

## Biocompatibility and efficiency of drug-loaded P(3)HB-microspheres

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### Introduction

The most promising drug delivery systems today are biodegradable microspheres and microcapsules. Microspheres can be injected subcutaneously or intramuscularly, adapted for oral administration or inhalation, and introduced into the bloodstream (Ueda 2003). The rapid development of microencapsulation has triggered an interest in biocompatible and biodegradable polyhydroxyalkanoates (PHAs), which can be processed in different physical states, by various methods. PHAs as materials for drug delivery systems have been investigated much less extensively than other biodegradable polymers, although they can successfully be used in constructing sustained drug delivery systems (Ammas 1998, Gürsel 2000). PHA microparticles have already been tested as carriers of antibiotics, hormones, and anticancer drugs. Researchers of the Institute of Biophysics of the Siberian Branch of the Russian Academy of Sciences have developed technologies of PHA synthesis, recovery, and purification to prepare high-purity polymers (Volova 2004). Specimens of these polymers were examined in comprehensive toxicological, medical, physiological, and histological investigations, which included experiments with cell cultures *in vitro* and with laboratory animals. High biological compatibility of PHAs and items from them was proved at the levels of cells, tissues, and organism. The tested PHAs can be safely used in medicine (Shishatskaya 2004 & 2005). Important aspects of developing drug delivery systems in the form of bio- and blood-compatible microparticles are careful choice and *in vivo* investigation of the route of drug administration and studying of bioresorption of the matrix.

The purpose of this study was to test biocompatibility of PHB microparticles with different ways of introduction and to estimate drug efficiency of antiproliferative drug-loaded microparticles *in vivo*.

### Material and methods

PHA-based microspheres were prepared by the solvent evaporation technique, using a triple emulsion of highly purified samples of poly(3)hydroxybutyrate (P(3)HB), synthesized at the Institute of Biophysics SB RAS (Trademark "BIOPLASTOTAN" 2006). The microspheres size distribution was measured using CASY TTC (Scharle system GmbH®, Germany). Separation of the microspheres into size fractions was performed using track filters with different pore sizes. Rubomicinum-loaded microspheres were prepared using the same fabrication method, with of drug solution added to the emulsion. For the investigation of biodistribution of microspheres in the animals' organisms, particles were prepared from <sup>14</sup>C-labelled PHB. Sterilization of samples was performed with UV during 40 min. All works with animals were carried out in accordance with the international and Russian regulations on laboratory animal care (Genin 2001, Guidelines for Care... 1990, Guide for the Care... 1996), and with permission of the IBP SB RAS Ethical Committee. Animals were kept in a vivarium and fed a standard diet. Experiments were conducted on Wistar rats (200-240 g each) and white Balb/c mice. For determination of microspheres biodistribution, rats of the treatment group (30 rats total) received a suspension of 2 mg <sup>14</sup>C-microspheres in 0.5 ml of physiological saline through the tail vein. At 3 h after injection and then every week, three animals were sacrificed by using an overdose of a volatile anesthetic. Their internal organs were examined macroscopically, weighed, and radioactivity counts were performed. A 100 mg sample of

tissues, which had been dried to stable weight, was placed into a plastic vial containing dioxane scintillation solution; radioactivity counts were performed using a TriCarb 2100TR automated liquid scintillation counter (Perkin Elmer/Packard, USA). Also, tissues of the organs were analyzed for polymer content to estimate the biodegradation of the polymer matrix. The polymer was extracted from the tissues with chloroform and precipitated with hexane (Fluka); then the polymer was methylated and fatty acid methyl esters were determined using a GCD plus chromatograph mass spectrometer (Hewlett Packard, USA); sensitivity was  $10^{-11}$  g. To estimate local tissue reaction, 20 mg of sterile microspheres of a mean diameter of  $10\pm 0.23$   $\mu\text{m}$  in 0.3 ml of a physiological saline, were injected intramuscularly into the femurs of rats. At 24 h after injection and then every week, three animals were sacrificed. The microspheres were removed with excess surrounding tissue; the samples for morphometric evaluation were prepared using conventional histological technique. The Carl Zeiss Jena Image Analysis System (Germany) was used for viewing microscopic images and analyzing morphometric characteristics of sections (ocular 10, objectives 10 and 40). To determine drug efficiency of the experimental delivery system of the antiproliferative drug – rubomycinum – its encapsulated form in the polyhydroxybutyrate microspheres was fabricated and an experiment was carried out on laboratory mice inoculated with Ehrlich ascites carcinoma (EAC). Mice were divided into two groups: control and treatment. EAC was implanted intraperitoneally to all mice – 100% lethal dose,  $3\times 10^6$  cells in 0.2 ml physiological saline per mouse. Mice of the treatment group additionally received 50 mg of rubomycin-loaded microparticles intraperitoneally. Animal mortality reduction, tumor volume decrease (ml) and cell concentration of the ascites were detected.

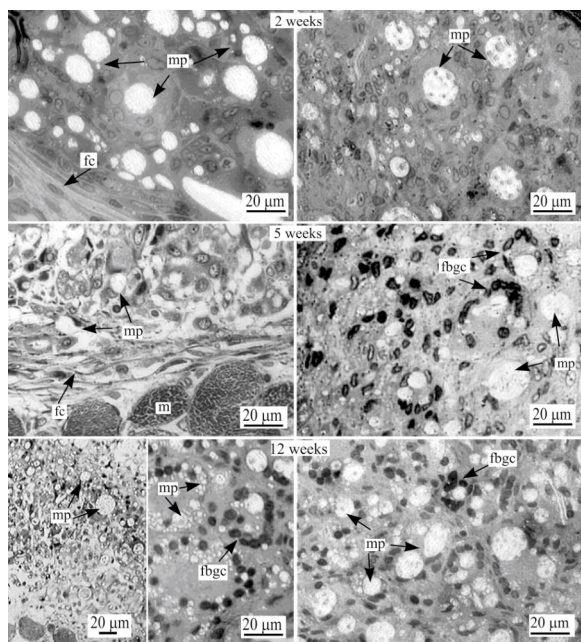
## Results and Discussion

The obtained microspheres were of regular spherical shape and had a well-developed “wrinkled” porous surface; their diameters were heterogeneous, from 1 to  $50\pm 0.13$   $\mu\text{m}$ .

**Muscular tissue reaction** After intramuscular injection no negative events on the macro- and microscopic levels were detected (Fig. 1). Negligible inflammation, expressed in slight tissue edema and polymorphonuclear leukocyte infiltration (20-25 cells in the field of view), was registered at 24 h after the injection. During the experiment, leucocyte infiltration gave place to mononuclear secretory-phagocytic macrophages, foreign body giant cells (FBGCs), and fibroblasts. At the end of the experiment, the tissue reaction was characterized by macrophage infiltration with a large number of poly-nuclear FBGCs. There were no fibrous capsules at the interface between the intact muscular tissue and the implanted microspheres. In some parts of the microsphere cluster there was polymeric detritus, and fragmented microparticles, as a degradation product of larger particles. For quite a long time, most of the microspheres persisted in the tissue undegraded, suggesting that *in vivo* bioresorption of microparticles must be rather a long process, which makes polyhydroxybutyrate a good candidate for fabricating a prolonged-action drug form intended for intramuscular injection.

**Microspheres Biodistribution** All animals in the treatment group, which had been injected intravenously with a  $^{14}\text{C}$ -microspheres suspension, were healthy and ate well throughout the experiment. Macroscopic examinations of the rats’ internal organs did not show any anomalies. Analysis of the radioactivity levels in rats’ organs revealed the heterogeneity of microspheres biodistribution during the observation period. Analysis of specific radioactivity of the organs’ tissues showed that the largest amounts of the label were contained in the liver, kidneys, and spleen. The amounts of radiocarbon registered in the tissues can comprise the radiocarbon of the undegraded polymer matrix of the microparticles and that of the polymer bioresorption C-products. To compare biodegradation of the polymer in different organs and to determine the *in vivo* “lifetime” of polymer microparticles, the tissues were subjected to chromatographic analysis. In the

course of the experiment, the contents of the analyzed polymer substance changed non-uniformly in all organs. The most pronounced drop in the polymer substance content was registered in the spleen, liver, and kidneys. All organs contained residual amounts of high-molecular-weight (undegraded) polymer, suggesting that part of microparticles remained intact. The registered presence of the polymer matrix in the analyzed organs suggested that microparticles remained undegraded up to 12 weeks.



**Figure 1:** Temporal changes in the microscopic picture of tissue at the site of microspheres implantation (semithin sections stained with methylene blue). Mp - microparticles, mph – macrophages, FBGCs – foreign body giant cells, mf – muscle fibers, fc – fibrous capsule. The bar is 20 µm

Organ	content ( $1 \times 10^{-4}$ mg/organ)		
	1 week	8 weeks	12 weeks
methyl esters of PHB monomers			
heart	157	132	75
lung	1694	111	56
liver	5861	4889	197
spleen	606	251	14
high-molecular-weight undegraded PHB			
heart	-	130	5
lung	600	5	6
liver	160	110	80
spleen	6	10	5
kidney	16	20	7

**Table 1 :** Results of chromatographic analysis of the tissues of rats' organs for methyl esters of PHB monomers and residual polymer  
 “-” denotes “not determined”.

**Drug efficiency** Analysis of the results suggests that in the control group mass dying of mice started at day 14, and all animals were dead within the following 8 days, i.e. by day 21, mortality had reached 100%. The average lifespan of tumor-bearing mice in the control group was 8 days. The mortality curve of the mice that received rubomycin encapsulated in polymer microparticles simultaneously with EAC transplantation was significantly different from that of the control mice. At day 21, when all the mice of the control group were dead, the survival of the mice that had received prolonged-action rubomycin amounted to 80% and their average lifespan was then 16 days, i.e. 2 times longer than that of the control animals. The follow-up observations for a period of 30 days showed that the death rate in the treatment group decreased and the mortality curve became flatter. In 55 days after the beginning of the experiment, the survivors (40%) showed no symptoms of EAC.

## Conclusions

Comparison of degradation of the polymer matrix in different organs showed that PHB-microspheres are resorbed at the highest rate in the spleen and the liver. Rubomycin encapsulated in polymer microparticles was shown to exhibit pronounced antitumor activity, inhibit proliferation

activity of EAC cells, and increase survival of tumor-bearing mice. Target delivery of the drug was achieved. The results of the study suggest that polyhydroxybutyrate is a good candidate for fabricating prolonged-action drugs in the form of microparticles intended for intramuscular and intravenous injection.

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