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Microparticles prepared from biodegradable polyhydroxyalkanoates as matrix for encapsulation of cytostatic drug

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Abstract Microparticles made from degradable polyhydroxyalkanoates of different chemical compositions a homopolymer of 3-hydroxybutyric acid, copolymers of 3-hydroxybutyric and 4-hydroxybutyric acids (P3HB/4HB), 3-hydroxybutyric and 3-hydroxyvaleric acids (P3HB/3HV), 3-hydroxybutyric and 3-hydroxyhexanoic acids (P3HB/3HHx) were prepared using the solvent evaporation technique, from double emulsions. The study addresses the influence of the chemical compositions on the size and ξ-potential of microparticles. P3HB microparticles loaded with doxorubicin have been prepared and investigated. Their average diameter and ξ-potential have been found to be dependent upon the level of loading (1, 5, and 10 % of the polymer mass). Investigation of the in vitro drug release behavior showed that the total drug released from the microparticle into the medium increased with mass concentration of the drug. In this study mouse fibroblast NIH 3T3 cells were cultivated on PHA microparticles, and results of using fluorescent DAPI DNA stain, and MTT assay showed that microparticles prepared from PHAs of different chemical compositions did not exhibit cytotoxicity to cells cultured on them and proved to be highly biocompatible. Cell attachment and proliferation on PHA microparticles were similar to those on polystyrene. The cytostatic drug encapsulated in P3HB/3HV microparticles has been proven to be effective against HeLa tumor cells.

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1 Introduction

Designing of controlled drug delivery systems (DDS) is a promising and rapidly developing line of biotechnology and experimental pharmacology. About 25 % of the drugs sold in the world at the present time are administered via transport/delivery systems [1].

DDS enable sustained release of the drugs, direct them to a specific organ or tissue, enhance their bioavailability, and reduce possible side effects of toxic drugs. It has been generally accepted that the most promising drug delivery systems are sustained-release DDS in the form of micro- and nanoparticles, which can be administered subcutaneously, intramuscularly, orally, and intravenously [2, 3].

Prior to construction of micro- and nanoparticle DDS, the properties of the biopolymers used in this process need to be examined in detail. By varying the properties of biopolymers, one can control the drug release rate. The choice of the biopolymer and the drug should be based on the knowledge of how they will interact and behave in the

organism. Thus, a comprehensive approach is needed to tackle the issue of drug delivery.

Among the wide range of biomaterials, a special position is occupied by linear polyesters of microbial origin, the so-called polyhydroxyalkanoates (PHAs). In recent years, PHAs have been increasingly used as materials to construct matrices for drug encapsulation and delivery and for cell and tissue engineering. The main advantage of PHAs is that they can consist of monomer units with different carbon chain lengths, making up polymers with different chemical structure. The most popular and the best studied PHA is a homopolymer of 3-hydroxybutyric acid (P3HB). PHA copolymers are more promising materials as their properties can vary within a fairly broad range, depending upon the proportions of different monomer units contained in them. The resulting materials have different properties—from high-crystallinity thermoplasts to construction elastomers [4–6]. There are, however, very few published studies on the use of PHA copolymers, whose synthesis is a complex technological task, for the construction of special devices such as drug micro-carriers.

By varying the parameters of the PHA matrix, one can get the unique opportunity to control drug release kinetics. Short-chain-length PHAs are degraded via surface erosion, which makes this type of PHAs the most attractive candidates for being used as drug carriers. The main advantages of microparticles based on short-chain-length PHAs are their crystallinity, hydrophobicity, and the presence of pores on their surface, which provides the most effective drug release from the degrading matrix [7].

At the present time, PHAs are used to prepare microparticles loaded with analgesics [8] and anti-inflammatory drugs; their release kinetics has been studied quite well [9–12]. PHA microparticles, films, and 3D matrices are promising carriers for antibiotics, enabling the sustained release of the drug [13–16]. Incorporation of protein compounds in composite microparticles consisting of PHAs and polyethylene glycol and polylactides was reported by Lionzo et al. [9].

Investigations performed at the Institute of Biophysics, Siberian Branch, Russian Academy of Sciences, revealed the high biocompatibility of high purity PHA samples at cellular and tissue levels, including contact with blood, as well as their applicability for the design of endoprostheses of various kinds, as matrices of functioning cells, and for deposition of drugs [17, 18].

The goal of this study was to compare polymer microparticles prepared from PHAs with different chemical composition and to investigate their biocompatibility and drug effectiveness in vitro.

2 Materials and methods

2.1 Materials

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 by cultivation hydrogen-oxidizing microorganisms (Table 1). The specimens were subjected to methanolysis, and PHA concentration and composition were analyzed 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 samples were performed using a D8 ADVANCE X-ray spectrometer (Bruker, Germany) (graphite monochromator on a reflected beam). Spectra were taken 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 × 40 μA.

Molecular weight and molecular-weight distribution of PHAs were examined using a gel permeation chromatograph ("Agilent Technologies" 1260 Infinity, USA) relative to reference polystyrenes from Fluka (Switzerland, Germany). The calculated parameters included the number average molecular weight (M_n), the weight average molecular weight (M_w), and polydispersity ($PD = M_w/M_n$), which provides an estimate of the proportions of fragments with different polymerization abilities in the polymer.

2.2 Preparation of microparticles

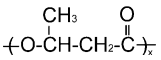
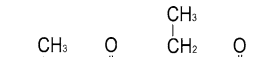
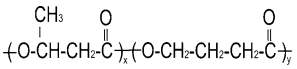
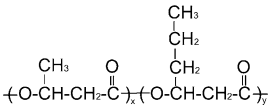
Microparticles were prepared by the solvent evaporation technique, using double (water/oil) emulsions. The double emulsion contained 0.4 g PHA in 10 ml of dichloromethane and 100 ml 0.5 % (w/v) PVA. The resulting double emulsion was mechanically agitated at 24,000 rpm (IKA Ultra-Turrax T25 digital high-performance homogenizer (Germany) until the solvent had completely evaporated.

All emulsions were continuously mixed mechanically for 24 h, until the solvent had completely evaporated. Microparticles were collected by centrifuging (at 10,000 rpm, for 5 min), rinsed 6 times in distilled water, and lyophilic dryer in an Alpha 1–2 LD plus (Christ®, Germany).

The yield of microparticles was calculated as percent of the mass of the polymer used to prepare them:

$$Y = \frac{M_p \times 100\,\%}{M_m}$$

Table 1 Biodegradable PHAs of different chemical compositions used microparticles for preparation

Polymer composition (mol%)	Structural formula	Polymer properties		
		M_n (kDa)	M_w (kDa)	PD
P3HB 100		710	1,200	1.71
P3HB/3HV 93.5/6.5		890	1,700	1.91
P3HB/3HV 89.5/10.5		840	1,500	1.79
P3HB/3HV 80/20		663	1,500	2.27
P3HB/3HV 63/37		1,026	2,000	2.00
P3HB/4HHx 93/7		270	950	3.52
P3HB/4HB 93.9/6.1		140	410	2.93
P3HB/4HB 84/16		370	970	2.62

where M_m is the mass of the prepared microparticles (mg) and M_p is the mass of the total polymer used for preparation of microparticles (mg).

The morphology of the particles was analyzed using an FEI Company Quanta 20 scanning electron microscope (USA). The size and size distributions of microspheres were determined using Zetasizer Nano ZS (Malvern, UK). Each sample was measured in triplicate. The obtained size distribution and mean diameters were used to describe the particle size. The surface charge of the microparticles was characterized in terms of zeta potential, which was determined the electrophoretic mobility and then applying the Henry equation using Zetasizer Nano ZS (Malvern, UK).

2.3 Preparation of drug-loaded polymer microparticles

Microparticles loaded with doxorubicin (DOX) were prepared using the solvent evaporation technique from the double emulsion. The DOX (4, 20 or 40 mg) was dissolved in 10 ml of dichloromethane containing 0.4 g P3HB or P3HB/3HV (6.5 mol%). Aqueous phase as a dispersion medium for the microparticles production was prepared by using 100 ml of a 0.5 % (w/v) PVA aqueous solution. The emulsion was agitated at 24,000 rpm (IKA Ultra-Turrax T25 digital high-performance homogenizer (Germany) until the solvent had completely evaporated. Microparticles were collected by centrifuging (at 10,000 rpm, for 5 min), rinsed six times in distilled water, and freeze dried in an LS-500 lyophilic dryer (Russia).

The amount of the drug loaded into the polymer matrix was determined spectrophotometrically (Uvicon 943, Italy), by measuring its initial and residual concentrations in the emulsion.

The encapsulation efficiency (E) was calculated using the following formula:

$$E = \frac{M_{init} \times 100\,\%}{M_{enc}}$$

where M_{enc} is the mass of the encapsulated drug in the polymer matrix (mg) and M_{init} is the mass of the initial amount of drug (mg).

2.4 In vitro drug release behavior

Drug-loaded PHA microparticles were sterilized using UV radiation for 20 min and then placed in sterile centrifuge tubes with caps, containing 5 ml of phosphate-buffered saline (PBS, pH 7.3); the tubes were incubated at 37 °C ($n = 3$). Microparticles were settled by centrifugation (for 5 min at 10,000 rpm), and samples were taken to determine the amount of the drug released into PBS using a Uvicon 943 spectrophotometer (Italy), based on absorption maxima at 580 nm.

Drug release (DR) into PBS was determined as follows:

$$DR = \frac{r \times 100\,\%}{e}$$

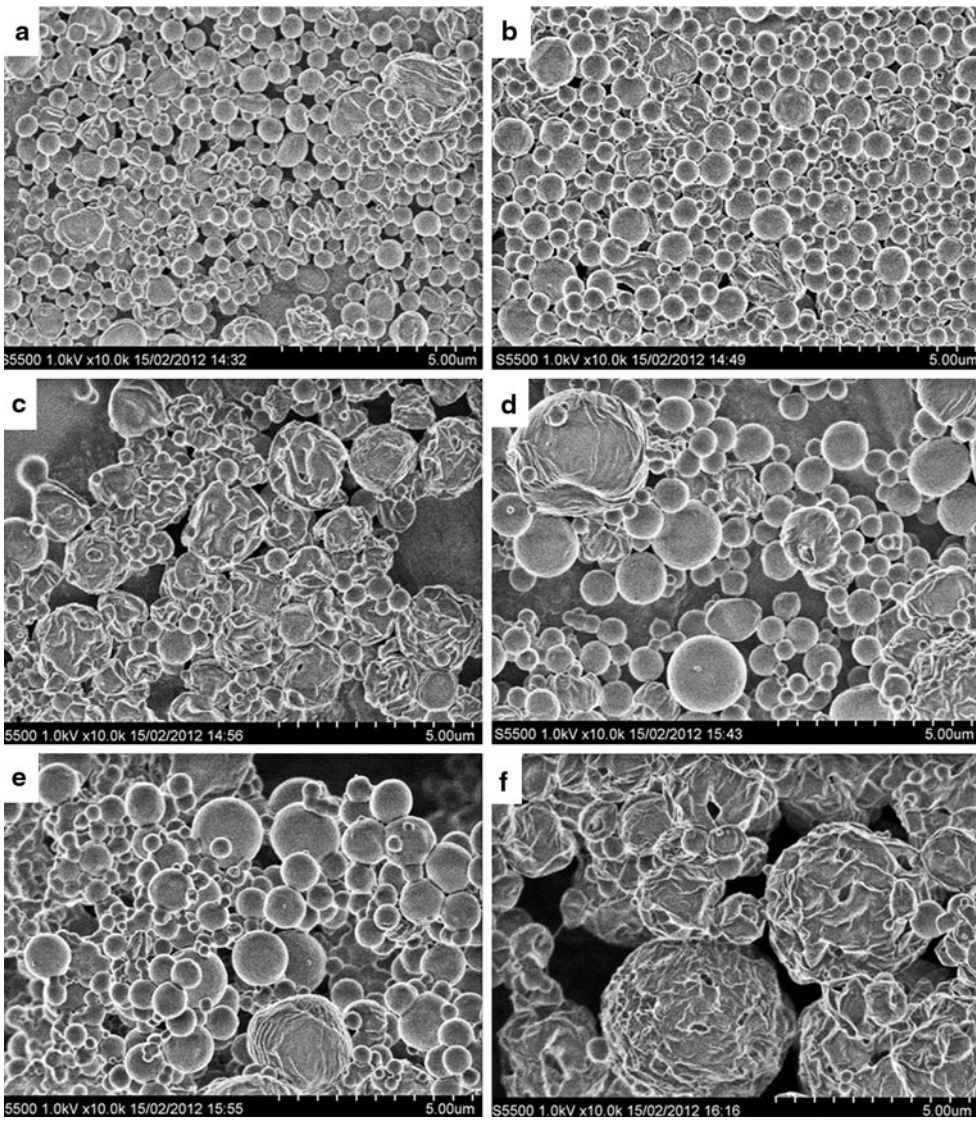


Fig. 1 SEM images of the microparticles prepared from PHAs of different chemical compositions: **a** poly-3-hydroxybutyrate, **b** poly-3-hydroxybutyrate-co-3-hydroxyvalerate (6.5 mol%), **c** poly-3-hydroxybutyrate-co-3-hydroxyvalerate (37 mol%), **d** poly-3-hydroxybutyrate-co-3-hydroxyhexanoate (7 mol%), **e** poly-3-hydroxybutyrate-co-4-hydroxybutyrate (6.1 mol%), **f** poly-3-hydroxybutyrate-co-4-hydroxybutyrate (16 mol%). The *bar* 5 μm

where e is the amount of the encapsulated drug (mg/mg) and r is released drug (mg/mg).

Theoretical analysis of the experimental data on drug release and quantification of the value of the drug diffusion coefficient in the polymer phase was performed through the graphic solution of the equations in coordinates $(m_t/m_\infty) - (t)^{0.5}$ and in semilogarithmic coordinates $\ln(1 - m_t/m_\infty)$ as described elsewhere [19, 20].

2.5 Cell cultivation

Determination of possible toxicity of PHA microparticles were investigated in experiments with mouse fibroblast NIH 3T3 cells, which were seeded onto microparticles (5×103 cells/cm^2) placed in 24-well plates, in accordance with the estimation as described Nakoaka R. [21]. To estimate influence the composition of the particles have, suspensions of the particles in PBS was prepared with the concentration 2 mg/ml; 100 μl of suspension of the particles of each type were put into 24-well culture plates (Cellstar, Greiner bio-one). The microparticles were sterilized using H_2O_2 plasma in a Sterrad NX sterilization system (Johnson&Johnson, USA) or autoclaving at 1 atm. Polystyrene plates (Orange Scientific) were used as controls.

Fibroblasts were cultured in Dulbecco's Minimal Eagle Medium (DMEM) supplemented with fetal bovine serum (10 % v/v) and a solution of antibiotics (streptomycin 100 μg/ml, penicillin 100 IU/ml) (Gibco, Invitrogen) in a CO_2 incubator with CO_2 level maintained at 5 %, at a temperature of 37 °C. The medium was replaced every three days.

Analysis of cell morphology and cell counting were performed in 1, 4, and 7 days after seeding on microparticles, using fluorescent stain DAPI (Sigma); cells were counted using an Axiovert 40 fluorescence microscope

(Carl Zeiss). Viability of cultured fibroblast NIH 3T3 cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) assay. Viability evaluation was based on the ability of dehydrogenases of living cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to formazan, which characterizes mitochondrial activity, estimates the abundance of living cells, and indirectly indicates the ability of cells to proliferate on the matrices. MTT solution (50 µl) and complete nutrient medium (950 µl) were added to each well containing a polymer. After 3.5 h incubation, the medium and MTT were replaced by DMSO to dissolve MTT-formazan crystals. After 30 min, the supernatant was transferred to the 96-well plate, and optical density was measured at wavelength 540 nm, using a Bio-Rad 680 microplate reader (Bio-Rad Laboratories Inc, USA). The number of cells was determined from the calibration graph.

2.6 Cytotoxicity of DOX-loaded PHA microparticles

For the experiment the polymer particles from P3HB/3HV (6.5 mol%) of 0.2 and 1.2 microns with various drug loading were prepared. Loading of the particles with the drug was done in such a way, that during introduction of the particles in the culture in the form of suspension the concentrations were as follows: 0.6; 3.2; 6.0 µg/ml.

Microparticles of mean diameters 0.2 and 1.1 µm were prepared using 1.2-µm and 0.25-µm-pore-size nitrocellulose membrane filters (Sartorius).

The cytostatic effect of microparticles loaded with DOX was estimated by the culture of tumor cells—HeLa. HeLa line cells were put into the cell culture on the basis 10 × 103 cells/well. The medium RPMI + FBS (10 %) + antibiotic (1 %) (streptomycin 100 µg/ml, penicillin 100 U/ml (Gibco, Invitrogen)). Suspension of sterile particles (2 mg of particles/200 µl of phosphate buffer) was introduced into each well of 24-well plate. Cultivation was done by the standard method in the humid medium during 3 days. Viability of cells was tested daily in MTT assay in relation to the positive reference (free DOX was introduced into the cells culture in the similar concentration: 0.6, 3.2 and 6.0 µg/ml).

2.7 Statistics

The results were analyzed statistically using the standard software package of Microsoft Excel and the StatPlus software. Arithmetic means, mean square error, and error of the arithmetic mean were calculated in all cases. Significant differences in average values were tested using the Mann–Whitney U test (significance level: $P = 0.05$).

3 Results and discussion

3.1 Characterization of PHAs used to prepare microparticles

Differences in the basic physical properties of the polymers under study (Table 1) influenced the characteristics of the microparticles. SEM images of the surface microstructure of microparticles prepared from PHAs that differed in their chemical composition and physicochemical properties showed certain dissimilarities (Fig. 1).

Whatever the PHA composition, microparticles were heterogeneous in their shape, and their surface structures were different. Microparticles prepared from P3HB and P3HB/3HV containing the lowest molar fraction of 3HV (6.5 %) were practically smooth and of a regular spherical shape, without surface deformation. Microparticles prepared from P3HB/3HV with a high molar fraction of 3HV (37 %) and P3HB/4HB (16 % 4HB) had a rough surface; some of the particles were irregularly shaped. Visual estimation showed that P3HB/4HB microparticles were of larger size. Microparticles prepared from P3HB/3HHx

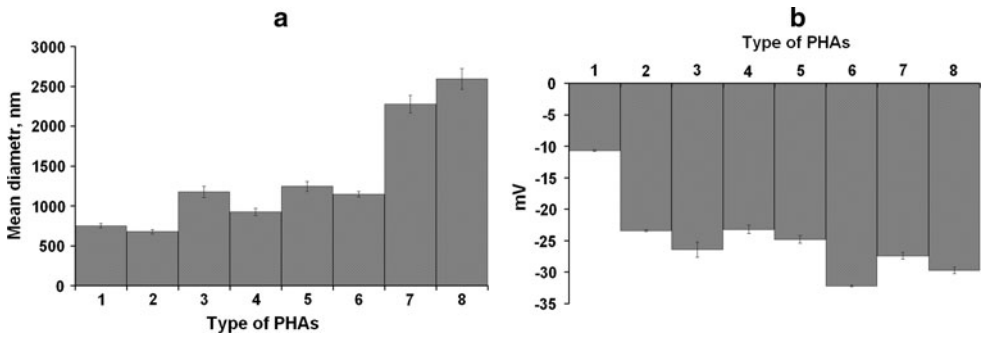


Fig. 2 Mean diameter and **a** ξ-potential **b** of the microparticles prepared from PHAs of different chemical compositions: *1* poly-3-hydroxybutyrate, *2* poly-3-hydroxybutyrate-co-3-hydroxyvalerate (6.5 mol%), *3* poly-3-hydroxybutyrate-co-3-hydroxyvalerate (10.5 mol%), *4* poly-3-hydroxybutyrate-co-3-hydroxyvalerate (20 mol%), *5* poly-3-hydroxybutyrate-co-3-hydroxyvalerate (37 mol%), *6* poly-3-hydroxybutyrate-co-3-hydroxyhexanoate (7 mol%), *7* poly-3-hydroxybutyrate-co-4-hydroxybutyrate (6.1 mol%), *8* poly-3-hydroxybutyrate-co-4-hydroxybutyrate (16 mol%)

(7 mol% HHx) and P3HB/4HB (6.1 mol% 4HB) had a spherical shape and smooth surface.

Important parameters determining the tissue specificity of the particles and their ability to cross biological barriers are their size and size distribution. Nanoparticles generally vary in size from 10 to 1,000 nm [22]. Microparticulated drug delivery systems of bigger size are very promising with various methods of administration: peroral (osmotic minipumps), parenteral (nanoparticles and nanocapsules), subcutaneous (implants), intracavitary (intrauterine inserts and various suppositoria), buccal, etc. [23].

The average diameter of microparticles prepared from PHA copolymers was larger than that of the particles prepared from the homopolymer (Fig. 2a) although the matrices prepared from P3HB/3HV (10 mol% 3HV) and those prepared from 3HB were similarly sized, and their average diameter was about 750–700 nm. The average diameter of microparticles prepared from the P3HB/3HV with the molar fraction of 3HV amounting to 37 % was almost twice greater, reaching 1.25 μm. The average diameter of P3HB/3HHx particles did not differ significantly from that of P3HB/3HV (37 mol% 3HV) ones: 1.14 μm. Microparticles prepared from P3HB/4HB containing 6.1 and 16 mol% 4HB were significantly larger than other copolymer microparticles, and their diameters were 2.3 and 2.6 μm, respectively (Fig. 2a).

Another important parameter is ξ-potential of microparticles, which characterizes stability or coagulation of the particles in the dispersion medium [24].

Determination of the zeta potential of microparticles prepared from PHAs with different chemical composition gave the following results (Fig 2b): the lowest ξ-potential was recorded for P3HB/3HHx microparticles (−32.2 mV); the second-lowest values of this parameter were recorded for P3HB/4HB (about −29–27 mV). P3HB microparticles had the highest ξ-potential (about −11 mV). Microparticles prepared from P3HB/3HV with different molar fractions of 3HV had a lower ξ-potential, which varied from −23 to −26 mV and was not influenced by the molar fraction of 3HV.

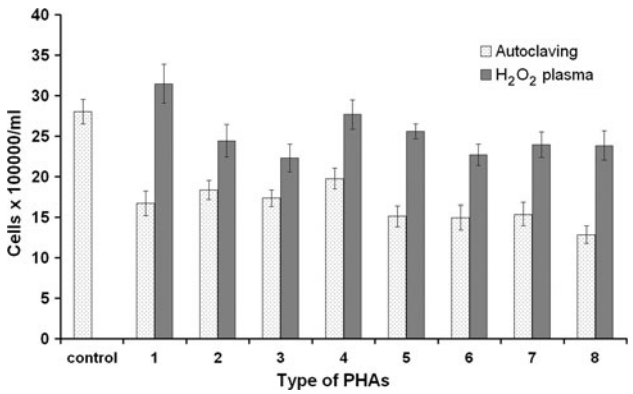


Fig. 3 Amount of cells adhered to the microparticles surface 3 h after seeding (numbers as in Fig. 2). Reference—polystyrene

3.2 Biocompatibility and adhesive properties of PHA microparticles in vitro

Figure 3 shows results of MTT assay: determination of viability of cells cultured in the presence of PHA microparticles treated with H_2O_2 plasma or by autoclaving on direct contact with fibroblast NIH 3T3 cells.

At 3 d after seeding, counts of attached cells showed that the number of cells on microparticles treated with H_2O_2 plasma was higher. The largest number of cells (up to 28–33 in the field of view) were attached to microparticles prepared from P3HB and P3HB/3HV with 20 mol% 3HV. That number was 1.4–1.8 times higher than the number of cells attached to the microparticles sterilized by autoclaving (Fig. 3). The number of cells attached to autoclaved microparticles prepared from P3HB/3HV (6.5, 10 and 37 mol% 3HV), P3HB/3HHx, and P3HB/4HB (6.1 and 16 mol% 4HB) was half that recorded on the corresponding microparticles treated with H_2O_2 plasma.

A possible explanation for this might be that treatment of polymer devices by physical methods (laser cutting or plasma) strengthens interphase adhesion joints, increasing surface hydrophilicity and, hence, improving its adhesion properties.

MTT assay did not reveal any cytotoxic effect of autoclaved or plasma-treated PHA microparticles. The number of viable cells adhering to the surface of the matrices treated with H_2O_2 plasma was higher than on the surface of the autoclaved ones in all treatments (Fig. 3).

Results of the cell counts obtained using the fluorescent DAPI DNA stain were as follows: at 3 d after fibroblast NIH 3T3 cells were seeded onto microparticles, the number of cells on PHA microparticles treated with H_2O_2 plasma was significantly higher than on autoclaved ones (Fig. 4).

On plasma treated microparticles prepared from PHAs with different chemical composition, cells spread well and formed a monolayer. On the corresponding PHA microparticles sterilized by autoclaving, the number of cells was 1.5–2 times lower, and they showed an irregular shape.

As differences in the number of cells proliferating on microparticles prepared from PHAs of different types are insignificant, all of the polymers investigated in this study are of good quality, showing high biocompatibility.

3.3 Preparation and investigation of DOX-loaded microparticles

Conditions of loading drugs (doxorubicin, DOX) into P3HB and P3HB/3HV (6.5 mol% 3HV) microparticles

were developed and investigated. The average diameter of the particles loaded with DOX was slightly, by 1.2 times, increased, whatever the composition of the particles (Fig. 5a).

The loading of DOX into P3HB/3HV (6.5 mol% 3HV) microparticles did not alter their surface structure. The DOX-loaded microparticles were of regular spherical shape and had a smooth surface.

The relationship between the DOX load and ξ-potential was studied using P3HB and P3HB/3HV (6.5 mol% 3HV) microparticles (Fig. 5b). In both cases, the values of ξ-potential of the drug-loaded particles were lower than those of the unloaded ones, and more pronounced decrease in this value was recorded for P3HB particles, whose ξ-potential decreased 1.8 times.

As P3HB/3HV (6.5 mol% 3HV) microparticles had a rather low ξ-potential, they were chosen for the further study, in which DOX-loaded particles were used to investigate their drug effectiveness.

DOX release kinetics was studied and found to be dependent upon the level of loading (Fig. 6).

The larger the amount of the DOX entrapped in the particles, the more that was released from P3HB/3HV microparticles. Independently of the extent of the load with the stain the curves had a typical 2-phase character—rapid drug release for short time periods and long segments with a nearly constant release rate. The initial output was more likely connected with solving and washing out of the drug from the microparticles' surface. In the first 12 h the release made 13.5 ± 1.4 and 8.9 ± 0.7 %, respectively, under the maximum and minimum loads of the microparticles. Then, for the following 5 days (120 h), DOX release rate increased dramatically, reaching 6.1 ± 0.6 and 8.92 ± 0.4 %, for microparticles that contained 1, 5, and

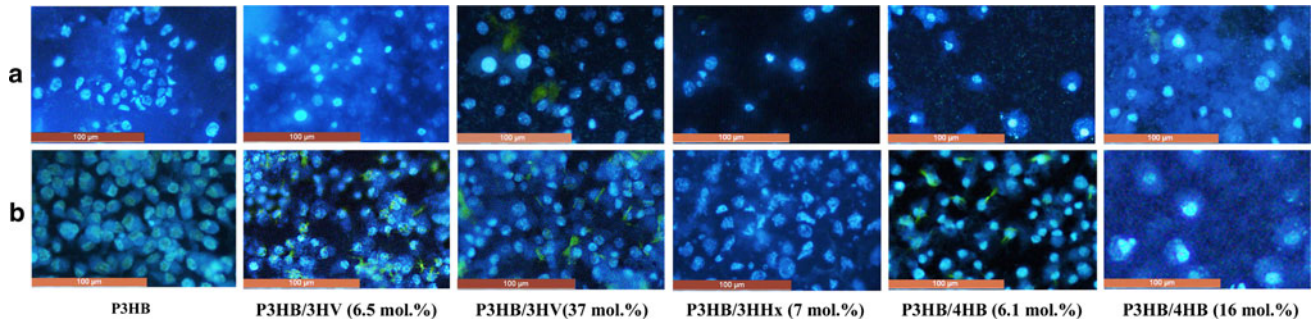


Fig. 4 DAPI staining of fibroblast NIH 3T3 cells on microparticles of different types sterilized with autoclaving (**a**) and H_2O_2 plasma (**b**), 7 days after seeding: *P3HB* poly-3-hydroxybutyrate, *P3HB/3HV* (6.5 mol%) poly-3-hydroxybutyrate-co-3-hydroxyvalerate (6.5 mol%), *P3HB/3HV* (37 mol%) poly-3-hydroxybutyrate-co-3-hydroxyvalerate (37 mol%), *P3HB/3HHx* (7 mol%) poly-3-hydroxybutyrate-co-3-hydroxyhexanoate (7 mol%), *P3HB/4HB* (6.1 mol%) poly-3-hydroxybutyrate-co-4-hydroxybutyrate (6.1 mol%), *P3HB/4HB* (16 mol%) poly-3-hydroxybutyrate-co-4-hydroxybutyrate (16 mol%)

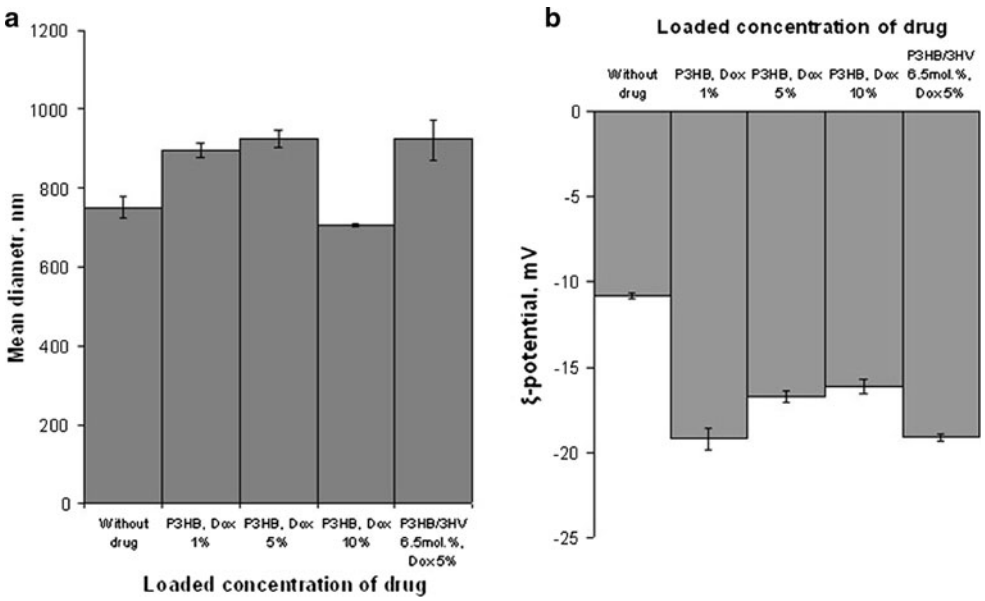


Fig. 5 Mean diameter and **a** ξ-potential **b** of the microparticles prepared from poly-3-hydroxybutyrate (P3HB) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate 6.5 mol% (P3HB/3HV 6.5 mol%) after loading different concentrations of drugs: *1* P3HB, without drug; *2* P3HB, 1 % loaded drug; *3* P3HB; 5 % loaded drug, *4* P3HB, 10 % loaded drug; *5* P3HB/3HV 6.5 mol%, 5 % loaded drug

10 % DOX. The DOX release from microparticles was gradual; during 528 h of the experiment the following content of the drug in the environment was registered: 23.61 ± 1.9, 28.15 ± 1.8, and 34.6 ± 2.3 %, respectively, with the initial load of the microparticles 1, 5, and 10 %.

Drug release kinetics from PHA microparticles can be described by diffusion-kinetic equations that were proposed by Livshits and coauthors [19] and Goreva and coauthors [20].

The graphic solution of the equations in coordinates

$$(m_t/m_\infty) - (t)^{0.5} \quad (1)$$

and in semilogarithmic coordinates

$$\ln(1 - m_t/m_\infty) \quad (2)$$

yielded quantification value of the drug diffusion coefficient in the polymer phase. Table 2 gives DOX diffusion coefficients in P3HB/3HV (6.5 mol% 3HV) microparticles. These results suggest a clear relationship between diffusion coefficients and the drug loading in the microparticles.

In the first phase, diffusion coefficient is 2 and 9 times higher for the microparticles with the greatest drug loading than for the microparticles with lower loading—5 and 10 % of the initial DOX content of the microparticles, respectively (Table 2, Eq. 1). In the second phase (when the curve reaches a plateau), diffusion coefficient drops by an order of magnitude, whatever DOX content of the microparticles.

In the first phase, drug release occurs due to the classical diffusion process. The linear phase of antibiotic release is recorded simultaneously with diffusion. Slopes of linear segments are close to each other and correspond to the constant of hydrolytic degradation of P3HB/3HV. These results prove that drug release from P3HB/3HV microparticles occurs due to the classical diffusion process.

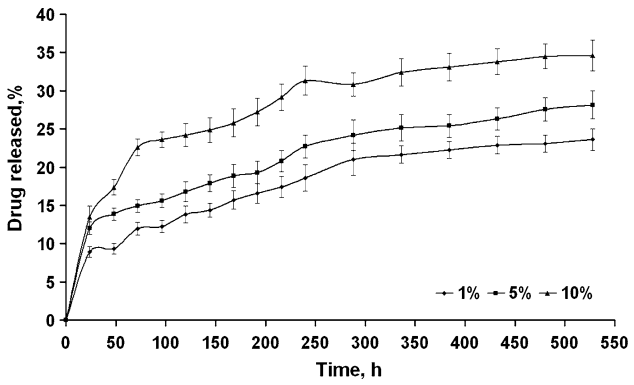


Fig. 6 Dynamics of DOX release from poly-3-hydroxybutyrate-co-3-hydroxyvalerate (6.5 mol%) microparticles with different levels of antibiotic loading

3.4 An in vitro study of the inhibiting effect of DOX-loaded microparticles

Figure 7 shows results of evaluation of the inhibiting effect produced by DOX-loaded microparticles on HeLa cell culture versus the effect of the free drug (Fig. 8).

At implementation of smaller particles (0.2 microns) with the highest load (0.6 μg/ml) the effect of the cytostatic drug depositing is comparable with free form as by the time of beginning of the action, so by the inhibiting effect on the cells. Particles loaded with the medium and lowest concentration (3.2 and 6.0 μg/ml) inhibited the growth of tumor cells only by the 3rd day of the experiment comparable with free DOX, but the beginning of the drug's action was late in time; the maximum inhibiting effect was observed on the 4th day. This is connected with the kinetics of the drug outflow from the polymer matrix into the culture at which in the first 2 days the release of the drug in the culture was low (at the level 0.09 and 0.07 μg/ml for the highest and the lowest concentration of DOX, correspondingly) and this concentration was insignificant for suppression of HeLa growth.

At implementation of larger polymer particles the effect of DOX depositing was more expressed (Fig. 7b). Delay in the inhibiting effect was registered only on the first day and only for the lowest and medium concentration of DOX (correspondingly, concentration of DOX in the culture made 0.08 and 0.28 μg/ml). Nevertheless, already on the second day the cytostatic effect of the deposited DOX was comparable with the action of the free drug.

These findings demonstrated the efficiency of the cytostatic drug deposited in the microparticles constructed from resorbing polymers in relation to the culure of HeLa tumor cells.

4 Discussion

In this study, for the first time, microparticles were prepared from four types of PHAs, containing different fractions of 3HV, 4HB, and 3HHx monomer units, and their

Table 2 Diffusion coefficients of DOX in P3HB/3HV (3HV 6.5 mol%) microparticles determining the initial and final stages of the diffusion process

Amount of encapsulated of DOX (%)	Diffusion coefficients D × 10^{-4} (cm/s)	
	At initial stage	At final stage
1	2.5	0.008
5	9.9	0.026
10	22.6	0.075

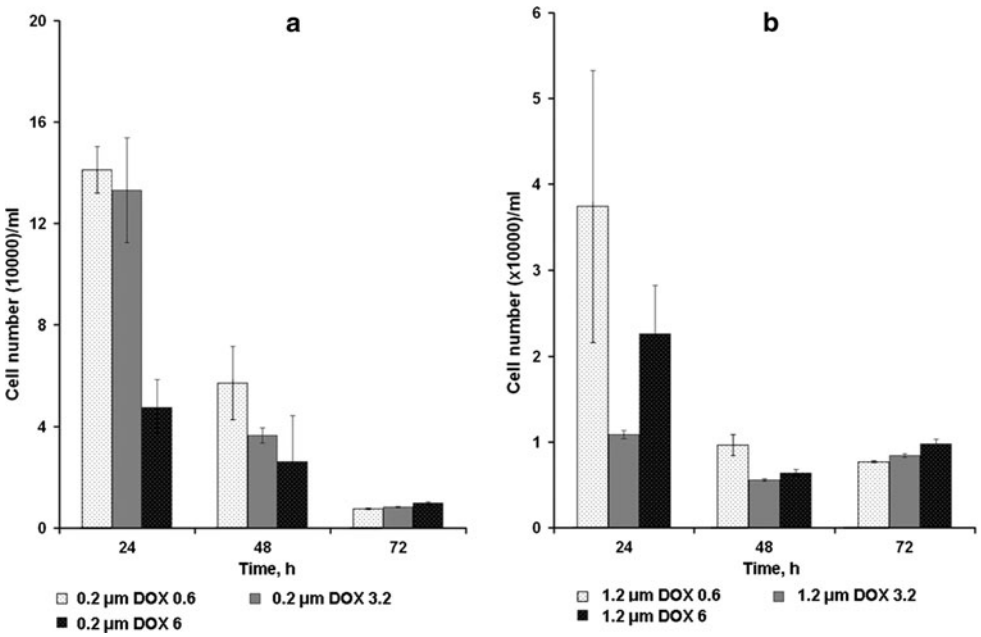


Fig. 7 MTT assay: the effect of DOX encapsulated in 0.2 μm (**a**) and 1.2 μm (**b**) polymer microparticles on the number of viable cells in HeLa cell culture

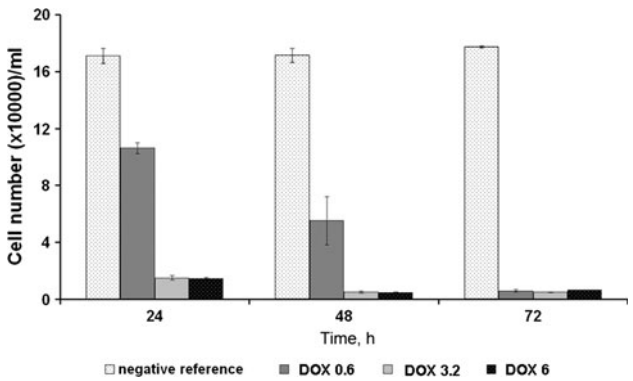


Fig. 8 The effect of free DOX concentration on the number of viable cells in HeLa cell culture: negative control, drug-free culture (DOX concentration)—0.6, 3.2, 6 μg/ml

comparative investigation was carried out. The studies reported in the available literature described PHA microparticles prepared from one or two types of PHAs, without discussing the effect of the chemical composition of the polymer on the properties of microparticles. The polymers used to prepare microparticles in those studies were P3HB/3HV with a low molar fraction of 3HV (6–15 %) [25–27]; P3HB/3HV containing 5 mol% 3HV and PHB3/HHx with 12 mol% 3HHx [28]; P3HB/3HV (12 and 33 mol% 3HV) and P3HB/4HB (6 and 20 mol% 4HB) with mPEG [29].

The present study revealed a significant effect of the chemical composition of the polymer on the average diameter and ξ-potential of microparticles. For instance, the surface of the particles prepared from copolymers with increased molar fractions of 3HV and 4HB was rougher and their average diameter was 1.7–2.5 times greater than that of P3HB particles.

There are very few literature data on ξ-potential of PHA microparticles. This study showed that the values of ξ-potential of microparticles prepared from different types of PHAs varied significantly. The lowest values of ξ-potential were recorded for PHB3/HHx microparticles (−32.2 mV) and the ξ-potential of P3HB was no higher than −11 mV.

An important part of this study is comparative evaluation of biocompatibility and adhesive properties of microparticles sterilized by different methods. MTT assay performed to determine viability of cells cultured in the presence of PHA microparticles did not show any toxic effect of PHA microparticles treated by autoclaving or with H_2O_2 plasma on direct contact with fibroblast NIH 3T3 cells. The number of viable cells adhering to the surface of the matrices treated with H_2O_2 plasma was higher than on the surface of the autoclaved ones in all treatments. Results of MTT assay and cell counts using the fluorescent DAPI DNA stain showed that microparticles prepared from PHAs of different chemical compositions and sterilized by autoclaving or with H_2O_2 plasma did not exhibit any cytotoxicity. These results are in good agreement with the data reported in the studies that evaluated biocompatibility of microparticles in NIH/3T3 cell cultures, in which microparticles were prepared from P3HB and copolymers P3HB/3HV (5 mol% 3HV) and P3HB/3HHx (12 mol% 3HHx) [28] and amphiphilic nanoparticles with mPEG were prepared from P3HB/3HV (33 mol% 3HV) and P3HB/4HB (20 mol% 3HHx) [29].

This study was the first to reveal the effect of loading P3HB and P3HB/3HV (6.5 mol% 3HV) microparticles with doxorubicin on the ξ-potential of microparticles. The ξ-potential of P3HB microparticles loaded with DOX (1, 5, and 10 % of the polymer mass) was lower than that of the

unloaded microparticles. Similar results were obtained for copolymer microparticles. Thus, DOX loading had a favorable effect on this parameter of the particles. Different levels of DOX loading also changed the average diameter of microparticles.

Studies published in the past few years suggest high potential of polymer microsystems for the delivery of drugs, including doxorubicin [30–32]. The formulations described in those studies include polymer micro- and nanoparticles, doxorubicin-polypeptide conjugates for thermally targeted delivery, polymer micelles with ionic crosslinking for conjugation with the drug, micelles, etc. [33–35].

The present study showed that doxorubicin was released from the microparticles without any burst effect. The study of DOX release kinetics as dependent upon the drug content of the matrix of microparticles showed that drug release rate became almost 1.5 times faster as DOX content of the matrix of microparticles was increased from 1 to 10 %. The results obtained in this study compare well with the data on the release kinetics of gentamicin from P3HB/3HV microparticles [36]; tramadol, piroxicam, and ibuprofen from P3HB microparticles [8, 10, 11].

The cytostatic effect of P3HB/3HV (6.5 mol% 3HV) microparticles loaded with DOX was estimated in the culture of tumor cells—HeLa, using microparticles with diameters 0.2 and 1.2 μm, containing 0.6, 3.2 and 6 μg/ml DOX. The use of the larger-sized particles resulted in a more pronounced effect of DOX. At day 3, however, the cytostatic effect of the drug embedded in the particles was comparable with the effect of free DOX.

5 Conclusion

Microparticles were prepared from different types of PHAs. The experiments showed that by varying the chemical composition of PHAs, one can prepare microparticles with different properties, which would be suitable for drug loading. The average diameter and ξ-potential of microparticles were found to be dependent on the level of loading (1, 5, and 10 % of the polymer mass). None of the high-purity PHAs directly contacting with NIH 3T3 fibroblast cells caused any toxic effect or impaired viability of these cells, i.e. all PHAs used in this study are biocompatible and suitable for biomedical use. The effectiveness of the cytostatic drug embedded in P3HB/3HV (6.5 mol% 3HV) microparticles was proved in the culture of tumor cells—HeLa. Results of the study provided a basis for experiments on animals.

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