Preparation and properties of polyhydroxybutyrate microspheres as drug carriers

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Introduction

Designing of controlled-release drug delivery systems is a promising and rapidly developing line of pharmacology. The main advantage of using drug delivery systems is that drug concentration in a patient's blood and/or tissues can be maintained at a target level for an extended time (Kost 2001). The principal requirement for fabricating prolonged-action controlled-release drug delivery systems is availability of an appropriate material, which must be absolutely harmless to an organism and possess the necessary physical-mechanical and biomedical properties, including degradability in biological media.

Polyhydroxyalkanoates (PHAs) are good candidates for fabrication of drug delivery systems (Ueda 2003, Piddubnyak 2004), because they possess all the properties required of materials for these systems. These polymers are biocompatible and inert towards animal tissues; in biological environments they are degraded to end products (CO_2 and H_2O). In contrast to other materials that are widely used in controlled drug delivery, such as gelatine, proteins, polylactide and poly(ethyleneglycol)-poly(D,L-lactide), PHAs are available in a chemically pure form (Gürsel 1995) and their degradation rate in biological media is low. By varying the chemical structure of PHAs or blending them with different materials, one can control porosity and degradation rate of the polymeric matrix and, hence, the rate of drug release.

The purpose of this study was to prepare microspheres from PHA and to test their *in vitro.*

Materials and method

Preparation of pure PHB polymer

The tested material was the PHB samples synthesized by the bacterium Ralstonia eutropha B5786 (Mw 340 000 Da, crystallinity 70-78%). The strain is registered in the Russian Collection of Industrial Microorganisms. The trademark of the material is Bioplastotan (TRADEMARK BIOPLASTOTAN^{TM}).

Preparation of microspheres

PHA-based microspheres were prepared by the solvent evaporation technique, using a triple emulsion. 600 mg of the polymer polyhydroxybutyrate (PHB) and 200 mg of polyethylene glycol (PEG40, molecular mass 40 kDa) were dissolved in 10 ml of dichloromethane at 40^{o}C. Then, 1 ml of a 6% gelatin solution (40^{o}C) was added and the mixture was shaken vigorously. The resulting (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 700 rpm, for 20 min, to obtain a triple (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 lyophilic dryer (Russia).Microspheres were sterelizated by autoclaving at 0.5 atm for 30 min.

PHA-based microspheres containing rubomycin, were prepared by the solvent evaporation technique, using double (water/oil, W/O) and triple emulsions (water/oil/wter W/O/W). Prior to loading microspheres with rubomycin, 2 mg of the drug was dissolved in dichloromethane and then

polymer was added to the solution; the subsequent procedure was performed as described above. In the course of encapsulation, the temperature of the medium was varied.

Microspheres characterization

The size and the size distribution of microspheres were determined with an Automatic Particle Counter + Analyser system (Casy TTC, Scharle System GmbH, Germany). The obtained size distribution was used to describe the particle size. The structure of microspheres surfaces was analyzed by electron microscopy (JEM-100C electron microscope with an EM-ASID-4 raster attachment, Japan).

In vitro cytotoxicity of microspheres

After sterilization, microspheres were tested for cytotoxicity towards the viability, growth, morphology and metabolism of fibroblasts. The cytotoxicity test was conducted according to the ISO 10993 standard. MEM Elution-Test on Extracts was conducted according to the ISO 10993- 5 standard: in this study, 100 mg of the dry microspheres (equals at least 120 cm^2) were extracted at 37^oC for 24 h in 20 ml of Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum. As a positive control we used red technical rubber (GOST). This standard corresponds to the European analogue of the cytotoxic positive control – Para rubber (Dijkhuizen-Radersma 2002). 60 cm^2 of polystyrene "Greiner bio-one" was used as a negative control. The maximum negative control was cells cultured in standard medium. The objects used were cultured cells: mouse fibroblast cell line NIH 3T3. Cells were cultured in a humidified atmosphere at 5% CO_2 at 37^oC. For cytological investigations, fixed and incompletely dried cells were Giemsa stained in astandard Jurr 65500 buffer at pH 6.8 and examined under a microscope with an H190/1.30 immersion objective and a PKIOX ocular. Preliminarily, cell viability was estimated by live staining with trypan blue (0.5% stain solution in 0.85% NaCl). Cell metabolic activity was measured at 3 days using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The number of living cells in the culture was determined from the MTT absorbance standard curve.

Statistics

Statistical analysis of the results was made using the standard software package of Microsoft Excel. Significant differences between mean values in control and treatment groups were tested using Student's t test (significance level: $p = 0.05$) by standard methods: using two sample equal variance, with two tail distribution. We used 6 samples per *in vitro* citotoxical experiment.

Results and Discussion

Microspheres characterization

Morphology of microspheres did not significantly depend upon the preparation procedure. The only difference was that microspheres prepared from the triple emulsion were of a more spherical shape and microspheres prepared using the double emulsion and ultrasound (US) were more spongy and, visually, more porous; the occurrence of deformed microparticles was more frequent among those microspheres (Figure 1).

Irregularly shaped large particles could have formed from smaller microspheres during the microsphere preparation procedure. Another possible explanation could be that when emulsion is formed at 40°C and US is used, the emulsion is heated, which leads to quick evaporation of the solvent and hardening of the polymer. The preparation procedure affected some of the properties of microspheres. The yield of the microspheres prepared from the double emulsion, using US, was higher than the yield of the microspheres prepared by the second encapsulation technique (W/O/W, stirring) and exceeded 75%. This parameter was obviously affected by the temperature at which microspheres were prepared.

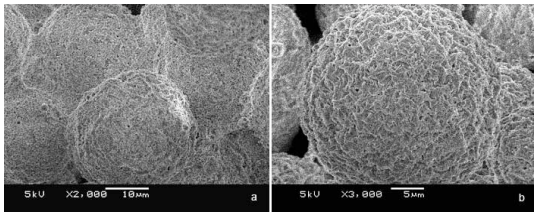

Figure 1: Micrographs of PHB microspheres prepared from two- (O/W) (a) and three-component (W/O/W) (b) emulsions

Preparation technique	Parameter:	
	O/W + rubomycin	W/O/W + rubomycin, (50°C)
Mean diameter, μm	6.9±0.9	8.8±0.7
Yield, %	76.2±1.3	52±1.2
Encapsulation efficiency, %	63±1.29	90±2.8

Table 1: Characterization of rubomycin-loaded PHB-microspheres

One of the most significant parameters of microspheres is their size. Drug release kinetics primarily depends on microsphere size, composition and shape. In addition, microsphere sizes play a crucial role when targeting a particular site in the body and this determines method of administering them. Both techniques used in this study yielded microspheres that were essentially uniform in diameter.

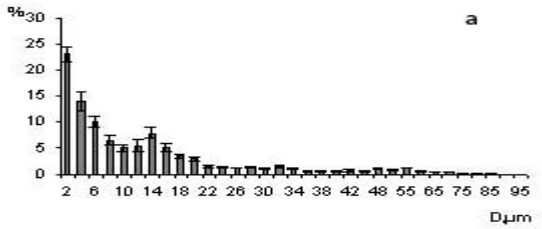

Figure 2a: Micrographs of PHB microspheres prepared from two- (O/W) (a) and three-component (W/O/W) (b) emulsions.

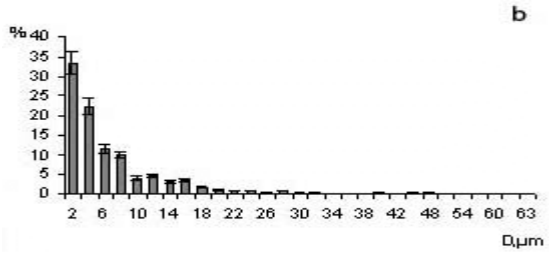

Figure 2b: Micrographs of PHB microspheres prepared from two- (O/W) (a) and three-component (W/O/W) (b) emulsions.

Microspheres of diameter about 2 μm are considered to be the best for clinical application. They constituted 23.0±1.4% and 33.3±2.7% in the first and second variants, respectively.

The largest diameter of microspheres prepared using the W/O emulsion and US was 95 μm versus 63 μm in the variant with W/O/W emulsion and stirring.The diameter of a microsphere increased when it was loaded with a drug. In our experiments, the size of the microspheres loaded with rubomycin increased, too, and microspheres of diameter 1-2 μm constituted 19.7±1.3% (Table 1). This could be accounted for by a decrease in surfactant power of the aqueous solution of polyvinyl alcohol with the drug added to it, which reduces stability of the ultrathin emulsion and increases the probability of small microspheres fusing into larger aggregates.

Cytotoxity

Evaluations of growth and metabolic parameters of the fibroblast cell culture in the treatment group and in the positive and negative control groups are presented in the Table 2. Mouse fibroblast cells cultured in the presence of polymeric (PHB) microspheres extracts similarly to the negative control (polystyrene extract) retained the morphology of normal cells, like those grown in the control, on polystyrene. Cell viability test, performed by the method of live staining with trypan blue, showed that 99.8±0.2% of the cultured cells did not incorporate the dye, i.e. remained highly viable, in contrast to the positive control (rubber extract), in which most of the cells died. The doubling time of fibroblasts corresponded to the generation time of the cells cultured on standard medium and experimental culture were 25.1±1.8 and 25±2 h; on negative and positive controls 24.9±2.1 and 168±21.3. The MTT test did not indicate any toxic effect of the polymer extract on the metabolic

activity of fibroblasts, either. It is well-known that only active dehydrogenases of living cells will convert MTT into insoluble formazan crystals.

Sample	Cell viability (%)	Cell doubling time, (h)	Change of cell morphology, in grades[a]	MTT test
PHB microspheres	99.8±0.2	25.0±2.0	0	< 10%
Polysterine (negative control)	98.8±0.2	24.9±2.1	0	< 10%
Industrial red rubber (positive control)	19.8±0.1	168±21.3	4	70–100%
Standard medium	99.4±0.15	25.1±1.8	0	< 10%

[a] in accordance with R. Dijkhuizen-Radersma et al., 2002

Table 2 : Evaluations of growth and metabolic parameters of the mouse fibroblast cells (NIH 3T3 line) culture in the treatment group and in the positive and negative control groups

Conclusion

A technique of fabricating microspheres by the solvent evaporation method from PHA has been developed, using double and triple emulsions; conditions have been determined for preparing microspheres of consistently good quality. In the in vitro experiments showed that polyhydroxybutyrate microspheres were biocompatible; they neither inhibited growth and metabolic activity of fibroblasts. The results of the study suggest that polyhydroxybutyrate is a good candidate as carrier for drugs in the form of microparticles.

References

J. Kost et al. (2001) *Responsive polymeric delivery systems*. Adv. Drug Deliv. Rev. 46 125-148.

H. Ueda et al. (2003) *Polyhydroxyalkanoate derivatives in current clinical applications and trials*. Adv. Drug Deliv.Rev. 55 501-518.

V. Piddubnyak et al. (2004) *Oligo-3-hydroxybutyrates as potential carriers for drug delivery.* Biomaterials 25 5271-5279.

Ih. Gürsel et al. (1995) *Properties and drug release behaviour of poly(3-hydroxybutyric acid) and various poly(3-hydroxybutyrate–hydroxyvalerate) copolymer microcapsules.* J.Microincapsulation. 12 185-193.

TRADEMARK "BIOPLASTOTAN™" Registration Certificate No. 315652 of the Federal Institute for Patent Examination for Application No. 2006703271/50, Priority of 15.02.2006.

GOST (The Russian Federation state standard) 5496-78.

R.Dijkhuizen-Radersma et al. (2002) *Biocompatability and degradation of poly(ether-ester) microspheres: in vitro and in vivo evaluation.* Biomaterials 23 4719-4729.