrounded mainly by monocytes (macrophages and lymphocytes), neutrophils and fibroblasts. The onset of the formation of a fibrous capsule around each matrix was observed at this stage. No foreign body giant cells (FBGCs) were detected, but extensive macrophage and fibroblast infiltration was observed at the PHA matrix/tissue interface. The initial tissue reaction to the implantation of PHA matrices was less pronounced than the reaction to matrices prepared from PLA; the number of neutrophils and lymphocytes around the reference implant (PLA) was higher. No destruction of PHA matrices was detected.

At day 30 after surgery, a thin fibrous capsule was formed around each matrix (Fig. 2). No penetration of connective tissue into the implant was observed. No necroses, hematomas, lymphohistiocytic infiltration or swelling was 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 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 P3HB homopolymer, and the thickest (42·36±3·43 μm) around P3HB/4HB matrices (Fig. 3*a*). The densest and the best developed capsule surrounded the control PLA matrix (56·75±4·5 μm). The number of cells infiltrating the capsules that were formed around all PHA matrices increased, mainly due to fibroblasts and active macrophages; there were a few inflammatory cells

Table 1 Characterisation of two-dimensional (2D) polymer matrices prepared from biodegradable PHAs of different chemical compositions

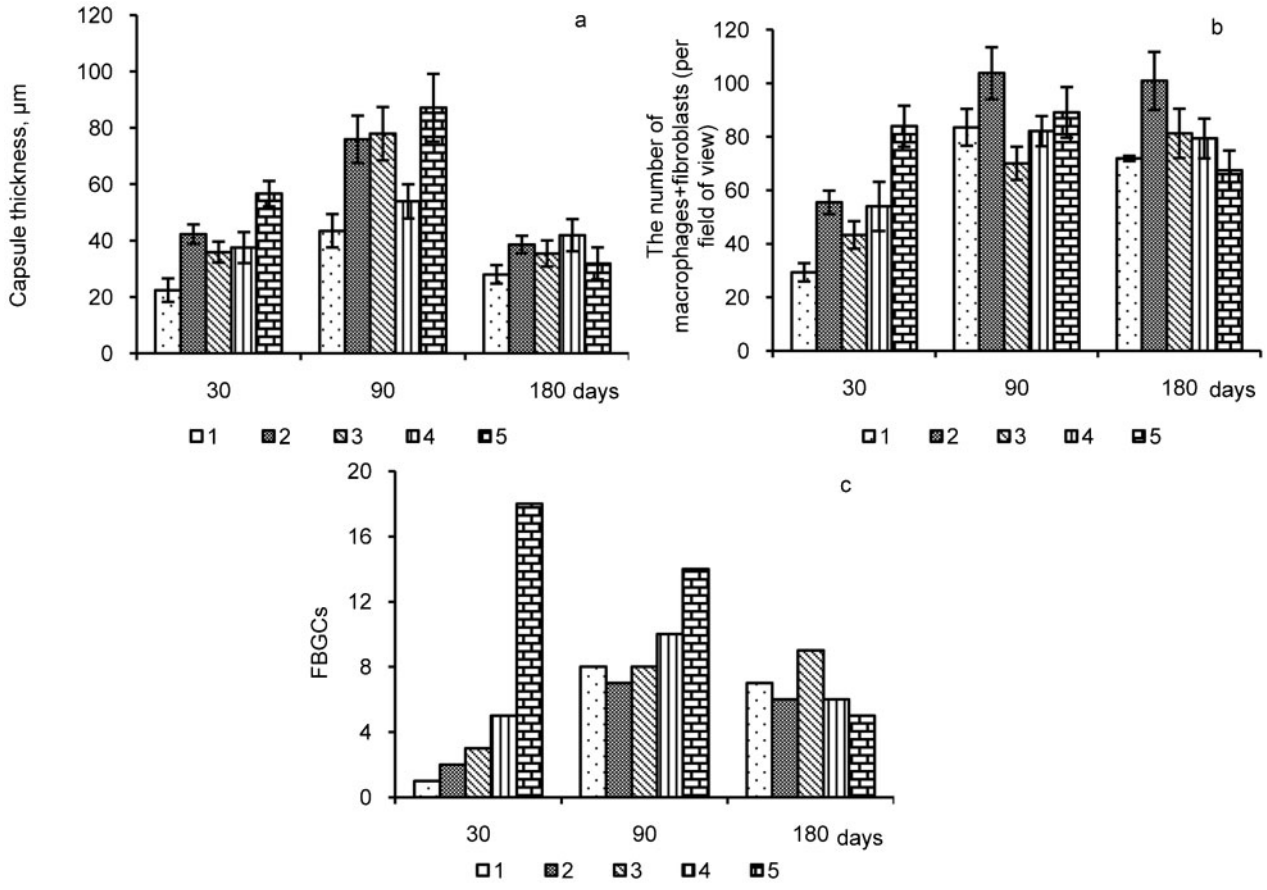
Parameter	2D matrix type, monomer composition (mol.-%)			
	P3HB (100)	P3HB/4HB (89·3/10·7)	P3HB/3HV (87/13)	P3HB/3HHx (93/7)
Weight average molecular weight M_w/kDa	652 000	634 000	603 000	315 000
Number average molecular weight M_n/kDa	144 889	285 586	208 650	152 174
Polydispersity *PD*	4·5	2·22	2·89	2·07
Crystallinity degree *Cx*/%	76	43	50	32
Melting point $T_{melt.}$/°C	179·7	172	162	158
Thermal decomposition temperature $T_{decomp.}$/°C	273	268	266	240
Water contact angle θ/°	70·0±0·4	57·4±0·6	60·3±2·8	60·9±1·6
Arithmetic mean value of roughness R_a/nm	93·200	92·909	98·682	99·12
Root mean squared roughness R_q/nm	109·390	113·062	107·931	111·1



2 State of 2D matrices prepared from polymers of different chemical compositions and morphology of surrounding tissues 30, 90 and 180 days after surgery; stained with hematoxylin–eosin; magnification ×400 (bar=40 μm)

(neutrophils and lymphocytes) in surrounding tissues. The number of neutrophils and lymphocytes in the capsule around the control matrix was several times greater. The number of FBGCs, which are agents of bioresorption of PLA and PHAs, increased considerably. No cracking or fragmentation of PHA matrices was recorded.

At day 60 after surgery, no significant changes were observed in the state of the tissues and structure of the fibrous capsules surrounding the experimental PHA matrices, although the number of FBGCs increased, while the number of inflammatory cells decreased. At day 90 after surgery, no necroses, hematomas, lymphohistiocytic infiltration or swelling was observed in the



1: P3HB; 2: P3HB/4HB; 3: P3HB/3HV; 4: P3HB/3HHx; 5: PLA

3 Morphometric characterisation of tissue response to implantation of 2D matrices prepared from polymers of different chemical compositions

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 (Fig. 2). The capsules consisted of two distinct layers. The inner layer comprised one third of the total thickness and was composed of loose fibrous tissue with numerous macrophages, fibroblasts and some FBGCs. The outer layer was composed of dense fibrous tissue consisting of collagen fiber bundles and fibrocytes adhering to them; there were fewer cells in the infiltrate. The collagen of the outer layer of the capsules was mostly mature, with fibrocytes adjacent to it; on the inside, there was a thin layer of fibroblasts with some macrophages, and the young fibrous tissue was moderately infiltrated by leukocytes. 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 (Fig. 3*c*). The matrices were significantly destroyed.

At day 180 after surgery, the capsules surrounding PHA matrices were substantially thinner (Fig. 2): 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 (Fig. 3*b*). There were macrophages 'lying' on the surface of polymer fibers; some FBGCs had 10–12 nuclei. Fibroblasts were mostly mature. No inflammatory cells were detected. Mature connective tissue represented by collagen fiber bundles and fibrocytes adhering to them was formed in the peripheral regions of the capsule. Active macrophages phagocytising the polymer and FBGCs were also observed there, suggesting migration of matrix disintegration products. Almost all matrices, except the PHB based ones, were considerably destroyed and broken into pieces. P3HB/3HHx, P3HB/4HB and PLA matrices were present as few fragments of destroyed implants.

Polyhydroxyalkanoate biodegradation

In addition to morphological examination of histological sections, destruction kinetics of matrices prepared from PHAs of different chemical compositions was also studied using measurements of residual polymer in tissues at different time points of the experiment and determination of parameters that characterise changes in PHAs during degradation.

The PLA (control) matrix was degraded at the highest rate. Surface defects could be observed at day 10; at day 30, PLA residual mass decreased to 60% of its initial value; at day 60, it dropped to 36%, and at day 90 to 10–15% of its initial value. At day 180, only trace amounts of the residual mass of the PLA matrices were detected. The second most actively degraded matrices were those prepared from P3HB/3HHx and P3HB/3HB. At day 30, their residual mass amounted to about 75–80% of their initial mass; at day 90, it dropped to 20 and 33%, decreasing to 10 and 20%, respectively, by the end of the experiment (day 180). Matrices prepared from P3HB/3HV with 13 and 27·5 mol.-% of 3HV were degraded in a similar fashion, and their degradation rates were lower than those of the copolymers described above. At day 90, their residual mass was ~40% of its initial value, and at day 180, it decreased to 30–35%. P3HB matrices were the most durable: their degradation became noticeable at day 90, when it reached 25%, and at day 180, the residual mass of these matrices amounted to 45% of its initial value.

Analysis of biodegradation of high molecular weight compounds involves investigations of changes in polymer molecular weight, including determination of such parameters as M_w, M_n and *PD* (Table 2) These parameters were studied for all PHA matrices and the PLA (control) matrix, using high performance liquid chromatography. During the course of the experiment, the weight average molecular weights M_w and the number average molecular weights M_n of all matrices decreased. The PLA (control) matrix, whose initial M_w and M_n were considerably lower than those of PHA matrices, showed the most rapid and significant decrease in its molecular weight. One month after implantation, the PLA M_w and M_n dropped by nearly half; by the end of the experiment (day 180), they had decreased to 13 and 12%, respectively, of their initial values. The M_w and M_n values of the matrices prepared from different PHAs, whose degradation occurred with dissimilar rates and was generally slower than that of PLA, also decreased consistently. The quickest and the most significant decrease in the M_w was recorded for P3HB/4HB matrices: to 58% of its initial value at day 30 and to 22% at day 180. The weight average molecular weight of the matrices prepared from P3HB/3HV and P3HB/3HHx decreased similarly: at day 30, M_w was somewhat higher than 60% of the initial M_w, and at day 180, it decreased to 26·5, 28·9 and 28·5% of the initial M_w values of 3-hydroxybutyrate/3-hydroxyvalerate (13·0 mol.-% 3HV and 27·6 mol.-% 3HV) and 3-hydroxybutyrate/3-hydroxyhexanoate, respectively.

Polyhydroxyalkanoate PD, which provides an estimate of the proportions of fragments with different polymerisation abilities in the polymer, decreased in almost all matrices, although it remained practically unchanged in PLA and P3HB/3HV (13 mol.-% 3HV). The decrease in PD of P3HB/4HB matrices was similar to that of P3HB/3HHx ones (by 1·3 times of the initial

Table 2 Comparative characterisation of PHA matrices in course of *in vivo* biodegradation

Materials		M_w/kDa	M_n/kDa	*PD* (M_w/M_n)
P3HB	Initiate	652	144	4·5
	30 days	619	176	3·5
	90 days	459	112	3·9
	180 days	210	63	1·9
P3HB/4HB 10%	Initiate	634	285	2·3
	30 days	370	169	2·18
	90 days	290	161	1·82
	180 days	140	83	1·7
P3HB/3HV 13%	Initiate	603	208	2·9
	30 days	380	96	3·94
	90 days	318	113	2·8
	180 days	160	51	3·1
P3HB/3HV 27·6%	Initiate	587	203	2·9
	30 days	360	144	2·5
	90 days	309	94	3·2
	180 days	170	95	1·8
P3HB/3HHx 7%	Initiate	315	152	2·07
	30 days	212	105	2·01
	90 days	170	95	1·8
	180 days	90	56	1·6
PLA	Initiate	90	50	1·8
	30 days	42	22	1·9
	90 days	38	20	1·85
	180 days	12	6	1·8

values); the drop in P3HB PD was more pronounced (2·3 times) (Table 2).

Thus, experiments with subcutaneously implanted 2D PHA matrices showed that the most rapidly degraded matrices were those prepared from copolymers containing 3-hydroxyhexanoate and 4-hydroxybutyrate. P3HB/3HV matrices were degraded more slowly, and P3HB matrices were the most durable. In the PHA matrices that were degraded more slowly, giant cell reaction developed later. The highest degradation rate was recorded for P3HB/P3HHx, followed in descending order by P3HB/4HB, P3HB/3HV and P3HB.

Discussion

In this work, we prepared and investigated matrices based on PHAs of different compositions. The main advantage of PHAs is that they can consist of monomers with different carbon chain lengths. However, a PHA containing not only hydroxybutyric acid but also other monomers needs to be thoroughly tested for biocompatibility. Analysis of the available literature showed that the published data on biological compatibility of PHAs with different chemical structures are incomplete. Moreover, authors of studies[12] consistently report that although short chain length PHAs (poly-3-hydroxybutyrate and copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate) are biocompatible, early tissue response to these PHAs is more pronounced than to, for example, PLAs. Other PHAs such as 3-hydroxybutyrate/4-hydroxybutyrate and 3-hydroxybutyrate/3-hyd roxyhexanoate copolymers are currently considered to be more promising materials. Few studies, however, address mechanisms of PHA *in vivo* degradation and interaction with tissues.

In the study performed by Qu *et al.*,[16] discs prepared from PLA, P3HB and P3HB/3HHx (the 3-hexanoate percentage being unreported) were subcutaneously implanted to rabbits. The results obtained by the authors showed that P3HB/3HHx was degraded *in vivo* faster than P3HB but slower than PLA. PLA exhibited more significant inflammatory response, which could be attributed to the fact that PLA degradation is associated with the leaching of lactic acid and, thus, acidification of tissues. They concluded that all materials studied exhibited high biocompatibility, without causing strong tissue response, with thin capsules formed around the implants and slight cellular infiltration.

Zhou *et al.*[17] compared tissue response to implantation of P3HB/3HHx (12 mol.-%HHx) and commercial matrices used for tarsal repair in upper eyelid reconstruction in rats. Tissue response was somewhat different from that observed in the study described above. Although P3HB/3HHx showed inflammatory tissue responses in the first 2 weeks, this material was found to be biocompatible: there was no adverse tissue response to the implantation of P3HB/3HHx matrices, and normal tissue formation was observed at the implantation site.

Ying *et al.*[22] studied bioabsorption behaviour of electrospun ultrathin fibers of P3HB homopolymer and copolymers of P3HB/3HHx (5 mol.-% 3HHx) and P3HB/4HB (7 and 97 mol.-% 4HB) and tissue response to their subcutaneous implantation. All implants showed comparable tissue response. The copolymer containing 97 mol.-% 4HB was destroyed within 4 weeks and was present as small fragments with a thin fibrous capsule surrounding them; the number of macrophages was greater than around implants prepared from other PHAs. After 12 weeks, the capsule became very thin, and no inflammatory cells were observed. Similar tissue reaction to implantation of P4HB was observed in other works.[23–24] The authors, however, did not observe a decrease in inflammatory cells around implants prepared from P3HB/3HHx (5 mol.-% 3HHx) and P3HB/4HB (7 mol.-% 4HB); inflammation resulted in the formation of a fibrous capsule around these implants.

The authors of the papers cited above[16–17,22] did not, however, report either the PHA recovery techniques or the degree of chemical purity of the specimens used in their studies. This is, though, a very significant issue as even trace amounts of macromolecules of bacterial biomass in the recovered polymer can cause pyrogenic and inflammatory reactions and activate the enzyme system of the blood. As Williams and Martin reported in their review,[5,25] polymers used in some PHA studies are not medically pure and contain bacterial cell debris and trace amounts of cell macromolecules. A number of studies performed by our team have shown that high purity P3HB and P3HB/3HV specimens exhibit high biocompatibility and can be used in contact with blood;[18] they can be effectively used as sutures in general surgery[23,26] and abdominal surgery;[24] they can also serve as a basis for preparing implants for bone replacement,[27] matrices for cell cultures,[28] and micro- and nanoparticles for drug delivery, which can be administered intraperitoneally, intramuscularly and intravenously.[29–30]

This study compared properties of 2D PHA matrices prepared from a homogenous polymer of 3-hydroxybutyric acid, 3-hydroxybutyrate/4-hydroxybutyrate, 3-hydroxybuty rate/3-hydroxyvalerate and 3-hydroxybutyric/3-hydroxy hexanoate copolymers, and those of the control–PLA matrices. All PHA specimens were of high purity and did not contain any organic impurities.

The 180 day experiment did not show any adverse effects of subcutaneously implanted PHA matrices on physiological and biochemical parameters of the rats. Tissue responses to implantation of PHA and PLA matrices were generally similar, but in the early after surgical period, the response to PHAs was less pronounced. Polyhydroxyalkanoate implants did not show such adverse responses as suppurative inflammation, necrosis, calcification or malignisation of fibrous capsules surrounding them at any time point of the 180 day experiment. 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 polymers of this kind.

In vivo degradation behaviours of matrices prepared from PHAs of different chemical compositions were not the same: the least crystalline PHA copolymers (P3HB/4HB and P3HB/3HHx) were degraded with the highest rates, while P3HB, a homopolymer, whose crystallinity was the highest, exhibited the slowest degradation (76%). Degradation of P3HB/3HV matrices was more pronounced than that of P3HB matrices and less pronounced than degradation of P3HB/4HB and P3HB/3HHx ones. All PHA matrices were degraded at

slower rates than PLA (control), and these results agree well with the data reported by other authors.[16] They also support the notion that enzymes preferentially attack hydrophilic regions of the PHA, which more readily absorb liquids, thus causing them to be faster degraded in biological media.[9,31] The decrease in the M_w and M_n of the PHAs implanted subcutaneously to laboratory animals for an extended period of time also supports the data reported by other authors, suggesting that PHA copolymers are more effectively degraded than the high crystallinity homogenous P3HB.[16,21]

Measurements of PD of different PHAs during the course of *in vivo* biological degradation showed that it decreased in almost all matrices, although it remained practically unchanged in PLA (control) and P3HB/3HV (13 mol.-% 3HV). The reason for this is that M_w and M_n decreased proportionally. A similar effect was described by Qu *et al.*[16] for P3HB matrices that were also implanted subcutaneously. The decrease in PD that we recorded in the matrices prepared from P3HB, P3HB/4HB, P3HB/3HHx and P3HB/3HV (27·6 mol.-% 3HV) indicates that during the course of degradation of these PHAs, low molecular weight fractions were removed and polymer mass became more homogenous.

Thus, matrices prepared from copolymer PHAs that, in addition to 3-hydroxybutyrate, contained 3-hydroxyhexanoate or 4-hydroxybutyrate exhibited the fastest degradation rates. P3HB/3HV matrices were degraded more slowly, and P3HB matrices were the most durable. In the PHA matrices that were degraded more slowly, giant cell reaction developed later. The degradation rates of PHAs decreased from P3HB/P3HHx to P3HB/4HB, P3HB/3HV and P3HB.

Conclusions

Experiments in vivo showed high biocompatibility of matrices prepared from PHAs of different chemical compositions: a homopolymer of 3-hydroxybutyric acid, copolymers of 3-hydroxybutyric and 4-hydroxybutyric acids, 3-hydroxybutyric and 3-hydroxyvaleric acids, 3-hydroxybutyric and 3-hydroxyhexanoic acids. No significant differences were found in tissue responses to the implantation of 2D matrices prepared from these PHAs; P3HB did not cause a more pronounced tissue response than the copolymers tested in this study. The chemical composition of a PHA determines its *in vivo* biodegradation. The highest degradation rate was recorded for P3HB/P3HHx copolymers, followed in descending order by P3HB/4HB, P3HB/3HV and P3HB.

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