The experimental model of lysozyme sustained release based on poly(3-hydroxybutyrate)-poly(ethylene glycol)/hydroxyapatite microparticles

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Abstract. Development of experimental models of proteins sustained release is a promising trend in modern biochemistry and biopharmacology. Application of proteins sustained release systems based on biopolymer microparticles allow to model prolonged protein action in vitro and in vivo. Moreover, the use of these devices in biopharmacology can eliminate the most of the drawbacks of traditional medicines: high toxicity, infectivity, substance instability, inconvenience of administration etc. For development of proteins sustained release system the biodegradable polymer poly(3-hydroxybutyrate) (PHB) and its copolymers, obtained biotechnologically in our laboratory with *Azotobacter chroococcum strain 7B*, with nanohydroxyapatite were used. Here we investigated microparticles loaded with composite of nanohydroxyapatite and model protein lysozyme that were produced by modificated two-step emulsification solid/oil/water (S/O/W) technique. For microparticles production we used a novel PHB copolymer poly(3-hydroxybutyrate)-poly(ethylene glycol) (PEG-PHB) with molecular weight of 250000, as well as PHB with molecular weight of 250000 for comparison. We have chosen lysozyme as model protein with positive net charge and enzymatic activity. A lysozyme sustained release from biopolymer microparticles was carried out *in vitro* in a phosphate buffer (pH 7,4) at 37°C. We showed that protein release from the microparticles occurs via two processes: diffusion and degradation of microparticles. The release kinetics of the protein was connected with polymer molecular weight and hydrophobicity. Therefore, to improve the performance of lysozyme sustained release we used a more hydrophilic PHB-PEG. As a result, the release kinetics of PHB-PEG microparticles was significantly more linear than that of PHB microparticles. The efficiency of protein encapsulating to PHB-PEG microparticles was also better. However, the encapsulated protein can lose its native structure and enzymatic activity during its release from polymer microparticles. To verify lysozyme stability we examined the lysozyme enzymatic activity. The obtained data demonstrated that the lysozyme do not lose their integrity and enzymatic activity for 14 days sustained release from microparticles. Thus, the developed technique for protein encapsulation in PHB-PEG microparticles can produce the experimental model of protein sustained release.

Keywords: PHB, PHB-PEG, microparticles, modeling, protein sustained release, lysozyme.

1 Introduction.

Nowadays, poly-3-hydroxybutyrate (PHB), as well as its copolymers, obtaining biotechnologically, attract attention as biodegradable and biocompatible polymers [1, 2]. A microbiological biosynthesis of PHB copolymers by incorporation of polymers of other origin into the PHB polymer chain can be successfully used for regulation of its physical-chemical properties [3-6]. PHB can be used for development of a wide range of polymer medical devices, such as microparticles for sustained release [7], scaffolds for tissue engineering [8], and other medical devices such as vascular stents, surgical thread, periodondal membranes, hernia meshes etc. [9]. Besides medical devices PHB can be used for development of sustained release pharmaceutical forms by encapsulation of various drugs in polymer microparticles: low molecular drugs [10-13], high molecular proteins [14] and inorganic nanoparticles [15]. The most promising trend in modern biopharmacology is development of proteins sustained release systems. Application of proteins sustained release systems based on biopolymer microparticles can eliminate the most of the drawbacks of traditional medicines: high toxicity, infectivity, substance instability, inconvenience of administration etc. Moreover, the use of these devices allows modelling prolonged protein action in vitro and in vivo. However for encapsulation of proteins in polymer microparticles should be used relatively more complex techniques. The main problem is that the level of encapsulated protein is rather low because of irreversible adsorption of protein to polymer matrix of a microparticle. In the case of globular proteins, they diffuse through hydrophobic microsurround of polymeric matrix [16]. Herewith, irreversible adsorption can appear and protein denatures. Application of principals of irreversible adsorption and denaturation is proved on PHB in vitro [17].This problem can be resolved by reducing of polymer hydrophobicity by copolymerization with an amphiphilic polymer, e.g. polyethylene glycol [4] that leads in particular to the better polymer biocompatibility in vitro [18, 19].

Thus, the purpose of our research was to develop the proteins sustained release systems for application in biochemistry as the novel experimental model and in biopharmacology as the novel biomedicines candidate.

2 Materials and Methods

2.1 Materials.

Poly(3-hydroxybutyrate)-polyethylene glycol (PHB-PEG) with M w = 250 000. Poly(3-hydroxybutyrate) (PHB) with M w 250 000 (the homopolymer was used as control to compare the current data with previously obtained data). PHB and PHB-PEG were obtained biotechnologically in our laboratory with Azotobacter chroococcum strain 7B [1]; chloroform (Ekos-1, Russia); lysozyme (Sigma Aldrich, Germany); FITC-labeled BSA (Sigma Aldrich, Germany); nanohydroxyapatite (HA) (Sigma Aldrich, Germany); polyvinylalcohol MW 72000 (MERK, Germany); phos-

phate buffered saline (PBS) (Sigma Aldrich, Germany); sodium azide (Sigma Aldrich, Germany). *Microccus lizodecticus ATCC No. 4698* (Sigma Aldrich, Germany);

2.2 Methods

2.2.1. Preparation of lysozyme–HA composite.

Composite of HA with lysozyme was prepared by mixing and following freeze-drying of two solutions: 1 ml (100 mg/ml) lysozyme in 25 mM PBS and 1 ml suspension of HA nanoparticles (900 mg/ml). The ratio of HA/lysozyme was selected as 90%/10%. The resulting mixture was freeze-dried overnight and kept at 4 °C.

2.2.2. Preparation of polymeric microspheres encapsulating HA-lysozyme composite.

A solid-in-oil-in-water (S/O/W) emulsion solvent evaporation method [15] was modified to prepare polymer–HA/lisozyme microspheres. Solutions of PHB 250 000 and PHB-PEG 250 000 with total concentration 30 mg of polymer in 1 ml of chloroform were prepared. Then the 10% dispersion HA/lysozyme in chloroform were prepared; and the resulting colloid was added to polymer solution at a w/w ratio of 10/90. The obtained mixture was poured into 50 ml of distilled water, containing 1.5% (w/v) polyvinylalcohol as an emulsifier and the resulting emulsion was then stirred on overhead stirrer for 2 h to evaporate the solvent under ambient temperature (25 °C). The microspheres were centrifuged, filtered, washed and freeze-dried. The final products were kept in a desiccator at 4 °C.

2.2.3. Microsphere characterization.

Microsphere size and morphology were obtained by scanning electron microscope (SEM; CamScan, USA; Quanta 200 3D, USA). Protein loading in microspheres was detected by confocal microscopy (Zeiss LSM 510 META) with using of lens C-Apochromat63x/1.2 W corr. For this investigation we used microspheres loaded by FITC-labeled BSA. The drug encapsulation efficiency was determined by comparing the quantity of released protein with total amount of encapsulated one.

2.2.4. In vitro release of lysozyme from microspheres .

The sustained release of lysozyme from PHB microspheres was carried out at conditions in vitro: in phosphate buffered saline at 37°C with continuous stirring. Freeze-dried lysozyme-loaded microspheres (20 mg) were dispersed in 2 ml PBS buffer (25 mM, pH 7.4) [7], and were put into a thermostat (TC-1/20, Russia) at 37°C with use of orbital shaker (OS-10 BIOSAN, Latvia). At specific time points, release medium was sampled completely by centrifugation of mixture at 14 000 rpm (5702 R centrifuge, Eppendorf, Germany) and assayed for protein concentration using the spectroscopic method. The release kinetics was then determined. The removed release medium was replaced with an equal volume of fresh medium after each sampling and was added to deposited microspheres.

2.2.7. Determination of enzymatic activity on released lysozyme.

The amount of active lysozyme in In vitro release samples was determined by measuring the turbidity change in a M. lysodeikticus bacterial cell suspension as previously reported [20]. Hundred microlitres of a lysozyme solution was added to 2.9 mL of a 0.015% w/v M. lysodeikticus suspension in TRIS–HCl (0.01 M, pH 7.4) buffer solution. After incubation (37 °C, 4 h), the absorbance was measured at 450 nm. The amount of active protein was calculated thanks to a standard curve.

3 Results and discussion

Firstly, we produced composite HA/lysozyme particles. Blending with HA was used to stabilize the protein for following use in S/O/W technique. Imaging of HA and HA/lysozyme particles are shown at fig. 1.

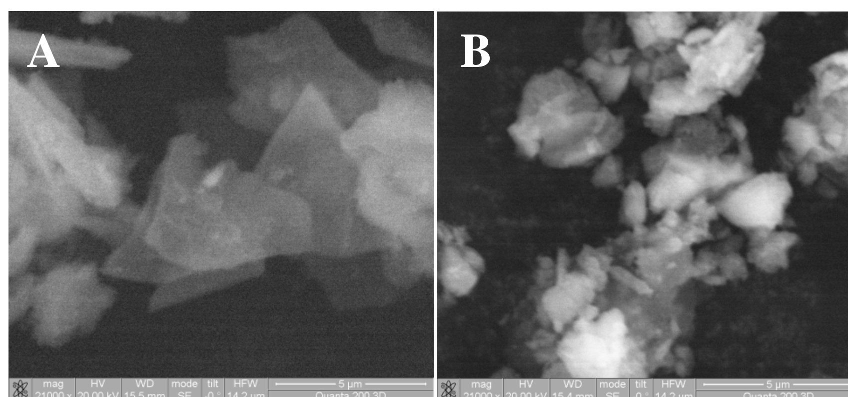

Fig. 1. SEM imaging of HA (A) and composite HA/lysozyme (B)

We chose copolymer PHB-PEG for microspheres production because copolymerization of PHB with PEG decreased polymer hydrophobicity [18]. Microparticles from PHB with the same molecular weight (Mw = 250000) were produced to compare with PHB-PEG microparticles. Images of lysozyme-loaded microparticles from PHB and PHB-PEG with hydroxyapatite are presented on Fig.2. The microparticles had spherical form with porous surface. Average size of these particles was 33±12 um; the surface of both samples was rough and the porosity was observed with porous size about 1 um (Fig. 2).

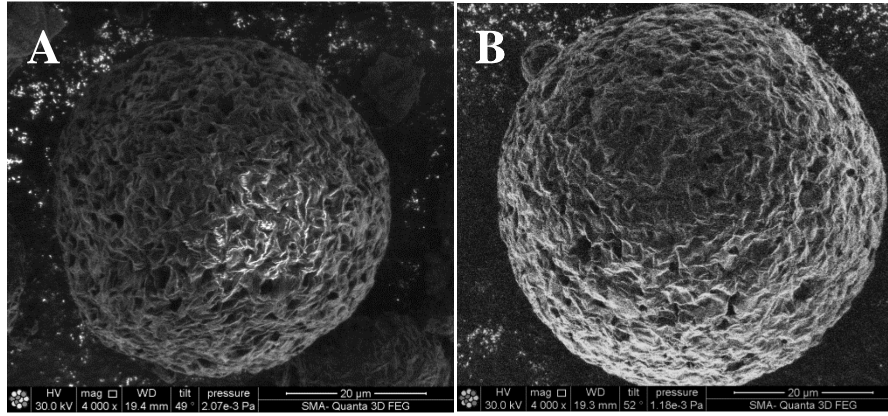

Fig. 2. SEM images of microparticles based on PHB-PEG (A), PHB 250 000 (B), obtained on Quanta 200 3D, USA

Protein encapsulation into polymer microparticles was confirmed by fluorescence microscopy (Fig. 4). With the help of imaging of fluorescence along the section on the sample we found that protein was incorporated in spheres irregularly. It is the promising feature for protein sustained release system functionality because protein clusters are more resistant to the hydrophobic polymer matrix microenvironment.

Then we carried out the *in vitro* sustained release test. The obtained data are shown at fig. 5 . It was shown that lysozyme release kinetics from PHB and PHB-PEG microparticles was rather different (fig. 4).

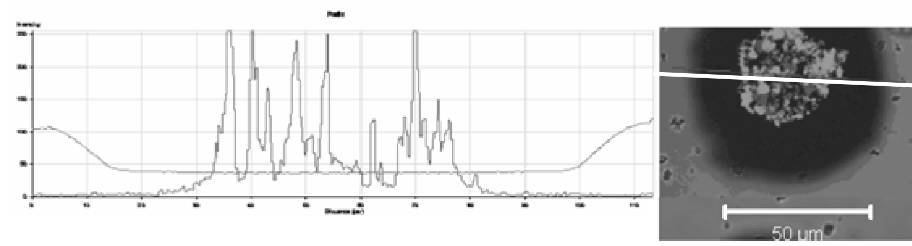

Fig. 3. Fluorescence along the section (is shown by white line) of the single microsphere loaded by FITC-BSA (confocal microscopy).

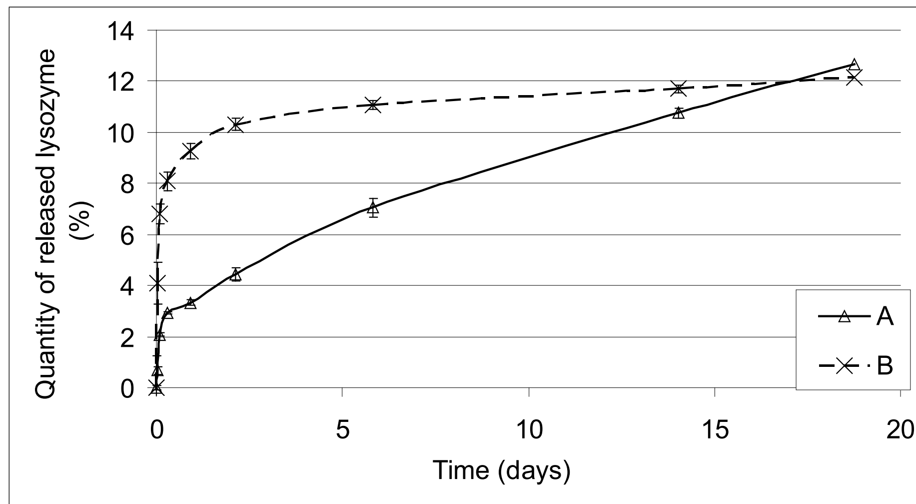

Fig. 4. The profile of lysozyme in vitro release from microspheres based on PHB-PEG 250 000 (A), and PHB 250 000 (B).

In the case of homopolymer the protein release was initially with high rate because of burst effect while the interval of linearly protein release was scarce. In opposite, from PHB-PEG microspheres the initial burst effect of lysozyme was rather low, when more than 70% of protein released with almost zero-order kinetics.

The main requirement for releasing protein is retention of its activity. To determine whether lysozyme activity was retained during its release from microparticles, the enzyme activity on samples obtained at the first hours, after 7 days and after 14 days of *in* vitro release was measured. To determine the samples activity we compared it with reference data. The obtained data are shown at fig. 6.

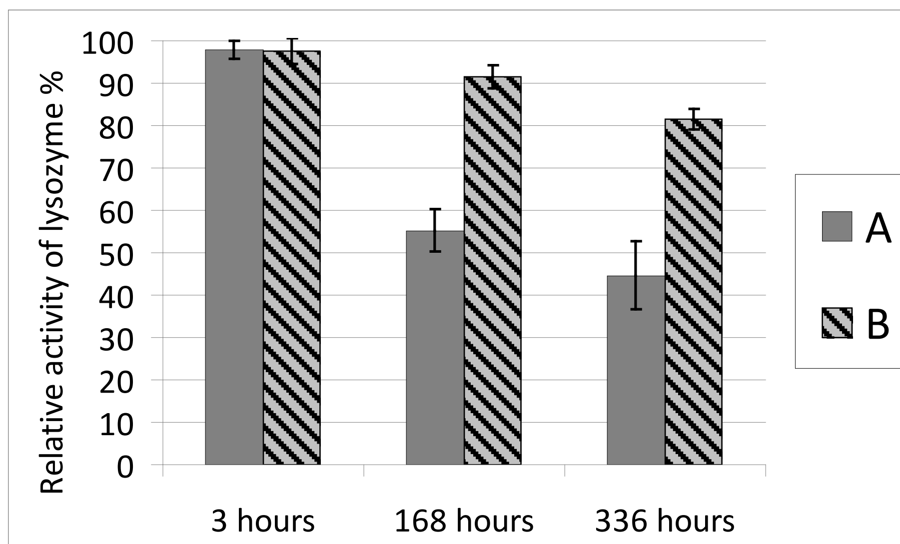

Fig. 5. The relative activity of lysozyme released from microparticles after 3, 168, and 336 hours of incubation. A for PHB 250 000; B for PHB-PEG 250 000.

So, as it is seen from the current diagram, the application of less hydrophobic copolymer PHB-PEG for microparticles production leaded to maintenance of significantly greater stability of protein released from polymer microparticles that was proposed previously.

4 Conclusions

Thus, the obtained data demonstrates that model protein lysozyme can release from polymer microparticles without lost of its enzymatic activity for 14 days of sustained release. The developed technique for protein encapsulation in polymer allow to model prolonged protein action in vitro and in vivo, as well as can be used for development of various therapeutic protein sustained release systems. The novel copolymer PHB-PEG had better features for protein encapsulation than homopolymer PHB.

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