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Biocompatibility and Resorption of Intravenously Administered Polymer Microparticles in Tissues of Internal Organs of Laboratory Animals

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Abstract

Specimens of ^{14}C-labeled polymer of 3-hydroxybutyric acid, P(3-HB), with different initial molecular weights, were used to prepare microparticles, whose morphology was not influenced by the M_w of the polymer. During the particle preparation process, P(3-HB) molecular weight decreased by 15–20%. Sterile microparticles (mean diameter 2.4 μm) were injected into the tail veins of Wistar rats (5 mg/rat). The effects of the particles administered to rats were studied based on the general response of animals and local response of internal organ tissues and blood morphology; no adverse effects on growth and development of the animals or unfavorable changes in the structure of the tissues of internal organs were observed. Measurements of radioactivity in tissues showed that ^{14}C concentrations are different in different organs, changing during the course of the experiment. The main targets for ^{14}C-labeled microparticles were tissues of the liver, spleen, and kidneys. Comparison of radioactivity levels and residual contents of high-molecular-weight matrix in tissues suggested that the most rapid metabolism and degradation of P(3-HB) occurred in the liver and spleen. Gel-permeation chromatography showed that at 3 h after the microparticles were injected into the bloodstream, polymer degradation started in all examined organs, except the lungs; at 12 weeks, the M_w of the polymer matrix was as low as 20–30% of its initial value. The presence of high-molecular-weight (undegraded) polymer in the tissues at 12 weeks after administration of the particles suggests that P(3-HB) is degraded in tissues of internal organs slowly and, hence, P(3-HB)-based microparticles can be used as sustained-release drug-delivery systems.

Keywords

Resorbable polyhydroxybutyrate, microparticles, intravenous administration, ^{14}C products of polymer resorption, tissues of internal organs

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1. Introduction

Designing controlled drug-delivery systems (CDDS) is a promising and rapidly developing line of modern pharmacology [1]. The mode of administration of these systems and their biocompatibility are very important aspects. The most promising CDDS seem to be those based on micro- and nanocarriers, which can be injected subcutaneously or intramuscularly, adapted for oral administration or inhalation, and infused into the bloodstream [2]. Microparticles, although containing a small amount of biomaterial, have a large surface area, which can cause stronger tissue reaction than implanted pellets, film matrices or rods [3, 4]. However, to date, there are only a few studies investigating *in vivo* effects of the CDDS, and scarcely any data are available regarding infusion of microcarriers. Finding materials for intravenous sustained-release drug delivery systems presents a special challenge. Blood-compatible materials must not induce thromboses, thromboembolisms, antigenic response, and destruction of blood constituents and plasma proteins; they must also retain their mechanical–physical properties [5, 6]. Thus, use of proper materials is the key aspect for constructing effective drug microcarriers. Materials used to construct CDDS must be absolutely harmless to an organism and possess the necessary physical–mechanical and biomedical properties, including degradability in biological media.

The effectiveness of microcarriers and their administration and delivery routes are determined, among other things, by their size and shape. Recently, this parameter has been given special consideration. Studies using silica, zinc oxide, and gold micro- and nano-particles showed that particles of these materials smaller than 100 nm exhibit new, unusual properties and biological effects. They can penetrate cells and be selectively accumulated in different types of cells and their compartments, are capable of transcytosis across epithelial and endothelial cells, can move along dendrites and axons, and cause oxidative stress and inflammation [7–9].

Thus, for designing CDDS the availability of proper material is a crucial issue. Recently, many studies have been published that address construction of CDDS based on biodegradable polymers [10]. Using biodegradable polymers as drug carriers (matrices) can be a very promising approach, as by varying the structure of polymeric carriers one can change pharmacological properties of the drug, including its biodistribution, membrane permeability, biotransformation and pharmacodynamics. At the present time, the most widely used and actively developed biomaterials are aliphatic polyesters, polyamides, segmental polyester urethanes, polymers of lactic and glycolic acids (polylactides and polyglycolactides), silicone and poly(ethylene terephthalate) (PET), which have recently been joined by polyhydroxyalkanoates (PHAs). PHAs are thermoplastic linear polyesters of hydroxy derivatives of alkanoic acids, which have been actively investigated as carriers of bioactive compounds and scaffolds for bioartificial organs and tissues [11, 12]. In contrast to polylactides and polyglycolactides, PHAs are bioresorbed slowly and their degradation in biological media does not change the pH of the medium [1, 13].

Comprehensive PHA studies conducted at the Institute of Biophysics (Siberian Branch of the Russian Academy of Sciences, Krasnoyarsk) proved that these polymers can be used in medicine, including blood-contacting applications [14], as a suture [15, 16], as bone replacement material [17], as a scaffold for functioning cells [18] and as a matrix for drug delivery [19].

The purpose of this study is to investigate the effects of intravenous administration of microparticles based on a polymer of 3-hydroxybutyric acid (poly(3-hydroxybutyrate), P(3-HB)), taking into account the response of tissues of internal organs, degradation of the polymer, changes in its molecular weight, and the distribution of ^{14}C-labeled polymer biodegradation products among different tissues.

2. Materials and Methods

2.1. Preparation of ^{14}C-Labeled PHB Polymer

The tested material was P(3-HB) ($C_4H_6O_2$), which is a homo-polymer of D(-)-3-β-HB, an isotactic polyester with regular $C_4H_6O_2$ units. The specimens were synthesized by the bacterium *Ralstonia eutropha* B5786. The strain is registered in the Russian Collection of Industrial Microorganisms. The culture technique has been described elsewhere [20].

The ^{14}C-labeled polymer was prepared by adding 1,2-[^{14}C]acetate (6×10^4 cpm/ml) as substrate to the bacterial culture in the polymer-synthesis phase. P(3-HB) is extractable from the bacterial biomass due to its ability to dissolve in organic solvents and to be then precipitated by alcohols. The polymer was extracted from the bacterial biomass with chloroform and precipitated with ethanol. The extraction of the polymer from biomass was conducted in several stages. In the first stage, to partially destroy the cell wall and attain a fuller extraction of lipids, bacterial biomass was centrifuged (for 15 min at 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 a backflow condenser for 30 min. Then the alcohol was removed, the biomass was covered with 86% ethanol and separated from alcohol by centrifuging. Then, 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 as a reagent. The procedure of re-dissolution and further precipitation of the polymer was repeated several times to prepare specimens that would not contain organic impurities of protein, carbohydrate or lipid nature. We have previously shown that this procedure can be used to prepare high-purity P(3-HB) specimens, which would not contain any organic impurities (components of bacterial biomass) and would exhibit excellent biocompatibility characteristics, allowing them to contact blood. The trademark of the material is Bioplastotan [21].

The chemical purity of the resulting specimens was tested by conventional biochemical methods. The presence of protein impurities was determined by the

Kjeldal micro-method [22] and carbohydrates by the anthranone method [23]. To determine the P(3-HB) composition, the specimens were subjected to methanolysis [24] and then analyzed using an Agilent 5975 Inert gas chromatograph-mass spectrometer (Agilent).

The radioactivity of the prepared P(3-HB) was 1.4×10^4 cpm/mg polymer. The radioactivity of the samples was measured using a Tri-Carb 2100TR scintillation counter (Packard BioScience).

2.2. Properties of the Polymers

P(3-HB) samples were subjected to X-ray structure analysis and crystallinity determination prior to the experiment and after 160 days of exposure to seawater. Measurements were conducted using a D8 Avance X-ray spectrometer (Bruker) (graphite monochromator on a reflected beam) in a scan-step mode, with a 0.04° step and exposure time 2 s, to measure intensity at point. The instrument was operating at 40 kV and 40 μA.

Integrated thermal analysis of the specimens was performed using a STA 449 Jupiter simultaneous thermal analyzer (Netzsch), which can simultaneously measure changes in the mass (thermogravimetry) and heat flows (differential scanning calorimetry), coupled with a QMS 403 Aeolus quadrupole mass spectrometer for analysis of gases released by heated samples.

Molecular weight and molecular-weight distribution of PHAs were examined using a gel-permeation chromatograph (Waters Breeze System) relative to reference polystyrenes from Sigma. The number-average molecular weight was calculated as follows:

$$M_{\mathrm{n}} = \Sigma((N_i \cdot M_i)/N),$$

where N_i is the number of molecules of mass I, N is the total number of molecules and M_i is the mass of molecules of length I.

The weight-average molecular weight of the PHA was determined as follows:

$$M_{\mathrm{w}} = \Sigma(w_i \cdot M_i),$$

where w_i is the portion of the mass ($w_i = N_i M_i / \Sigma(N_i \cdot M_i)$).

Polydispersity, which provides an estimate of the proportions of fragments with different polymerization abilities in the polymer, was calculated from the formula:

$$\mathrm{PD} = M_{\mathrm{w}}/M_{\mathrm{n}}.$$

2.3. Preparation of Microspheres

Microparticles were prepared from P(3-HB) specimens of different M_{w}, 340 000 and 860 000, with PD 3.3 and 2.7, respectively.

The ^{14}C-labeled microparticles were prepared by the solvent evaporation technique, using a triple emulsion. Polymer (600 mg) and 200 mg poly(ethylene glycol) (PEG40, molecular weight 40 000) were dissolved in 10 ml dichloromethane. Then,

1 ml of a 6% gelatin solution (40°C) was added and the mixture was shaken vigorously. The resulting water/oil (W/O) double emulsion was allowed to cool to room temperature and then it was gradually poured into 150 ml of a 0.5% PVA solution, which was stirred with a three-blade propeller (at 2000 rpm, for 20 min) (Heidolph), to obtain a triple (water/oil/water, W/O/W) emulsion. The emulsion was continuously mixed mechanically for 24 h, 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 freeze dryer (Prointex).

2.4. Microsphere Characterization

The size of microspheres with a diameter larger than 3 μm was determined using an Automatic Particle Counter + Analyser system (Casy TTC, Scharle System). The obtained size distribution was used to describe the particle size.

For intravenous injections, a fraction of microspheres with a diameter smaller than 3.8 μm was selected, using a track filter. The size of particles was evaluated using the Scan master program of a CMM-2000 scanning multimicroscope, taking into account the parameters that determined the image size.

The diameter of the particles used in the experiment was not larger than 3.8 μm. Particles taken for intravenous injection ranged from 0.5 to 3.8 μm. The microparticles used in the experiment had an average diameter of 2.4 ± 0.21 μm.

Electron microscopy was performed using an FEI Company Quanta 200 scanning electron microscope coupled to an EDAX attachment for X-ray microanalysis with nitrogen-free cooling (Genesis XM 2 60 Imaging SEM with Apollo 10).

2.5. Animal Model

Experiments were conducted on 36 adult female Wistar rats (200–240 g each) 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 [25]. The rats were divided into two experimental groups, 18 rats per group. Microparticles prepared from P(3-HB) with an initial M_w of 340 000 were injected to rats of the first group and microparticles prepared from P(3-HB) with an initial M_w of 860 000 to the rats of the second group. A sterile suspension of microspheres (5 mg/0.5 ml physiological saline, 5×10^4 cpm/g) was injected to rats of both groups through the tail vein, without any anesthesia. Intact animals were used as control.

Three h after the injection, 24 h after the injection, and then every week, 3 animals were killed by an overdose of a volatile anesthetic. Their internal organs were removed, examined macroscopically and histologically, weighed, dried and ground; then, radioactivity counts were performed. A 100 mg sample was placed into a plastic vial (PerkinElmer/Packard) containing 15 ml dioxane scintillation solution; 1 l dioxane contained 10 g 2,5-diphenyloxazole, 0.25 g 1,3-di-2,5-phenyloxazolyl

benzene and 100 g naphthalene. Radioactivity counts were performed in a Tri-Carb 2100TR scintillation counter.

Radiocarbon was measured in the heart, lungs, liver, kidneys, bone marrow and blood, without taking into account the radioactivity of soft and hard tissues, excreted metabolic products and the brain, assuming that microparticles bigger than 3.8 μm in diameter cannot cross the blood–brain barrier.

To study the ^{14}C-containing P(3-HB) degradation products in the form of 3-HB oligo-, tetra-, di- and monomers, samples of the dried tissues were subjected to methanolysis, and fatty acid methyl esters were determined using a GCD plus chromatograph mass spectrometer. To determine high-molecular-weight (undegraded) polymer in the organs, it was extracted from the tissues with chloroform and precipitated with hexane. Then the polymer was methylated and chromatographed as described above; the sensitivity was 10^{-11} g.

Dynamics of biodegradation of the polymer matrix was studied by analyzing molecular weight of P(3-HB) microparticles. The polymer extracted from the tissues of rats' organs was purified, dried, dissolved in chloroform, and analyzed as described in Section 2.2.

2.6. Histological Examination

The general tissue reaction to intravenous injection of microparticles was investigated using conventional histological techniques. Sections of tissues of internal organs were excised; the samples were fixed in 10% formalin and embedded in paraffin; 5–10 μm thick microtome sections were prepared. The tissues excised from one rat usually yielded 10 to 18 serial sections, which were stained with hematoxylin and eosin. A Carl Zeiss Image Analysis system was used for viewing microscopic images and analyzing morphometric characteristics of sections (ocular 10, objectives 10 and 40×).

2.7. Statistics

The results were analyzed statistically using the standard software package of Microsoft Excel. Arithmetic means and standard deviations were found. Significant differences between average values in control and treatment groups were tested using the Mann–Whitney U-test (significance level $P < 0.05$).

3. Results

3.1. Microsphere Characterization

Microparticles were prepared from P(3-HB) specimens with different M_w, whose physical properties were slightly different. The polymer specimen with the initial M_w of 340 000 (PD 3.3) had a crystallinity of 78%, and its melting point and degradation onset temperature were 178°C and 290°C, respectively. The polymer specimen with the initial M_w of 860 000 (PD 2.7) had a crystallinity of 76%, and its

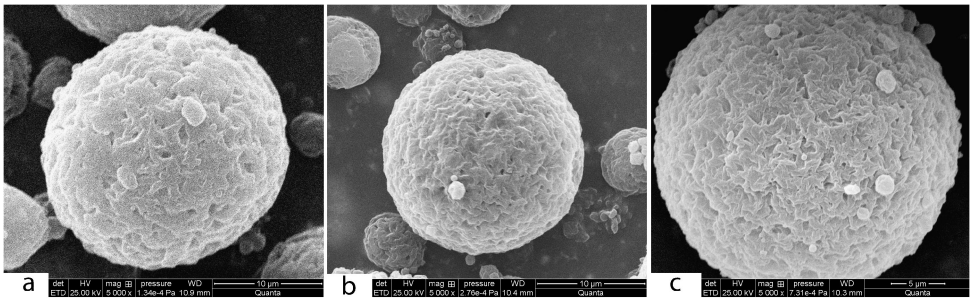

Figure 1. Micrographs of microparticles prepared from polyhydroxybutyrate (a, P(3-HB); b, ^{14}C-labeled P(3-HB) with M_w 340 000; c, ^{14}C-labeled P(3-HB) with M_w 860 000).

melting point and degradation onset temperature were 178°C and 284°C, respectively.

The initial molecular weight of P(3-HB) did not affect either the production yield of microparticles from emulsion or their properties. The mean sizes of microparticles prepared using polymers with different molecular weights were similar. In both cases, microparticles were of regular spherical shape and had a wrinkled surface with dimples and pores. Comparison of microparticles prepared from the ^{14}C-labeled polymer and the unlabeled one did not reveal any differences either (Fig. 1).

Micronization of polymer emulsion in the course of microparticle preparation changed the molecular weight of the microparticle polymer (Fig. 2). The M_w of the polymer matrix of microparticles was 15–20% lower than that of the initial P(3-HB), 289 000 and 693 000, respectively (Fig. 2).

3.2. General Response of Animals and Local Tissue Response to Intravenous Administration of Microparticles

All animals that had been injected with a microsphere suspension were healthy and ate well throughout the experiment. Their body mass and masses of their internal organs were similar to those of the rats in the control group (Table 1). The morphology of peripheral blood of experimental animals was the same as that of intact ones (Table 2).

Macroscopic examination of the rats' internal organs and histological studies of the tissue sections did not show any unfavorable changes in them throughout the observation period. Conventional histological examination did not show any adverse changes in the structure and state of the tissues of internal organs. Analysis of tissue specimens did not reveal any necrosis, clotting, or other unfavorable morphological changes in the tissues (Figs 3 and 4). The structure of internal organ tissues of all experimental animals was identical to that of intact rats throughout the observation period. Figure 4 shows sections of the rats' internal organs at the end of the experiment (after 12 weeks of observation).

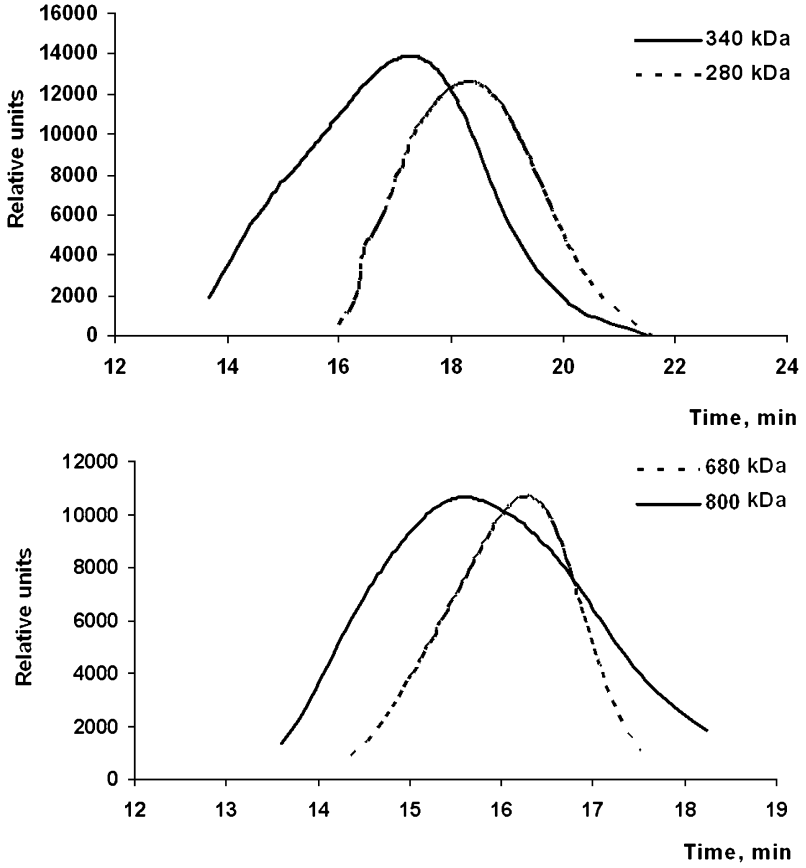

Figure 2. Chromatograms of molecular weight distribution of PHA specimens of two types: (—) initial polymer; (- - -) microparticles.

3.3. Distribution of ^{14}C in Internal Organs

The dynamics of ^{14}C distribution among tissues of internal organs were similar in both groups of rats, irrespective of the molecular weight of P(3-HB) used to prepare microparticles, and the patterns of ^{14}C accumulation varied in different tissues.

At 3 h after the injection of the microparticles into the bloodstream of the animals, the largest ^{14}C activity (14 680 ± 417 cpm/g) was registered in heart tissue. The second largest radioactivity was determined in kidney tissue (7520 ± 81 cpm/g) and the third in lung tissue (5280 ± 65 cpm/g) (Fig. 4). These results were confirmed by histological examination (Fig. 5), clearly showing the presence of microparticles in these tissues. Liver and spleen tissue contained similar ^{14}C activity (4400 ± 58 cpm/g). The lowest radioactivity levels were registered in blood (1820 ± 43 cpm/g) and bone marrow (580 ± 75 cpm/g).

At 24 h, the pattern of radioactivity distribution was radically different: the activity in heart tissue declined by 50%, while the ^{14}C level in liver tissues increased

Table 1.
Dynamics of the mass of rats' internal organs during the experiment

Group	Time (days)	Mass of organs (g)				
		Heart	Lung	Liver	Spleen	Kidney
Control	1	1.05 ± 0.2	1.4 ± 0.3	10.95 ± 1.4	0.98 ± 0.4	2.39 ± 0.2
	7	1.06 ± 0.3	1.51 ± 0.2	8.89 ± 1.1	1.1 ± 0.3	2.12 ± 0.3
	28	1.2 ± 0.1	1.67 ± 0.4	9.11 ± 1.3	1.2 ± 0.2	2.1 ± 0.1
	60	1.4 ± 0.4	1.9 ± 0.1	10.95 ± 1.2	1.2 ± 0.2	2.17 ± 0.2
	120	1.32 ± 0.3	2 ± 0.3	11.05 ± 1.4	1.18 ± 0.3	2 ± 0.3
Microparticles (M_w 289 000)	1	1.03 ± 0.2	1.56 ± 0.4	8.71 ± 1.2	0.9 ± 50.3	1.99 ± 0.1
	7	1.09 ± 0.2	1.32 ± 0.1	8.89 ± 1.2	0.99 ± 0.1	2.08 ± 0.4
	28	1.1 ± 0.4	1.67 ± 0.2	10.01 ± 1.4	1.08 ± 0.2	2.12 ± 0.3
	60	1.28 ± 0.5	1.79 ± 0.5	10.86 ± 1.3	1.18 ± 0.4	2.2 ± 0.2
	120	1.33 ± 0.3	2.2 ± 0.4	11.1 ± 1.2	1.19 ± 0.2	2.2 ± 0.2

Table 2.
Morphology of rats' peripheral blood during the experiment

Group	Time (days)	ESR (ml)	Leucocytes (10^9/l)	Erythrocytes (10^{12}/l)	Haemoglobin (g/l)
Control	1	1.5 ± 0.2	11.0 ± 0.0	6.8 ± 0.0	128 ± 17
	7	2.0 ± 0.2	10.5 ± 1.3	7.0 ± 1.1	123 ± 17.5
	28	1.8 ± 0.3	10.0 ± 1.6	6.7 ± 1.2	118 ± 19
	60	2.1 ± 0.1	10.2 ± 1.1	6.8 ± 0.9	118 ± 18
	120	1.9 ± 0.2	10.3 ± 1.2	6.3 ± 1.1	120 ± 17
Microparticles (M_w 289 000)	1	1.6 ± 0.1	11.7 ± 1.1	7.1 ± 0.9	118 ± 16
	7	2.0 ± 0.2	10.2 ± 1.1	6.9 ± 1.1	124 ± 16
	28	1.8 ± 0.3	10 ± 1.2	6.7 ± 1.1	121 ± 19
	60	1.7 ± 0.2	10.1 ± 1.3	7.1 ± 1.1	124 ± 18
	120	2.2 ± 0.2	10.5 ± 1.2	6.8 ± 1.3	123 ± 17

ESR, erythrocyte sedimentation rate.

sharply (almost 5.5 times). ^{14}C concentration in spleen tissues increased 1.8 times, practically reaching the concentration of the label in the heart at the same time point. At 7 days after the injection, the ^{14}C concentration in the liver increased again, at the same time decreasing in the heart and lungs. At 1 month, radioactivity significantly decreased in the tissues of all organs, except the spleen, which may be due to the fact that the spleen accumulates carbon-containing P(3-HB) degradation products: this organ contains not only hydrolytic enzymes but also active macrophage-type cells, which, as is well known, resorb P(3-HB) [26]. At 2 months after the injection, the distribution and concentration of microparticles was the same as at 1 month. By the end of the experiment, 84 days after the injection of the par-

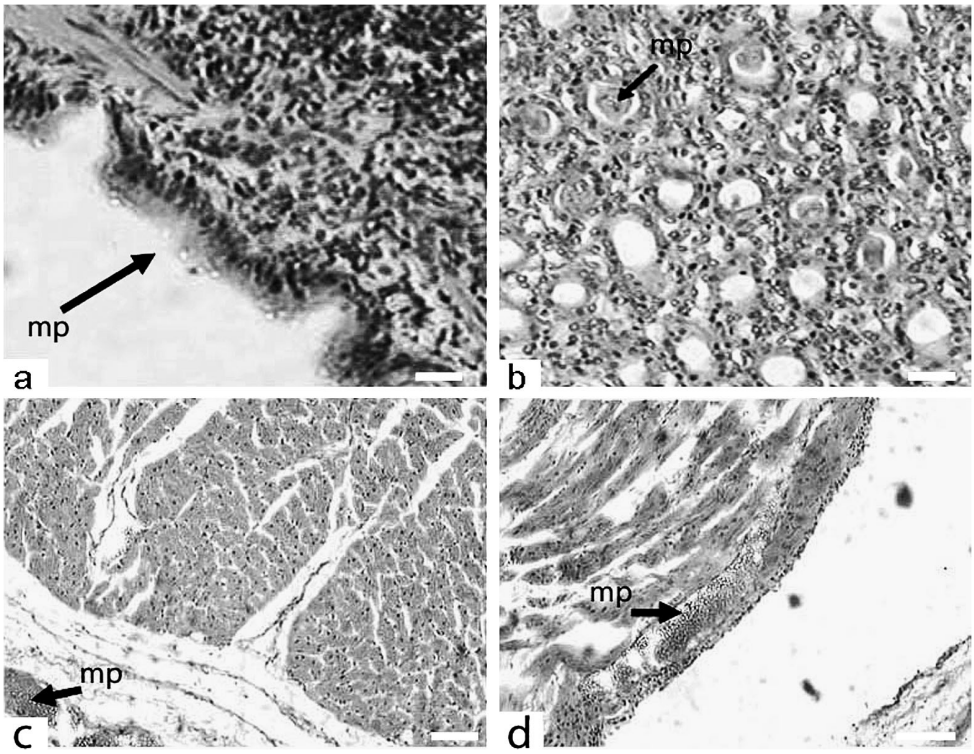

Figure 3. Microscopy of tissues of internal organs at 1 day after administration of polyhydroxybutyrate microparticles (M_w of microparticles 289 000) (hematoxylin and eosin staining). (a) Lungs, (b) kidneys, (c, d) heart; mp, microparticles. Scale bars = 10 μm (c, d) and 20 μm (a, b).

ticles, the radioactivity of heart tissues had dropped nearly 6.5-fold and that of the lungs 8-fold, compared to the initial levels, amounting to just 2126 ± 671 cpm/g and 687 ± 581 cpm/g, respectively. At the same time, the radioactivity of liver tissues still amounted to $12\,200 \pm 788$ cpm/g. The ^{14}C levels in blood and bone marrow were low, under 100 cpm/g. A similar distribution of ^{14}C-labeled microparticles was recorded in the experiment with the high-molecular-weight (693 000) polymer: accumulation of high levels of radioactivity in lung and heart tissues immediately after the injection of the particles into the bloodstream, was followed by accumulation of ^{14}C in liver, spleen and kidney tissue, and a drop in its concentration in the lungs.

We should note here that the recorded amounts of radiocarbon in the tissues do not quite accurately correspond to the accumulation of microparticles in the organs: P(3-HB), like other PHAs, is a biodegradable polyester and is bioresorbed *in vivo* by the enzymes and cell elements of blood and tissues; carbon-containing polymer degradation products get involved in biological cycles and leave the body with metabolites [26].

Our results show that, irrespective of the molecular weight of the microparticle polymer matrix, the radioactivity of the tissue of every organ that we analyzed

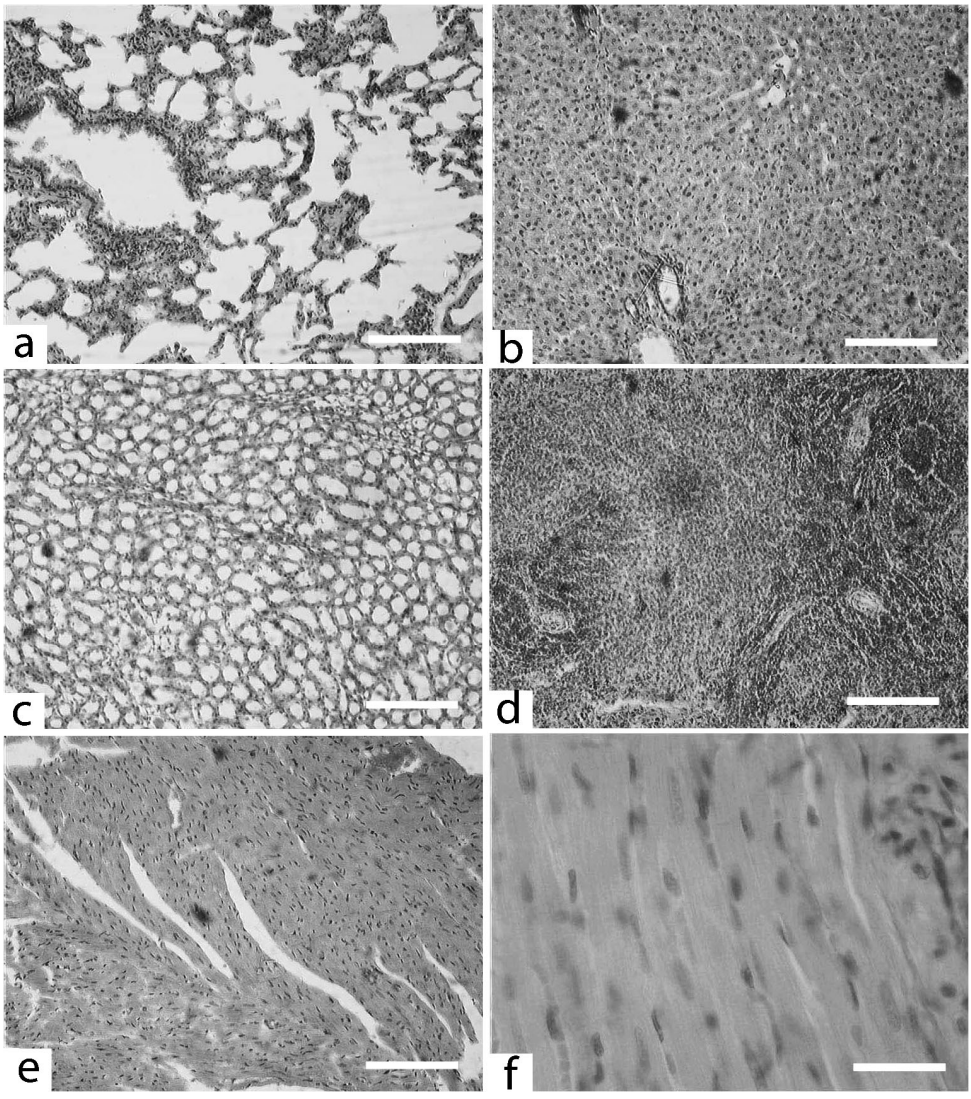

Figure 4. Microscopy of tissues of internal organs at 12 weeks after administration of pP(3-HB) (M_w of microparticles 289 000) (hematoxylin and eosin staining). (a) Lungs, (b) liver, (c) kidneys, (d) spleen, (e, f) heart. Scale bar = 10 μm (a–e) and 20 μm (f).

decreased, indirectly suggesting gradual biodegradation of the polymer matrix of microparticles. At the same time, low-molecular-weight products of polymer matrix degradation could be eliminated from the tissues of the rats' organs.

3.4. P(3-HB) Biodegradation in Internal Organs

In addition to monitoring of tissue radioactivity, the *in vivo* study of degradation dynamics of the particle polymer matrix involved chromatographic examination of the residual polymer in the rats' organs and measurements of the molecular weight of the polymer extracted from the tissues at different time points of the experiment.

As the monomer forming P(3-HB), 3-HB, is known to be a natural metabolic product of higher animals and humans, which is present in their blood (up to 3–10 mg/100 ml) [27], we examined the tissues of the intact rats' organs for the possible presence of 3-HB, in order to make our experiment more accurate. Chromatographic examination of these tissues did not detect any analytically significant concentrations of 3-HB; the tissues contained only trace amounts of 3-HB (Table 3).

To find out whether the rats' organs contained residual, i.e., undegraded, polymer, the polymer was extracted from dried tissue specimens with chloroform and precipitated with ethanol. Oligomers and monomers stayed dissolved and, thus, were not analyzed in the samples. The extracted polymer was thoroughly purified and dried and then subjected to methanolysis and chromatography, to determine concentrations of undegraded polymer in rats' organs at different time points. Table 4 shows the tissue contents of P(3-HB)s with different initial molecular weights. At 1 week after microparticles were injected into the bloodstream of rats, the highest P(3-HB) contents were recorded in the heart and lungs; P(3-HB) contents in the liver were an order of magnitude lower and polymer contents in the spleen and kidneys were two orders of magnitude lower. At 8 weeks, P(3-HB) content in the

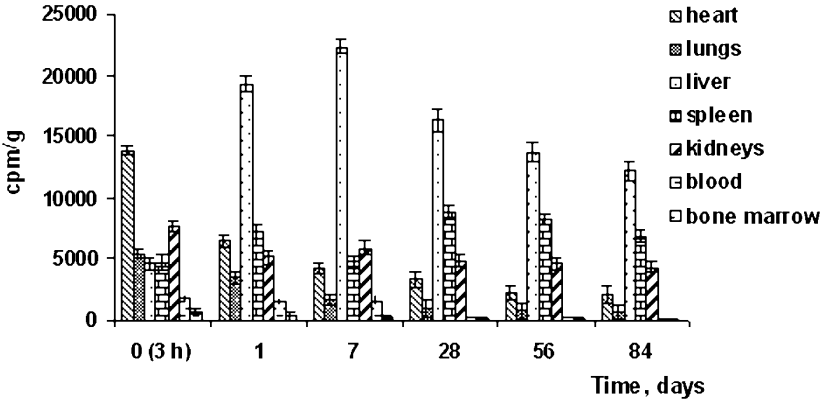

Figure 5. Specific radioactivity dynamics in tissues of rats' internal organs after intravenous injection of ^{14}C-labeled polymer microparticles (M_w of microparticles 289 000).

Table 3.
Content of 3-HB in tissues of an intact rat

Organ (% of dry weight)	3-HB (%)
Lungs	0.01
Heart	0.01
Liver	0.02
Kidneys	0.01
Spleen	0.02
Blood	0

heart and lungs dropped dramatically. By the end of the experiment (12 weeks after the microparticles were injected into the bloodstream), residual polymer contents in all organs had become equal, amounting to (4–8) $\times$ 10^{-4} mg/g for the particles prepared from the P(3-HB) with initial M_w 340 000 and (12–19) $\times$ 10^{-4} mg/g for the particles prepared from the P(3-HB) with initial M_w 860 000. The fact that the examined organs, especially the liver and the spleen, contained low amounts of the residual high-molecular-weight (undegraded) P(3-HB), while radioactivity of the tissues was high (Fig. 5), can be indicative of the rapid degradation of the polymer in these organs. The high concentrations of radiocarbon in these organs can, in our opinion, be accounted for by the presence of both residual polymer and carbon-containing P(3-HB) biodegradation products, both formed in these organs and brought from other tissues through the bloodstream.

Treatment of biological tissues with chloroform results in the extraction of both P(3-HB) with high molecular weight and 3-HB oligomers and monomers. Therefore, we performed an additional experiment, in which the tissues of the rats' organs were subjected to methanolysis followed by chromatography, and obtained the contents of 3-HB monomers in the tissues (Table 5). Concentrations of 3-HB changed during the course of the experiment, regardless of the M_w of the polymer used to prepare microparticles. Determination of 3-HB incorporated in P(3-HB) with the lower M_w (of the polymer 340 000, of microparticles 289 000) showed that in 1 week after the injection of microparticles, the largest amounts of the monomers were found in the heart and lung tissues, was more than twice the content of the monomers in the liver and spleen tissues. The lowest monomer content was recorded in kidney tissue. At 8 weeks, 3-HB monomer content in the heart and lungs decreased almost by a factor 10, compared with week 1. At 12 weeks after the injection of microparticles, the content of monomers decreased almost 30-fold in the lungs and liver, 20-fold in the heart and 45-fold in the spleen; the smallest (6-fold) decrease was recorded in kidneys. Similar dynamics of 3-HB monomer content were recorded in the tissues of the rats that were injected with micropar-

Table 4.
Residual high-molecular-weight polymer in tissues of rats' internal organs (M_w of microparticles 289 000)

Organ	PHB content (1×10^{-4} mg/organ)		
	1 week	8 weeks	12 weeks
Heart	420	30	5
Lung	600	5	6
Liver	360	110	80
Spleen	6	10	5
Kidney	16	20	7

Table 5.
Results of chromatographic analysis of methyl esters of hydroxybutyrate monomers contained in tissues of rats' internal organs (M_w of microparticles 289 000)

Organ	PHB content (1×10^{-4} mg/organ)		
	1 week	8 weeks	12 weeks
Heart	1527	132	75
Lung	1694	111	56
Liver	5861	4889	197
Spleen	606	251	14
Kidney	189	124	43

ticles prepared from the P(3-HB) with the higher M_w (of the polymer 860 000, of microparticles 289 000).

Comparison of results listed in Tables 4 and 5 with the data on tissue radioactivity changes in the course of the experiment suggests the following conclusions. First, there is a considerable disagreement between the data in Tables 4 and 5 for the time point at 12 weeks after the injection of microparticles: the polymer content determined by chromatography of the tissues of different organs was much larger (Table 5) than the amount of high-molecular-weight polymer extracted from the rats' organs (Table 4). Thus, at the end of the experiment, the major portion of radioactive polymer was present *in vivo* as low-molecular-weight polymer degradation products, including 3-HB monomers, and, probably, smaller molecules.

Another important fact suggested by the data in Table 4 is that rats' organs contained high-molecular-weight polymer, i.e., probably, parts of undegraded microparticles, at different time points of the experiment. Finally, the relatively low polymer content in different organs, liver and spleen in particular, on the one hand, and the high radioactivity of these organs, on the other, suggest rapid degradation of the polymer matrix.

An accurate picture of biodegradation of the microparticle polymer matrix in rats' internal organs was obtained from determination of molecular weight of P(3-HB) extracted from the tissues at different time points of the experiment, using gel-permeation chromatography (Fig. 6). During the experiment, we recorded a decrease in the molecular weight of microparticle polymer in all the organs, whatever the initial molecular weight of the P(3-HB) used to prepare microparticles.

At three hours after the injection of microparticles, the M_w of the polymer extracted from most of the organs decreased, indicating the breakage of polymer chains. In the spleen, M_w decreased to 53% of the initial value, which was the most significant drop, whereas the decrease was 43% in the liver and 38% in the heart. The M_w of the polymer remained almost unchanged in the lungs, where P(3-HB) metabolism is the slowest. At 2 weeks after the injection, molecular weight of

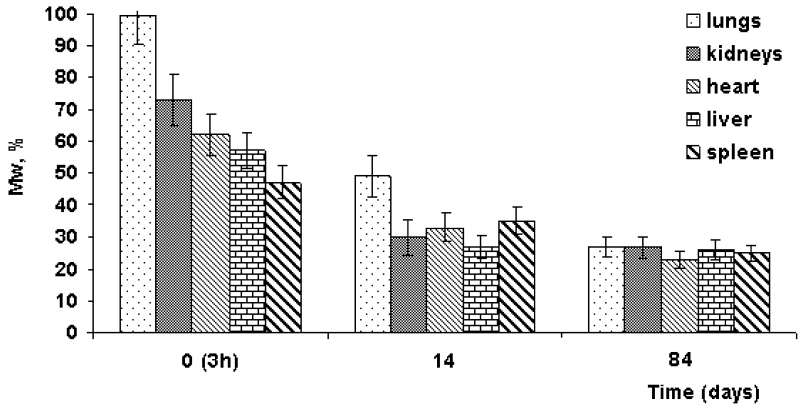

Figure 6. Dynamics of molecular weight of intravenously injected polymer microparticles in tissues of rats' internal organs (M_w of microparticles 693 000).

P(3-HB) of the microparticle polymer matrix was at least 70% lower than the initial M_w in liver, kidney, heart and spleen tissues; a less dramatic decrease (49%) was recorded in the lungs. At 12 weeks after the injection of the particles, the M_w of the polymer of microparticles extracted from all organs did not exceed 23–27% of its initial value. By the end of the experiment, the molecular weight of the microparticles prepared from the polymer with initial M_w 360 000 (289 000 for the initial microparticles) had dropped by 88–90%. At the end of the experiment, the PD of the polymers of microparticles was on average half of that at the beginning, suggesting a more uniform composition of the polymer matrix, maybe due to washout of low-molecular-weight fragments.

4. Discussion

In this paper we have for the first time presented results of an integrated study addressing the effects of infused radioactive microparticles prepared from ^{14}C-labeled polymer of 3-hydroxybutyric acid (3-HB), specifically investigating responses of the organism and tissues of internal organs, dynamics of ^{14}C distribution, and process of *in vivo* biodegradation of the polymer matrix.

Although polymer-based microparticles have recently received much attention as drug carriers, there are very few data in the available literature regarding biocompatibility of microparticles injected into the bloodstream. Kanke and co-authors [28] reported that distribution of ^{141}Ce-labeled polystyrene/divinylbenzene microparticles intravenously injected into Beagle dogs depended on the size of the particles: larger particles (over 7 μm) were retained for long periods of time (up to 4 weeks) in the lungs, while smaller (3–5 μm) ones were retained in the liver and spleen. The authors observed vascular occlusions around clusters of microspheres and pinpoint necrotic sites without tissue destruction. Studies published between the mid-1980s and early 1990s and more recent studies [7, 29, 30] that investigated the distrib-

ution of microparticles prepared from different polymeric materials in tissues of internal organs of animals (mainly rats or mice) did not address the effects of administration of the microparticles. This study shows that microparticles less than 3.6 μm in diameter, prepared from high-purity specimens of ^{14}C-labeled P(3-HB), can be intravenously injected to laboratory rats, with no adverse effects occurring in the animals. Peripheral blood counts performed at different time points of the experiment, macroscopic and histological examination of the tissues of rats' internal organs (heart, lungs, kidneys, spleen and liver) did not reveal any unfavorable response to the administration of microparticles.

Another object of this study was to examine *in vivo* distribution of microparticles prepared from P(3-HB) and their carbon-containing degradation products. As is well known, the size of microparticles is one of their main parameters, determining their distribution and accumulation in tissues of internal organs [31]. This aspect of controlled drug delivery has recently received considerable attention from scientists and designers of microcarriers. For example, bovine serum albumin-based microparticles of mean diameter 2.58 μm were intravenously injected into mice; in 24 h after injection, 11.74% of the particles were found in the liver, 8.5% in the kidneys, 7.69% in the lungs, and 5.67% in the spleen [32]. Smaller (<50 nm in diameter) triglyceride particles surrounded by a surfactant shell (2-hydroxy-stearate of PEG and lecithin) labeled with rhenium and technetium were injected into rats; at 30 min of the experiment, they were localized in the heart but at 1 h the maximum radioactivity was recorded in the liver and spleen. However, at the 24-h time point the remaining activities in the liver and spleen were 5% and 2.5% of the initial value, respectively [33]. Intravenously injected 5-μm microparticles prepared from water-soluble poly(2-methyl-2-oxazoline) were not retained in the tissues but were rapidly excreted by the kidneys. The lowest concentration of the polymer was found in the brain, indicating that poly(2-methyl-2-oxazoline) does not cross the blood–brain barrier [34]. As noted by Owens and Peppas in their review [35], analysis of the data on *in vivo* distribution of particles prepared from different materials shows that the main targets of intravenously injected particles are organs of the reticuloendothelial system, namely the liver and the spleen.

Rather few *in vivo* investigations of the distribution of microparticles based on P(3-HB) or any other PHA have been reported in the available literature. Bissery and co-authors [36] studied the distribution of 1–12-μm microparticles based on ^{14}C-labeled P(3-HB) intravenously injected to mice. At 30 min after injection, 47% of the total radioactivity was recorded in the lungs, 14% in the liver, and 2.1% in the spleen. However, at 24 h, ^{14}C increased to 60% in the lungs and to 24% in the liver; in 7 d after the injection, this distribution pattern remained generally unchanged, except for a slight ^{14}C decrease in the lungs. The dynamics of ^{14}C distribution reported in this study was similar to the results obtained in experiments with polystyrene/divinylbenzene microparticles injected to dogs [28], but is different from the data presented by Saito and coauthors [37]. Saito and co-authors injected smaller particles (0.3–0.5 μm in diameter; polymer granules recovered

from *Zoogloea ramigera* cells grown on ^{14}C-labeled glucose) into larger animals (rats) through the tail vein and obtained a different picture: at 2.5 h after the injection of the granules, 86% of the radioactivity was found in the liver, while the lungs and the spleen contained just 2.5% each. After 1 day, the radioactivity in the liver decreased to 66%. During 8 weeks, the radioactivity decreased slowly in most tissues examined, which allowed the authors to conclude that the polymer matrix was slowly degraded. Saito and co-authors suggested that the differences between their results and those obtained by Bissery and co-authors might be due to the difference in animal species used or diameter of the particles.

The pattern of the distribution of P(3-HB) particles under 3.8 μm in diameter in internal organs of laboratory animals that we obtained in our experiment is generally comparable with the data reported by other authors, who used different polymers. Except for the distribution recorded at the first time point (3 h after the injection of microparticles into rats' bloodstream), the highest percentages of ^{14}C were found in the liver, spleen and kidneys. Whatever the molecular weight of the P(3-HB) used to prepare microparticles, the patterns of the distribution of radioactivity were similar. The radioactivity in the tissues decreased slowly, and even after 12 weeks, the examined organs retained radioactivity, which suggested that they contained polymer degradation products and undegraded high-molecular-weight P(3-HB). This fact was proven in a special experiment, which was performed for the first time and which revealed the presence of undegraded high-molecular-weight polymer in the tissues.

An important point of this study is investigation of degradation of microparticle polymer matrix in tissues of rats' internal organs, taking into account P(3-HB) molecular weight dynamics. There are very few published studies addressing this issue. Leach and Takahashi [38] studied the decrease in molecular weight of microparticles prepared from polylactide and showed that it was affected by the manner and route of administration of the particles (subcutaneous or intramuscular). However, van Dijkhuizen-Radersma *et al.* [39] did not detect any influence of the manner in which polyanhydride/polylactide-based microparticles were administered subcutaneously or intramuscularly on their degradation rates. The authors showed that the choice of administration route was irrelevant to degradation process; in one month after injection, molecular weight of the microparticle polymer was found to decrease slightly (to about 20 000); after 2 months, however, the molecular weight of the polymer matrix dropped dramatically (more than 3-fold, to 6000). Sandor and Harris [40] studied degradation of poly(fumaric-co-sebacic acid) microparticles loaded in polyethylene pouches, which were implanted to rats intramuscularly, subcutaneously, and intraperitoneally. The molecular weight of the polymer was found to decrease dramatically (by up to 90% of its initial value) after 72 h of implantation.

No literature data is available concerning *in vivo* degradation dynamics of microparticles prepared from P(3-HB) or any other PHA. In most of the studies addressing PHA biodegradation, it is investigated using large implants in the form

of films, 3D matrices, rods, etc. Freier and Kunze [41] implanted P(3-HB) films into rats intraperitoneally and recorded a 30% decrease in the polymer molecular weight after 2 weeks of implantation and a 60% decrease after one month. Similar results were obtained by Qu and Wu [42], who implanted P(3-HB) and P(3-HB)-co-P(3-HV) discs to rabbits and recorded a 60% decrease in the polymer molecular weight after 6 months of implantation.

In a recent study, addressing the response of muscular tissue to implantation of P(3-HB) microparticles [19], we showed that biodegradation of polymer microparticles is a slow process and that it is performed by macrophages and foreign body giant cells. Biodegradation of the particles was evaluated based on results of the analysis of particle sizes, from the data obtained by morphometric examination of tissue sections and the level of macrophage response in the cluster of implanted microparticles. As the mean diameter of microparticles was reduced from 15 μm to 7.8 μm, at 12 weeks after implantation we recorded an increased number of smaller particles, which resulted from degradation of larger ones, and a few (about 3% of the initial number) undegraded large (10–15 μm) particles.

In this study we, for the first time, investigated changes in the M_w of the P(3-HB) matrix of microparticles in tissues of internal organs. The study showed that degradation rates of P(3-HB) were determined by the localization of the particles, regardless of the M_w of the polymer used to prepare them. At 3 h after the microparticles were injected, there was a dramatic decrease in the M_w of the polymer extracted from all examined organs, except the lungs: the kidneys (73% decrease), the heart (62%), the liver (57%), and the spleen (47%). Then the P(3-HB) M_w decreased slower: 27–40% (for different organs) after 2 weeks and about 23–27% after 12 weeks. At 12 weeks after the microparticles were injected into the bloodstream, residual polymer concentrations in all organs were equal, amounting to $(4–8) \times 10^{-4}$ mg/g. The presence of high-molecular-weight (undegraded) polymer in the tissues at 12 weeks after administration of the particles suggests that P(3-HB) is degraded in tissues of internal organs slowly and, hence, P(3-HB)-based microparticles can be used as sustained-release drug-delivery systems.

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