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A comparative investigation of biodegradable polyhydroxyalkanoate films as matrices for *in vitro* cell cultures

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The paper describes the production and investigation of flexible films made of high-purity polyhydroxyalkanoates (PHAs) – polyhydroxybutyrate [poly-(3HB)] and poly-3-hydroxybutyrate-*co*-poly-3-hydroxyvalerate [poly(3HB-*co*-3HV)], containing 4–30 mol % hydroxyvalerate. Poly(3HB-*co*-3HV) films have a more porous structure than poly-(3HB) films, which are more compact, but their surface properties, such as wettability and surface and interface energies, are the same. Sterilisation of the PHA films by conventional methods (heat treatment and γ-irradiation) did not impair their strength. Cells cultured on PHA films exhibited high levels of cell adhesion. Cell morphology, protein synthesis and DNA synthesis were estimated by extent of ^{3}H-thymidine incorporation into the animal cell cultures of various origins (fibroblasts, endothelium cells, and isolated hepatocytes) in direct contact with PHAs. The investigation showed that this material can be used to make matrices for *in vitro* proliferous cells. The investigated properties of poly-(3HB) and poly(3HB-*co*-3HV) films proved to be fundamentally similar.
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1. Introduction

Polyhydroxyalkanoates (PHAs) are a class of naturally occurring biodegradable and biocompatible polyesters that are produced by a wide variety of different microorganisms [1, 2]. PHAs are promising candidates to be widely applied in various spheres, including medicine and pharmacology [3]. In recent years PHAs have become one of the leading classes of biomaterials investigated for the development of tissue-engineered cardiovascular products because they can offer properties not available in existing synthetic absorbable polymers [4]. Today there are more than 100 known PHAs [2], which have different structures and, consequently, significantly differ in their physical-mechanical properties and kinetics of biodestruction. Among them are high-crystallinity and thermoplastic materials as well as elastic and rubber-like polymers with low melting points [5]. The first and most thoroughly investigated, of the PHA family is the high-crystallinity polymer of β-hydroxybutyric acid [polyhydroxybutyrate, poly-(3HB)]. It has ideal biocompatibility because the polymer as such, and its derivatives, are the only products of cell metabolism and are present in blood and tissues [6]. However, due to brittleness and low mechanical strength of polyhydroxybutyrate, its application is significantly limited. At the present time, although the known PHAs are quite diverse, only few of them are being investigated: isomers and copolymers hydroxybutyrate, poly-3-hydroxybutyrate [poly-(3HB)], poly-4-hydroxybutyrate [poly-(4HB)], poly-3-hydroxybutyrate-*co*-poly-4-hydroxybutyrate [poly(3HB-*co*-4HB)]; poly-3-hydroxyoctanoate-co-poly-3-hydroxyhexanoate [poly(3HO-*co*-3HH)] and poly-3-hydroxybutyrate-*co*-poly-3-hydroxyvalerate [poly(3HB-*co*-3HV)] [4]. The copolymers poly(3HB-*co*-3HV) feature good strength properties, which vary widely depending on monomer proportions; hence they have potential use for many applications [7]. This PHA, however, contains hydroxybutyric acid and also other monomers, for example, hydroxyvalerate, and, hence, toxicological properties of the material must be tested thoroughly.

The results of toxicological investigations of poly(3HB-*co*-3HV) in animal cell cultures *in vitro* and on test animals *in vivo* show that there may be cytotoxic and inflammatory reactions of different intensity, depending on the hydroxyvalerate content of the PHA [8, 9]. However, the reaction of cells and tissues to PHAs depends not only on the chemical composition of the material but also on the degree of purity [10], methods of processing and form of items [11, 12], properties of the surface and methods of its treatment [13–15].

We conducted chemical investigations of poly-(3HB) and poly(3HB-*co*-3HV) and found significant differences in some data: pH shift, spectroscopy of PHA aqueous extracts in the UV range, and permanganate test. We assumed that the material contained impurities of biological nature in the form of residual components of bacterial cells. This was confirmed in the investigations

of the material for its possible application in contact with blood [16]. An express analysis of PHA hemocompatibility was carried out *in vitro* to quantify the platelets attached to polymer film surfaces and to determine their morphology as well as the plasma recalcification time and the reaction of the complement. The estimates for the two last-mentioned parameters suggested an activation of haemostasis systems. A detailed chemical analysis of the PHAs revealed the presence of long-chain fatty acids, which can cause inflammatory and pyrogenic reactions. The subsequent investigations of additionally purified PHAs, free of these acids, and tests for haemocompatibility did not register any adverse reactions [16].

These results suggest that in biological investigations of PHAs, primary consideration should be given to the degree of purity of the material. Other important aspects are a possible effect of the chemical composition of the material as such, methods of processing the material into items, and physical-mechanical properties of these items.

The purpose of this work was to produce films of high-purity poly-(3HB) and poly(3HB-*co*-3HV), to compare their properties, and to assess their suitability for *in vitro* cell culturing.

2. Materials and methods

2.1. Preparation of pure PHA polymers

The tested material was the PHA samples synthesized by the bacteria *Ralstonia eutropha*. The strain of *R. eutropha* B578 is registered in the Russian Collection of Industrial Microorganisms. The bacteria were grown under autotrophic conditions in batch cultures in a 10-l laboratory fermenter equipped with a turbine-type mixer at 1000 rpm; the culture contained the mineral salts medium, and carbon dioxide and hydrogen as sources of carbon and energy. A diaphragm-type compressor continuously pumped the gas mixture through the culture at a rate of 8–12 l/min, the volumetric efficiency being 0.3. The initial volume proportions of CO_2, O_2, and H_2 in the control were 1 : 2 : 7, respectively. To attain maximum accumulation of a PHA, we used a two-stage batch cultivation mode: in the first stage, the bacteria were grown in a nitrogen-deficient medium (for 25–30 h) and in the second, in nitrogen-free medium at pH 7.0 and temperature 30 °C (for 20–30 h). Carbon dioxide was the carbon source in the nutrient medium used to synthesize poly-(3HB), and the medium used to synthesize the poly(3HB-co-3HV) copolymers also contained sodium valerate at a concentration of 2 g/l.

PHAs are extractable from bacterial biomass due to their ability to dissolve in organic solvents and to be then precipitated by alcohols. The PHAs were extracted from bacterial biomass with chloroform and precipitated with ethanol. The extraction of PHAs from biomass was conducted in several stages. In the first stage, to partially destroy the cell wall and attain a fuller extraction of lipids, the 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 backflow condenser for 30 min. The alcohol was then removed, the biomass was covered with 86% ethanol and separated from alcohol by centrifuging. In the next stage 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 polymers was repeated several times to prepare specimens that would not contain organic impurities of protein, carbohydrate, or lipid nature. All the organic solvents used in the procedure were distilled earlier to remove impurities.

The chemical purity of the resulting specimen was estimated by conventional biochemical methods. The presence of protein impurities was determined by the Kjeldal micro-method [17] and carbohydrates by the anthranone method [18].

To determine the PHA composition, approximately 4 mg of PHA were reacted in a small flask, using the backflow condenser, with a solution containing 1 ml chloroform, 0.85 ml methanol, and 0.15 ml sulfuric acid for 140 min at 100 °C in thermostatically regulated bath [19]. This method degraded PHA by methanolysis to its constituent β-hydroxycarboxylic acid methyl esters (FAME). After the reaction, 0.5 ml of distilled water was added and the tube was shaken for 1 min. After phase separation, the organic phase was removed, transferred into a vial and used for analysis. FAMEs were analysed with gas chromatograph-mass spectrometer (GC/MS, model GCD Plus, Hewlett Packard, USA), equipped with a 30 m × 0.25 mm HP-5 (5% diphenyl and 95% dimethylpolysiloxane) fused silica capillary column. Chromatographic conditions were: carrier gas helium; flow rate 1 ml/min; sample input temperature 220 °C; initial temperature 70 °C, programmed to 230 °C at a rate of 8 °C/min; interface temperature 250 °C; ion source temperature 175 °C; electron impact mode 70 eV; scanning from 45 to 450 amu at 0.5 s/scan.

2.2. Investigation of physical and chemical properties of PHAs

The molecular mass of the polymer (M_w) was measured in an Ubellode viscometer, with capillary diameter 0.34 mm, at 30 °C. PHA solutions in chloroform were used, with polymer concentration (C g/l) 0.25–1.0. Experimental measurements of the flow time of the solvent and the polymer solutions of various concentrations were used to calculate the corresponding values of the relative (η_{rel}) and specific (η_{sp}) viscosity and the η_{sp}/C ratio. Then a graph was constructed, which showed the relationship between η_{sp}/C and polymer concentration in the solution (C). The results were extrapolated to C equal to 0, and intrinsic viscosity $[\eta]$, proportional to M_w, was obtained.

The degree of crystallinity was measured on a D8 ADANCE X-ray spectrometer (Bruker, Germany) (graphite monochromator on a reflected beam) in a scan-step mode, with step 0.04°, exposure time 2′. The operating mode of the instrument was 40 kV × 40 μA.

2.3. Polymer film casting

Films were prepared by casting chloroform solution (from 5% to 15% w/v) on degreased glass and subsequent drying at room temperature for two to three days in a dust-free box. Film thickness was measured with an MKO-25 micrometer (Russia) at sensitivity 0.01 mm. The film thickness (average of 10 measurements) was 5.507 μm (the error was 0.026). Segments of equal thickness were selected and disks of diameter 15 mm were cut out to be further used in the experiments.

2.4. Scanning electron microscopy

The microstructures of film surfaces were analysed by electron microscopy. The samples were placed on the microscopic stage, coated with carbon and aluminium in a JEE-4C vacuum evaporator and studied using a JEM-100C electron microscope with an EM-ASID-4 raster attachment (Japan).

2.5. Properties of film surfaces

The following characteristics of film surfaces were calculated by measuring contact angles for water in air (θ, degrees), using known equations [20]: free surface energy (γ_S), free interface energy (γ_{SL}), and cohesive force (W_{SL}) (erg/cm^2). To determine contact angles for water in air, film samples were placed on the microscopic stage, and distilled water was dropped on them with an automatic macropipette – 100, 200, and 300 μl, 10 drops of each volume. With a digital camera a computer image of the drop was obtained, and the angular value was determined (the average angular value was calculated from the measurement of 10 drops of each volume). Free surface energy of polymer films (erg/cm^2) was found from the equation $\gamma_s = \gamma_L(1+\cos\theta)^2/4$, where γ_L is free water surface tension, 72.8 erg/cm^2. Free ''polymer-water'' interface energy (γ_{SL}, erg/cm^2) was found from the equation $\gamma_{SL} = \gamma_S + \gamma_L - W_{SL}$, where γ_{SL} is the criterion of the residual interface energy; γ_S and γ_L are free energies of film and water surfaces, respectively. The cohesive force, which characterises the strength of the interface adhesion seam, was calculated from the relationship: $W_{SL} \approx 2\sqrt{\gamma_S\gamma_L}$ (erg/cm^2).

2.6. Cell culture

Cells used in this study: mouse fibroblast cell line NIH 3T3, which belongs to the least transformed cell lines, and retains many features of normal diploid cells and primary cultures of parenchymal cells (hepatocytes) and non-parenchymal (mostly endothelial) cells of the mouse liver. Hepatocytes were extracted by the two-stage (non-enzymatic and enzymatic) non-recirculation perfusion of the liver of anesthetised mice. *Clostridium histoliticum* collagenase (Serva, USA) was used at a concentration allowing the maximum harvest of live hepatocytes (up to 96% cells not incorporating trypan blue at pH 7.2; 0.5% stain solution in 0.85% NaCl). After perfusion the liver was placed in perfusion solution and cut with blood vessel scissors into small pieces, which were then destroyed further and rubbed through a $50 \times 50\,\mu m^2$-pore-sized nylon filter. Cell suspension was centrifuged under mild conditions (50 g, 3 min) through a Metrizamid cushion to separate parenchymal cells (hepatocytes) from non-parenchymal (endothelial) cells.

2.7. Growth of the cells on polymer films

Fibroblasts of line NIH 3T3 were cultured in DMEM medium supplemented with 10% FCS (RPA Vector, Novosibirsk), 1.0 mM L-glutamine, 10 mM HEPES, and 100 μg/ml kanamycin sulfate (BDSL, UK). Hepatocytes were re-suspended in the mixture DMEM + NCTC-135 (1 : 1) (BDSL, UK) that contained 5% FCS inactivated at 56 °C, 0.1% glucose, 0.2% serum albumin for cell cultures, 0.5 μg/ml of insulin, 50 ng/ml of dexamethasone, 0.2 μg/ml of glucagons (Sigma, USA), 0.02 mM β-mercaptoethanol, 10 mM HEPES, and 100 μg/ml of kanamycin sulfate. Endothelial cells were re-suspended in Ham's F12 medium (BDSL, UK), containing 10% FCS. The cell count increased after the cells were passaged two or three times using 0.2% collagenase solution diluted with a 0.02% versene solution. All stages were conducted under sterile conditions.

Cells were plated into penicillin vials (five vials per test site) containing either PHA films or coverslips (control) in 2 ml. Cells were cultured in a humidified atmosphere at 5% CO_2 at 37 °C. The duration of experiments ranged from 4 h to three days. To investigate the adhesion of cells on films and their morphology, samples were taken every hour; to determine protein concentration in the culture, cells were grown for three days and sampling was made once a day. To detect possible toxicity of poly-(3HB) and poly(3HB-*co*-3HV) with 15 and 28 mol % hydroxyvalerate, we investigated DNA synthesis in cell cultures grown on polymer films and compared it with control. The concentrations of both fibroblasts and endothelial cells were 30 000 cells/ml, and that of hepatocytes was 50 000 cells/ml. We used a radioisotope method. The culture medium was supplemented with ^{3}H-thymidine as a ^{3}H-labelled substrate. The reagent dose was 37 kBq/ml of culture or 0.004 mM ^{3}H-thymidine/ml (0.0008 mM ^{3}H-thymidine added to each sample). Cells were cultivated for 16 h and sampled every 2 h for radioactivity count.

2.8. Cytological and biochemical investigations

For cytological investigations, fixed and incompletely dried cells were Giemsa stained in a standard Jurr 65 500 buffer at pH 6.8 and examined under a microscope with an H190/1.30 immersion objective and a PKIOX ocular. The ability of cells to adhere to polymer films was estimated *vs.* control (coverslips) by plating 10^3 cells/ml and counting the attached cells in 100 fields of vision, in 1–4 h, using an inverted-stage microscope at 10 × 3.5 magnification. Preliminarily, cell viability was estimated by live staining with trypan blue (0.5% stain solution in 0.85% NaCl). Whole protein concentration in cell culture was determined spectrophotometrically (λ 540 nm), according to Bredford [21].

Radioactivity of samples was measured by a standard method of liquid scintillation counting [22]. The samples were prepared as follows. A sample contained in one vial

was filtered through a membrane. The collected cells were rinsed in a funnel with a 0.1% HCl solution and then with a 3% cold solution of trichloroacetic acid; then they were dried. The dried sample was placed into a 25-ml vial and 5 ml of scintillation liquid was added. The main scintillator was diphenyloxazole (PPO) and the secondary one was di-VSB-p-di(o-methylstyrene)-benzene (POPOP). The label (^3H) that was incorporated into the insoluble precipitate of trichloroacetic acid was counted using a ''Beta-1'' scintillation counter (''Medapparatura'', Ukraine) simultaneously with a standard sample and a reference sample. Radioactivity of samples was determined as pulse per minute (pulse/min) per unit volume of solvent (ml) [23].

2.9. Statistics

All measurements in the *in vitro* tests were carried out in duplicate. Statistical analysis of the results was carried out using the standard software package of Microsoft Excel. The results were expressed as arithmetic means with standard deviations. Significant differences between mean values in control and test groups were tested using student's *t*-test (significance level: $p = 0.05$) by standard methods.

3. Results

The PHA samples used to make films had the following characteristics: poly-(3HB) – M_w 340 000 Da, crystallinity 70–78%, and copolymers of poly(3HB-*co*-3HV) containing from 4 to 30 mol % hydroxyvalerate – M_w 295 000–360 000 Da, crystallinity 50–60%. The degree of purity of the PHA samples taken for investigation was up to 99.9%. No trace amounts of protein, carbohydrate and lipid compounds were registered in them; there were no long-chain fatty acids either. Among impurities we identified mineral elements and metals, which appear in the polymer as a result of using certain reagents (alkalis, chloroform, ethanol) in the extraction process. The total content of mineral elements in the PHA samples was below 0.1%: Na constituted 0.036–0.013%, Ca 0.005%, Mg 0.036–0.013%, Fe 0.0052–0.009%, Cu 0.0046–0.0145%, and Zn 0.0019–0.0045% of absolutely dry matter.

To estimate the feasibility of producing poly-(3HB) and poly(3HB-*co*-3HV) films, we investigated how these polymers behave when dissolved at various concentrations in a number of solvents (chloroform, dimethyl formamide, dichloroethane, and a mixture of chloroform and toluene). The investigated solutions contained 5, 10, and 15% PHAs. Of the tested variants, only 15% PHA chloroform, dichloroethane, and dimethyl formamide solutions formed a bound gel. However, when a PHA was dissolved in dimethyl formamide, its molecular mass decreased almost twice, while in dichloroethane and chloroform the polymer M_w did not change.

Films derived from the 15% PHA solutions were at least 0.1 ± 0.01 mm thick. Examination by SEM showed that the surfaces of polymer films of different composition vary somewhat topographically (Fig. 1). The more elastic films of a less crystalline copolymer material, poly(3HB-*co*-3HV), had a more porous and laminar structure than more crystalline and compact poly-(3HB) films.

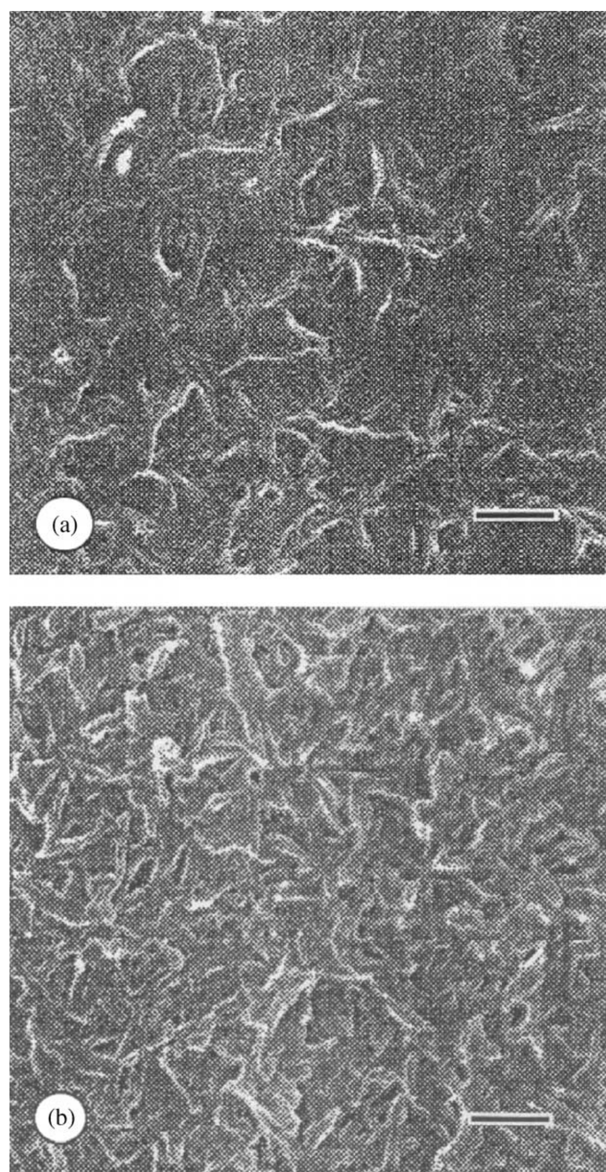

Figure 1 Topography of film surfaces: (a) poly-(3HB), (b) poly(3HB-*co*-3HV) (containing 15 mol % hydroxyvalerate). Marker 1 μm.

The results of investigations of film surface properties are listed in Table I. It has been found that the chemical composition of a polymer, that is, the presence of hydroxyvalerate, bears little influence on the tested surface properties of the material, such as the contact angle for water in air (θ), surface tension (γ), free interface energy and cohesive forces (W_{SL}). No statistically significant differences have been recorded between the film surfaces that front towards the air or the glass.

The hydrophobic surfaces of films prepared from the PHAs of both types are similar to those of synthetic polyesters (polyethylene terephthalate, polymethyl methacrylate, polyvinyl chloride, and polyethylene).

In principle, PHAs, which are thermoplastic and radiation resistant, can be sterilised by various methods. However, the available data on PHA items' tolerance of sterilization methods are fragmentary and ambiguous. In the work by Lootz *et al.* [24] it has been shown that experimental polyhydroxybutyrate items treated with ethylene oxide and formalin vapour, and autoclaved do

TABLE I Characterisation of PHA film surfaces

Parameter	PHAs			
	Poly-(3HB)	Poly(3HB-*co*-3HV) (containing 3HV mol %):		
		4	18	30
Contact angle for water in air (θ, degrees)	67°06′	65°60′	67°59′	67°33′
Surface tension (γ, erg/cm^2)	36.16	36.18	34.66	34.91
Free interface energy (γ_{SL}, erg/cm^2)	6.77	6.35	7.00	6.89
Cohesive forces, (W_{SL}, erg/cm^2)	101.19	102.63	100.46	100.82

not decompose; neither do the molecular weight and strength of the items change; however, the items sterilised with a γ-source (40 kGy) feature a significant decrease in M_w and in hardness. Williams and Miller [25], however, observed a sharp decline in strength properties of monofilaments made of polyhydroxybutyrate and copolymers of hydroxybutyrate and hydroxyvalerate γ-irradiated with a much lower dose (10 kGy); however, still lower doses (2.5 and 5.0 Mrad) produced little, if any, effect on the structure and strength properties.

To estimate resistance of polymer films to the action of sterilising agents, we investigated the effect produced by thermal and chemical sterilisation methods on their physical-mechanical properties (Table II). Films were sterilised by heat treatment (in a heat-drying cabinet for 24 h at 120 and 140 °C), by autoclaving at 120 °C for 45 min (1.1 atm), by γ-irradiation (2.5 Mrad), and by keeping in disinfecting solutions – 96% ethanol (24 h) and 6% hydrogen peroxide (6 h).

The molecular mass of the polymer film material was not significantly influenced by the method of sterilisation, but the strength properties of poly-(3HB) films somewhat depreciated after autoclaving and thermal air-drying at 140 °C. The films became brittle, and their strength somewhat decreased (by less than 20%). The tested methods of sterilisation did not affect the strength properties of the poly(3HB-*co*-3HV) films.

The results of investigating the adhesion of NIH 3T3 fibroblasts on sterilised polymer film surfaces *vs*. control (coverslips) are presented in Fig. 2. Fibroblasts were cultured for 4 h in the medium with 10% FCS in direct contact with the polymer films that had been treated by the above methods; samples were taken every hour to count the attached cells. It is worth noting, however, that the sterilized films had low wettability and had to be kept for at least 2 h in distilled water before cells were plated on them, as cells are poorly attached to a hydrophobic surface. During the first hours, the number of cells attached to the polymer films that had been autoclaved by thermal methods was lower than in the control; the effect was particularly manifest on poly-(3HB) films. However, by the end of the experiment, in 4 h after the samples had been sterilised with air-drying at 140 °C, autoclaving, and γ-irradiation (2.5 Mrad) and treated with alcohol, the number of cells attached to the polymer film surfaces poly-(3HB) and poly(3HB-*co*-3HV) (Fig. 2) was comparable to that of the control cells (on coverslips) Thus, the PHA films sterilised by different methods have a high adhesive capacity for the cultured mouse fibroblasts.

Contradictions in the literature data on cytotoxicity of polyhydroxyalkanoates containing hydroxyvalerate have encouraged us to conduct an investigation and compare toxicological properties of poly(3HB-*co*-3HV) and poly-(3HB) in animal cell culture *in vitro*.

The NIH 3T3 fibroblasts cultured on polymer films of any composition retained cell morphology characteristic of normal (control) cells, cultured on coverslips. Live staining with tryptan blue demonstrated that $99.8 \pm 0.2\%$ cells cultured on poly-(3HB) and poly(3HB-*co*-3HV) films (containing 4 to 30 mol % hydroxyvalerate) did not incorporate the stain, i.e. remained highly viable. The cultivation of fibroblasts on polymer films for three days did not affect protein synthesis in the culture (Fig. 3). The doubling time of the fibroblasts cultured on the polymer films of all types corresponded to the generation time of the control cells cultured on coverslips – 25 ± 2 h.

Since the template activity is one of the most important indications of cell viability, we have studied the dynamics of DNA synthesis in the cell culture *in vitro* to detect possible cytotoxicity of PHAs. It has been known that the template processes are most intensive in the previously resting cells stimulated into proliferation. These cells are most sensitive to the damaging effect of cytotoxic factors. The materials used were cultured mouse fibroblasts (mouse fibroblast cell line NIH 3T3), which belong to the least transformed cell lines, and primary cultures of parenchymal cells (hepatocytes) and non-parenchymal (mostly endothelial) cells of the mouse liver. These cultures were chosen for the following reasons: fibroblasts are readily involved in the inflammation process and encapsulation of foreign inclusions in mice, whereas liver cells, in addition to their detoxifying function, are more sensitive to the effect of cytotoxic factors than other cells.

TABLE II Effect of sterilisation method on properties of PHA films

Sterilisation method	Poly-(3HB)		Poly(3HB-*co*-3HV)	
	M_w (kD)	Strength (kg/mm^2)	M_w, (kD)	Strength, (kg/mm^2)
Without treatment (control)	340 ± 25	3.8 ± 0.6	260 ± 19	4.0 ± 0.3
120 °C, 24 h	330 ± 19	3.6 ± 0.3	258 ± 22	4.2 ± 0.2
140 °C, 24 h	300 ± 21	3.4 ± 0.4	240 ± 15	4.2 ± 0.4
γ-irradiation (2.5 Mrad)	342 ± 21	3.7 ± 0.3	264 ± 20	4.0 ± 0.2
Autoclaving (1.1 atm)	306 ± 28	3.2 ± 0.5	250 ± 31	4.1 ± 0.5
Ethanol, 96%	340 ± 19	3.8 ± 0.2	265 ± 23	4.1 ± 0.3
H_2O_2, 6%	329 ± 23	3.7 ± 0.24	259 ± 22	4.0 ± 0.2

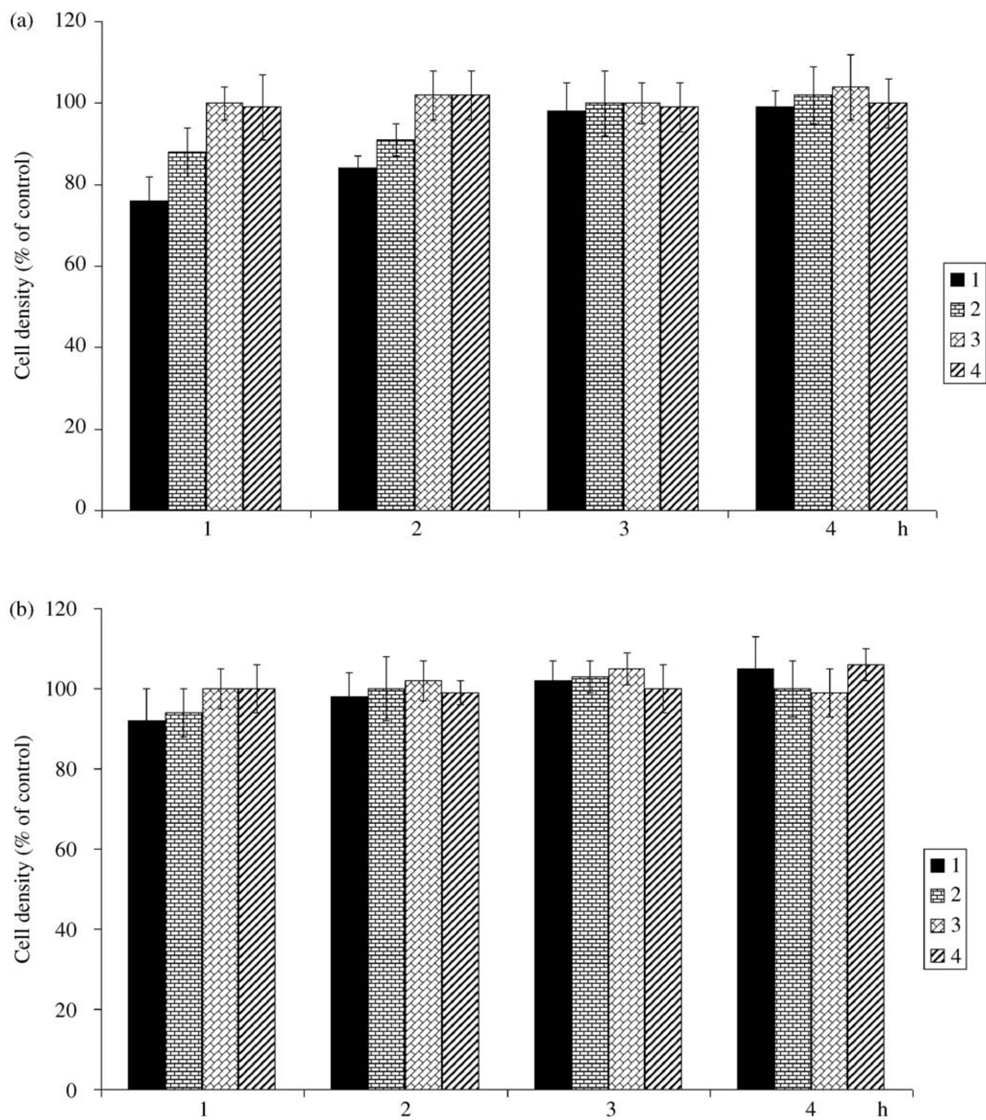

Figure 2 Effect of sterilisation method on the adhesion dynamics of mouse fibroblasts line NIH 3T3 onto poly(3HB) (a) and poly(3HB-*co*-3HV) (b) film surfaces after sterilisation: (1) air-drying at 140 °C, (2) autoclaving, (3) γ-irradiation (2.5 Mrad), (4) treated with alcohol. Along the vertical axis is the average number of cells, percentage of control (coverslips).

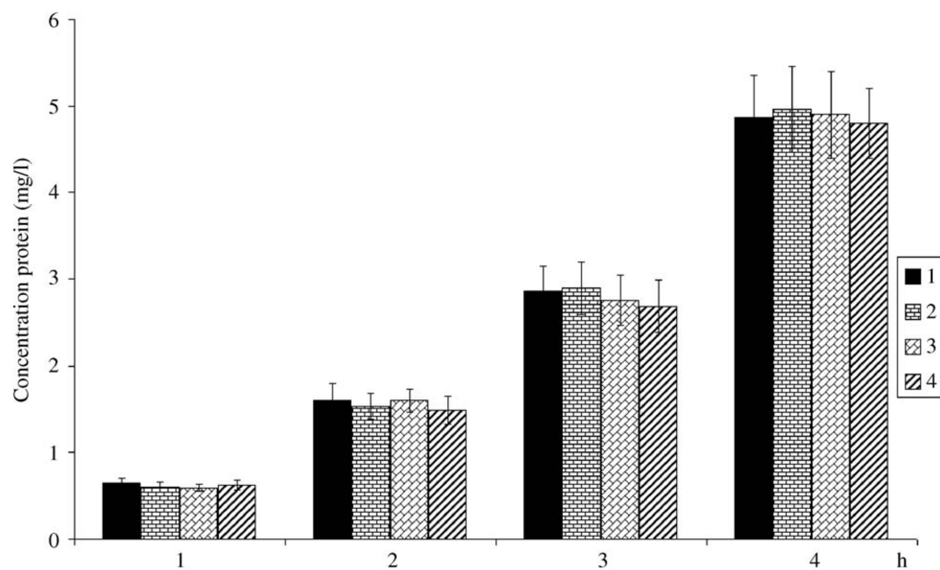

Figure 3 Dynamics of protein synthesis in the fibroblasts line NIH 3T3 cultured on: (1) coverslips (control), (2) poly-(3HB), (3) and (4) poly(3HB-*co*-3HV) with 15 and 28 mol % hydroxyvalerate films.

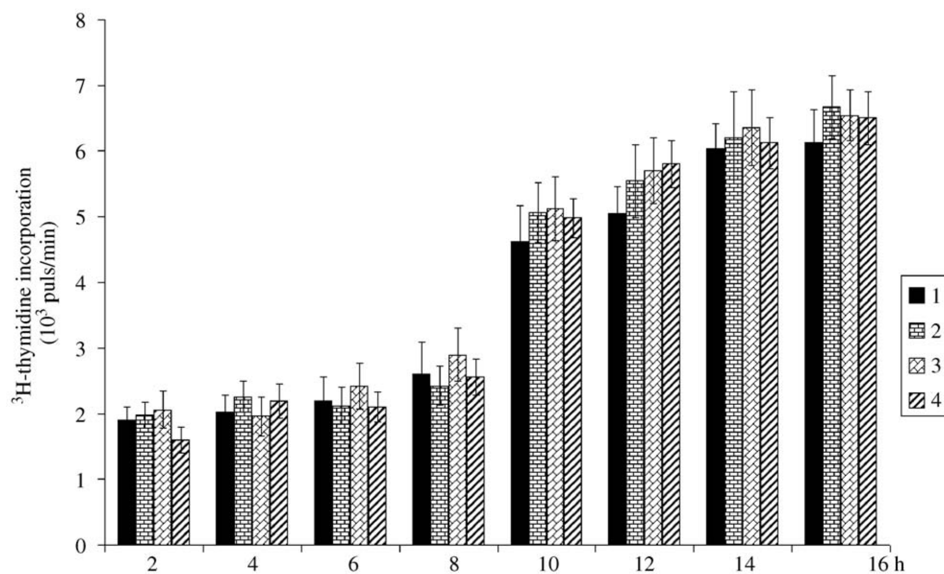

Figure 4 Kinetics of ^{3}H-thymidine incorporation into the fibroblasts line NIH 3T3 cultured on: (1) coverslips (control), (2) poly-(3HB), (3) and (4) poly(3HB-*co*-3HV) with 15 and 28 mol % hydroxyvalerate films in the medium containing 10% FCS. Label is cumulative.

The ^{3}H- thymidine incorporated into the stimulated NIH 3T3 fibroblasts cultured on poly-(3HB) and poly(3HB-*co*-3HV) with 15 and 28 mol % hydroxyvalerate films was not reduced (Fig. 4). Hence, direct contact of cells with a PHA of any composition did not result in the inhibition of DNA synthesis. The label incorporated into fibroblasts grown on polymers was similar to the incorporation observed in the control group.

The proliferative activity of cells of mesenchymal origin (endothelial cells of the mouse liver) grown on polymer films also remained unchanged. Fig. 5 shows the intensity of ^{3}H-thymidine incorporation into the endothelial cell nuclei that proliferated on poly-(3HB) and poly(3HB-*co*-3HV) with 28 mol % hydroxyvalerate films and on coverslips (control). The template activity dynamics and intensity were similar in cells grown on the polymer films of both types and in the control group.

Hepatocytes have a high sensitivity to the effect of cytotoxic factors. The results of hepatocyte culturing on polymer films are presented in Fig. 6. Hepatocytes are known to proliferate *in vitro* only in the attached state. In preliminary experiments, when they were cultivated on a glass surface, which is not adhesive for these cells, the label incorporation was lower (two to three times) than the one observed when hepatocytes were cultivated on either collagen-treated coverslips or polymeric films. Thus, we used collagen-treated coverslips as controls. The intensities of ^{3}H- thymidine incorporation into hepatocytes grown on polymer films of all types and on control were the same (Fig. 6).

Thus, our results indicate that the films of the purified poly-(3HB) and poly(3HB-*co*-3HV) containing up to 28 mol % hydroxyvalerate and free of organic micro-impurities can be sterilised by conventional methods and used to cultivate cells *in vitro*.

4. Discussion

Biodegradable natural polyesters, PHAs, are being actively investigated as candidates for various biome-

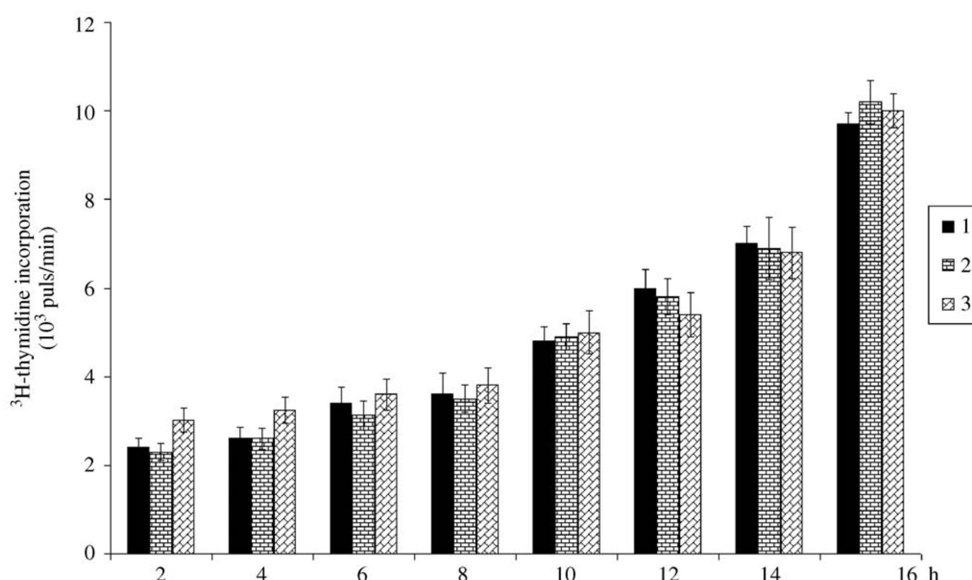

Figure 5 Kinetics of ^{3}H-thymidine incorporation into the endothelial cells of the mouse liver cultured on: (1) coverslips (control), (2) poly-(3HB), (3) poly(3HB-*co*-3HV) with 28 mol % hydroxyvalerate in the medium containing 10% FCS. Label is cumulative.

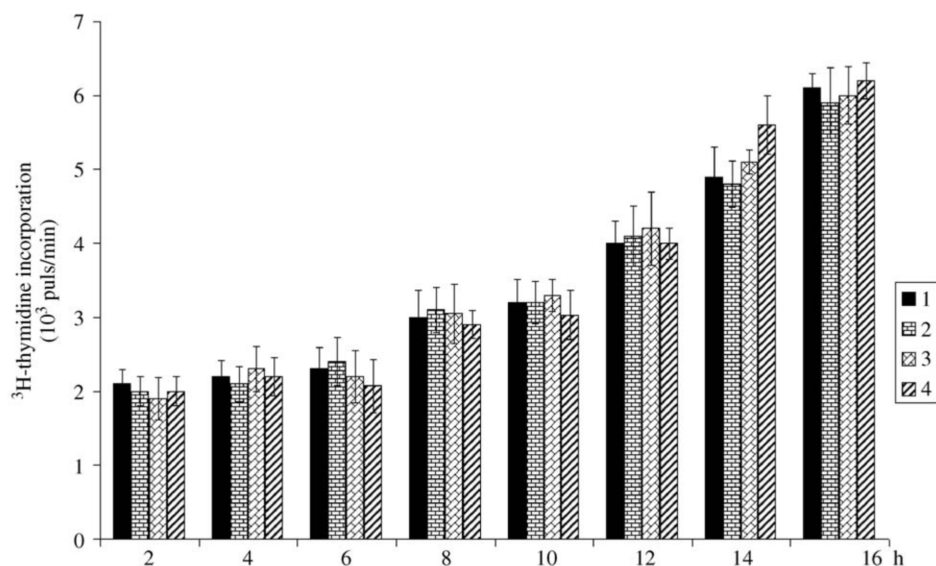

Figure 6 Kinetics of ^{3}H-thymidine incorporation into the cells of the primary culture of the mouse hepatocytes cultured on: (1) collagen-treated coverslips (control), (2) poly-(3HB), (3) and (4) poly(3HB-*co*-3HV) with 15 and 28 mol % hydroxyvalerate in the medium containing 10% FCS. Label is cumulative.

dical applications. The available data suggest that the physical-mechanical properties and biocompatibility of PHA-based products can be determined not only by the structure and basic physicochemical properties of the starting material, but also, and to a large extent, by the purity of samples and the method of production. Chaput *et al*. [8] have studied cytotoxicity of poly(3HB-*co*-3HV) on fibroblasts cultivated either in direct contact with the polymer, or on agar supplemented with aqueous extracts of variously produced PHAs. When the portion of hydroxyvalerate in the polymer increased from 7 to 22 mol %, a slight cytotoxic effect was recorded, but the cytotoxicity of extracts also depended on the conditions of their production (temperature, duration of extraction, etc.). In a series of works it was shown that block copolymers of poly-(3PHB) and poly(3HB-*co*-3HV) and polyurethanes (DegraPol®) are good materials for the growth of animal cells of various types (macrophages, fibroblasts, Kupffer cells, osteoblasts, and hepatocytes [26–28]. Yet, when the concentration of PHA particles in the medium increases (from 2 to 20 mg/l or from 40 to 400 pg/cell) and they grow in size (from 1 to 20 μm), the growth rates and metabolite production (collagen, fibronectin, etc.) of various cells decrease to a different extent [29]. It has been established that when the surfaces of PHA films and membranes are subjected to laser cutting [14], enzymatic (lipase) treatment, or chemical hydrolysis (NaOH) [13], which affect their porosity and hydrophilic property, their adhesive capacity improves greatly and fibroblasts grow better.

In the course of toxicological investigations of PHAs it was found that commercially available PHAs contained endotoxins in the form of lipopolysaccharide complexes of cell membranes released by bacteria producing polymers. The endotoxin levels can reach 100 U and more [10]. However, special treatment can decrease the endotoxin level to 20 U and make the material suitable for biomedical applications [11].

As has been mentioned above, in our earlier investigations of poly-(3HB) and poly-(3HB-*co*-3HV) we detected a negative effect of the both PHAs on some parameters [16]. Our sanitary-chemical investigation of the properties of PHA aqueous extracts, which was aimed to determine whether chemical components of polymers migrate into the medium, yielded widely varying results (pH shift and dichromate and permanganate oxidisation). Moreover, these values were higher than the international standards for materials for medical devices [30, 31]. Preliminary analysis of PHA haemocompatibility revealed activation of blood enzyme systems (plasma recalcification time and complement activation) by the surface of PHA films [16]. Two possible reasons for this could be either that blood enzyme systems are activated by the polymer material itself – poly(PHB) and poly(PHB-*co*-PHV) – or that the undesirable reactions could be caused by the action of biologically active substances of the bacterial biomass present in the samples.

It is well known that components of bacterial cell walls contain endotoxins and toxic macromolecules, which can cause negative reactions of tissues [32, 33]. Since the bacteria *Ralstonia eutropha* producing the study PHAs are gram-negative microorganisms and since lipopolysaccharides of these bacteria are known to include lipid A, which contains long-chain and saturated β-hydroxy acids that can cause pyrogenic and other adverse reactions [34], we have focussed our search for possible undesirable impurities on the lipid constituent. We have detected the presence of long-chain fatty acids (0.5 to 1.0–1.5 mol %), for the most part (up to 70% of the total) represented by β-hydroxypalmitic (C_{16}) acid. We have also found saturated β-hydroxytetradecanoic acid (up to 0.06 mol %). The investigation of additionally purified PHAs, free of these acids, did not register any of these adverse responses and showed that poly-(3HB) and poly(3HB-*co*-3HV) could be used in contact with blood [16].

Thus, to produce films and investigate them as candidates for cell culture matrices, we took high-purity PHA samples, which contained no organic micro-impurities, including long-chain and saturated fatty acids. The resulting films retained their physical-

mechanical properties in the process of sterilisation and had a good adhesive capacity. The results of investigation of cell morphology and protein and DNA synthesis in various animal cells on their direct contact with PHAs suggest a conclusion that this material can be used to make matrices for *in vitro* functioning cells. None of the samples under investigation was cytotoxic. The properties of poly-(3HB) and poly(3HB-*co*-3HV) films proved to be fundamentally similar.

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