

Microcapsules of Poly(3-Hydroxybutyrate) for Sustained Protein Release

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The aim of investigation was to develop a system for protein sustained release based on the use of poly(3-hydroxybutyrate) (PHB) microcapsules loaded with bovine serum albumin (BSA).

Materials and Methods. To develop microcapsule we used PHB obtained microbiologically by a strain-producer *Azotobacter chroococcum* 7B. Microcapsules loaded with model protein BSA were produced by double emulsion technique "water/oil/water". Morphology of microcapsules was investigated by methods of confocal and scanning electron microscopy, when the loading and release of BSA was examined spectrophotometrically. *In vivo* biocompatibility of microcapsules was studied in accordance with intramuscular implantation and histology findings.

Results. The study of BSA incorporation and its sustained release from microcapsules for more than 190 h demonstrated the efficacy of proposed system. The mechanism of protein release was found to occur due to the rupture of polymer walls. Moderate tissue response to the implantation of obtained microcapsules was demonstrated.

Conclusion. Developed PHB microcapsules loaded with BSA are good model of long acting protein drugs.

Key words: biodegradable polymers; polyhydroxyalkanoates; poly(3-hydroxybutyrate); prolonged release; microencapsulation; biocompatibility.

Nowadays, poly(3-hydroxybutyrate) (PHB) as well as its biotechnologically prepared copolymers attract high attention as biodegradable and biocompatible materials. PHB is used to develop a wide range of polymer products for biomedical applications such as vascular stents, surgical sutures, periodontal membranes [1–3]. In addition to medical products PHB can be used for development of sustained release drugs by incorporating medicinal substances into polymer microparticles: low

molecular compounds [4–6], bimolecules with high molecular weight [7] and inorganic nanoparticles [8].

One of the most promising trend in modern biopharmacology is producing sustained protein release systems. Application of biopolymer microparticle-based systems can eliminate most of drawbacks of traditional medications: high toxicity, ineffectiveness of active ingredients, substance instability, inconvenience of administration, etc. There are a lot of complex

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techniques are required to encapsulate proteins into polymeric microparticles, double emulsion technique being one of the most effective ones. It is used to obtain protein-loaded microcapsules and microparticles, since in this case the protein is not soluble in the same solvent with polymers. There are several modifications of this technique that differ in emulsification steps and their composition [9].

Biocompatibility is an important property of polymer sustained release systems. There is a number of techniques both *in vitro* and *in vivo* for its assessment. The former are a series of cell culture-based models [10], while the latter imply subcutaneous [11] or intramuscular [12] implantation of a polymer product into laboratory animals with the following histologic study.

The aim of investigation was to develop a system for sustained protein release based on use of poly(3-hydroxybutyrate) microcapsules loaded with bovine serum albumin.

Materials and Methods. To develop the system we applied PHB with molecular weight of 826 kDa, produced microbiologically by cultivating *Azotobacter chroococcum* strain 7B [13] in the Laboratory of Nitrogen Fixation Biochemistry and Nitrogen Metabolism at the Research Center of Biotechnology RAS; methylene chloride (dichloromethane, CH₂Cl₂, Ecos 1, Russia); bovine serum albumin (BSA) (Sigma Aldrich, Germany); fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (Sigma Aldrich, Germany); polyvinyl alcohol (MERK, 72,000 Da); potassium phosphate buffer (Chimmed, Russia); sodium azide (NaN₃) (Sigma Aldrich, Germany); Coomassie brilliant blue G-250 (Astra, Russia); phosphoric acid (H₃PO₄, Chimmed, Russia); ethyl alcohol (C₂H₅OH, Chimmed, Russia); diethyl ether (C₄H₁₀O, Chimmed, Russia).

Microcapsule preparation. Microcapsules were obtained by double emulsion technique "water/oil/water" (W/O/W) [14]. This method involves 1) emulsifying aqueous solution of the protein in chloroform polymer solution; 2) emulsification obtained emulsion in water. The final emulsifier was removed using distilled water and the microspheres were isolated [9].

Emulsion of the protein aqueous solution containing 10 mg of BSA in the polymer (PHB with molecular weight of 826 kDa) solution with concentration of 10 mg/ml in the organic solvent in total volume of 4 ml was gradually added to 50 ml of polyvinyl alcohol at 1.5% concentration. It was mixed with a R2R 2021 overhead stirrer (Heidolph, Germany) at 2,000 rpm. When organic solvent has been completely evaporated, microcapsules were isolated by centrifugation (10 min at 4,400 rpm) performed in a 5707R centrifuge (Eppendorf, Germany) and then rinsed 3 times in distilled water to completely remove emulsifier. Then the microcapsules were lyophilized (New Brunswick Scientific, USA) prefrozen in liquid nitrogen. The percentage of protein incorporation was determined by measuring the protein

mass during the operational analysis of its release from the polymer.

Microcapsule morphology. Microcapsule morphology was studied by scanning electron-ion microscopy without ion sputtering with a Quanta 200 3D device (FEI Company, USA). To achieve a normal distribution of microparticles by size the images were processed with the ImageJ software. The images, demonstrating the character of the protein incorporation in the structure, were studied using a Zeiss LSM 510 META confocal microscope (Germany), with C-Apochromat 63x/1.2 W corr lens. Fluorescence excitation was performed with laser of 488 nm wavelength, radius of the confocal aperture of 1 Airy disk (112 μm). The LP 505 nm filter was used. The microparticles were examined similarly after the experiment on sustained release [14].

The study of BSA sustained release from polymer microstructures in vitro. Protein sustained release from microstructures was performed at 37°C in a TC 1/20 thermostat (Russia) in 25 mM potassium phosphate buffer (pH=7.4) with 0.004% sodium azide as an antimicrobial agent: 10 mg of microcapsules in 1 ml of buffer were stirred at 350 rpm in the OS-10 shaker (BIOSAN, Latvia). In the study of the BSA release kinetics over the given time intervals we separated microcapsules from the buffer by centrifugation at 10,000 rpm in a 5702 R centrifuge (Eppendorf, Germany), sampled the supernatant and added 1 ml of fresh buffer. The amount of protein in the obtained samples was estimated.

Spectrophotometrical determination of BSA in the obtained samples. The BSA content in the obtained samples was determined spectrophotometrically by Bradford staining, as well as measuring protein concentration in a solution with wavelength of 280 nm. Measurements were performed with a UV-1601PC spectrophotometer (Shimadzu, Japan) at λ=595 nm and λ=280 nm [14].

The study of obtained microparticles biocompatibility in vivo. The study of obtained microparticle biocompatibility *in vivo* was performed on 18 Wistar male rats weighing 300±50 g. Animals were kept in the vivarium of Central Research Laboratory, Nizhny Novgorod State Medical Academy at 15 daylight hours at +22°C. All manipulations were carried out in accordance with order of the Health Ministry No.708n from 23.08.2010 "On approval of the guidelines of good laboratory practice". 1 ml of microparticle suspension in a phosphate buffered saline (PBS) (50 mg/ml) was administered intramuscularly in the hind leg of a laboratory animal under anesthesia (zoletil 10 mg/kg; rometar 6 mg/kg). On days 7, 20 and 60 post-injection animals were taken out from experiment (by 6 animals), and after that muscle tissue samples containing microcapsules were taken. Then the candidate material was fixed in a 4% formaldehyde solution in PBS for 24 h. Further obtained biopsy samples were rinsed in water, dehydrated in a graded set of ethanol solution (50, 60,

70, 80, 96% and absolute ethanol for 1.5 h in each concentration), rinsed in chloroform for 40 min, soaked in a 45% solution of paraffin in chloroform (37°C, 12 h) and poured into a mold. The 6 μm thick sections were cut out from the paraffin blocks with a MC 2 microtome (Tochmedpribor, Ukraine), deparaffinized, stained with hematoxylin and eosin followed by light microscopy examination.

Image analysis was performed using a Biomed 1 microscope (Biomed, Russia) and the Zeiss LSM Image Browser 4.2.0 open source software (Carl Zeiss MicroImaging, Germany).

Results and Discussion

Microcapsule morphology. We used the W/O/W technique to obtain microcapsules from 826 kDa PHB loaded with BSA model protein. In the micrograph of obtained polymer microcapsules (Figure 1) we can clearly see their rough surface with multiple strands, which seem to be caused by the condensation of polymer chains on each other when evaporation of the

solvent, as described in some studies on determination polyhydroxyalkanoates morphology and other partially crystalline polymer folding [15]. It should be mentioned that microcapsules are hollow particles with protein placed inside. This is quite visible at the consideration of a particle which was dissected by an ion beam under a Quanta 200 3D microscope (Figure 2): the cavity inside the particle is limited by a monolithic wall where protein is kept. Particle size distribution (Figure 3) demonstrates a typical particle size — from 5 to 30 μm with the mean diameter of 17 μm.

Protein encapsulation. Confocal microscopy was chosen to study the character of protein incorporation in the polymer matrix. For this purpose FITC-labeled BSA particles were prepared. The images (Figures 4 and 5) clearly showed a diffuse protein arrangement inside microcapsules while there are clusters of adsorbed protein on the microparticle surface. Such protein adsorbed on the particle surface is released first, whereas the encapsulated BSA provides sustained



Figure 1. Poly(3-hydroxybutyrate) microcapsules with encapsulated protein

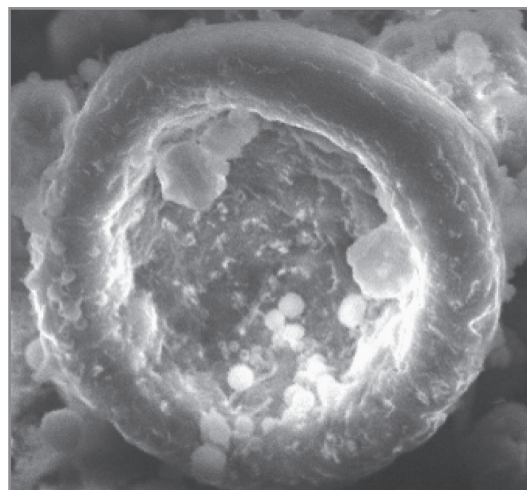


Figure 2. Sample section of poly(3-hydroxybutyrate) microcapsules with encapsulated protein

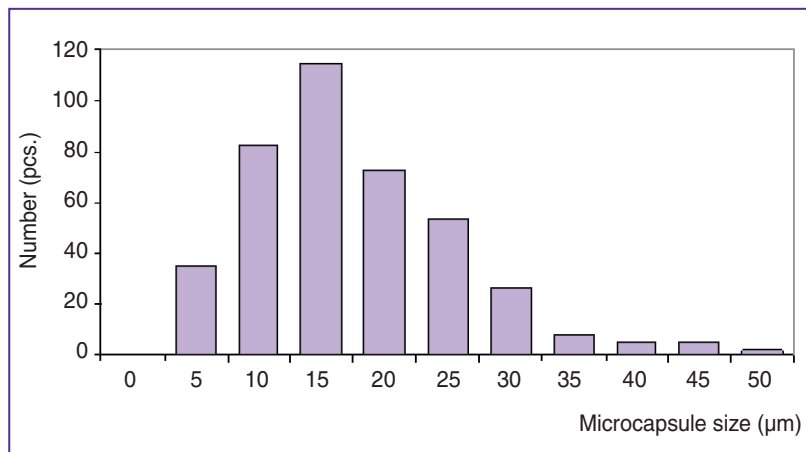


Figure 3. Size distribution of poly(3-hydroxybutyrate) microcapsules with encapsulated protein

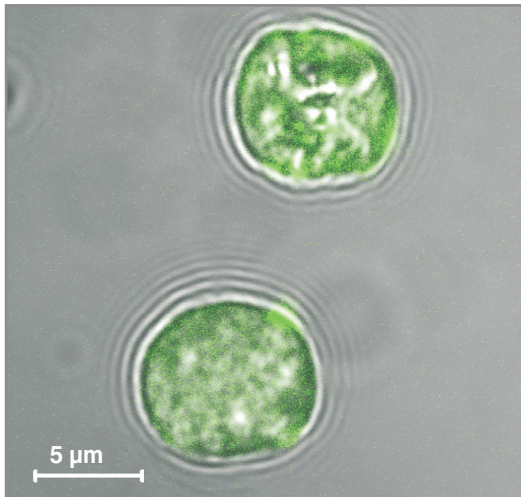


Figure 4. Individual microcapsules, prepared from poly(3-hydroxybutyrate) with FITC-BSA by the W/O/W technique, obtained with confocal microscope. Protein distribution in structures is shown by green curve

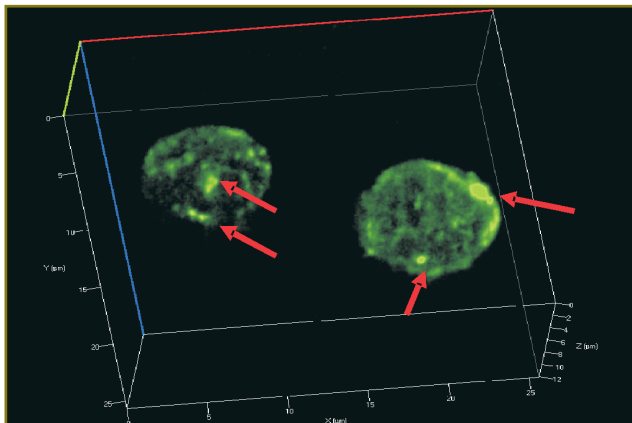


Figure 5. Reconstruction of 3D models of individual microcapsules, prepared from poly(3-hydroxybutyrate) with FITC-BSA by the W/O/W technique, taken with confocal microscope. Arrows indicate areas of protein accumulation

release. This phenomenon will be further described in details in the study of protein release from particles in the experiment *in vitro*.

Protein release *in vitro*. Obtained PHB particles loaded with protein were used in the experiment of BSA release *in vitro* (Figure 6). It was carried out for more than 190 h (8 days) in conventionally physiological conditions up to the point when released protein was not determined any more. The total released protein was taken for 100% because it was not possible to estimate the total encapsulated protein (due to the fact that when protein adsorption irreversible, BSA and PHB form conglomerate that is not soluble neither in water nor in chloroform). In other words, we were interested only in the useful protein, reversibly adsorbed onto polymer and then released from polymer structures. We neglected denatured, irreversibly adsorbed BSA.

According to the experimental data, we calculated parameters of BSA encapsulating:

- encapsulation efficiency — $1.92 \pm 0.51\%$;
- microcapsules yield — $46.62 \pm 5.96\%$;
- burst effect — $49.94 \pm 3.50\%$.

Apparently, encapsulation efficiency was rather low — about 1.9%. This is due to the technique, when protein initially being in the form of a solution is exposed to heavy impacts. So this technique can be further applied only to systems where protein is relatively cheap and simple to be produced, such as lysozyme, insulin, and so on. Another parameters of particles were quite satisfactorily. The value of burst effect, characterizing the initial protein release from produced particles and being of around 50%, means that particles will be able to provide both the target initial delivery of drug in the required dose and subsequent long-term maintenance of a constant therapeutic concentration in the internal environment [16]. The value of polymer microstructures yield is also acceptable.

Figure 6 clearly shows that kinetic release profile

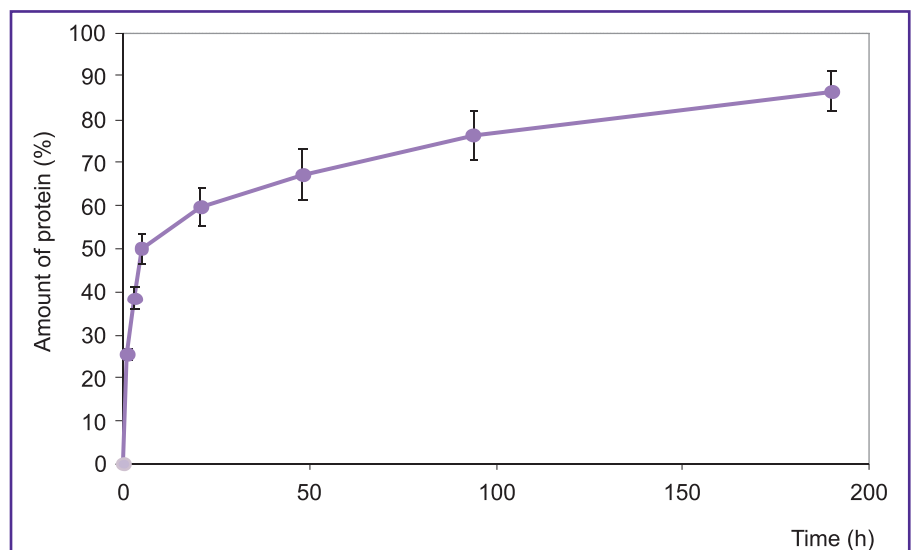


Figure 6. Kinetic profile of protein release from poly(3-hydroxybutyrate) microcapsules

can be divided into two steps. In the first step of release (from 0 to 5 h) we determined burst effect characterized by a strong initial release of protein. The substance desorption from the particle surface described above is supposed to take place at this time. Then (5–190 h) we observe a sustained protein release. This time period of the most interesting, because of processes taking place on it provide BSA sustained release from polymer microcapsules. To understand the release mechanism we carried out various morphological studies of incubated particles.

Determination of the mechanism of protein sustained release. Thus, as established by confocal microscopy, desorption of unconjugated protein from the capsule surface occurs in the step of initial burst protein release. The next step of our research was to study morphology of incubated particles by scanning

electron (Figure 7) and confocal (Figure 8) microscopy. For this purpose, particle samples were frozen and then lyophilized after the completion of protein release experiment *in vitro*.

As demonstrated in images, release mechanism of protein from microstructures obtained by proposed technique presents rupture of polymer walls with the following discharge of the protein, it easily going into surrounding medium. Scanning confocal microscopy (See Figure 8) showed the gap on the surface of microcapsules. Apparently FITC-labeled BSA have been releasing through this holes as they were formed. However, fluorescence at the site of rupture of polymer membrane is much higher than inside the particle. It seems to be connected with emergence of defects in material known to have a semi-crystalline structure. Due to the change in folding of PHB chains, adsorption



Figure 7. Samples of poly(3-hydroxybutyrate) microcapsules with encapsulated protein after the *in vitro* experiment for 200 h according to scanning electron microscopy

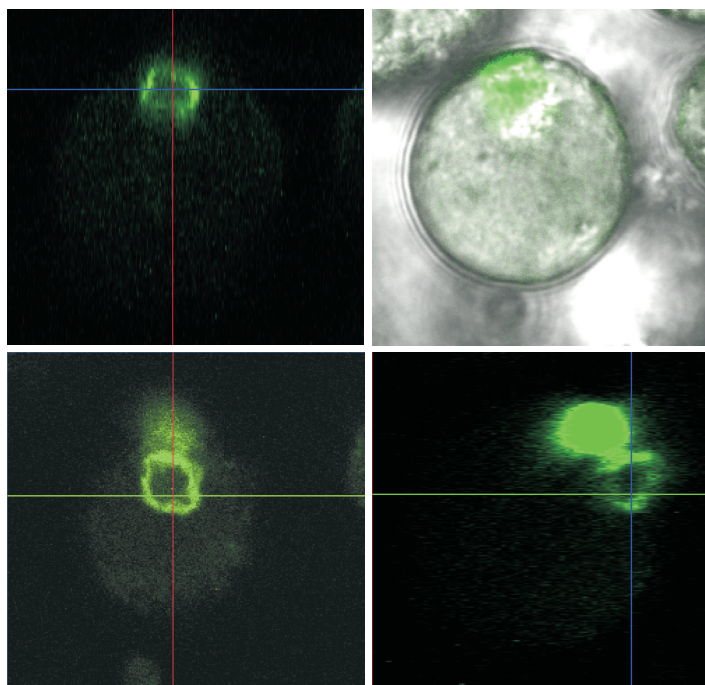


Figure 8. Image and orthogonal projections of a separate microcapsule according to confocal microscopy

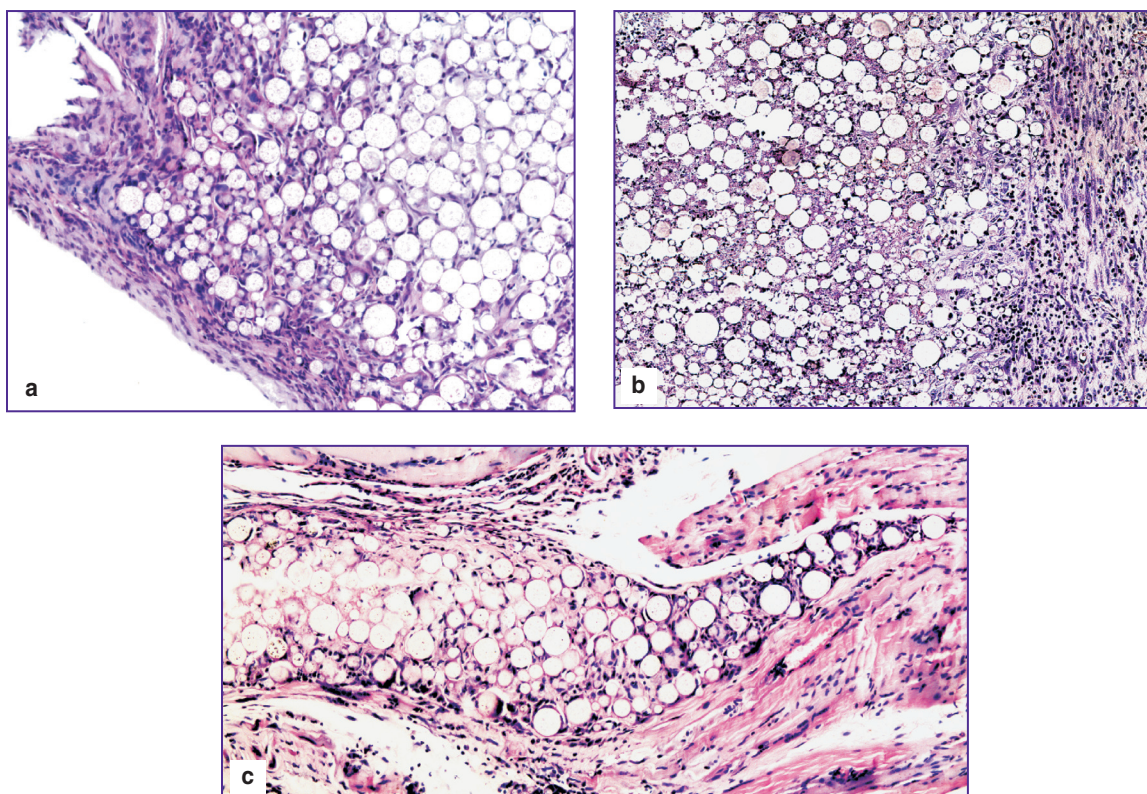


Figure 9. Images of areas of the muscle tissue straight at the site of microcapsule implantation after 7 days (a), 21 days (b), 60 days (c)

of released protein onto material locally increases. This process has not yet been described in detail, and requires further study.

Determining biocompatibility of microparticles *in vivo*. Implantation of microcapsules in rats did not cause significant inflammation or septic foci in them. On day 7 we observed an increase in blood supply at the site of implantation and formation of a connective tissue capsule around microparticles.

Histological sections (Figure 9) clearly have shown forming capsules of 100–150 μm thick, which reach the final size after 60 days after implantation. We also marked the spread of surrounding tissue between microparticles. The presence of white blood cells and fibroblasts around microparticles indicates moderate inflammation and is a normal response to the implant, replaced by the body tissue, and due to the process of polymer degradation [12]. The microcapsule surface becomes markedly smoothed by day 60 which indicates a significant PHB bioresorption. It conforms to results obtained previously in our laboratory [11].

Thus, poly(3-hydroxybutyrate) microcapsules with encapsulated model protein represent a good example of creating a prolonged form of a protein preparation. And microcapsules demonstrated quite good results in experiments on biocompatibility *in vivo*. A detailed study of such polymer particles will contribute to a better understanding of the process of protein release from

them and the body's response to protein release system. It also allows to choose the right trend in the further development of research on the given subject.

Conclusion. The developed system of prolonged protein release based on the use of a new biodegradable polymer — poly(3-hydroxybutyrate) as microcapsules, and bovine serum albumin as a model protein, can serve as a basis for the development of new formulations of therapeutic proteins.

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Conflicts of Interest. Authors have no conflicts of interest.

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